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The use of ketogenic diet therapy in the era of individualized therapy

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The use of ketogenic diet therapy in the era of individualized therapy

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Editorial: The use of ketogenic diet therapy in the era of individualized therapy

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KEYWORDS

individualized, personalized, ketogenic diet, therapy, precision

Editorial on the Research Topic
The use of ketogenic diet therapy in the era of individualized therapy

The term individualized medicine refers to truly personalized treatment that tries to treat each patient according to their individual biology.

"Personalized" medicine is a model of N-of-1, in which each patient is considered the only patient treated. "Precision" medicine, on the other hand, resembles the 1-in-N model, allowing a more traditional Western medicine approach to conducting research on groups and sub¬groups and treating the patient's specific subgroup (1, 2). Thus, individualized medicine can go one step further and be considered as personalized medicine.

This Research Topic aimed to assess ketogenic diet usage, acceptability, and efficacy of individualized medicine.

In this special e-collection, we tried to collect and combine the articles that will help us understand the effects and mechanisms of ketogenic diet therapy in special patients and/or patient groups in which they are used individually.

Ketogenic dietary therapies are well-established, safe, non-pharmacologic treatments used for children and adults with drug-resistant epilepsy and other neurological disorders (3). Ketone body levels are recognized as helpful to check compliance to the ketogenic diet therapy and to attempt titration of the diet according to individualized needs. Possible variations in glycemia and keton bodys blood levels according to the menstrual cycle have not been systematically assessed yet, but this time window deserves special attention because of hormonal and metabolic-related changes. Pasca et al. aimed at searching for subtle changes in the ketone body's blood level during the menstrual cycle in female patients. A significant increase in glucose blood levels during menstruation was found in the entire cohort. As far as the keton bodys blood levels, an inversely proportional trend compared to glycemia was noted.

The classic ketogenic diet is an isocaloric, high-fat, low-carbohydrate diet that induces the production of keton bodys. High consumption of dietary fatty acids, particularly long-chain saturated fatty acids, could impair nutritional status and increase cardiovascular risk. De Amicis et al. evaluated the long-term effects of a 5-year classic ketogenic diet on body composition, resting energy expenditure, and biochemical parameters in children affected by Glucose Transporter 1 Deficiency Syndrome (GLUT1DS). Long-term adherence to the classic ketogenic diet showed a good safety profile on anthropometric measurements, body composition, resting energy expenditure, and biochemical parameters. Ketogenicdietary treatments are to date the gold-standard treatment for GLUT1DS. Administration of ketogenic diet therapy is generally per os; however, in some conditions including the acute

gastro-enteric post-surgical setting, short-term parenteral administration might be needed. De Giorgis et al. reported the first pediatric patient with GLUT1DS in chronic treatment with ketogenic diet therapy efficiently treated with exclusive parenteral nutrition for 5 days. This case reports on real-world management and the ideal recommendations for parenteral-ketogenic diet therapy in an acute surgical setting.

COVID-19 is associated with subclinical myocardial injury. Exogenous ketone esters acutely improve left myocardial function in healthy participants and patients with heart failure, but the effects have not been investigated in participants previously hospitalized for COVID-19. Wodschow et al. performed a randomized placebo-controlled double-blind crossover study. They concluded that in patients previously hospitalized with COVID-19, a single oral dose of ketone ester had no effect on left ventricular ejection fraction (LVEF), cardiac output or blood oxygen saturation, but increased global longitudinal strain (GLS) acutely.

The ketogenic diet, as a dietary intervention, has gained importance in the treatment of solid organ structural remodeling, but its role in renal fibrosis has not been explored. Qiu et al. demonstrated that a ketogenic diet significantly enhanced serum β-OHB levels in mice. Histological analysis revealed that the ketogenic diet alleviated structural destruction and fibrosis in obstructed kidneys and reduced the expression of the fibrosis protein markers α-SMA, Col1a1, and Col3a1. Their results highlight that the ketogenic diet attenuates unilateral ureteral obstruction (UUO)-induced renal fibrosis by enhancing fatty acid oxidation (FAO) via the free fatty acid receptor 3 (FFAR3)-dependent pathway, which provides a promising dietary therapy for renal fibrosis.

High carbohydrate, low fat (HCLF) diets have been the predominant nutrition strategy for athletic performance. Highly trained competitive middle-aged athletes underwent two 31-day isocaloric diets (HCLF or LCHF) in a randomized, counterbalanced, and crossover design while controlling calories and training load. These results: (i) challenge whether higher carbohydrate intake is superior for athletic performance, even during shorter-duration, higher-intensity exercise; (ii) lower carbohydrate intake may be a therapeutic strategy to independently improve glycemic control, particularly in those at risk for diabetes; (iii) unique relationship between continuous glycemic parameters and systemic metabolism (Prins et al.).

Sub-acute sclerosing panencephalitis (SSPE) is a chronic, progressive neurodegenerative disorder, commonly seen in measles-endemic countries leading to progressive neuronal loss and death. Currently, there is no proven cure for this devastating disease. Ibrahim and Farooq evaluated 12 children whoes low glycemic index diet (LGIT) was started with a confirmed diagnosis of SSPE. Seven (58.3%) children showed a >50% reduction in myoclonic jerks with three (25%) having a 100% reduction. LGIT may play an effective role in the management of SSPE.

Glioblastoma Multiforme is an aggressive brain cancer affecting children and adults frequently resulting in a short life expectancy. The analysis revealed several limitations of the ketogenic diet as an intervention. The effectiveness is more robust in mice than in human studies. Furthermore, tolerability is marginally supported in human studies requiring more reproducible research to validate that the intervention is manageable and effective in patients with glioblastoma (Clontz). Mast cell tumors (MCT) are common neoplasms in dogs and are similar to most other malignant cancers in requiring glucose for growth, regardless of histological grade. Ketogenic metabolic therapy (KMT) is emerging as a non-toxic nutritional intervention for cancer management in animals and humans alike. Seyfried et al. reported the case of a 7 years-old Pit Bull terrier that presented in 2011 with a cutaneous mast cell tumor under the right nostril. The resolution of the tumor in this canine patient could have been due to the diet-induced energy stress and the restriction of glucose-driven aerobic fermentation that is essential for the growth of most malignant tumors.

In summary this Research Topic support that ketogenic diet therapies are effective and safe treatment for many diseases and is promising as an individualized treatment.

Author contributions

AÜ: Writing—review and editing. BÜ: Writing—review and editing. EA: Writing—review and editing.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Low and high carbohydrate isocaloric diets on performance, fat oxidation, glucose and cardiometabolic health in middle age males

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High carbohydrate, low fat (HCLF) diets have been the predominant nutrition strategy for athletic performance, but recent evidence following multi-week habituation has challenged the superiority of HCLF over low carbohydrate, high fat (LCHF) diets, along with growing interest in the potential health and disease implications of dietary choice. Highly trained competitive middle-aged athletes underwent two 31-day isocaloric diets (HCLF or LCHF) in a randomized, counterbalanced, and crossover design while controlling calories and training load. Performance, body composition, substrate oxidation, cardiometabolic, and 31-day minute-by-minute glucose (CGM) biomarkers were assessed. We demonstrated: (i) equivalent high-intensity performance (@~85%VO_{2max}), fasting insulin, hsCRP, and HbA_{1c} without significant body composition changes across groups; (ii) record high peak fat oxidation rates (LCHF:1.58 ± 0.33g/min @ 86.40 ± 6.24%VO_{2max}; 30% subjects > 1.85 g/min); (iii) higher total, LDL, and HDL cholesterol on LCHF; (iv) reduced glucose mean/median and variability on LCHF. We also found that the 31-day mean glucose on HCLF predicted 31-day glucose reductions on LCHF, and the 31-day glucose reduction on LCHF predicted LCHF peak fat oxidation rates. Interestingly, 30% of athletes had 31-day mean, median and fasting glucose > 100 mg/dL on HCLF (range: 111.68-115.19 mg/dL; consistent with pre-diabetes), also had the largest glycemic and fat oxidation response to carbohydrate restriction. These results: (i) challenge whether higher carbohydrate intake is superior for athletic performance, even during shorter-duration, higher-intensity exercise;

(ii) demonstrate that lower carbohydrate intake may be a therapeutic strategy to independently improve glycemic control, particularly in those at risk for diabetes; (iii) demonstrate a unique relationship between continuous glycemic parameters and systemic metabolism.

KEYWORDS

high fat diet, low-carbohydrate, high-carbohydrate, fat oxidation, carbohydrate oxidation, prediabetes

Introduction

From 1896 to 2008, athletes competing in the Olympics demonstrated trends for increased carbohydrate intake in 1976 and a predominant shift toward high-carbohydrate low-fat (HCLF) diets in the 1996 Olympic games (1–3). This shift in athlete food preference toward carbohydrates was cited to be driven by (i) increased user consciousness of healthy food choice to optimized performance (1, 4, 5); (ii) the importance of muscle glycogen as the preferable metabolic fuel during exercise of either high intensity or long duration low intensity (6–12) following the emergence of muscle biopsy techniques in 1960s (13); (iii) anaplerotic theory in which depleted muscle glycogen attenuates mitochondrial oxaloacetate concentrations and thus reduced mitochondrial capacity to oxidize fatty acids (14); (iv) multiple studies illustrating carbohydrate ingestion delayed or reversed fatigue by maintaining blood glucose homeostasis (15–17); (v) "cross-over effect" (18–20) in which exercise of increasing intensity becomes increasingly dependent on carbohydrate oxidation since fat oxidation effectively ceases at any exercise intensity $\geq$ 85% VO_{2max} (18–22); (vi) clinical trials of high-fat diets resulting in impaired performance in both recreational (23) and elite athletes (i.e., Olympic class) (24–26).

Countering this evidence is a growing body of data demonstrating that extended habituation to a low-carbohydrate high-fat (LCHF) diet can shift the "cross-over" set-point in favor of greater fat oxidation, even at much higher intensities [($<$ 85% VO_{2max}); (27, 28)], and dramatically increase the rates of peak fat oxidation at moderate intensities (i.e., 60% VO_{2max}) (29). Rates of fat oxidation during exercise across these LCHF studies are amongst the highest yet measured (24, 25, 28, 30–32) even though they were measured during progressive exercise to exhaustion (e.g., minutes), rather than more prolonged exercise (e.g., hours). These studies opened key questions of whether performance-equivalence would still hold (i) if exercise intensity was increased (i.e., running trial 800-1,609 m) and (ii) if high-intensity interval sessions would facilitate more muscle glycogen depletion in those habituated to LCHF which would become more apparent as the number of intervals increased.

Paramount to both athletes and the general population is the potential health and disease implication of their dietary choice, particularly as individuals advance in age. Multiple studies have illustrated beneficial shifts in glucose, insulin, hemoglobin A_{1c} (HbA_{1c}), inflammation, and oxidative stress biomarkers, along with proposed alterations in diabetes, cancer, neurological, and other disease clinical outcomes resulting from LCHF diets and their resulting metabolite shifts (33–40). While there is evidence for optimism, some dating back over a century (36, 38), there has been a call for more high-quality evidence (41). Thus, leveraging a randomized within-subject controlled cross-over design in middle-aged competitive athletes, we sought to rigorously address these gaps and evaluate the effects of 4-week habituation to either an LCHF or HCLF diet while controlling calories and training volume across groups on (i) high-intensity short duration exercise performance, (ii) body composition, (iii) metabolite oxidation rates at graded exercise intensities, and the (iv) continuous glycemic, insulin and overall cardiometabolic biomarker changes which accompanied these multiweek dietary patterns. We hypothesized that LCHF habituation would result in reduced high-intensity exercise performance. Interestingly, without changes in calories or training load, or significant changes in body composition across groups, the results found herein challenge the current superiority of high-carbohydrate diets (even during high-intensity exercise) for performance, illustrate record high rates of fat oxidation, demonstrate unique and consistent changes in continuous glucose which predict systemic fat oxidation rates, and a surprising incidence of glycemic values consistent with pre-diabetes phenotype in this middle-aged competitive athlete cohort during high carbohydrate consumption which was therapeutically addressed with carbohydrate restriction (**Figure 1**).

Materials and methods

Experimental design

Highly trained competitive middle-aged athletes underwent two 31-day isocaloric diet periods (HCLF or LCHF) in a randomized (www.randomizer.org), counterbalanced, crossover design with a two-week washout period between dietary interventions without feeding limitations. We assessed both diets while controlling calories and training load. Primary outcomes were performance, substrate oxidation during exercise, continuous glucose and cardiometabolic biomarkers. Each subject visited the laboratory on ten separate occasions, performing testing before (PRE) and at the completion (POST) of each 31-day dietary intervention (**Figure 2**). Visit one and two consisted of a familiarization of measurement instruments, equipment, perceptual measurements (42, 43), consent, VO_2max test (31), and continuous glucose monitoring (CGM; Freestyle Libre 2, Abbott Diabetes Care Inc; Almeda, CA) sensor application. One-mile [1,609 m] running time trial (TT) and repeated sprint protocol (RSP; 6 $\times$ 800 m) were performed twice on each dietary intervention (Pre and Post). One-mile TT was performed on Day –4 and Day 28. Three days later, subjects performed the RSP on Day –1 and Day 31. Gas exchange and perceptual changes were recorded throughout each performance trial Pre and Post each dietary intervention.

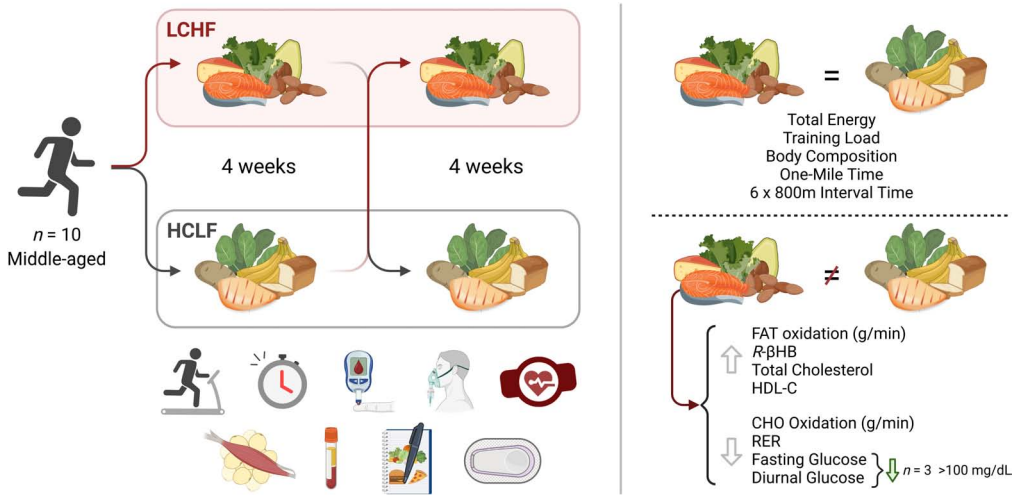


FIGURE 1
Low and high carbohydrate isocaloric diets on performance, fat oxidation, glucose and cardiometabolic health in middle age males. Highly trained competitive middle-aged male athletes underwent two 31-day isocaloric diets (HCLF or LCHF) in a randomized, counterbalanced, and crossover design while controlling calories and training load. Performance, body composition, substrate oxidation, cardiometabolic, and 31-day minute-by-minute glucose (CGM) biomarkers were assessed. We demonstrated: (i) equivalent high-intensity performance (@∼85%VO2max), fasting insulin, hsCRP, and HbA1c without significant body composition changes across groups; (ii) record high peak fat oxidation rates (LCHF:1.58 ± 0.33g/min) at 86.40 ± 6.24%VO2max with 30% subjects > 1.85g/min; (iii) higher total, LDL, and HDL cholesterol on LCHF; (iv) reduced glucose mean, median, and variability on LCHF. We also found that the 31-day mean glucose on HCLF predicted 31-day glucose reductions on LCHF, and the 31-day glucose reduction on LCHF predicted LCHF peak fat oxidation rates. 30% of athletes had 31-day mean, median and fasting glucose > 100mg/dL on HCLF (range:111.68-115.19 mg/dL; consistent with pre-diabetes), also had the largest glycemic and fat oxidation response to carbohydrate restriction. CHO, Carbohydrate; HCLF, High-Carbohydrate Low-Fat Diet; HDL-C, High Density Lipoprotein Cholesterol; LCHF, Low-Carbohydrate High-Fat Diet; *R*-βHB, *R*-β-Hydroxybutyrate; RER, Respiratory Exchange Ratio.

Body composition and cardiometabolic parameters were measured Pre and Post each dietary intervention. Capillary and interstitial metabolite concentrations were measured throughout each 31-day dietary intervention period. Testing sessions were conducted at the same time of day in an environment with controlled temperature and humidity (19-21 ^{0}C, humidity = 35-40%) within the Exercise Science Laboratory of Grove City College. The experimental protocol was approved by the Institutional Review Board of Grove City College prior to implementation (IRB number 110-2021).

Subjects

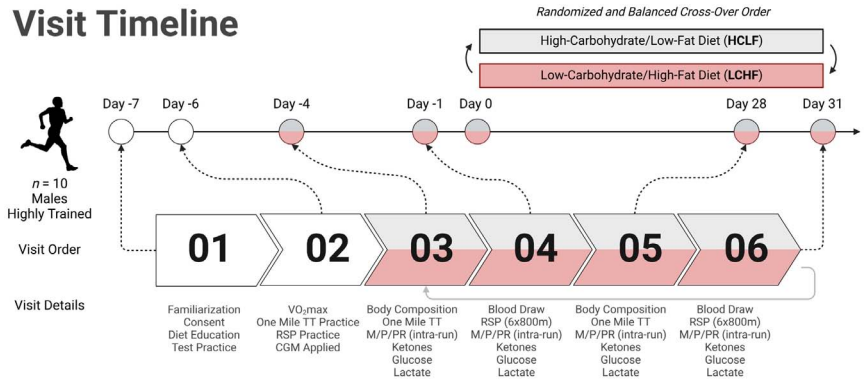
Ten middle-aged competitive distance runners (Figure 2) were recruited directly from local running organizations and by advertising within the local community. Inclusion and exclusion criteria were screened utilizing medical history/Physical Activity Readiness Questionnaire (PAR-Q). Inclusion criteria included: (1) 30 to 50-years old males; (2) completion of one-mile (1,609 meter) run in under seven minutes; (3) running ≥ 20 miles (32 kilometers) per week; (4) > 2 years of running experience; (5) currently consuming a carbohydrate-based diet (> 50% kcals). Exclusion Criteria included: (1) cigarette smoker; (2) metabolic or cardiovascular disease, (3) orthopedic, musculoskeletal, neurological, and/or any medical conditions that prohibit exercise; (4) psychiatric disorder; (5) pharmaceutical drugs affecting measurement parameters. Subjects were prohibited from using any ergogenic aids/supplements one month prior to study initiation to study completion. Subjects were instructed to refrain from caffeine and alcohol consumption for 48 h, and racing or training for 24 h before each performance test.

Dietary interventions

Using direct counseling and prepared educational handouts, a registered dietitian taught and guided each athlete prior to the experimental phase on how to implement the LCHF and HCLF diets at home. Instructional handouts included: (i) summary of key aspects of each diet, (ii) 31-day LCHF and HCLF meal plan but were advised to use this meal plan as a guide rather than a strict protocol; (iii) detailed guide on acceptable low-carbohydrate foods as well as a recommended list of nutritious fat and protein-rich foods. The primary macronutrient targets for LCHF and HCLF were expressed as both a percentage of total daily energy intake and daily gram intake: LCHF: < 50 g/day carbohydrate, 75–80% fat, 15–20% protein; HCLF: 60–65% carbohydrate, 20% fat, 15–20% protein (Table 1). Additionally, the LCHF group was recommended to supplement their diets with added salt to taste at mealtime and supplement 1-2 g/day of sodium from bouillon cubes, or homemade broth (44).

Weekly energy intake and relative macronutrient distribution were monitored and estimated via 3-day weighed food records, capturing two consecutive weekdays and a weekend day via the online smartphone application, MyFitnessPal. Subjects used digital kitchen scales to measure the weights of food portions for total energy intake estimates. Researchers administrated subject's MyFitnessPal user accounts and therefore had the ability to assess and modify the subject's macronutrient and micronutrient intake throughout the intervention (45). A two-week recovery macrocycle was incorporated between each dietary intervention without dietary restriction.

Verification of compliance to the LCHF diet was done via capillary blood ketone concentrations on days 3, 7, 14, 21, and 28 before ingesting breakfast and exercising. Capillary blood ketone concentrations (*R*-β-hydroxybutyrate; Precision Xtra, Abbott



Physical Characteristics

Variable	Mean ± SD
Age (years)	39.9 ± 5.1
Height (cm)	181.7 ± 8.8
Weight (kg)	86.7 ± 12.5
Body Fat (%)	15.7 ± 5.1
Fat-Free Mass (kg)	72.6 ± 7.9
Fat Mass (kg)	14.1 ± 5.9
Body Mass Index (kg/m^2)	26.1 ± 2.6
Mean Running Distance per Week (km)	49.7 ± 16.3
Running Experience (years)	9.7 ± 6.1
Maximum Oxygen Consumption (ml/kg/min)	58.7 ± 5.2

FIGURE 2
Experimental timeline and participant characteristics. Highly trained middle-aged male runners (n = 10) underwent a battery of tests throughout the study. The Pre-diet study tasks included familiarization with the study protocol, time trial (TT) practice runs, VO2max assessment, and continuous glucose monitor (CGM) application for measurement of interstitial fluid glucose (visits 01 and 02). Ketones (R-β-hydroxybutyrate) and lactate were analyzed enzymatically using finger-sticks. All participants were assessed for body composition changes; metabolic, physiological, and perceptual assessments (M/P/PR); venous blood draws; ketone/glucose/lactate measurements; one mile TT; and repeat sprint protocol (RSP; 6 × 800m) from visit 03 through 06. Diet order was randomized and balanced to explore the between/within-participant cross-over effects. Once a first diet was completed, subjects crossed over to the other dietary treatment and repeated visits 03 through 06 (i.e., visits 07 through 10). The table illustrates the mean (SD) participant age, anthropometry, and training status variables collected at visit 01. TT = time trial; RSP = repeated sprint protocol; LCHF = low-carbohydrate/high-fat diet; HCLF = high-carbohydrate/low-fat diet; M/P/PR = metabolic, physiological, and perceptual. Figure was created with BioRender.com.

Diabetes Care Inc., Almeda, CA) was also measured immediately before and after the LCHF diet by the primary researcher. The results from the dietary intake and ketone measurements enabled the registered dietician to individually fine-tune participants' diets, if necessary, via phone or email, thus ensuring continuous nutritional ketosis while on the LCHF diet.

Physical activity monitoring

Participants were instructed to maintain a training log (mode, duration, and intensity of each workout) during the study intervention without increasing or decreasing the training load (46). Training load was calculated by using the session-RPE method (RPE x session duration [min]) (47).

Body mass and body composition monitoring

Participants reported to the laboratory at least ≥ 3 h post-prandial, refraining from exercise for 48 h. The measurements of body mass were performed on a medical scale with a precision of 0.1 kg. Body composition was evaluated using the electrical impedance technique (Tanita® MC-980U, Tanita Corporation, Inc., Tokyo, Japan).

Maximal exercise testing

On the second visit, subjects performed an incremental test to exhaustion on a motorized treadmill (Trackmaster TMX425C treadmill, Newton, KS) utilizing the modified Astrand treadmill

TABLE 1 Dietary composition.

Variable	LCHF	HCLF	*P*-value
Energy (kcal/day)	2545.3 ± 503.6	2595.7 ± 443.1	0.785
Carbohydrate (g)	40.9 ± 8.7	384.7 ± 65.3	<0.001
Protein (g)	141.0 ± 26.0	110.6 ± 20.8	0.010
Fat (g)	198.2 ± 45.2	67.6 ± 17.6	<0.001
Carbohydrate (%)	7.98 ± 3.49	63.2 ± 3.88	<0.001
Protein (%)	26.7 ± 7.32	17.7 ± 2.61	0.001
Fat (%)	64.4 ± 9.33	18.4 ± 6.12	<0.001
Cholesterol (mg)	839.7 ± 193.8	180.9 ± 66.2	<0.001
Fiber (g)	13.7 ± 6.51	31.2 ± 5.71	<0.001
Sugar (g)	13.1 ± 4.09	129.1 ± 37.1	<0.001

Participant dietary composition on low-carbohydrate high-fat (LCHF) and high-carbohydrate low-fat (HCLF) diets were captured using a 3-day food records including 1 weekend day using MyFitnessPal. Paired t-tests were conducted on 31-day averages. Every comparison was significant between-diet difference for energy intake. Every other variable demonstrated significant differences. n = 10. Values are Mean ± SD.

protocol. The treadmill was calibrated before each exercise test according to the manufacturer's instructions. Oxygen consumption (VO_2) and carbon dioxide production (VCO_2) was assessed using an automated metabolic analyzer system (TrueOne 2400, ParvoMedics, Sandy, UT) calibrated prior to each exercise test using standard calibration gases (16% O_2 and 4% CO_2). Subjects wore a Polar heart rate monitor (Polar Electro, Kempele, Finland) during exercise to measure heart rate. After a thorough explanation of the experimental procedures, each subject was instructed to walk on the treadmill for 3 min as a warm-up at a self-selected speed (0% grade). Immediately following the 3-min warm-up, the speed was increased to 8–13 $km.hr^{-1}$ for 3 min (0% grade) to achieve the subjects' comfortable running pace. After 3 min of running at 0% grade, the grade was increased 2.5% every 2 min throughout the test protocol while speed was kept constant. At the end of the test, the highest VO_2 value recorded during the last 30 seconds of exercise was considered the subject's VO_{2max} (48). Some or all of the following criteria were used to determine if a physiologically valid VO_{2max} had been attained: (a) a plateau in VO_2 with increasing exercise intensity ($<$ 150 ml/min or $<$ 2.1 ml/kg/min), (b) a respiratory exchange ratio (RER) of $\geq$ 1.1, (c) and volitional termination due to exhaustion.

One-mile time trial

Participants arrived at the laboratory in the morning at least $\geq$ 3 h post-prandial and performed a one-mile TT on a motorized treadmill (TMX425C treadmill, Trackmaster, Newton, KS, USA). Subjects performed a warmup run consisting of 5 min at 45% VO_{2max} followed by 5 min at 65% VO_{2max} (10 min total). After a 5-min passive rest period, subjects then initiated the one-mile TT (1,609 m). Prior to the TT, the treadmill was brought to a standstill (0 $km.hr^{-1}$), all timing devices were reset, the distance covered on the treadmill monitor was reset, a 5-s count down was given, and the TT began. Each TT commenced with a 30-second rolling start at a running speed approximating 80% VO_{2max}, after which subjects could freely adjust their speed. Running speed and time was not visible to subjects during the TT. Subjects were verbally informed of the distance they had covered at 200m intervals. The treadmill gradient was set to 1% throughout the exercise (49). Subjects were instructed to finish the TT as fast as possible and were not informed of the overall performance time until completion of the final testing session. Heart rate (Polar Electro, Kempele, Finland), RPE (RPE-Overall; RPE-Chest; RPE-Legs) and affect (Feeling Scale) were recorded at 200 m intervals during the TT. Lastly, RPE and affect for the entire exercise session (session RPE and session affect) were obtained 5 min following the one-mile TT. Expired respiratory gases were continuously collected during the entire time trial and capillary blood was collected Pre- and Post-TT.

Repeated sprint performance

Participants arrived at the laboratory in the morning at least $\geq$ 3 h post-prandial. Upon arrival to the laboratory subjects completed a 10 min self-paced warm-up on the treadmill. The RSP prolonged high intensity interval protocol was chosen because it requires a high level of aerobic oxidative, as well as anaerobic glycolytic contributions (50). The RSP was performed on a treadmill and consisted of 6 $\times$ 800 m (0.5 miles) sprints, with a three-minute passive recovery interval. Subjects were instructed to finish each 800 m sprint as fast as possible. The time it takes to complete each sprint was recorded. No feedback on sprint times was given to the participants during trials. Verbal encouragement was provided during maximum effort sprints in a standardized fashion throughout each visit. Heart rate, blood samples, RPE, affect, and metabolic gases were collected throughout.

Respiratory gas exchange

Respiratory gas exchange was recorded using an automated metabolic analyzer system (TrueOne 2400, ParvoMedics, Sandy, UT, United States). Prior to each experimental session, the device was calibrated using procedures according to manufacturer instructions. The breath-by-breath measurements were performed for oxygen uptake (VO_2), carbon dioxide production (VCO_2), and respiratory exchange ratio (RER) and was measured continuously throughout trials (one-mile TT and RSP). The average values for VO_2 (L/min) and VCO_2 (L/min) were calculated over the last minute of each 2-min exercise stage in the maximal exercise test, and each minute of the one-mile TT. Whole-body rates (g/min) of CHO and fat oxidation were calculated using intensity dependent equations that assume negligible protein contribution to energy expenditure (51).

Blood metabolites

Blood samples were measured via fingertip blood samples collected using a lancet following cleaning of the fingertip with an alcohol swab and then dried. The first droplet was wiped away with a cotton swab to remove any alcohol and the subsequent droplets were used for analysis. Samples were immediately processed for measurement of blood lactate (Lactate Plus, Nova Biomedical), ketones (*R*-β-hydroxybutyrate; Precision Xtra, Abbott Diabetes Care Inc., Almeda, CA) and glucose (Precision Xtra, Abbott Diabetes Care Inc., Almeda, CA) concentrations.

Biochemical assays

Blood samples were collected using a validated dried blood spot (DBS) card technique (52). DBS cards were allowed to dry for 30 min, and then stored at −30°C prior to shipment to ZRT CLIA-approved Laboratory (Beaverton, OR) for immunoassay analyses as previously described (52, 53). DBS were assayed for HbA_{1c}, total cholesterol, low-density lipoprotein cholesterol (LDL-C), very low-density lipoprotein cholesterol (VLDL-C), high-density lipoprotein cholesterol (HDL-C), triglycerides, insulin, and high-sensitivity C-reactive protein (hsCRP). Dried blood spot testing has shown a strong correlation with conventional serum tests, making it a reliable and convenient tool for screening cardiometabolic risk factors (52).

Continuous glucose monitoring

Participants' interstitial glucose concentrations were measured throughout each 31-day dietary intervention via CGM (Freestyle

Libre 2, Abbott Diabetes Care Inc; Almeda, CA) utilizing Levels, Inc., mobile application software to capture glucose response and trends in real-time (Levels Health, Levels, Inc., New York, New York). CGM tracks long-term to HbA_{1c} (54–56), short term CGM readings (10-14d) are good estimates of 3-month CGM averages (57), and (iii) CGM can also capture both fasting and post-prandial differences in glucose (validated diagnostic tools). Participants were instructed to apply a 14-day sensor to the back of the arm following the manufacturer's written instructions and demonstration video. Participants were instructed to place it in the same location throughout. CGM subcutaneous sensor measured interstitial glucose values every 15 min which was transmitted using near-field communications. Participants obtained their glucose values after scanning for up to 8 h of data. All 24-hour CGM readings were included into daily and 31-day statistics. All CGM sensors were obtained in batch to control for any potential manufacturing discrepancies across different sensor batches.

Statistical analysis

Statistical analyses were performed using SPSS version 26.0 (SPSS Inc., Chicago, IL), and MATLAB 2022a (MathWorks Inc., Natick, MA). Statistical significance was set *a priori* at $p < 0.05$. Descriptive statistics were calculated for all variables. Normality and absence of large outliers were verified by using the Shapiro-Wilks test, observing the normality plots, and residual plots. Repeated measure analyses of variance (ANOVA) were utilized to assess physiologic, metabolic, respiratory, perceptual, and performance time, treatment, and interaction effects. Bonferroni *post hoc* was utilized to control for multiple comparisons when main effects were observed. A paired samples *t*-test was used to analyze differences in macronutrient composition and 31-day glycemic variable averages between the two dietary interventions. A one-way repeated measures analysis of variance was used to analyze differences over time for training load. The assumption of sphericity was confirmed using Mauchly's test. Greenhouse-Geisser epsilon corrections were used when the sphericity assumption was violated. Partial-eta squared (η2p) was used to report the effect sizes for the above metrics, where appropriate. Simple linear regression analyses were run to determine the relationship between glycemic, substrate oxidation, and biochemical parameters. Slope intercept ($y = mx + b$), r^2, and p values represent best-fit equation, the goodness of fit, and slope significantly non-zero values, respectively. To avoid (58) assumptions of normality in continuous data, a kruskal Wallis (KW) test with Dunn's correction for multiple comparisons was utilized to determine differences across the entire 24 h circadian window for the all data in the 31d CGM response window. Time points every 4th h across the entire 24 h period were included in the statistical comparison, corresponding to ∼1 point maximum per ultradian cycle (59, 60). All data are reported as Median ± SD, with exception of circadian glucose patterns presented as Median, 25th, and 75th percentile. Sample size ($n = 10$) was determined based on prior studies where one-mile TT performance differences were observed using dietary interventions in elite runners which was adjusted for the expected increase in one-mile TT performance time variance across cohort in non-elite athletes (61).

Results

Dietary and exercise adherence

Daily dietary nutrient intakes are summarized in **Table 1**. All participants ($n = 10$) completed all the required study duties. The energy intake between LCHF and HCLF treatments remained isocaloric. Significant differences were detected for every diet composition variable, notably for carbohydrate and fat intake. LCHF consumed less absolute and relative carbohydrates compared to HCLF, largely driven by the 10-fold reduction in simple sugars and approximately one-half the dietary fiber. Conversely, LCHF consumed significantly more dietary fat and cholesterol, both as absolute and relative amounts, compared to HCLF. While attempts were made to control protein intake across groups, athletes consumed an extra 31 grams of protein during the LCHF treatment ($p < 0.01$), likely due to the close association between dietary fat and protein.

Average capillary *R*-βHB (mean ± SD: 0.76 ± 0.04 mM; range: 0.3 – 2.2 mM) during LCHF increased significantly from baseline and remained within the range of nutritional ketosis (≥ 0.5 mM *R*-βHB) throughout the intervention (**Figure 3**).

There were no significant differences for weekly training load, either within- or between dietary phases (**Table 2**).

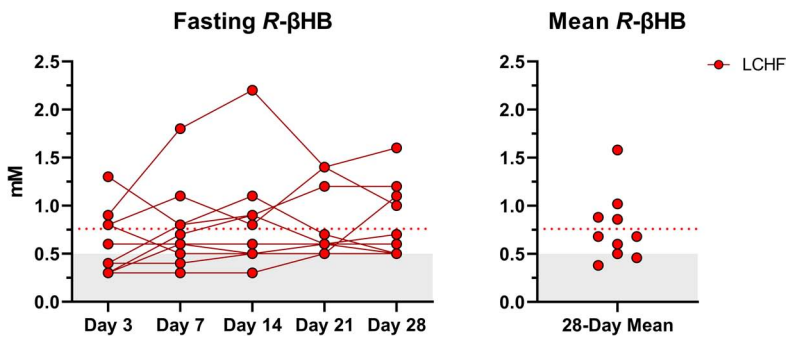


FIGURE 3
Daily capillary ketones. *y*-axis values ≥ 0.5 mM define a range characterized as nutritional ketosis. The dotted line denotes the low carbohydrate high fat (LCHF) *R*-beta-hydroxybutyrate (*R*-βHB) mean. Eight out of ten participants consistently remained in nutritional ketosis throughout the LCHF intervention, whereas two participants were marginally below threshold. One-Way ANOVA revealed that there were no significant time effects from day 3 and thereafter, meaning that ketosis was rapidly induced and maintained over four-weeks. $n = 10$.

Body composition data

Participants started each diet at similar weight and body composition. There were no significant treatment or interaction effects for weight or body composition during either LCHF or HCLF (Table 3). Overall changes in weight and body composition on each diet were similar. Significant time effects were detected for weight and BMI in both LCHF and HCLF treatments. Approximately ∼80% of weight loss composition was derived from fat mass (Δ: 1.8 ± 0.6 kg; $p = 0.05$) in 4:1 ratio fat:fat-free mass, while the remaining portion of weight loss was derived non-significantly from fat-free mass (Δ: 0.6 ± 0.2 kg).

Physiological, metabolic, respiratory, perceptual, and performance data collected during the one-mile time trial

All 10 participants completed the treadmill one-mile time trial (1,609 m) at a self-selected pace before initiating either diet. No significant baseline (Pre) differences were observed across physiological, metabolic, respiratory, perceptual, or performance parameters. Significant differences were detected within-diet and between-treatments at the post-timepoint (Table 4).

Within-diet, LCHF mean respiratory exchange rate (Δ: −0.08 ± 0.02; −6%), and mean carbohydrate oxidation (Δ:−1.06 ± 0.36 g/min; −20%) decreased significantly from Pre to Post, whereas heart rate (Δ: 4 ± 1 bpm; 3%) and mean fat oxidation (Δ: 0.44 ± 0.16 g/min; 190%) increased significantly from pre to post (all $p < 0.05$). The significant decrease in respiratory exchange rate from Pre to Post-LCHF revealed a decrease in carbohydrate reliance (91 to 73%) and an increase in fat utilization (9 to 27%). Additionally, these effects were corroborated by the absolute (g/min) carbohydrate and fat oxidation rates, which revealed that for every 1 g/min decrease in carbohydrate oxidation there was an expected 0.42 g/min of increase in fat oxidation during LCHF. There were no significant changes in substrate oxidation and respiratory exchange rate detected pre- to post-HCLF.

Significant interactions revealed that Post-LCHF athletes had a higher heart rate (Δ: 6 ± 2 bpm), mean fat oxidation rate (Δ: 0.62 ± 0.21 g/min), mean respiratory rate (Δ: 1.0 ± 0.3 bpm), and a lower carbohydrate oxidation rate (Δ: 1.75 ± 0.60 g/min) compared to Post-HCLF (all $p < 0.05$).

Physiological, metabolic, espiratory, perceptual, and performance data collected during the repeated sprint protocol

All 10 participants completed the treadmill series of 6-sets of 800m sprints at a self-selected pace. Before each dietary phase there were no significant differences in physiological, metabolic, respiratory, perceptual, or performance parameters. Additionally, neither diet influenced repeated sprints running performance post-diet intervention (Table 5 and Figure 4).

During RSP, LCHF induced very high rates of fat oxidation which peaked at 1.58 ± 0.33g/min during 86.40 ± 6.24%VO_{2max} (range: 0.99 to 2.01 g/min) compared to 0.69 ± 0.24g/min on HCLF during 79.67 ± 3.15%VO_{2max} (range: 0.32 to 1.13 g/min). Interestingly, 30% of the subjects on LCHF had peak fat oxidation rates > 1.85 g/min (1.95 ± 0.08 g/min; range: 1.86 to 2.01 g/min). To our knowledge, these are the highest rates of fat oxidation ever recorded. Within-diet, LCHF mean respiratory exchange rate (Δ:−0.06 ± 0.02; −7%),

TABLE 2 Training load.

	Diet treatment	Week 1	Week 2	Week 3	Week 4	Grand mean	Within-diet effect (*P*-value)	Between-diet effect (*P*-value)
Training Load (RPE x min)	LCHF	2928 ± 2057	2543 ± 2172	2343 ± 1822	2143 ± 1917	2489 ± 2425	0.14	0.94
	HCLF	2077 ± 1161	2630 ± 1507	2489 ± 1669	2503 ± 1488	2425 ± 1398	0.18	

Weekly training load were captured on low carbohydrate high fat (LCHF) and high carbohydrate low fat (HCLF) diets. 2 (Treatment) x 4 (Time) ANOVA revealed no significant differences in training load. $n = 10$. Values are Mean ± SD. RPE, rate of perceived exertion. LCHF = low-carbohydrate/high-fat diet; HCLF = high-carbohydrate/low-fat diet.

TABLE 3 Body composition.

Variable	LCHF			HCLF			*P-value*; η 2p		
	Pre Day −4	Post Day 28	Change Mean (95% CI)	Pre Day −4	Post Day 28	Change Mean (95% CI)	Treatment	Time	Interaction
Mean data									
Weight (kg)	85.2 ± 12.5	81.9 ± 10.9#	−3.2 (5.2, 1.3)	84.8 ± 10.2	83.4 ± 9.4#	−1.4 (3.4, −0.6)	0.676; 0.020	**0.012**; 0.519	0.071; 0.318
BMI (kg/m^2)	25.6 ± 2.23	24.7 ± 1.99#	−0.89 (1.5, 0.2)	25.7 ± 2.38	25.3 ± 1.84#	−0.44 (1.0, −0.1)	0.379; 0.087	**0.017**; 0.487	0.165; 0.202
Body Fat (%)	14.5 ± 4.80	11.9 ± 3.90	−2.5 (5.2, −0.2)	13.3 ± 5.14	13.0 ± 3.84	−0.26 (1.5, −1.0)	0.938; 0.001	0.080; 0.302	0.104; 0.267
Fat Mass (kg)	12.7 ± 5.26	9.99 ± 3.97#	−2.7 (5.3, 0.0)	11.5 ± 5.00	10.7 ± 4.02#	−0.78 (−2.2, 0.7)	0.849; 0.040	**0.050**; 0.363	0.127; 0.239
Fat Free Mass (kg)	72.5 ± 8.69	71.9 ± 8.74	−0.61 (2.3, −1.1)	73.3 ± 8.21	72.7 ± 8.15	−0.63 (1.6, −0.4)	0.111; 0.258	0.256; 0.140	0.976; 0.000

Body composition was assessed using bioelectrical impedance pre and post low-carbohydrate/high-fat (LCHF) and high-carbohydrate/low-fat diet (HCLF). 2 (Treatment) x 2 (Time) Repeated Measures ANOVA demonstrated no differences across treatments. A main effect of time was observed within LCHF and HCLF treatments for weight, body mass index (BMI) and fat mass (FM), and a trend in body fat percentage. Fat free mass (FFM) did not change over time. No significant interaction effects were observed, although trends were observed across LCHF and HCLF for weight (kg; $p = 0.071$). $n = 10$. Mean ± SD.
= significant difference for Pre vs. Post within-diet ($p \leq 0.05$). Bold values represent the $p < 0.05$.

TABLE 4 One-mile time trial performance, gas exchange, and perception.

Variable	LCHF			HCLF			*P-value*; η 2p		
	Pre Day −4	Post Day 28	Change Mean (95% CI)	Pre Day −4	Post Day 28	Change Mean (95% CI)	Treatment	Time	Interaction
Time (sec)	381.4 ± 30.6	367.1 ± 37.5$^{\#}$	−14.3 (26.4, 2.2)	374.1 ± 38.6	374.0 ± 31.5$^{\#}$	−0.10 (11.1, −10.9)	0.958; 0.000	**0.009**; 0.553	0.159; 0.208
Mean Carbohydrate Oxidation (g/min)	5.22 ± 1.07	4.16 ± 1.48†	−1.06 (1.8, 0.3)	5.50 ± 2.14	5.91 ± 1.07	0.4 (0.7, −1.5)	0.099; 0.273	0.335; 0.103	**0.028**; 0.433
Mean Fat Oxidation (g/min)	0.23 ± 0.29	0.67 ± 0.45$^{\#\star\dagger}$	0.44 (−0.3, −0.6)	0.21 ± 0.29	0.05 ± 0.11$^{\#}$	−0.15 (0.32, −0.01)	**0.023**; 0.454	**0.029**; 0.427	**0.001**; 0.756
Heart Rate (b.min-1)	168.8 ± 8.7	173.4 ± 8.8$^{\#\star\dagger}$	4.46 (−0.8, −8.2)	167.7 ± 10.8	167.1 ± 11.1	−0.59 (4.2, −2.9)	**0.003**; 0.644	0.145; 0.220	**0.040**; 0.389
Mean VO_2 (ml/kg/min)	50.2 ± 5.63	52.9 ± 6.84	2.72 (0.5, −5.9)	48.4 ± 8.98	50.0 ± 6.19	1.67 (2.7, −6.0)	0.070; 0.319	0.116; 0.252	0.651; 0.024
Mean VO_2 (L/min)	4.23 ± 0.44	4.34 ± 0.66*	0.11 (0.1, −0.3)	4.03 ± 0.62	4.17 ± 0.62*	0.14 (0.2, −0.5)	**0.013**; 0.516	0.257; 0.140	0.869; 0.003
Mean VCO_2 (L/min)	4.13 ± 0.46	3.97 ± 0.64	−0.15 (0.4, −0.1)	4.05 ± 0.82	4.24 ± 0.61	0.19 (0.3, −0.7)	0.580; 0.035	0.904; 0.002	0.119; 0.249
Mean V_E (L/min)	122.5 ± 13.4	123.6 ± 18.5	1.13 (6.1, −8.4)	116.8 ± 16.9	123.4 ± 15.0	6.67 (7.3, −20.6)	0.296; 0.120	0.332; 0.105	0.396; 0.081
Mean RR (bpm)	41.7 ± 6.47	43.9 ± 5.83	2.19 (−0.1, −4.3)	42.0 ± 3.98	42.9 ± 6.52	0.93 (3.0, −4.9)	0.712; 0.016	0.154; 0.212	0.534; 0.045
Mean RER	0.97 ± 0.05	0.91 ± 0.07*†	−0.06 (0.10, 0.01)	1.00 ± 0.09	1.01 ± 0.05*	0.01 (0.03, −0.06)	**0.045**; 0.376	0.110; 0.259	**0.021**; 0.466
Session RPE	7.40 ± 1.64	7.90 ± 1.59	0.5 (0.3, −1.3)	7.60 ± 1.42	7.70 ± 1.49	0.1 (0.8, −1.0)	1.000; 0.000	0.081; 0.300	0.574; 0.036
RPE-O	6.70 ± 1.14	6.72 ± 1.02	0.02 (0.4, −0.5)	6.64 ± 0.89	6.69 ± 1.09	0.05 (0.5, −0.6)	0.871; 0.003	0.868; 0.003	0.897; 0.002
Session affect	−0.10 ± 2.96	−0.30 ± 2.98	−0.2 (0.5, −0.1)	0.00 ± 3.01	−0.50 ± 2.87	−0.50 (2.1, −1.1)	0.935; 0.001	0.322; 0.109	0.703; 0.017
Affect	0.04 ± 2.16	0.51 ± 1.93$^{\#}$	0.47 (0.15, −1.1)	0.41 ± 1.73	0.79 ± 1.76$^{\#}$	0.38 (0.2, −1.0)	0.379; 0.087	**0.017**; 0.487	0.852; 0.004

One-mile time trial performance, gas exchange, and perception metrics were gathered pre and post-low carbohydrate high fat (LCHF) and high carbohydrate low fat treatments. n = 10. Mean ± SD. RPE-O = RPE for overall body; RPE = rating of perceived exertion (OMNI rating of exertion); RER = Respiratory exchange ratio; VO_2 = oxygen consumption; VCO_2 = carbon dioxide production; VE = ventilation; RR = Respiratory Rate; LCHF = low carbohydrate high fat diet; HCLF = high carbohydrate low fat diet.

$^{\#}$ = significant difference from Pre vs. Post ($p \leq 0.05$).

* = significant difference between treatment ($p \leq 0.05$); † = significant difference between LCHF and HCLF at Post ($p \leq 0.05$). Bold values represent the $p < 0.05$.

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TABLE 5 Repeated sprint protocol performance, gas exchange, and perception.

Variable	LCHF			HCLF			*P-value*; η 2p		
	Pre Day −1	Post Day 31	Change Mean (95% CI)	Pre Day −1	Post Day 31	Change Mean (95% CI)	Treatment	Time	Interaction
Total time (sec)	1267.0 ± 90.3	1236.1 ± 69.2	−30.9 (84.9, −23.1)	1267.1 ± 93.3	1254.0 ± 101.2	−13.1 (32.2, −6.0)	0.695; 0.018	0.064; 0.331	0.556; 0.040
Mean carbohydrate oxidation (g/min)	3.13 ± 1.08	1.44 ± 0.84$^{\#\star\dagger}$	−1.69 (2.7, 0.7)	3.66 ± 1.19	3.66 ± 0.52$^{\#\star}$	−0.00 (0.8, −0.8)	**0.004**; 0.611	**0.011**; 0.532	**0.023**; 0.456
Mean fat oxidation (g/min)	0.75 ± 0.36	1.44 ± 0.41$^{\#\star\dagger}$	0.69 (−0.3, −1.1)	0.61 ± 0.31	0.53 ± 0.22$^{\#\star}$	−0.08 (0.4, −0.2)	**0.002**; 0.683	**0.012**; 0.526	**0.007**; 0.577
Heart rate (b.min-1)	160.5 ± 9.2	163.2 ± 13.5	2.64 (2.7, −8.0)	162.0 ± 7.6	159.6 ± 8.4	−2.44 (5.0, −0.2)	0.700; 0.017	0.931; 0.001	0.125; 0.242
Mean VO_2 (ml/kg/min)	45.8 ± 5.75	48.9 ± 3.89	3.09 (−0.3, −5.9)	46.4 ± 8.02	46.1 ± 5.15	−0.31 (3.8, −3.2)	0.457; 0.063	0.259; 0.139	0.070; 0.320
Mean VO_2 (L/min)	3.87 ± 0.41	3.99 ± 0.41	0.12 (0.1, −0.3)	3.86 ± 0.49	3.80 ± 0.50	−0.06 (0.3, −0.2)	0.366; 0.091	0.662; 0.022	0.191; 0.182
Mean VCO_2 (L/min)	3.34 ± 0.39	3.20 ± 0.35	−0.14 (0.5, −0.2)	3.53 ± 0.57	3.50 ± 0.42	−0.03 (0.3, −0.2)	0.083; 0.297	0.388; 0.084	0.504; 0.051
Mean V_E (L/min)	107.0 ± 10.4	106.8 ± 11.3	−0.18 (5.0, −4.7)	106.9 ± 13.0	106.7 ± 10.2	−0.24 (8.7, −8.2)	0.960; 0.000	0.918; 0.001	0.990; 0.000
Mean RR (bpm)	40.8 ± 7.51	39.7 ± 6.88	−1.08 (2.6, −0.5)	40.3 ± 7.82	40.7 ± 7.38	0.41 (2.1, −2.9)	0.796; 0.008	0.627; 0.027	0.272; 0.132
Mean RER	0.86 ± 0.05	0.80 ± 0.04$^{\#\star\dagger}$	−0.06 (0.15, 0.05)	0.91 ± 0.05	0.92 ± 0.04$^{\#\star}$	0.01 (0.0, −0.1)	**0.001**; 0.716	**0.005**; 0.596	**0.010**; 0.536
Session RPE	7.20 ± 1.03	7.10 ± 1.37	−0.10 (0.8, −0.6)	7.10 ± 0.99	7.60 ± 1.26	0.50 (0.0, −1.0)	0.534; 0.044	0.399; 0.080	0.081; 0.300
RPE-O	7.16 ± 0.93	6.87 ± 1.08	−0.29 (0.7, −0.1)	6.91 ± 0.84	7.16 ± 0.75	0.25 (0.3, −0.8)	0.927; 0.001	0.894; 0.002	0.095; 0.279
Session affect	0.50 ± 2.55	0.00 ± 2.75$^{\#}$	−0.50 (1.1, −0.1)	0.30 ± 3.05	−0.10 ± 3.07$^{\#}$	−0.40 (1.1, −0.3)	0.678; 0.020	**0.041**; 0.386	0.823; 0.006
Affect	0.16 ± 2.52	0.18 ± 2.59	0.02 (0.8, −0.9)	0.34 ± 2.45	−0.01 ± 2.57	−0.35 (0.9, −0.2)	0.984; 0.000	0.347; 0.099	0.507; 0.050

Repeated sprint protocol performance, gas exchange, and perception after low carbohydrate high fat (LCHF) and high carbohydrate low fat (HCLF) treatments. LCHF lowered mean carbohydrate and respiratory exchange ratio, and lowered mean fat oxidation but remained unchanged pre to post-HCLF. Both LCHF and HCLF lowered session affect. n = 10. Mean ± SD. Total time = time to complete all sprints; RPE-O = RPE for overall body; RPE = rating of perceived exertion (OMNI rating of exertion); RER = Respiratory exchange ratio; VO_2 = oxygen consumption; VCO_2 = carbon dioxide production; V_E = ventilation; RR = Respiratory Rate; LCHF = low carbohydrate high fat diet; HCLF = high carbohydrate low fat diet.

$^{\#}$ = significant difference from Pre vs. Post ($p \leq 0.05$).

* = significant difference between treatment ($p \leq 0.05$); † = significant difference between LCHF and HCLF at Post ($p \leq 0.05$). Bold values represent the $p < 0.05$.

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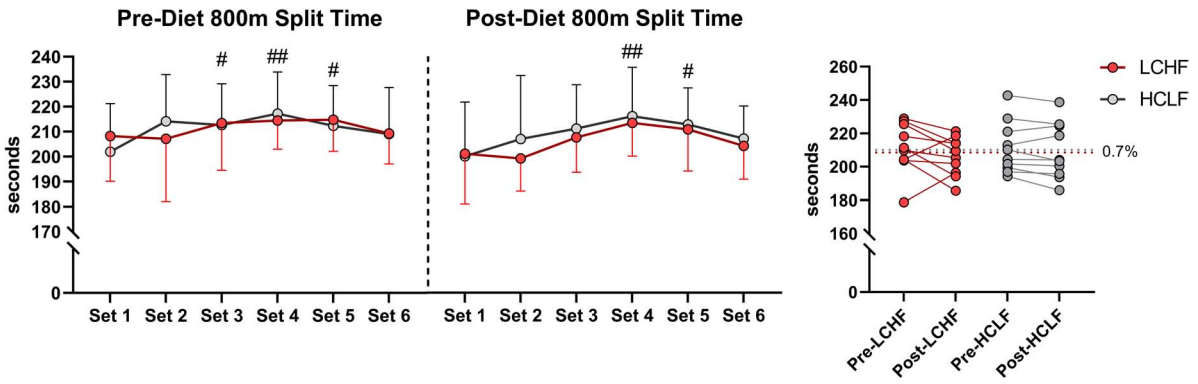

FIGURE 4
Repeated sprint running performance. Diet did not significantly alter running times between or within treatments. There was a main effect of time for running performance reflected in higher running split time at set 3, 4, and 5 during pre-diet phase. Post-diet phase showed significant increase in split time during set 4 and 5 only. n = 10. Mean ± SD. LCHF = low-carbohydrate/high-fat diet; HCLF = high-carbohydrate/low-fat diet. $^{\#,\#\#}$ are $p < 0.05$ and 0.01 significantly different from Set 1, respectively.

and mean carbohydrate oxidation (Δ:−1.69 ± 0.57 g/min; −54%) decreased significantly from pre to post, whereas mean fat oxidation (Δ: 0.69 ± 0.23 g/min; 92%) increased significantly from pre to post (all $p < 0.05$). The significant decrease in respiratory exchange rate indicated decreased carbohydrate reliance (53 to 35%) toward fat utilization (47 to 65%). Additionally, the absolute carbohydrate and fat oxidation rates (g/min) demonstrated that for every 1 g/min decrease in carbohydrate oxidation there was 0.41 g/min increase in fat oxidation. No significant Pre-Post changes were detected within-HCLF (Figure 5 and Supplementary Table 1).

There was a trend for greater mean relative VO_2 consumption ($p = 0.07$) and lower rate-of-perceived exertion, both as overall body RPE-O ($p = 0.095$) and session RPE ($p = 0.08$), when comparing post-LCHF to post-HCLF. This increased relative oxygen consumption after LCHF is largely explained by weight-loss, and partly explained by the non-significant increase in heart rate (i.e., greater cardiac output) and fat oxidation, altogether increasing tissue demand for oxygen and thereby augmenting relative VO_2.

Blood metabolite data

One-mile time trial (TT; 1,609 m)

Prior to dietary intervention, capillary blood *R*-βHB was below the limit of nutritional ketosis pre-diet and pre-TT (mean ± SD: 0.2 ± 0.1 mmol/L) and remained unaltered pre-diet post one-mile TT across groups. Post-LCHF treatment significantly increased *R*-βHB from baseline (0.21 ± 0.11 vs. 0.67 ± 0.30 mmol/L *R*-βHB; $p < 0.001$). *R*-βHB concentrations demonstrated a slight decrease after exercise, however, the effect was non-significant (Δ = −0.14 ± 0.10 mmol/L; $p = 0.23$).

Capillary blood glucose concentrations were similar Pre- and Post-diet between LCHF and HCLF treatments (LCHF average: 87.4 ± 9.6 mg/dL vs. HCLF average: 90.7 ± 10.5 mg/dL; $p = 0.27$) Pre-one-mile TT. There was a main effect of time observed Post one-mile TT that raised blood glucose from baseline (89.1 ± 10.0 to 126.8 ± 20.2 mg/dL; + 42%; $p < 0.001$), independent of diet.

Capillary blood lactate before the one-mile TT was at the same concentration Pre- and Post-diet in both LCHF and HCLF treatments (LCHF average: 1.1 ± 0.5 vs. HCLF average: 1.3 ± 0.7 mmol/L; $p = 0.48$). There was a main effect of time induced by exercise that increased blood lactate significantly from baseline (1.2 ± 0.6 to 8.0 ± 2.4 mg/dL; + 557%; $p < 0.001$), independent of diet (Figure 6 and Supplementary Table 2).

Repeated sprint protocol (RSP; 6 × 800 m)

Capillary blood *R*-βHB was below the limit of nutritional ketosis Pre-diet and Pre-RSP (0.14 ± 0.05 mmol/L). LCHF significantly increased *R*-βHB into nutritional ketosis compared to pre-diet concentrations (0.19 ± 0.12 vs. 0.72 ± 0.73 mmol/L; 279%; $p < 0.001$) whereas HCLF did not meaningfully influence *R*-βHB. Over the course of the RSP, *R*-βHB decreased by approximately 0.05 mmol/L between sets and was significantly lower from Pre- to Post-RSP (Δ = −0.26 ± 0.09 mmol/L; $p = 0.001$), denoting increased ketone oxidation rates after every sprint.

Capillary blood glucose concentrations Pre-diet and Pre-RSP (99.9 ± 6.2 mg/dL) increased significantly by set 5 (106.2 ± 25.7 mg/dL; $p = 0.012$) and Post (107.2 ± 26.4 mg/dL; $p = 0.006$). There was a treatment-dependent effect that revealed lower blood glucose levels during LCHF diet compared to HCLF diet (Δ = −8.5 ± 3.8 mg/dL; 8%; $p = 0.03$).

Capillary lactate concentrations Pre-diet and Pre-RSP (1.2 ± 0.9 mmol/L) increased significantly after set 1 (4.6 ± 2.2 mmol/L; $p = 0.001$) and thereafter. Peak lactate concentrations (6.0 ± 2.8 mmol/L; $p = 0.001$) were recorded at set 6 (i.e., post-set). Diet did not significantly influence the rate of lactate appearance in the blood nor peak lactate (Figure 7 and Supplementary Table 3).

Cardiometabolic indices

There were no significant changes over time in any of the variables of interest (Figure 8 and Supplementary Table 4). Between-condition effects reveal higher total cholesterol (Δ: 20.7 ± 3.3 mg/dL; $p = 0.001$) and LDL-C (Δ: 10.7 ± 4.6 mg/dL; $p = 0.03$) after LCHF. Interaction effects revealed total cholesterol (Δ: 29.5 ± 6.3 mg/dL; $p = 0.007$) and HDL-C (Δ: 11.4 ± 3.3 mg/dL; $p = 0.045$) were significantly greater Post-diet on LCHF treatment.

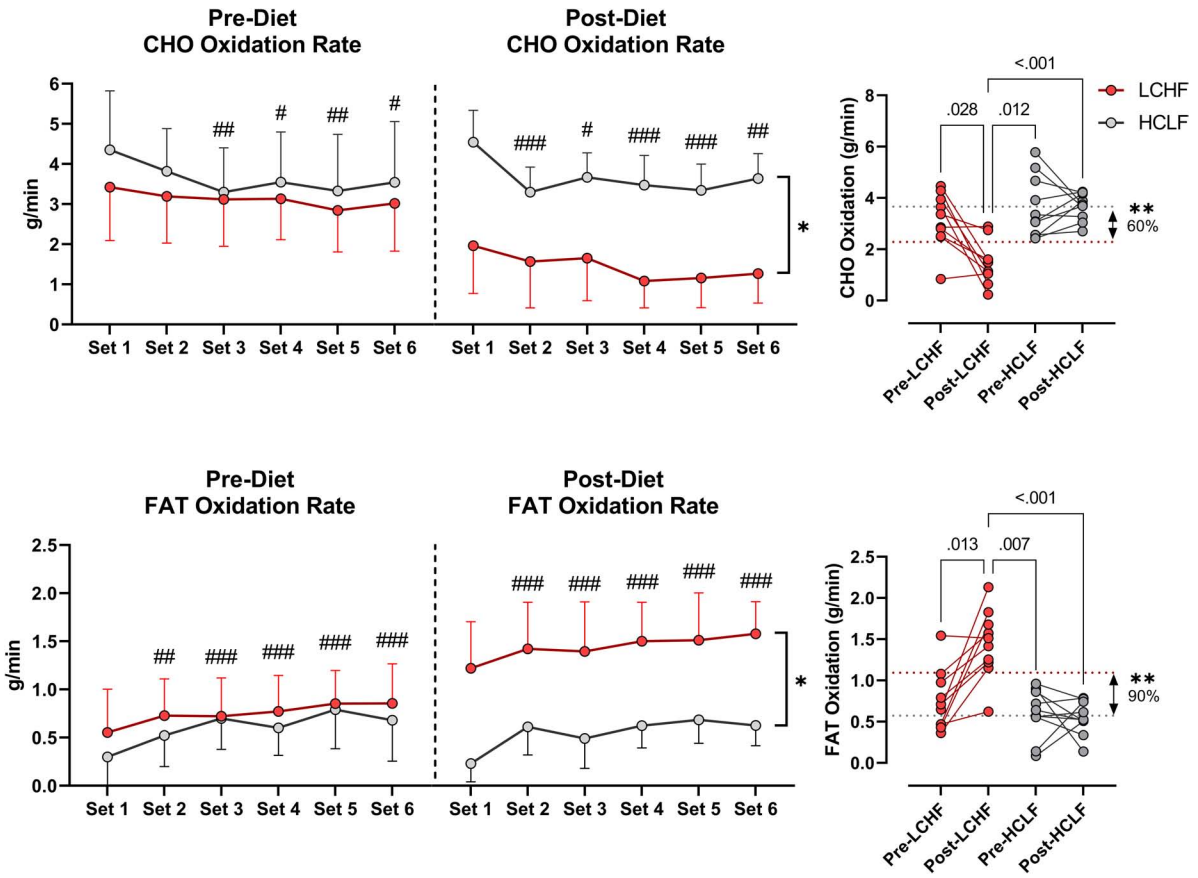

FIGURE 5
Substrate oxidation rates. Carbohydrate (CHO) and lipid (FAT) oxidation rates (g/min) were calculated using intensity-dependent formulas. Pre-diet CHO and FAT oxidation were comparable between the high-carbohydrate/low-fat (HCLF) and low-carbohydrate/high-fat (LCHF) treatment from set 1 and thereafter. There was a main effect of time for CHO and FAT oxidation reflected in lower CHO ($p < 0.05$) and higher FAT ($p < 0.001$) oxidation by set 6, respectively. Post-diet CHO and FAT oxidation were significantly altered by diet. Within-LCHF treatment there was a significant decrease in CHO oxidation (−54%; $p < 0.05$) and a significant increase in FAT oxidation (93%; $p < 0.05$) from Pre to Post-diet. No Pre-Post substrate oxidation changes were detected for HCLF. $n = 10$. Mean ± SD. $^{\#}$, $^{\#\#}$, and $^{\#\#\#}$ are $p < 0.05$, 0.01, and 0.001 significantly different from Set 1, respectively. *, ** are $p < 0.05$, 0.01 significantly different between diets, respectively.

Glycemic control

All glycemic parameters significantly improved on LCHF (Figures 9, 10). Average glucose was significantly lower during LCHF treatment starting day 8, and remained lower on day 13, 15-20, and 22 (Figure 9). 31-day mean and median glucose levels were reduced −15.0% (range: −34.3 to 0.7%; $p = 0.0057$) and −15.2% (range: −33.2% to 0.4%; $p = 0.0058$) on LCHF treatment, respectively. 31-day time in range (70-110 mg/dL) increased 35.4% (range: 1.1 to 122.3%), standard deviation reduced −34.9% (range: −57.5 to 5.7%), and coefficient of variance reduced −25.4% (range: −41.0 to 12.4%) on LCHF. 31-day daily glucose minimum and maximums were also reduced on LCHF. 31-day median glucose was significantly lower throughout fasting (00:00 to 07:00) and feeding (07:01 to 23:59) windows ($p = 0.004$; Figure 10). Interestingly, 30% of subjects had 31-day mean and median glucose (mean glucose range: 111.7-115.2 mg/dL; Figure 9) and fasting glucose > 100 mg/dL (Figure 10) throughout HCLF treatment, which is consistent with prediabetes glycemic values (62). These subjects were also the greatest responders to carbohydrate restriction (range: −21.5 to −34.3% mean glucose). When observing whether there was a significant relationship between the mean glucose while on HCLF versus the percentage change in mean glucose between LCHF and HCLF, we observed a significant ($p = 0.0077$) interaction with a large effect size ($r^2 = 0.6094$) indicating that those individuals with a higher mean glucose, are more responsive to carbohydrate restriction treatment (Figure 9). Importantly, 30% of subjects who had a 31-day average mean, median, and fasting glucose > 100 mg/dL on HCLF (range: 111.68-115.19 mg/dL), were also the largest glycemic responders to carbohydrate restriction and also reported the highest fat oxidation rates during LCHF, with a large inverse relationship ($p = 0.0069$; $r^2 = 0.6194$) across the entire cohort between the percent change in mean glucose when switching to LCHF and the peak fat oxidation rate at 86.4% VO_{2max} indicating that those individuals with the greatest change in glycemic control also had the greatest shift in global metrics of systemic metabolic adaptation to diet. To explore whether peak fat oxidation rates on LCHF were associated with circulating lipids, we found that higher peak fat oxidation ($p = 0.0034$; $r^2 = 0.6775$) predicted higher total cholesterol, with trends for triglycerides ($p = 0.0730$; $r^2 = 0.3474$; X = Peak Fat Oxidation on LCHF; Y = Triglycerides on LCHF; $Y = 29.06^{*}X + 24.62$), suggesting a potential relationship between changes in fat oxidation rates and circulating lipid metabolism. These findings in the 30% of subjects with pre-diabetes in our study could not be explained by

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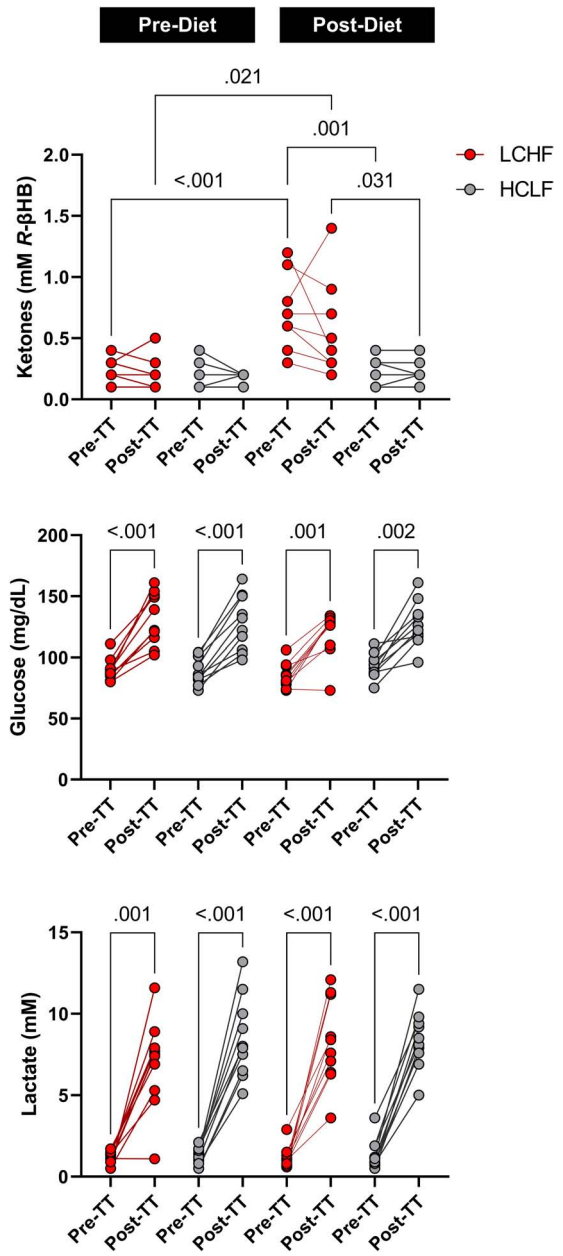

FIGURE 6
One-mile time-trial metabolite impact. Capillary ketones, glucose and lactate were measured immediately pre- and post-one mile time trial (TT) to evaluate the within- and between-diet effects in the context of exercise. There were no significant Pre-diet and Pre-TT differences. A significant post-diet effect was detected in capillary ketones Pre-TT. Glucose and lactate were significantly elevated over time, independent of diet and dependent on TT. n = 10. Mean ± SD.
LCHF = low-carbohydrate/high-fat diet;
HCLF = high-carbohydrate/low-fat diet.

underlying demographic, body composition or running experience as these subjects with glycemic values consistent with pre-diabetes (62) had near equivalent age (pre-diabetic: 41.67 y/o; cohort: 39.3 y/o), running experience (pre-diabetic: 8.67 y; cohort: 9.70 y), lower weight (pre-diabetic: 84.03 kg; cohort: 86.70 kg), BMI (pre-diabetic: 25.37 kg/m^2; cohort: 26.2 kg/m^2), body fat [% (pre-diabetic: 14.8%; cohort: 15.7%) & kg (pre-diabetic: 12.7 kg; cohort: 14.1 kg)], and relative VO_{2max} (pre-diabetic: 60.97 mL/kg/min; cohort: 58.70 mL/kg/min). Additionally, this prediabetic phenotype was present in these subjects despite them losing weight on both nutritional strategies (LCHF: −2.3 ± 1.3 kg; HCLF: −2.1 ± 3.2 kg).

Discussion

There are four key findings of this study (Figure 1). (i) Athletes achieved equivalent exercise performances during a 1,609 m time trial and a 6 × 800 m interval session after a 31-day habituation to LCHF or HCLF diets when controlling calories, training load, and body composition changes across groups. (ii) During the latter stages of the 6 × 800m interval session, athletes achieved the highest rates of fat oxidation yet reported. According to current understanding, this is paradoxical since these high rates were measured in subjects exercising at an intensity (86.4 ± 6.24% VO_{2max}) at which the rate of fat oxidation should be approaching zero (25, 26, 30), not increasing. (iii) 31-days on each diet produced equivalent fasting insulin, hsCRP, and HbA_{1c}, with elevated total, low-density lipoprotein, and high-density cholesterol on LCHF. (iv) LCHF consistently reduced glucose levels and variability with a large inverse relationship observed between mean glucose on HCLF and the percent change in mean glucose when switching to LCHF. Importantly, 30% of subjects who had a 31-day mean, median, and fasting glucose > 100 mg/dL on HCLF were also the largest responders (i.e., glycemic change over diet and peak fat oxidation rates) to carbohydrate restriction. No subjects on LCHF had a 31-day average mean glucose > 100 mg/dL. Additionally, relationships were observed between glycemic change, peak fat oxidation, and circulating lipids, as the larger the reduction in mean glucose on LCHF the larger the peak fat oxidation on LCHF, and the larger the peak fat oxidation on LCHF and the higher circulating lipids were. These results challenge the existing paradigm that diets with higher carbohydrate intake are superior for athletic performance, even during shorter-duration, higher-intensity exercise. Critically, these results demonstrate that lower carbohydrate intake may be a therapeutic strategy, even in athletes, to improve glycemic control, particularly in those with, or at risk for diabetes, without requiring changes in body composition or physical activity. Interestingly, these results also demonstrate a unique association between glycemic responsiveness to carbohydrate restriction, fat oxidation rates, and circulating lipids, suggesting an important relationship between continuous glycemic parameters and systemic metabolic responsiveness.

Unaltered athletic performance during the 1,609 meter time trial and during the interval session of 6 × 800 m

Performance during the 1,609 m time trials was the same when athletes ate HCLF or LCHF diets. This is in keeping with our previous study (31) in which the 5-km time trial performances of athletes, similar in ability to those studied here, were equivalent on either diet. It adds further weight to the conclusions from two recent meta-analyses (63, 64) that that LCHF and HCLF diets produce equivalent performances across a wide range of athletic events.

Further, to search for a performance difference between dietary interventions, we asked the athletes to perform an interval session involving six repetitions of 800m at a pace equivalent to an exercise

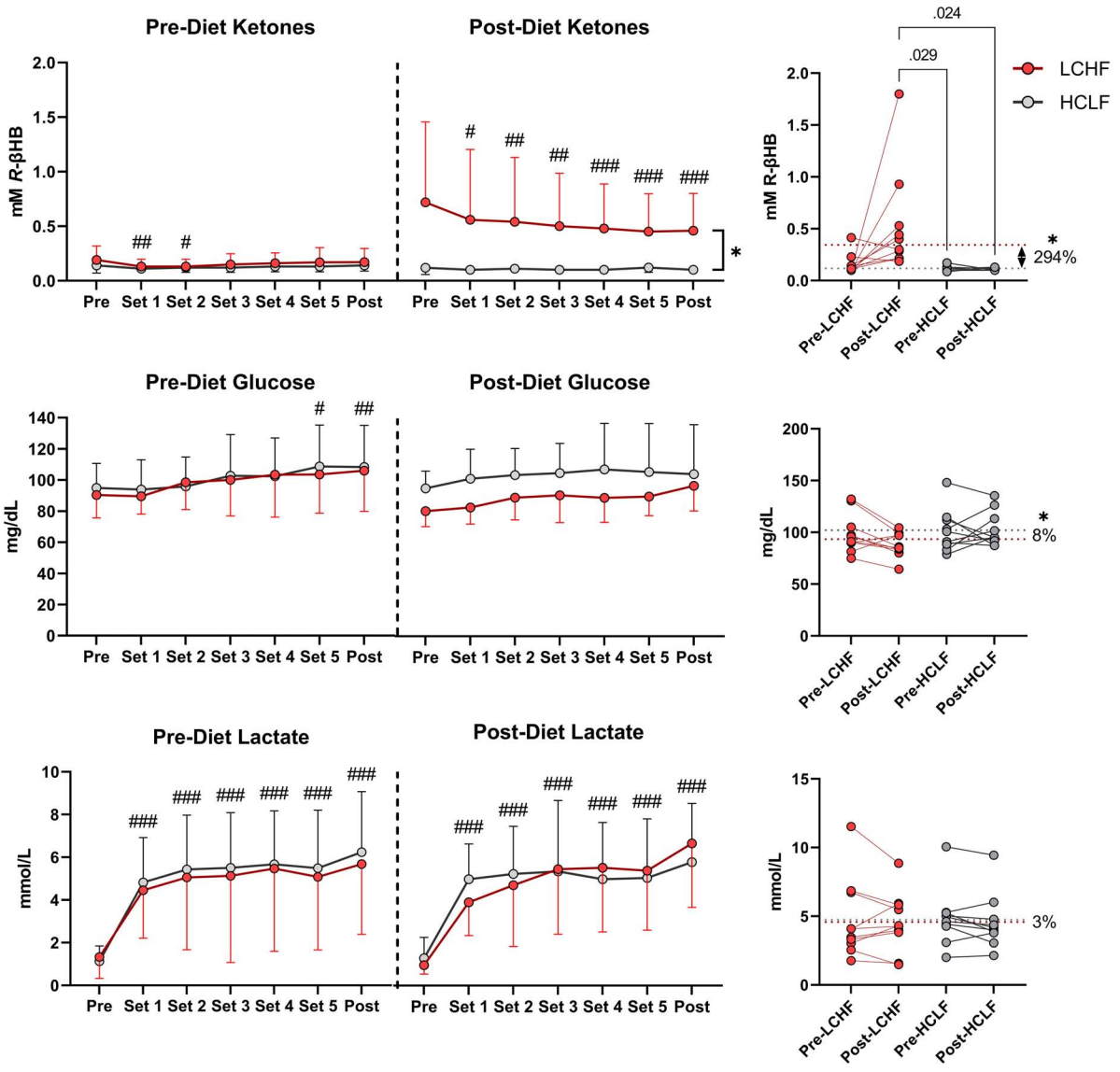

FIGURE 7
Repeated sprint performance metabolite impact. Capillary R-βHB, glucose, and lactate were measured in capillary blood immediately after each repeated sprint performance (RSP) set. There were no significant between-treatment differences pre-diet. The significant time effects were detected in lower R-βHB values at set 1 and 2, higher glucose values at set 5 and post, and significantly higher lactate values from set 1 and thereafter. Post-diet ketones were significantly influenced by low carbohydrate high fat (LCHF) treatment, with 3-fold higher R-βeta-Hydroxybutyrate (R-βHB) concentrations throughout the sets compared to HCLF. *R*-βHB decreased between each set by a total of 46% from pre-post ($p < 0.001$). Glucose was overall 8% lower during LCHF compared to HCLF ($p = 0.03$). Lactate was not affected significantly by diet. $n = 10$. Mean ± SD. $^{\#}$, $^{\#\#}$, and $^{\#\#\#}$ are $p < 0.05$, 0.01, and 0.001 significantly different from the Pre timepoint. * is $p < 0.05$ between diet overall effect.

intensity of ∼85% VO_{2max}. Our reasoning was that if the pre-exercise muscle glycogen stores are a critical determinant of exercise performance and if the LCHF diet is associated with lower muscle glycogen concentrations in recreational athletes (28) (but perhaps not in highly competitive athletes (65)), and since very high rates of muscle glycogen use are measured during 800m repetitions (66) so that, if significant muscle glycogen depletion can be produced by a high intensity interval session, then any impaired performance of athletes eating the LCHF diet should become apparent in the latter intervals of that session.

For example, Impey et al. reported rates of muscle glycogen use of 12.7 and 7.0 mmol/min in the gastrocnemius and vastus lateralis muscles respectively of recreational male athletes performing 800m repetitions at 100% VO_{2max} (66). Webster et al. reported that pre-exercise vastus lateralis glycogen concentrations were 85 mmol/kg in well-trained recreational athletes eating the LCHF diet (28). Whilst appreciating that rates of muscle glycogen use are reduced in those eating the LCHF diet (28, 65), according to these data a starting muscle glycogen concentration of 85 mmol/kg in the vastus lateralis muscle would be depleted after just 12 minutes of high intensity exercise. Our athletes exercised for ∼21 minutes during the 6 × 800m repetitions; sufficient time to produce substantial glycogen depletion.

In contrast to our expectation, based on this prediction that significant muscle glycogen depletion would occur in athletes following the LCHF diet and this would impair their performance,

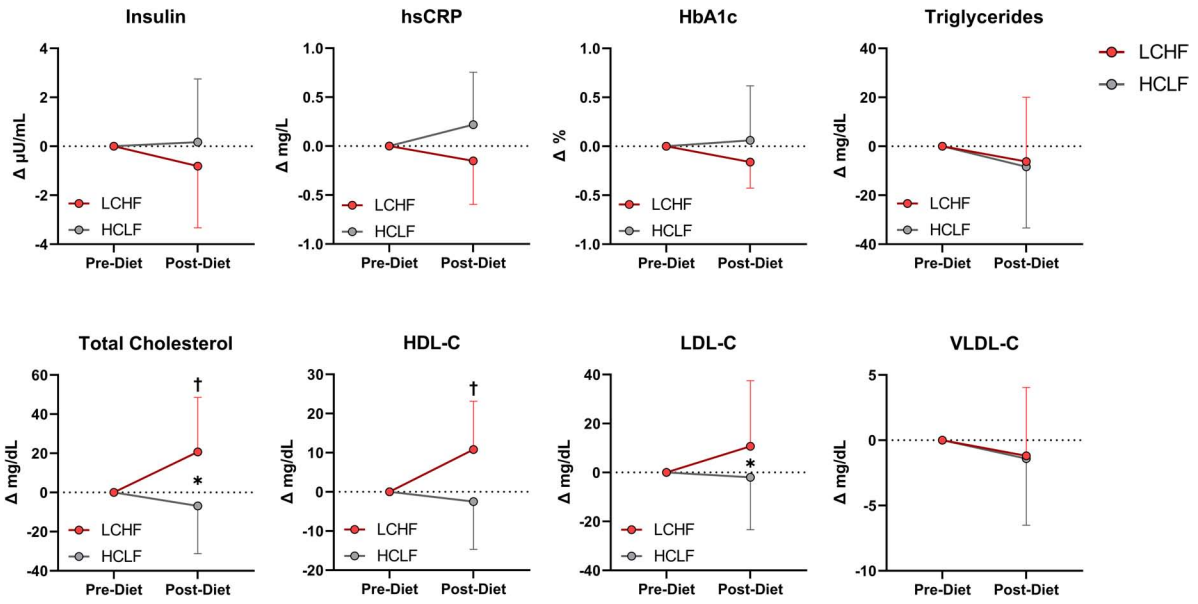

FIGURE 8
Cardiometabolic scores. Statistics were conducted on absolute values and are presented as mean change from Pre-diet. There were no cardiometabolic differences Pre-diet or significant time effects. Between-diet effects revealed greater total cholesterol and LDL-C concentrations during the LCHF versus HCLF treatment. The significant interaction revealed greater total cholesterol and HDL-C concentrations Post-LCHF treatment. n = 10. Mean ± SD. * = $p < 0.05$ between-diet main effect. † = significant interaction Post-diet ($p < 0.05$).

in fact exercise performance was identical across all the intervals on either diet (**Tables 4**, **5**; **Figure 4** and **Supplementary Table 1**).

These finding raise the important question of why our two studies have failed to detect diet-induced differences in performance whereas prior meticulously conducted studies (16–18) detected meaningful differences in their studies of Olympic standard race walkers. Five key factors may have contributed to these differences: randomization, dietary controls during exercise, training load, body composition, and dietary habituation timeline. Prior studies with differing results allowed subjects to choose the diet they preferred (16–18). In addition, during the final performance trial involving simulated races of up to 25-km, subjects on the LCHF diet were provided with carbohydrate-free "non-caloric fluid (water or artificially sweetened drinks)" (16) and "LCHF cookies" (17) whereas subjects on the HCLF diet received "sports drink, sports gels and confectionary" providing "∼60g CHO" every hour (16, 17). As a result, blood glucose levels were lower in the LCHF group in the two trials (16, 17) in which it was measured, with a trend toward a progressive hypoglycemia in one trial [figure 5A from (17)]. As the authors of those studies appreciate, even in the absence of hypoglycemia, carbohydrate ingestion alone can have an ergogenic effect even if the carbohydrate is not ingested (67). Thus, these trials did not control for the potential effects of carbohydrate ingestion during exercise. The potential role of hypoglycemia in explaining differences in exercise performance has recently been revisited (29). The intensified training load and across group differences in body composition in these trials (16–18) also illustrates key differences as increased physical activity levels (68) and body weight reductions (69) both illustrate biological stressors requiring adaptation and may independently impact performance. The increased physical activity across groups and more significant reductions in bodyweight in LCHF arm (16–18), on top of introducing a diet which requires systemic metabolic reprogramming (70), illustrate three co-administered biological stressors all requiring adaptions and which may influence performance. Lastly, our analyses allowed for a 4-week adaptation timeline, which is ≥ 33% longer than prior trials describing negative performance impacts (16–18). Thus, it is not surprising that when we controlled randomization (within-subject), dietary controls during performance testing, calories, training load, and body compositional changes across groups to allow for the isolation of diet-induced changes across these key parameters, we observed different results from prior observations (16–18). Of note, when Burke et al. (16) attempted to repeat their findings (18), they did not detect differences between LCHF and HCLF in real world race performance via IAAF points [figure 5C from; (16)], a point acknowledged by the authors in the abstract.

Subjects achieved amongst the highest rates of fat oxidation yet measured during the latter stages of the interval session when eating the LCHF diet

The described method for measuring maximal rates of fat oxidation during exercise is to have subjects exercise for short periods of approximately 3 minutes at exercise intensities that gradually increase (22–24). Using this method, the highest rates of fat oxidation are generally achieved at submaximal exercise intensities of between 55-72% VO_{2max}. Maximal rates of fat oxidation measured with this method are usually in the range of 0.5-0.6 g/min. Importantly, at higher exercise intensities rates of fat oxidation fall precipitously, reported reaching 0% at exercise intensities > 85% VO_{2max}.

Higher rates of fat oxidation have been measured in athletes adapting to the LCHF diet. Volek et al. measured rates of 1.2 g/min in elite ultra-marathon runners performing prolonged exercise (180 minutes) at 64% VO_{2max} with peak fat oxidation of 1.54 g/min

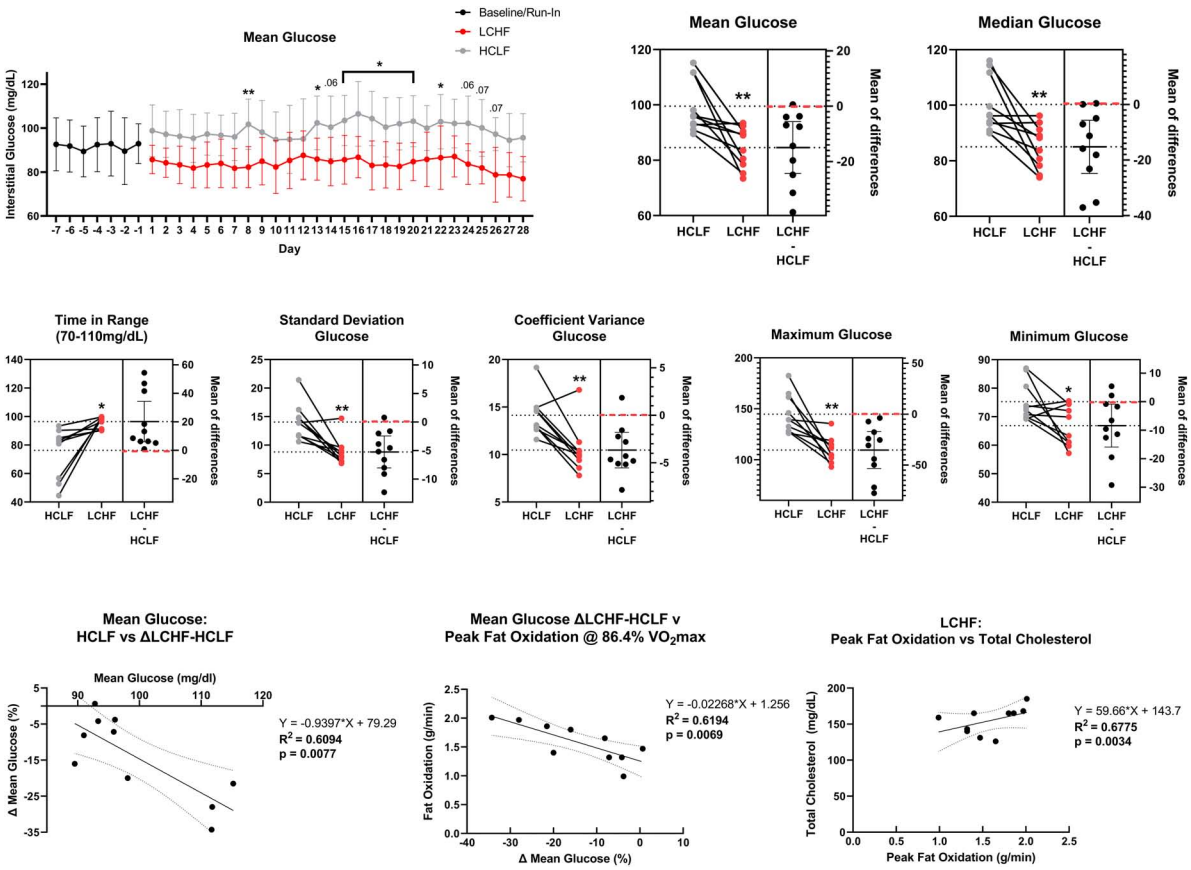

FIGURE 9
Continuous glucose monitoring. Interstitial glucose values were continuously gathered every 15 min over the duration of the sensor life prior to treatment (baseline/run-in), and during both low carbohydrate high fat (LCHF) and high carbohydrate low fat (HCLF) diets. Average glucose was significantly lower on LCHF on day 8, 13, 15-20, and 22. All glycemic parameters over the 31-days dietary intervention were significantly improved during LCHF. Across all variables, only 0-2/10 subjects favored HCLF. The 31-day mean glucose predicted the percent change in mean glucose between LCHF and HCLF diets. 30% of subjects had mean and median glucose > 100 mg/dL throughout HCLF treatment. These subjects were the largest responders to LCHF with no subject > 100 mg/dL during LCHF treatment. These same subjects also reported the highest peak fat oxidation rate as the percent change in mean glucose between LCHF and HCLF diets predicted the peak oxidation rates across the entire cohort. Peak fat oxidation rates on LCHF were also associated with higher cholesterol demonstrating a potential interaction between oxidation rates and global lipid metabolism. n = 10. Mean ± SD. *, ** and $p < 0.05$, $p < 0.01$ are significant difference between LCHF and HCLF at same time point.

at 70.3% VO_{2max} (65). Webster et al. measured identical values in well-trained recreational athletes during 120 minutes' exercise at 55% of peak power output (28); whereas Burke et al. measured rates in excess of 1.4-1.5 g/min during the final km of 25-km time trials in Olympic class race walkers (16, 17). In a case study of an elite Ironman triathlete, Webster et al. reported a peak fat oxidation rate of 1.6 g/min in a single cyclist for the full duration of a 100-km cycle trial performed at an average power output of 224 W (28, 71). Shaw et al. demonstrate fat oxidation ranging from 0.88 to 1.51 g/min following 31-day of LCHF habituation (72). Our data is in line with these prior studies showing elevated fat oxidation rates (LCHF: 1.58 ± 0.33 g/min).

Thus, the values for fat oxidation rates measured in these recreational athletes are unusually high, particularly when they were measured under experimental conditions expected to produce values close to 0g/min. However, what is particularly unique in our findings is that we observed the peak fat oxidation rates (LCHF: 1.58 ± 0.33 g/min) at 86.40 ± 6.24% VO_{2max}. Additionally, 30% of subjects reported record high fat oxidation rates > 1.85 g/min (1.95 ± 0.08 g/min), ranging from 1.86 to 2.01 g/min, which to our knowledge is the highest rates ever recorded. These unique findings may be a result of subject age and athletic status and/or due to the unique controls incorporated into this study to isolate diet-induced effects (i.e., randomization, calories, training load, and body composition changes controlled across groups).

Cardiometabolic impact of LCHF and HCLF

Three out of eight cardiometabolic markers were significantly modulated by diet, most notably post-diet LCHF vs. HCLF total cholesterol (238 vs. 208 mg/dL, 14%; p = 0.007) and HDL-C (68 vs. 57 mg/dL, 20%; p = 0.045) concentrations. Although the overall LDL-C condition effect was significant (p = 0.03), the LCHF diet did not increase LDL-C concentrations beyond HCLF after four-weeks (155 vs. 137 mg/dL; 18% p = 0.30), implying that post-diet hypercholesterolemia effects were predominantly determined by changes in HDL-C fraction. We anticipated based on our prior work (73) and others (65, 74, 75) that competitive runners would experience significant blood lipid changes within weeks after starting a LCHF designed to induce nutritional ketosis (≥ 0.5 mM R-βHB).

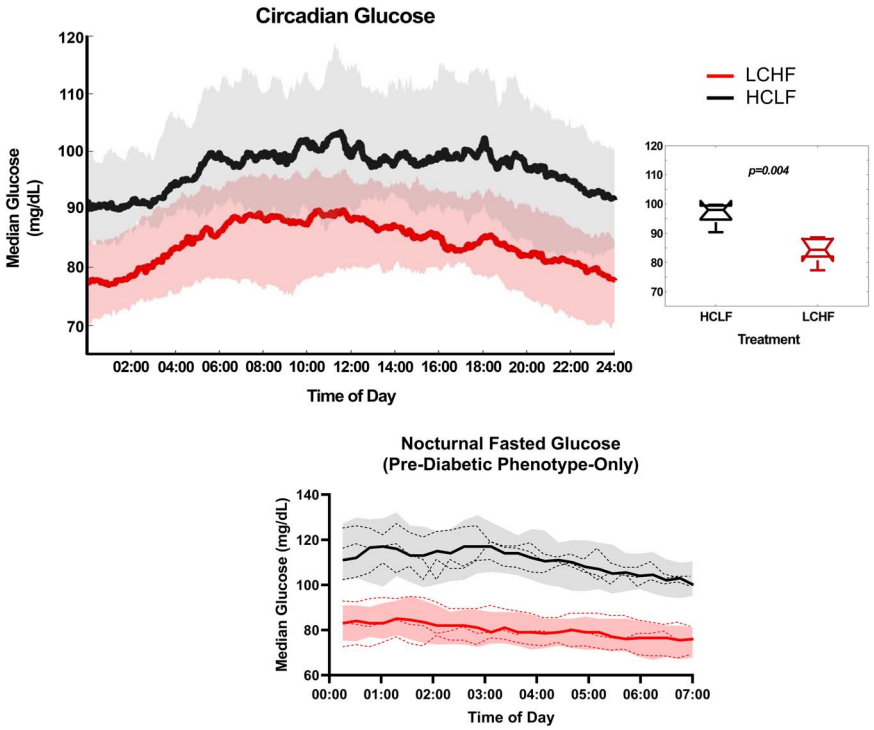

FIGURE 10
Circadian glucose patterns. Interstitial glucose values were continuously gathered every 15 minutes over the duration of the sensor life prior to treatment (baseline/run-in), and during both low carbohydrate high fat (LCHF) and high carbohydrate low fat (HCLF) diets. 31-day glucose values were plotted over 24-h time of day (circadian glucose patterns). Median glucose was significantly lower across the entire day during LCHF (p = 0.004). 31-day nocturnal fasting glucose (00:00 to 07:00) for the 30% of subjects with pre-diabetic phenotype (mean and median glucose > 100 mg/dL), all had fasting glucose values > 100 mg/dL. These subjects with pre-diabetic phenotype all reduced their fasting glucose < 100 mg/dL on LCHF. n = 10. Median ± 25 to 75% Percentiles. Red, LCHF. Black, HCLF. Dashed line, individual pre-diabetic subject circadian glucose patterns.

The significant main effects in this study were directly attributable to the between-diet differences in dietary fat and fat composition, however, the fact that more than half of participants had borderline elevated total cholesterol, LDL-C, and HDL-C at baseline was somewhat unexpected. While carryover effects were ruled out by identical concentrations at baseline (i.e., both conditions started the same), this effect may have indirectly revealed that a peak or plateau had not been reached over our four-week intervention, making blood lipid trends harder to speculate. Moreover, it is unclear if similar dietary interventions in mildly hypercholesterolemic athletes will exert any significant impact on cardiometabolic indices that are beyond the effects induced by their habitual diet. Individual cardiometabolic responses are available for review in the supplement (**Supplementary Figure 1**).

We detected a small, but significant change in weight over time, primarily derived from fat mass. Despite energy intake and training load being similar between LCHF and HCLF diets, weight-loss and diet did not modulate triglycerides, insulin/HbA_{1c}, and inflammation (hsCRP). Based on prior evidence (73, 76) the LCHF diet was projected to lower cardiometabolic markers beyond a HCLF diet, even in the absence of weight-loss (77); however, we did not observe these results with our between-diet isocaloric feeding design. Additionally, it is important to acknowledge that HbA_{1c} is a 2–3-month biomarker that we quantified to predict directional trends rather than significant changes over four weeks. Based on our findings, we expect that these markers will continue decreasing after four-weeks of LCHF, similar to isocaloric HCLF feeding when duration, energy intake, and weight are controlled between conditions.

Improved glycemic control when eating the LCHF diet

Continuous glucose monitors are unique tools which allow researchers to extract changes in glycemic control every ≤ 15 min over extended periods of time without relying on the limitations at a single timepoint which may only provide limited biological feedback (78, 79). Importantly, (i) CGM tracks long-term with HbA_{1c} (54–56), (ii) shorter term CGM readings (10-14d) are good estimates of 3-month CGM averages (57), and (iii) can also capture both fasting and post-prandial differences in glucose (validated diagnostic tool) demonstrating a powerful monitoring tool for glycemic control, particularly in interventions not long enough to confirm alterations in HbA_{1c} (< 2-3 months in length). The value of these tools has been shown in previous observations utilizing lower carbohydrate (80) or ketogenic interventions (81) where continuous glucose patterns found key shifts in glycemic control before and/or in the absence of changes in traditional biochemical cardiometabolic biomarkers. When measuring continuous glucose levels every 15 min over a 31-day period, we observed improvements across all glycemic parameters in virtually all subjects on the LCHF diet, with initial significant differences in mean glucose observed on day 8 of dietary habituation. Carbohydrate restriction is a known therapeutic strategy

to help facilitate improvements in glycemic control and other key metabolic parameters in other clinical conditions such as obesity (82), type-1 diabetes (36), and type-2 diabetes (33, 35). The improved glycemic mean, median, and variability on the LCHF diet in middle-aged athletes was observed in a rigorous, randomized cross-over study design without the confounding influence of caloric, training load/physical activity, and body composition differences across diets. These illustrate critical controls allowing us to extract diet-induced impact on glycemic parameters as prior observations have shown that caloric intake and changes in body weight both influence glucose levels regardless of diet (83, 84). Additionally, prior evaluations have found that intensified training programs can disrupt not only glycaemia and mitochondrial function, but also performance (68).

While there have been small short-term investigations exploring glycemic control during exercise in athletes (79, 85), very few studies have investigated the relationship between the long-term (i.e., $\geq$ 1 month) 24-h glycemic control while also observing performance. Nolan et al. reported the impact of a ketogenic diet on an individual type-1 diabetic cyclist during a 20-day, 4011-km race (86). This case report demonstrated remarkable glycemic control for a Type-1 Diabetic compared to historical glycemic norms for Type-1 Diabetics during this 20d race window, but nature of the report did not allow for the comparison of performance on- and off-diet.

While all subjects were "healthy," with normal bodyweight and BMI, and competitive middle-aged athletes without any medical diagnoses, we observed that when continuously monitoring glucose parameters over a 31-day period, 30% of subjects on a HCLF diet had mean, median, and fasting BG > 100 mg/dL, consistent with pre-diabetes interstitial glucose values using analogous technology (62). This is consistent with a prior analysis which found that 30% of sub-elite endurance athletes exercising > 6-hour per week had undetected pre-diabetes when measured via continuous glucose monitoring devices (87). These subjects fitting the pre-diabetes glycemic phenotype in our study could not be explained by underlying demographics, body composition or physical activity differences as these pre-diabetic subjects had near equivalent age (pre-diabetic: 41.67 y/o; cohort: 39.3 y/o), running experience (pre-diabetic: 8.67 y; cohort: 9.70 y), lower weight (pre-diabetic: 84.03 kg; cohort: 86.70 kg), BMI (pre-diabetic: 25.37 kg/m2; cohort: 26.2 kg/m^2) and body fat [% (pre-diabetic: 14.8%; cohort: 15.7%) & kg (pre-diabetic: 12.7 kg; cohort: 14.1 kg)], and higher VO_{2max} (pre-diabetic: 60.97 mL/kg/min; cohort: 58.70 mL/kg/min) when compared to the entire cohort. This is in line with the understanding that multiple factors contribute to diabetes onset (88, 89), some of which may go undetected until overt diagnosis. Potential explanations for early pathogenic progression of diabetic dysglycemia include genetic predisposition, adiposity-induced insulin resistance, fasting insulin, and beta-cell dysfunction (88). However, markers of elevated adiposity were not higher in the prediabetic group. In fact, this sub-cohort lost weight on both dietary protocols. Additionally, circulating lipids tended to be lower on the HCLF diet suggesting lipids could not explain dysglycemia on HCLF. Fasting insulin was not different across diet groups, nor were baseline or post-HCLF diet fasting insulin levels associated with elevated mean glucose on HCLF (p = 0.4048). However, this does not exclude the possibility that dynamic changes in post-prandial insulin or beta-cell function couldn't be contributing to this effect which we did not measure herein. While intense exercise overtraining has also been demonstrated to acutely disrupt mitochondrial and glycemic function (68), this dysfunction was reversed following reduction in activity and cannot explain our results as our subjects did not increase or decrease physical activity levels. While genetic predisposition may explain why 30% of healthy, active and normal weight individuals had pre-diabetes on HCLF, it cannot explain across treatment effects as the crossover design controlled for this variable. Our results demonstrate that all these subjects were able to reduce their mean and median glucose < 100 mg/dL on LCHF diet. While prior observations have demonstrated the ability to improve glycemic control in individuals with established pre-diabetes and obesity in the absence of exercise consumed a low-carbohydrate diet (< 100 g carbohydrates/day) (62), to our knowledge, this is the first observation to demonstrate the ability to detect and resolve pre-diabetes without changes in activity or changes across groups in body composition using carbohydrate restriction in competitive middle aged athletes. Importantly, Al-Ozairi et al. found that a 6-day LCHF diet in Type-2 Diabetic subjects who kept calories and bodyweight controlled were unable to find differences in mean and post-prandial glycaemia utilizing CGM devices (90). This could be due to the short treatment duration as we observed significant differences on day 8 of the isocaloric HCLF and LCHF diets. Alternatively, it may be explained by the influence of engaging in physical exercise regularly as Moholdt et al. found a 5% reduction in mean glucose levels using CGM without changes in bodyweight following a 5-day lower carbohydrate diet (i.e., 15% kcal carbohydrates) and exercise in obese subjects who were of similar age to our cohort (80). Our larger reduction in mean (15%) and median glucose (15.2%) compared to Moholdt's (5%) is likely explained by Moholdt's higher percentage of carbohydrate (15%), shorter duration of diet (5-days), and different metabolic phenotype in their cohort (i.e., sedentary; BMI > 30 kg/m^2). Our observation of consistent improvements in glycaemia in middle-aged athletes (30% pre-diabetic glycemic phenotype), without impairing performance, illustrates a promising therapeutic strategy for improving glycemic control, without requiring body composition and physical activity change.

Higher mean glucose levels predict glycemic response to carbohydrate restriction, glycemic response to carbohydrate restriction predicts peak fat oxidation, and peak fat oxidation predicts circulating lipids

Our study found that 30% of subjects who had a 31-day average mean and median glucose > 100 mg/dL on HCLF (range: 111.68 – 115.19 mg/dL; pre-diabetic phenotype), were also the largest glycemic responders to carbohydrate restriction. Importantly, when looking to observe if the entire cohort also observed a relationship between 31-day average mean glucose on HCLF diet and percentage change in mean glucose between LCHF and HCLF diet, we observed a large significant inverse relationship, indicating that those individuals with a higher mean glucose, are more responsive to carbohydrate restriction treatment, not just those with pre-diabetic glycemic phenotypes. As our study and prior literature suggests this change is in response to diet and not other factors (i.e., calories, body composition, altered activity levels, cardiometabolic factors), thus indicating that as individuals develop increasing levels of mean BG, they may become more responsive to therapeutic carbohydrate

restriction. Interestingly, the 30% of subjects who had a 31-day average mean and median glucose > 100 mg/dL on HCLF (range: 111.68 – 115.19 mg/dL; pre-diabetic phenotype), also reported the highest fat oxidation rates during LCHF, with a large inverse relationship ($r^2 = 0.6194$; $p = 0.0069$) across the entire cohort between the percent change in mean glucose when switching to LCHF and the peak fat oxidation rate at 86.4% VO_2max indicating that those individuals with the greatest change in glycemic control also had the greatest shift in global metrics of systemic metabolic adaptation to diet. While multiple studies have shown reductions in glucose (35, 80, 83) and elevations in fat oxidation (16–18, 28, 31, 91) on a LCHF diet, we are unaware of any data which has demonstrated that the magnitude of glycemic changes across diet predicted the magnitude of peak fat oxidation rates. Interestingly, we also observed that higher peak fat oxidation levels on LCHF predicted higher total cholesterol on LCHF suggesting a potential interaction between higher rates of fat turnover and higher levels of circulating lipids while on a diet that restricts carbohydrates and increases fat intake. While elevated fat oxidation rates have been observed on LCHF diet in the absence in changes of insulin or calories, explained by elevated fat intake, (91), they did not see a change in glucose levels nor did they explore whether the magnitude of fat oxidation rate was associated with glucose or lipid parameters. In line with our data, there has been a report demonstrating that individuals with healthy bodyweight undergoing a LCHF diet can have elevated circulating lipid (i.e., LDL-C) (92). While this prior observation did not look at either total cholesterol or fat oxidation rates, in light of our data, there remains a possibility that these individuals (92), have elevated levels of systemic fat oxidation which requires further analyses. However, in disagreement with these findings we did not observe a relationship between fat oxidation and LDL, a relationship between baseline Trig/HDL predict LDL-C, nor any LDL-C levels "hyper-responders" (LDL-C: > 200 mg/dL) which indicates our findings may not translate to this unique cohort (92). The ability for (i) 31-d mean glucose on HCLF to predict changes in mean glucose following carbohydrate restriction, (ii) changes in mean glucose with carbohydrate restriction to predict peak oxidation rates, and (iii) peak fat oxidation to predict total cholesterol suggests a unique predictable physiologic relationship between glycemia, substrate oxidation, and circulating lipids biomarkers which requires further validation.

Limitations

This study had middle-aged competitive male athletes which may limit our understanding of the translatability of these findings to female athletes due to potential differences across sex on the magnitude of metabolic response (93–95), particularly for those women in middle age during pre-menopause and post-menopause who may benefit most due to elevated risk for cardiovascular and metabolic disease (96, 97). While our short-duration high-intensity exercise (6 × 800 m) would be sufficient to reduce muscle glycogen content based on prior work, (28, 65, 66) we did not measure muscle glycogen content so we cannot say for certain what levels of muscle glycogen were achieved and if they were associate with elevated fat oxidation levels during exercise. While HbA_{1c} is gold-standard for diagnosing diabetic phenotype due to its established role in diabetes, our dietary intervention was 4 weeks in length, an insufficient time to observe the full diet-induced impact on HbA_{1c} which requires a minimum of 8-12 weeks (98, 99). We utilized CGM to capture the 4-week 24-h glycemic control as (i) CGM tracks long-term to HbA_{1c} (54–56), (ii) shorter term CGM readings (10-14d) are good estimates of 3-month CGM averages (57), and (iii) can also capture both fasting and post-prandial differences in glucose which is a validated diagnostic tool (**Figures 9**, **10**). Although limitation have been cited when looking different CGM technology and different insertion sites, both technology and insertion site were controlled in our analyses (100). However, it is important to note the clear limitation of HbA_{1c} and oral glucose tolerance test (OGTT) in our present analyses and why CGM was the primary glycemic metric. It is well-established that for a given HbA_{1c} value, there is a wide-range of mean glucose concentrations, and for any given mean glucose concentration, there is a wide-range of HbA_{1c} values, suggesting some limitation around this biomarker (101). Thus, some expert consensus has argued for moving beyond just HbA_{1c} at the individual levels (102). Additionally, it has been known for decades that OGTT is inappropriate for individuals not adhering to an HCLF diet as this test was only validated under high-carbohydrate consumption (103). While we feel confident that our 24-h 4-week glycemic values across subjects accurately capture the glycemic impact over our study duration, future studies with benefit from longer dietary interventions 2-3 m in duration to capture changes in HbA_{1c}.

Conclusion

We demonstrated that a habituating to a LCHF for ≥ 4weeks in 30 to 50-years old competitive male athletes resulted in equivalent short duration, high-intensity performance without differences in calories, training load, and body composition across groups. We observed record high peak oxidation rates with elevations in cholesterol in LCHF. Interestingly, we found a 30% incidence of pre-diabetic glycemic phenotype in seemingly healthy athletes consuming a high carbohydrate diet without detectable risk factors for pre-diabetes. All individuals experienced reductions in 31-day average glucose means, median, and variability with carbohydrate restriction (LCHF) which resolved the pre-diabetic phenotype across all subjects without requiring caloric restriction, increased physical activity, or significant changes in body composition across groups. Interestingly, the average glucose during high carbohydrate consumption predicted the degree of glycemic response to carbohydrate restriction suggesting that individuals with higher starting glucose may benefit most from carbohydrate restriction. Surprisingly, we also found that the magnitude of glucose reduction during carbohydrate restriction predicted the elevation in fat oxidation rates during exercise suggesting that glucose response is linked to systemic fat oxidation. Taken together, LCHF may represent a therapeutic strategy to improve glucose levels, particularly in those at risk for diabetes, without compromising high intensity exercise performance in middle-aged athletes. Future studies should evaluate the impact of these dietary strategies in middle-aged women who are at elevated risk for cardiovascular and metabolic disease.

Data availability statement

The original contributions presented in this study are included in this article/**Supplementary material**, further inquiries can be directed to the corresponding authors.

Ethics statement

The studies involving human participants were reviewed and approved by Institutional Review Board of Grove City College (IRB number 110-2021). The patients/participants provided their written informed consent to participate in this study.

Author contributions

PP and TN conceived the original study design. PP, JB, DJ, NT, HG, AJ, KJ, and DD'A conducted the participant testing and collected all the data. KH designed the diets and provided the nutritional counseling. PP, AB, and AK conducted the data analysis. PP, TN, AK, and AB drafted the final manuscript. All authors have read and agreed to the published version of the manuscript.

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Conflict of interest

TN and JV were authors of low-carbohydrate nutrition books. TN book royalties go to The Noakes Foundation which contributes to the Eat Better South Africa Campaign. JV receives royalties from book sale; is a founder, and has equity in, Virta Health; and is a science advisor for Simply Good Foods and Cook Keto. DD'A is an inventor of patents on the use of exogenous ketones, advisor for Levels Health, Readout Health, and co-owner of Ketone Technologies LLC, which does consulting and public speaking events. AK was a patent inventor and has consulted for Simply Good Foods.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2023.1084021/full#supplementary-material

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Oral ketone esters acutely improve myocardial contractility in post-hospitalized COVID-19 patients: A randomized placebo-controlled double-blind crossover study

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Background: COVID-19 is associated with subclinical myocardial injury. Exogenous ketone esters acutely improve left myocardial function in healthy participants and patients with heart failure, but the effects have not been investigated in participants previously hospitalized for COVID-19.

Methods: This is a randomized placebo-controlled double-blind crossover study comparing a single oral ketone ester dose of 395 mg/kg with placebo. Fasting participants were randomized to either placebo in the morning and oral ketone ester in the afternoon or vice versa. Echocardiography was performed immediately after intake of the corresponding treatment. Primary outcome was left ventricular ejection fraction (LVEF). Secondary outcomes were absolute global longitudinal strain (GLS), cardiac output and blood oxygen saturation. Linear mixed effects models were used to assess differences.

Results: We included 12 participants previously hospitalized for COVID-19 with a mean (±SD) age of 60 ± 10 years. The mean time from hospitalization was 18 ± 5 months. Oral ketone esters did not increase LVEF between placebo and oral ketone ester [mean difference: −0.7% (95% CI −4.0 to 2.6%), $p = 0.66$], but increased GLS [1.9% (95% CI: 0.1 to 3.6%), $p = 0.04$] and cardiac output [1.2 L/min (95% CI: −0.1 to 2.4 L/min), $p = 0.07$], although non-significant. The differences in GLS remained significant after adjustment for change in heart rate ($p = 0.01$). There was no difference in blood oxygen saturation. Oral ketone esters increased blood ketones over time (peak level 3.1 ± 4.9 mmol/L, $p < 0.01$). Ketone esters increased blood insulin, c-peptide, and creatinine, and decreased glucose and FFA (all $p \leq 0.01$) but did not affect glucagon, pro-BNP, or troponin I levels (all $p > 0.05$).

Conclusion: In patients previously hospitalized with COVID-19, a single oral dose of ketone ester had no effect on LVEF, cardiac output or blood oxygen saturation, but increased GLS acutely.

Clinical trial registration: https://clinicaltrials.gov/, identifier NCT04377035.

KEYWORDS

ketone bodies, post COVID-19, myocardial metabolism, myocardial contractility, subclinical myocardial injury

Introduction

COVID-19 is associated with myocardial injury (1–3) and has been observed in hospitalized COVID-19 patients as impaired left ventricular- and right ventricular systolic function assessed by echocardiography (4–7), and even in patients that did not require hospitalization assessed by magnetic resonance imaging (8). The degree of impairment in left ventricular function, quantified by absolute global longitudinal strain (GLS), is associated with higher mortality (5, 9, 10) also in the presence of normal left ventricular ejection fraction (LVEF) (11), the recommended parameter for measuring left ventricular systolic function (12). In recovered post-hospitalized COVID-19 patients, GLS, and RVLS are still impaired (13, 14) and an increased risk of cardiovascular disease persists 1 year after infection (15). This indicates a need for improving ventricular function, especially left ventricular function, in post-hospitalized COVID-19 patients.

Several treatment principles for COVID-19 have been suggested. Corticosteroids reduce mortality in patients hospitalized for COVID-19 (16), and reduce the risk of severe disease course (17). Moreover, post-recovery intensive steroid treatment is shown to have positive effects on self-reported long-term symptoms. The antiviral agent, Remdesivir, is widely used in treatment as it shortens recovery time in hospitalized COVID-19 patients (18). Tocilizumab has also been proposed, but was not able to reduce biomarkers of cardiac injury during hospitalization (19). One study on cardiovascular rehabilitation concerning exercise in post-hospitalized COVID-19 patients has shown positive results on physical performance (20). This study did, however, not include objective assessment of cardiac function, and though treatment of acute COVID-19 infection is extensively covered, studies on treatment of cardiovascular sequela and prognostic prevention are lacking. New interventions are needed.

Intravenous ketone body infusion acutely increases LVEF, GLS, and cardiac output in patients with heart failure with reduced ejection fraction (21). To our knowledge, the effects of ketone bodies on myocardial function have never been investigated with an oral intervention in subjects with subclinical myocardial dysfunction, as can be seen following COVID-19. The aim of the present study was to assess the acute effects of oral ketone ester on LVEF, GLS, cardiac output, and peripheral blood oxygen saturation in post-hospitalized COVID-19 patients with the hypothesis that these measures can be improved. To gain mechanistic insight, we exploratively investigated the effects of additional echocardiographic parameters, blood biochemistry, and vital values.

Abbreviations: ACE, angiotensin-converting enzyme; ATP, adenosine triphosphate; BHB, beta-hydroxybutyrate; BMI, body mass index; Bpm, beats per minute; FFA, free fatty acids; GLS, absolute global longitudinal strain; IQR, interquartile range; LVEF, left ventricular ejection fraction; MV, mitral valve; N, number; Pro-BNP, pro-brain natriuretic peptide; SD, standard deviation; SGLT2, sodium-glucose cotransporter 2; TAPSE, tricuspid annular plane systolic excursion.

Materials and methods

Study design

This randomized, placebo-controlled, double-blind cross-over acute intervention study was a sub-study of the ECHOVID-19 study (4, 22), a prospective longitudinal study investigating echocardiographic parameters in adults hospitalized with COVID-19. Participants were randomly allocated in a 1:1 ratio to either placebo in the morning and oral ketone ester in the afternoon (sequence A) or vice versa (sequence B). The study was conducted from May to December 2021 and was carried out at the Department of Endocrinology, Copenhagen University Hospital – Bispebjerg and Frederiksberg Hospital.

Participants

Twelve post-hospitalized COVID-19 patients were enrolled from the ECHOVID-19 study. Inclusion criteria for ECHOVID-19 were adults hospitalized in hospitals of the Capital- and Zealand regions with a laboratory confirmed diagnosis of COVID-19. Exclusion criteria for ECHOVID-19 were patients unable to understand and sign informed consent, or too sick to cooperate. Additional exclusion criteria for the present study were a diagnosis of chronic obstructive pulmonary disease or asthma, active treatment with sodium-glucose 2 inhibitors, eGFR < 15 ml/min/1.73 m^2 or insulin-dependent diabetes. All participants gave informed consent. The study was performed in accordance with the Second Declaration of Helsinki and approved by the regional ethics board (H-20021500). The ECHOVID-19 study is registered at Clinicaltrials.gov (NCT04377035) and the present sub-study likewise (NCT04573764).

Study procedures

Participants arrived between 7.45 and 8.00 am after an overnight fast and remained fasting until the end of the visit. For the intervention, a single oral ketone ester (KetoneAid Inc., Falls Church, VA) dose of 395 mg/kg bodyweight, containing primary the D-isoform, was compared with taste- and volume-matched placebo

provided by the same company. The intervention (ketone esters and placebo) was consumed using a sipping method over 60 min, the purpose of which was to promote steady state and increase the timespan that ketones would be elevated (23): the first third of the intervention was consumed at baseline (t0), the second third after 20 min (t20), and the last third after 60 min (t60). To prevent carry-over, the design included a washout period of 2 h leading to the last part of the morning intervention being ingested 3.5 h before commencing the afternoon intervention. A bedside echocardiography was performed immediately after the last sip of the corresponding treatment (**Supplementary Figure 1**). Blood samples were taken every half hour from t0 to the end of the intervention at t150. Vitals were measured before blood sampling at t0 and t60 on the same arm of the subject: blood pressure and pulse were measured using a standard hospital cuff, peripheral blood oxygen saturation using a standard finger pulse oximeter. A continuous infusion of glucose (10% solution, 50 mM potassium-chloride) was initiated before baseline at 50 ml/h to maintain euglycemia.

Characteristics and COVID-19 complications were achieved through patient journals and information on medicine through self-report. BMI was calculated on the day of the trial.

Outcomes

The primary outcome was left ventricular ejection fraction (LVEF). The secondary outcomes were absolute global longitudinal strain (GLS), cardiac output, and peripheral blood oxygen saturation. Exploratory outcomes were additional echocardiographic measures of systolic- and diastolic function, blood sample biochemistry, and vital values.

Echocardiography

Echocardiography was performed bedside with a portable Vivid IQ Ultrasound System (GE Healthcare, Horten, Norway). Examinations were performed according to a pre-determined protocol by two trained sonographers who were blinded to the intervention. The recordings were analyzed offline using commercially available post-processing software (ECHOPAC Version 203, GE Vingmed Ultrasound AS). Each parameter was analyzed separately by one of two trained investigators blinded to all clinical information. All echocardiographic measurements were performed and analyzed according to existing guidelines (12). Abnormal echocardiographic findings were assessed from the echocardiography performed during the placebo-intervention.

Left ventricular volumes and LVEF were measured using Simpson's biplane method. For GLS, two-dimensional speckle tracking was performed offline in the three apical views (four-chamber, two-chamber, and three-chamber view) of the left ventricle. Frame rate was optimized for speckle tracking analysis. The myocardial wall was traced with a semi-automatic function and manually adjusted in case of inaccurate tracing. GLS was measured by dividing each of the three projections into six segments and integrating the segments into a global 18-segment model of the LV. Global values were obtained by averaging the strain values of all included segments. Segments deemed untraceable by the investigators were excluded. Cardiac output was measured from left ventricular outflow tract. TAPSE was measured by M-mode in the apical four-chamber view. Peak mitral inflow velocity (E-wave, A-wave, and E/A ratio) and deceleration time of E were measured with pulsed-wave Doppler in the four-chamber view. Color tissue Doppler velocities (a', e', and s') were measured at the septal and lateral wall of the mitral annulus in the apical four-chamber view. E-wave was indexed to e' to estimate E/e'. Left atrial volume was measured by the area-length method in the apical two-chamber and four-chamber view. Abnormal LVEF (<52% for males and <54% for females), GLS (<16%) and TAPSE (<1.7 cm) were defined according to existing guidelines (12).

Blood samples

Blood samples for glucose and BHB analyses were collected at six timepoints every half hour from t0-t150 for the morning and afternoon periods, respectively, while samples for free fatty acids (FFA), insulin, troponin I, creatinine, pro-brain natriuretic peptide (pro-BNP) and glucagon were collected at baseline (morning t0), t60 morning and t60 afternoon.

FFA, creatinine, troponin T, pro-BNP, glucose and BHB were stored at −20 degrees Celsius, and insulin and glucagon were stored at −80 degrees Celsius until analysis. FFA and Insulin were analyzed from serum and the rest from EDTA or Li/hep plasma. Total BHB was analyzed from whole blood using hydrophilic interaction liquid chromatography tandem mass spectrometry. Insulin and glucagon were measured with ELISA (Mercodia, Sweden). FFA was quantified by *in vitro* enzymatic colorimetric method assay (Trichem, Denmark). Pro-BNP was analyzed by chemiluminescent microparticle immunoassay on an Abbott Architect i2000SR (Abbott, Germany), and c-peptide by direct chemiluminescent immunoassay (Atellica IM, USA). Troponin I was analyzed by an immunoassay kit (Cobas; Roche Diagnostics GmbH, Germany). Glucose and creatinine were analyzed by enzymatic absorption photometry (Atellica CH, USA). All analyses were performed by blinded personnel.

Statistical methods and sample size calculation

Data are reported as means ± standard deviation (SD) or, if skewed distributions, as medians with interquartile range [interquartile range (IQR)]. GLS is reported as the absolute value.

A *general linear mixed model* (R package "LMMstar") was used to estimate the difference between intervention and placebo. Time (morning versus afternoon) and intervention (placebo versus ketone esters) were used as fixed effects. Participant ID was used as random effect. A p-value of <0.05 was considered significant. For the echocardiographic outcomes, significant associations were further adjusted for change in heart rate, as this might affect results. Echocardiographic outcomes were adjusted for sequence *post hoc*. Vitals and blood values were adjusted for baseline values. Comparison between the ECHOVID-19 follow-up cohort and participants in the present study were calculated by t-test and Fisher's exact test. Percentage is calculated from number of measured valuables (not including missing values). All statistical analyses and graphical illustrations were carried out using R Statistical Software [R version 4.1.0; R Core Team (24)].

The randomization list was generated by an unrelated study nurse and was performed in fixed blocks of six (allocating three participants to each sequence ketone-placebo vs. placebo-ketone)

using the "blockrand" package in R. Personnel and study participants involved in the study were blinded to the randomization code until the end of the last participant's last visit.

The power calculation was based on a study demonstrating improved LVEF in patients with heart failure with reduced ejection fraction after continuous intravenous BHB infusion where mean ± SD LVEF changed from 35 ± 7% to 43 ± 9% (21). Assuming oral ketone esters would change LVEF 8% with a mean SD of 8%, the sample size required to demonstrate a significant effect with a power of 80% and type 1 error of 5% was 10 participants. To account for missing data and errors, 12 participants were included. Power calculation was performed using the power statement implemented in the SAS enterprise software 7.1 (SAS Institute, Cary, NC, USA).

Protocol changes

The study was initially meant to include twelve hospitalized patients with COVID-19 recruited from the ECHOVID-19 trial but was changed to include post-hospitalized patients because the in-hospital setting proved unfeasible for the study procedures. Blood gas analyses and urine creatinine clearance were study outcomes originally but turned out to be unfeasible and removed.

Results

Study participants and intervention

The ECHOVID-19-study counted 215 patients of whom 43 did not survive. From the remaining 172 participants, 91 participated in a follow-up visit 2–3 months after hospitalization and were assessed for eligibility for this sub-study. Forty-two did not meet the additional criteria for participation, 16 were not interested, 3 dropped-out before randomization due to personal health issues, and 18 were never asked because 12 participants had completed the study (Figure 1).

We included 12 participants (5 women) with a mean ± SD age of 60 ± 10 years. All women were by chance randomized to sequence B. The mean time from hospitalization was 18 ± 5 months, 5 (42%) had a diagnosis of post-COVID-19 sequela, of whom 2 had radiological signs of fibrosis (without self-reported effect on daily life), and 3 had post-viral mental fatigue or self-reported cognitive decline. All participants were Caucasians and non-smokers (Table 1). Compared to ECHOVID-19, participants included in this study had higher systolic blood pressure and lower heart rate compared with those not included from the follow-up cohort, but where otherwise comparable (Supplementary Table 1). The participants with GLS < 16% during hospitalization ($n = 3$) also had a GLS < 16% in the present study.

Total blood BHB increased with ketone esters vs. placebo over time ($p < 0.01$) and reached the highest mean levels at t90 (3.1 ± 4.9 mmol/L) (Figure 2A). Mean plasma glucose decreased between 0.53 and 0.97 mmol/l from t60-t150 with ketone esters vs. placebo ($p = 0.01$) (Figure 2B).

Effect of ketone esters on echocardiographic parameters

Compared with placebo, oral ketone esters had no effect on LVEF with a mean difference of −0.7 (95% CI: −4.0 to 2.6)%, $p = 0.66$ (Figure 3A). GLS was significantly improved after oral ketone esters compared with placebo, with a mean GLS of 16.7 ± 3.4% after placebo and 18.6 ± 3.5% after oral ketone esters. This corresponded to a mean difference of 1.9 (95% CI: 0.1 to 3.6)%, $p = 0.04$ (Figure 3B). The difference remained significant after adjustment for change in heart rate ($p < 0.01$). Cardiac output was increased, although not significantly, by oral ketone esters, with a mean cardiac output of 4.3 ± 1.1 L/min after placebo and 5.4 ± 1.9 after oral ketone esters which correspond to a mean difference of 1.2 (95% CI: −0.1 to 2.4) L/min, $p = 0.07$ (Figure 3C). Stroke volume increased with 11 ml with ketones versus placebo, but the change was not significant ($p = 0.2$). Right ventricular systolic function did not improve as assessed by TAPSE ($p > 0.05$), nor did diastolic function as assessed by mitral

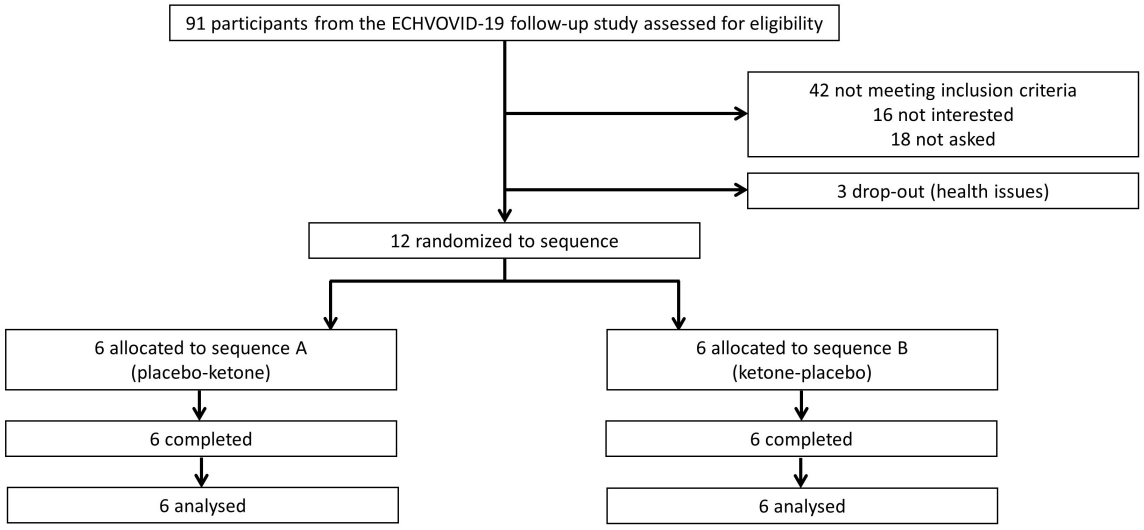

FIGURE 1
Participant flow diagram. Three dropped out due to personal health issues (one broken foot, one had progression in cancer, one due to worsening of general health).

valve (MV) E/e' or MV E/A ratio or MV E-wave deceleration time ($p > 0.05$) (Table 2). When adjusting for sequence, cardiac output and cardiac index increased significantly (both $p < 0.05$).

When stratified according to normal (>16%) and abnormal GLS (<16%), GLS was increased on average by 3.6% for participants with abnormal GLS and 0.9% for participants with normal GLS, though insignificant (p for interaction = 0.2). Ketone esters elevated GLS to above the cut-off value of 16% for 2 out of 4 participants with abnormal GLS, yet results were insignificant ($p = 0.6$) (Supplementary Figure 2). We found no association between blood levels of BHB and GLS increase with an estimate of −0.001 (95% CI: −0.004 to 0.001)%, $p = 0.3$.

Effect of ketone esters on blood values and vitals

Oral ketone esters increased blood insulin by 36 (95% CI: 21 to 50) pmol/L, c-peptide with 265 (95% CI: 156 to 374) pmol/L, and creatinine with 2 (95% CI: 1 to 4) μmol/L compared with placebo, and decreased FFA with 0.26 (95% CI: −0.33 to −0.18) mmol/L. Ketone esters did not affect glucagon, pro-BNP, or troponin I levels (all $p > 0.05$) (Table 3).

Oral ketone ester increased heart rate 10 (95% CI: 7 to 13) bpm. Ketone esters had no effect on systolic blood pressure ($p > 0.05$) but lowered diastolic blood pressure by 3.4 (95% CI: −5.1 to −1.8) mmHg. Ketone esters had no effect on peripheral blood oxygen saturation ($p > 0.05$).

Discussion

In this study, we found no effect of oral ketone esters on LVEF in post-hospitalized COVID-19 patients, but we are the first to demonstrate that a single oral dose of ketone esters could increase GLS acutely compared with placebo in post-hospitalized COVID-19 patients. Oral ketone esters had no effect on cardiac output or peripheral blood oxygen saturation, but increased heart rate and lowered diastolic blood pressure. Blood ketones, insulin, c-peptide, and creatinine were increased while glucose and free fatty acids were decreased compared with placebo.

Improvement in cardiac function following treatment with oral ketone esters

In hospitalized COVID-19 patients, reduced GLS is independently associated with death (4). In patients with heart failure with reduced ejection fraction (LVEF: 35 ± 7%), intravenous infusion of ketone salts to 3.3 mmol/L has been shown to improve left ventricular systolic function assessed as an increase in LVEF by 8%, GLS by 2%, and cardiac output by 2 L/min. Right ventricular systolic function was also improved assessed as an increase in TAPSE by 0.2 cm after ketones (21). In the present study on patients with suspected COVID-19-induced myocardial damage, we similarly found significant improvements in GLS at comparable BHB levels (2.5–3.1 mmol/L), however, in a population with only subclinical or normal cardiac function as assessed by GLS and, importantly, using an oral intervention which is a more feasible approach to achieve ketosis. We did not observe an increase in LVEF, which might be explained by our study participants having a normal

TABLE 1 Clinical characteristics.

	Placebo-ketone (A)	Ketone-placebo (B)	Total
n	6	6	12
Characteristics			
Age (years)	63 ± 5	55 ± 8	59 ± 8
Male sex, *n* (%)	6 (100%)	1 (17%)	7 (58%)
BMI (kg/m^2)	26.0 ± 4.2	28.5 ± 7.9	27.3 ± 6.2
Smoking	0	0	0
Hypertension, *n* (%)	2 (33%)	1 (17%)	3 (25%)
Hyperlipidemia, *n* (%)	4 (66%)	3 (50%)	7 (58%)
Prevalent heart failure, *n* (%)	0	0	0
Ischemic heart disease, *n* (%)	1 (17%)	1 (17%)	2 (17%)
COVID-19 complications			
Time from hospitalization (months)	18 ± 4	17 ± 7	17 ± 5
Length of hospitalization (days)	9 [5; 11]	6 [2; 19]	8 [4; 14]
Admission to intensive care unit, *n* (%)	1 (17%)	2 (33%)	3 (25%)
Acute respiratory distress syndrome, *n* (%)	0	1 (17%)	1 (8%)
Venous thromboembolic event, *n* (%)	1 (17%)	0	1 (8%)
Diagnosis of post-COVID-19 sequela, *n* (%)	2 (33%)	3 (50%)	5 (42%)
Medicine, *n* (%)			
Direct oral anticoagulants	3 (50%)	0	3 (25%)
Acetylsalicylic acid	0	3 (50%)	3 (25%)
Lipid-lowering agents	4 (67%)	2 (33%)	6 (50%)
Beta-blocker	3 (50%)	1 (17%)	4 (33%)
Calcium-antagonist	1 (17%)	0	1 (8%)
ACE-inhibitor	1 (17%)	0	1 (8%)
Baseline values			
Systolic blood pressure (mmHg)	145 ± 13	128 ± 10	136 ± 14
Diastolic blood pressure (mmHg)	85 ± 12	79 ± 6	82 ± 10
Heart rate (bpm)	58 ± 7	65 ± 9	62 ± 9
Oxygen saturation (%)	99.1 ± 0.8	98.3 ± 1.1	98.9 ± 0.9
Cardiac involvement, *n* (%)			
Abnormal LVEF	1 (17%)	1 (17%)	2 (17%)
Abnormal GLS	1 (17%)	3 (50%)	4 (33%)
Abnormal TAPSE	0	0	0

Data for all participants are divided by sequence and presented as either mean ± SD, median [IQR], and *n* (%). Abnormal echocardiographic findings were assessed from the echocardiography performed during the placebo-intervention. ACE, angiotensin-converting enzyme; BMI, body mass index; bpm, beats per minute; GLS, global longitudinal strain; LVEF, left ventricular ejection fraction; TAPSE, tricuspid annular plane systolic excursion.

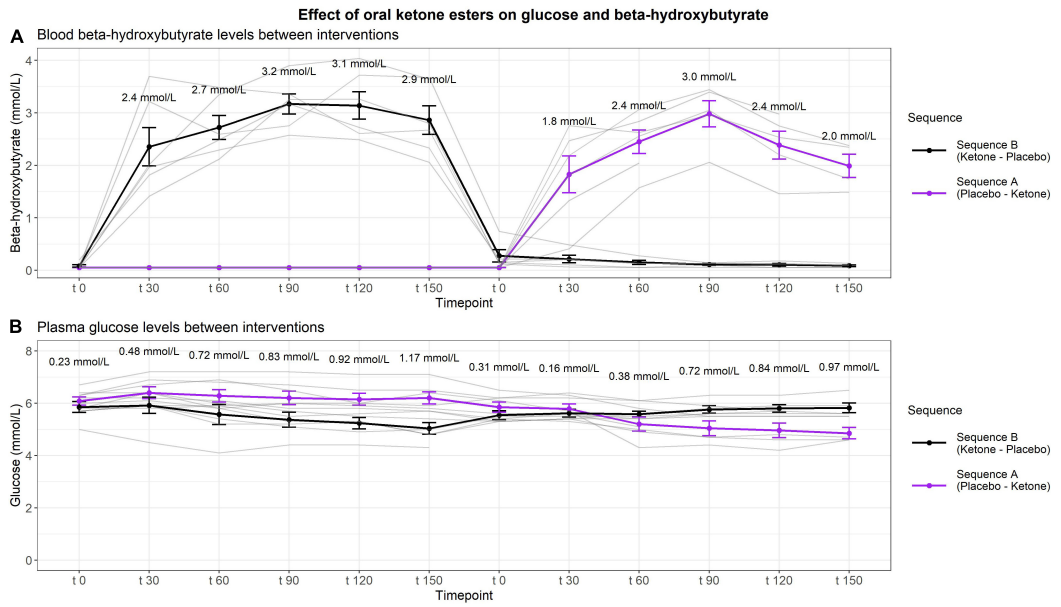

FIGURE 2
Effect of ketone esters on BHB and glucose levels. The figure depicts the changes in BHB levels assessed from whole blood and shows the average peak BHB-levels at the different timepoints **(A)** and depicts the absolute changes in glucose assessed from plasma **(B)** between interventions given as mean ± SEM. Data was analyzed by general linear mixed model adjusted for time of day, t0 and interaction between timepoints and intervention. Blood samples were collected at six timepoints every half hour from t0 to t150 for morning and afternoon, respectively.

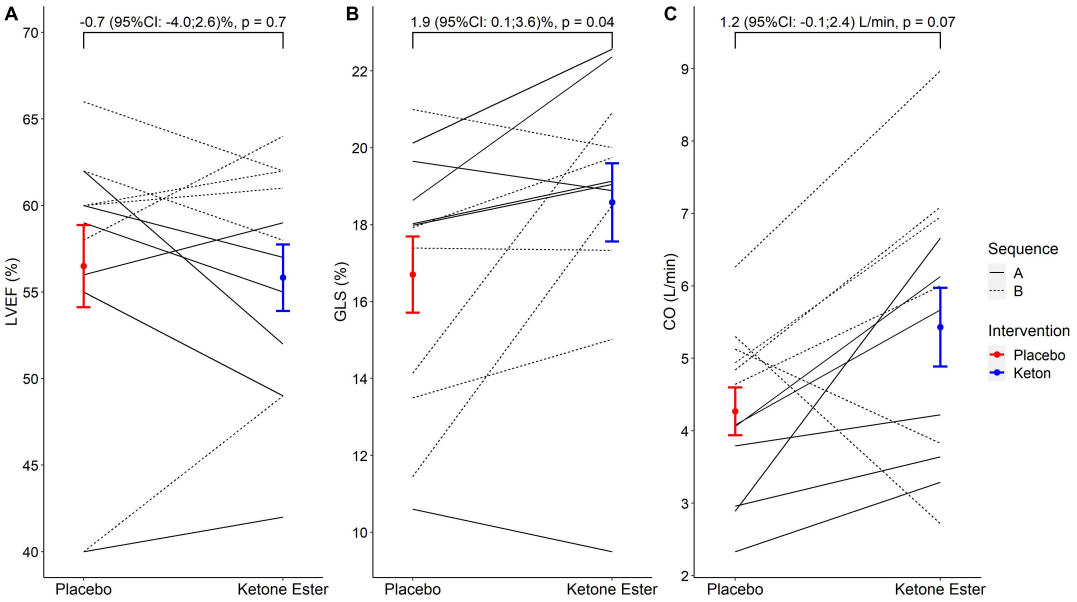

FIGURE 3
The effects of oral ketone esters on LVEF, GLS, and cardiac output. The figure depicts the absolute changes (mean ± SEM) between placebo and oral ketone esters for LVEF **(A)**, GLS **(B)**, and cardiac output **(C)**, respectively. The brackets report unadjusted mean differences (95% CI) with *p*-values. LVEF, left ventricular ejection fraction; GLS, absolute global longitudinal strain.

baseline LVEF (57% ± 8). In patients with heart failure, intravenous ketone esters increase cardiac output in a dose-dependent manner (21), and in a study on healthy participants, a single oral dose of ketone esters reaching higher BHB levels than in the present study [median blood-BHB concentration: 3.23 mmol/L (IQR: 2.40–4.97)] LVEF was increased by 3.1%, GLS by 2.0%, and right ventricle S' by 1.1 cm (25). Myocardial BHB utilization is furthermore increased in proportion to blood ketone levels (26), indicating that our failure to report an increase in LVEF and cardiac output could also be dose dependent. On the contrary, we were not able to demonstrate an association between blood levels of BHB and a GLS increase, which does not support a dose-response relationship. This could, however, be due to the small sample size. In the present study we did not find an improvement in right ventricular systolic function as assessed by TAPSE and right ventricular longitudinal strain was excluded due to lacking data. The insignificant results of TAPSE

are probably due to the same mechanisms as for LVEF. Diastolic function was not found to be impaired compared with controls in a study with hospitalized COVID-19 patients (4) but was, however, impaired in hospitalized COVID-19 patients with simultaneous elevated high-sensitive troponin levels or classified as being severely ill (6, 7). In the present study we did not find any effect of ketone esters on diastolic function as assess by MV E/A ratio, MV E/e', or MV E-wave deceleration time. To our knowledge, no effect of ketone esters on diastolic function has previously been reported (21, 25). With oral ketone esters elevating GLS to 18.6 ± 3.5% in the present study, we propose that oral ketone esters could normalize impaired GLS in in patients with asymptomatic subclinical myocardial dysfunction. In fact, ketones esters did indeed raise GLS for 2 out of 4 of the participants with GLS < 16% to within the normal range. Although insignificant, probably due to the small sample size, this suggests a larger effect in those with abnormal GLS. Interestingly, endogenous BHB concentrations rise in response to the degree of cardiac dysfunction (27), and one dose of oral ketone esters enhanced myocardial BHB extraction in patients with heart failure with reduced ejection fraction compared with controls and correlates with the degree of cardiac dysfunction (28). These findings support the hypothesis that increased BHB utilization in the failing heart could be an adaptive mechanism. The effects of BHB on different degrees of myocardial malfunction from subclinical

TABLE 2 Differences in echocardiographic parameters between oral ketone esters and placebo.

Variable	Placebo	Ketone	Mean difference [CI]	*P*-value	Adjusted *P*-value
Left ventricular systolic function					
Left ventricular ejection fraction, (%)	57 ± 8	56 ± 7	−0.7 [−4.0; 2.6]	$p = 0.66$	
Absolute global longitudinal strain, (%)	16.7 ± 3.4	18.6 ± 3.5	1.9 [0.1; 3.7]	$p = 0.04$	$p < 0.01$
Cardiac output, (L/min)	4.3 ± 1.1	5.4 ± 1.9	1.2 [−0.1; 2.4]	$p = 0.07$	
Cardiac index, (L/min/m^2)	2.1 ± 0.7	2.7 ± 1.1	0.6 [−0.1; 1.2]	$p = 0.08$	
Left ventricular end-diastolic volume, (mL)	94.0 ± 22.6	88.1 ± 25.9	−5.9 [−15.7; 3.9]	$p = 0.21$	
Left ventricular end-systolic volume, (mL)	41.0 ± 12.1	38.9 ± 12.6	−2.1 [−7.2; 3.0]	$p = 0.38$	
Left atrial end-systolic volume, (mL)	45.3 ± 16.6	47.9 ± 14.6	2.8 [−10.0; 15.5]	$p = 0.63$	
Stroke volume, (mL)	71.3 ± 12.4	82.0 ± 29.0	10.7 [−6.5; 28.0]	$p = 0.20$	
Diastolic function					
MV E/e'	7.1 ± 1.3	6.9 ± 1.4	−0.3 [−1.3; 0.8]	$p = 0.55$	
MV E/A ratio	1.1 ± 0.3	1.0 ± 0.3	−0.1 [−0.2; 0.0]	$p = 0.11$	
MV E-wave deceleration time, ms	275 ± 89	238 ± 92	−37 [−85; 11]	$p = 0.12$	
Right ventricular function					
TAPSE, (mm)	2.4 ± 0.6	2.4 ± 0.6	0.05 [−0.4; 0.4]	$p = 0.80$	

Data are mean ± SD and mean differences with 95% CI. Data are analyzed by general linear mixed model; significant differences were adjusted for change in heart rate. There was one missing datapoint for left atrial end-systolic volume, three for MV E/e', and one for TAPSE. Right ventricular longitudinal strain was excluded (four missing datapoints and three outliers). MV, mitral valve; TAPSE, tricuspid annular plane systolic excursion.

TABLE 3 Effect of ketone esters on blood values and vitals.

Variable	Baseline	Placebo	Ketone	Mean difference [CI]	*P*-value
Blood values					
Insulin, (pmol/L)	48 ± 23	51 ± 26	87 ± 40	36 [21; 50]	$p < 0.01$
C-peptide, (pmol/L)	696 ± 226	760 ± 290	1025 ± 355	265 [156; 374]	$p < 0.01$
Free fatty acids, (mmol/L)	0.42 ± 0.20	0.35 ± 0.16	0.09 ± 0.05	−0.26 [−0.33; −0.18]	$p < 0.01$
Glucagon, (pmol/L)	8.2 ± 2.8	6.6 ± 2.1	5.8 ± 2.2	−0.8 [−2.0; 0.4]	$p = 0.16$
Creatinin, (μmol/L)	78 ± 20	73 ± 22	75 ± 19	2 [1; 4]	$p < 0.01$
pro-BNP, (pmol/L)	10.1 [6.0; 27.8]	11.9 [6.4; 30.6]	11.2 [7.5; 30.6]	−0.2 [−3.1; 2.6]	$p = 0.87$
Troponin I, (ng/L)	4.5 [3.0; 5.8]	4.5 [3.8; 6.5]	5.0 [3.8; 6.0]	0 [−0.4; 0.4]	$p = 1.00$
Vitals					
Heart rate, (bpm)	61 ± 9	59 ± 9	66 ± 7	10 [7.4; 13.2]	$p < 0.01$
Systolic blood pressure, (mmHg)	135 ± 15	134 ± 14	133 ± 16	−2.8 [−8.1; 2.5]	$p = 0.26$
Diastolic blood pressure, (mmHg)	81 ± 9	81 ± 10	78 ± 11	−3.4 [−5.1; −1.8]	$p < 0.01$
Saturation, (%)	98.9 ± 0.9	98.7 ± 0.5	98.7 ± 1.4	0.3 [−0.2; 0.8]	$p = 0.19$

Data are mean ± SD or median [IQR]. Data are analyzed by general linear mixed model. Blood samples were taken at baseline (morning t0) and at the end of each intervention (t60). Mean difference and 95% CI were adjusted for baseline values and time of day. Vitals were measured at t0 and t60 for each intervention. Mean difference and 95% CI were adjusted for the respective t0 value and time of day. The reported baseline values for vitals are the average of both t0 measurements. Bpm, beats per minute.

lowered myocardial function to manifest heart failure should be explored in future studies.

Metabolic changes in heart failure

Both right and left ventricular function are impaired in patients formerly hospitalized for COVID-19 (13, 14). The healthy human heart utilizes both fatty acids, glucose, and ketone bodies (29, 30), but prefers fatty acids over glucose. In heart failure, BHB utilization along with the rate-limiting enzyme in ketone oxidation are increased (26, 31), which could be a beneficial adaptive mechanism, since ketone oxidation is highly energy efficient compared to fatty acids (32), and is utilized in proportion to blood-levels (26), replace myocardial glucose uptake and increase myocardial blood flow (33), and can occur independent of insulin (34). Sodium-glucose cotransporter 2 (SGLT2) inhibitors induce ketosis in patients with diabetes mellitus type 2 (5.6 $\pm$ 6.0 μmol/L when fasting and 3.3 $\pm$ 3.8 μmol/L when fed), but not in patients without diabetes (35) and reduce the risk of cardiovascular death and hospitalization for heart failure in both groups (36, 37). It has been suggested that ketone bodies might have a direct metabolic cardioprotective effect (38). In patients with T2D without heart disease, treatment with SGLT2 inhibitors increase GLS but only in the group with subclinical myocardial dysfunction (GLS $<$ 16.5%) (39). This supports the proposed hypothesis that the effect of ketone esters on myocardial contractility depends on the degree of cardiac dysfunction. Our data indicates an improvement in left ventricular systolic function following consumption of ketone esters and thus supports the hypothesis that BHB is a competitive, efficient, and thrifty substrate for the struggling heart by increasing myocardial tissue energy availability.

In the present study we demonstrated a decrease in free fatty acids and glucose, and an increase in insulin-production and plasma-creatinine under constant glucose infusion. Exogenous ketosis decreases free fatty acids and blood-glucose and increases plasma-insulin (23, 33, 40) in contrast to endogenous ketosis which is characterized by low levels of insulin and glucose, and high free fatty acids (23). High blood ketone levels have been shown to inhibit lipolysis by negative feedback, and lower glucose levels through inhibiting gluconeogenesis (41) and by increasing peripheral glucose uptake and glycogen synthesis in muscles (40). Accumulation of toxic lipid intermediates is associated with myocardial insulin-resistance in heart failure, and active unloading leads to improved cardiac insulin signaling (42). In theory, ketone bodies might therefore be cardioprotective by inhibition of free fatty acids. To our knowledge, there are no studies testing the effect of exogenous ketone esters on toxic lipid accumulation in the heart, and studies on associations between post-infectious sequela and ketosis are severely lacking.

Besides their metabolic effects, ketone bodies also serve as signaling molecules and have been shown to reduce oxidative stress and inflammation in several tissues (43). COVID-19 virus infection is known to induce an inflammatory response and even months after infection, myocardial inflammation can be detected in some patients (44). It could therefore be speculated that the improved cardiac function found following ketone treatment is partly related to anti-inflammatory properties of ketone bodies.

Ketone esters have been shown to increase heart rate and decrease systemic vascular resistance while having no effect on blood-pressure (21). Similar findings were observed in the present study.

Implications and further studies

The present study is the first to demonstrate that oral ketone esters acutely improve left ventricular systolic function in post-hospitalized COVID-19 patients and that oral ketone esters may represent a novel treatment principle in combatting the impending rise in COVID-19 sequela following the pandemic and other conditions with subclinical lowered myocardial function. Our study supports the hypothesis that increased BHB utilization in the failing heart is an adaptive mechanism and might help explain the cardioprotective effects of SGLT2 inhibitors. Studies are needed to investigate if ketone bodies have mechanisms other than increasing energy availability such as possible effects on toxic lipid accumulation in the heart, and if the effect of ketone bodies increases with the degree of heart decompensation. Studies on the chronic effects of ketone bodies are warranted.

Strengths and limitations

A limitation is the relatively small sample size, although we did have power to demonstrate effects on GLS. The sample size was calculated from a population with reduced ejection fraction which theoretically should have a greater effect of ketone esters than in the cohort of the present study. The study might therefore be underpowered which could explain the negative results on the primary outcome. Participants in the present study had significantly higher systolic blood pressure and lower heart rate compared with the follow-up cohort though they were comparable on all other parameters including no difference in odds for hypertension, ischemic heart disease or prevalent heart failure. The cohort in the present study was therefore deemed representative for participants in the main study. Participants were not stratified for the outcome of previous echo-examinations and the below-expected values of GLS are assumed to be COVID-19-related, just as patients recovered from COVID-19 have significantly impaired left and right ventricular function compared to matched controls (14). However, there is no definite proof that COVID-19 and subclinical systolic dysfunction are related in our study population and therefore, the results of the present study should be interpreted as the effect of oral ketone esters on patients with asymptomatic subclinical myocardial dysfunction and not specifically in patients previously hospitalized with COVID-19. Five outcomes were significant for time: insulin, c-peptide and creatinine were higher in the morning while heart rate and cardiac output were higher in the afternoon. The observed periodic effect was accommodated by adjusting for time. A possible carry-over effect was accommodated by including a wash-out period of 2 h leading to the last part of the morning intervention being ingested 4 h before commencing the afternoon intervention. For orally ingested ketone esters, the time taken to reach the maximal concentration is approximately 1-h, coinciding with the echocardiography. Elimination is non-linear and follows first order elimination kinetics, eliminating approximately 130 mmol/min by peripheral oxidation (23). Hence, the design should hereby have made any carry-over effect negligible. Another limitation was the unequal distribution of sex between the two sequences with all women by chance being randomized to sequence B and with 50% being characterized by abnormal GLS opposed to only 17% of sequence A (**Table 1**). This distribution

was accommodated by the paired design. When adjusting for sequence, cardiac output and cardiac index became significant (both $p < 0.05$) while having no impact on the other results. This indicates that the observed effect of oral ketone esters on cardiac function was not driven by differences in the sequence groups.

Strengths of the study include that both interventions were conducted on the same day making the circumstances for the individual participant as identical as possible and therefore avoiding day-to-day variations. The study was performed according to standardized procedures.

Conclusion

In patients previously admitted with COVID-19, moderate ketosis achieved by a single oral dose of ketone esters increased GLS acutely and independently of heart rate but had no effect on LVEF, cardiac output or blood oxygen saturation. Our study suggests a direct improvement in left ventricular myocardial function in subjects previously hospitalized for COVID-19. Oral ketone esters may represent a novel treatment principle in combatting the impending rise in COVID-19 sequela following the pandemic and other conditions with subclinical cardiac injury.

Data availability statement

The original contributions presented in this study are included in the article/**Supplementary material**, further inquiries can be directed to the corresponding author.

Ethics statement

The studies involving human participants were reviewed and approved by the Danish Ethical Committee on Health Research Ethics, Copenhagen, Denmark. The patients/participants provided their written informed consent to participate in this study.

Author contributions

HW: formal analysis, investigation, resources, data curation, writing – original draft, writing – review and editing, visualization, and project administration. FD: software, data curation, and writing – review and editing. JC: software, investigation, data curation, and writing – review and editing. ML, KS, and HN: writing – review and editing. NM: conceptualization, validation, and writing – review and editing. JR and PR: conceptualization, validation, resources, writing – review and editing, and supervision. TB-S: conceptualization, validation, resources, and writing – review and editing. NJ: conceptualization, methodology, validation, resources, writing – review and editing, and funding acquisition. JL: conceptualization, methodology, validation, investigation, resources, data curation, writing – review and editing, and funding acquisition. All authors contributed to the article and approved the submitted version.

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Conflict of interest

FD reports having served as a consultant for Bayer. TB-S reports serving as steering committee member of the Amgen financed GALACTIC-HF trial, chief investigator and steering committee chair of the Sanofi Pasteur financed "NUDGE-FLU" trial, chief investigator and steering committee chair of the Sanofi Pasteur financed "DANFLU-1" trial, chief investigator and steering committee chair of the Sanofi Pasteur financed "DANFLU-2" trial, steering committee member of "LUX-Dx TRENDS Evaluates Diagnostics Sensors in Heart Failure Patients Receiving Boston Scientific's Investigational ICM System" trial. Being on the advisory board for Sanofi Pasteur, Amgen, and GSK. Receiving speaker honorarium from Bayer, Novartis, Sanofi Pasteur, and GSK and research grants from GE HealthCare and Sanofi Pasteur. PR reports having received research grants from AstraZeneca and Novo Nordisk and given lectures for AstraZeneca, Mundipharma, and Boehringer Ingelheim and has served as a consultant for AstraZeneca, Bayer, Eli Lilly, Boehringer Ingelheim, Astellas, Gilead, Sanofi Aventis Vifor, and Novo Nordisk, all fees given to Steno Diabetes Center Copenhagen. JL reports receiving a speaker honorarium from Boehringer Ingelheim, all fees given to Steno Diabetes Centre Copenhagen.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2023.1131192/full#supplementary-material

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Classic ketogenic diet in parenteral nutrition in a GLUT1DS patient: Doing more with less in an acute surgical setting

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Ketogenic Dietary Treatments (KDTs) are to date the gold-standard treatment for glucose transporter type 1 (GLUT1) deficiency syndrome. Administration of KDTs is generally *per os*; however, in some conditions including the acute gastro-enteric post-surgical setting, short-term parenteral (PN) administration might be needed. We report the case of a 14-year-old GLUT1DS patient, following classic KDT for many years, who underwent urgent laparoscopic appendectomy. PN-KDT was required, after 1 day of fasting. No *ad hoc* PN-KDTs products were available and the patient received infusions of OLIMEL N4 (Baxter). On the sixth day postoperatively enteral nutrition was progressively reintroduced. The outcome was optimal with rapid recovery and no exacerbation of neurological manifestations. Our patient is the first pediatric patient with GLUT1DS in chronic treatment with KDT efficiently treated with exclusive PN for five days. This case reports on real-word management and the ideal recommendations for PN-KDT in an acute surgical setting.

KEYWORDS

GLUT1 deficiency syndrome, classic ketogenic diet, ketone bodies, parenteral nutrition, appendicitis, surgery

1. Introduction

Glucose Transporter Type 1 Syndrome (GLUT1DS) is a rare genetically determined neurometabolic disorder causing impaired glucose transport through the blood-brain barrier. The GLUT1DS key clinical features are mainly represented by eye-head movement abnormalities, epileptic seizures, neurodevelopmental impairment, deceleration of head growth, and movement disorders (1). Ketogenic dietary therapies (KDTs) -high-fat, carbohydrate-restricted, adequate proteins- are, to date, the gold standard treatment for the syndrome (2): ketone bodies can cross the blood-brain barrier and can be used as an alternative fuel for brain metabolism.

Ketogenic dietary therapies are generally administered *per os*; however, a variety of acute conditions, causing transient intestinal failure or in an immediate gastro-enteric post-surgical setting, might require complete bowel rest, thus requiring short-term parenteral administration as a bridge to the reintroduction of enteral KDTs (3). However, there are currently extremely

limited data, with less than 40 patients described up-to-date in literature, focusing on the feasibility and efficacy of parenteral nutrition (PN) KDTs (PN-KDTs) (4).

Herein, we present the first pediatric patient with GLUT1DS in chronic treatment with classic KDT (cKDT) efficiently treated with exclusive PN for 5 days.

2. Case report

We report the case of a 14 years old boy affected by GLUT1DS, whose clinical picture encompassed mild cognitive impairment, generalized epileptic seizures (atypical absences) at the age of three, partially controlled with valproic acid, and a paroxysmal movement disorder (characterized by paroxysmal exercised induced dystonia and episodes with choreoathetosis features). At the age of 5 years, genetic diagnosis of GLUT1DS was provided, and a classic ketogenic diet (cKD) with a 2.6:1 (fat to protein + carbohydrate) ratio was administered with complete resolution of epileptic seizures and movement disorder for more than 9 years. At the time of the event, the patient was not receiving any concomitant treatment. During the years, regular ketone monitoring revealed mean values of beta-hydroxybutyrate ranging from 1.6 to 2 mmol/L. Adequate minerals and vitamin supplementation were associated with KDT. The patient was regularly followed at Mondino Neurological Institute (Pavia) to monitor neurological and nutritional aspects, according to recent guidelines for KDTs (2).

No adverse events were derived from KDT implementation. At the last follow-up 2 months before the acute event, a stable clinical picture was documented, with an EEG substantially within normal limits.

Due to abdominal pain and vomiting, the patient was admitted to the Pediatric Emergency Department of Del Ponte Hospital (Varese). After surgical evaluation, the patient underwent laparoscopic appendectomy, which revealed a clinical picture of acute perforated gangrenous appendicitis with diffuse peritonitis. Broad-spectrum antibiotic combination therapy saline solution was administered as per internal Hospital protocol. The patient recovered well from surgery; however, after 1 day of fasting, based on daily consultation between surgeons and the patient's referral Keto-Team, PN-KDT was started as the boy was unable to tolerate enteral nutrition postoperatively. Data on parenteral and enteral nutrition management are shown in **Table 1**. PN was provided through a central venous catheter. Given the unavailability of ketogenic formulated products and of *ad hoc* galenic formulation production for PN-KDT in an emergency setting, where treatments with KDTs are not commonly used, the patient received infusion of OLIMEL N4 (Baxter). From the second to the fifth day of PN only OLIMEL N4 solution was administered, and the quantity was increased from 500 ml/day (equal to 560 kcal/day) to 1,440 ml/day (equal to 1,008 kcal). Since OLIMEL N4 (Baxter) is a non-ketogenic product, a rapid and conspicuous drop in ketonemia was expected. To partially overcome this inconvenience, the caloric intake was calculated to be lower than expected, to stimulate fasting ketones production. Hydration was administered according to water balance. Ketonemia was monitored every 6 h, with ketone levels in range (2.7 mmol/L) during the first day of fasting, and subsequent reduction ranging from 1 to 1.2 mmol/L. On the sixth day, enteral nutrition was progressively reintroduced through a ketogenic formulated product (Ketocal powder 3:1, Nutricia) (**Table 1**) *via* a nasogastric (NG) tube. The product was given in doses of 30 g, diluted in 120 ml of water, three times a day in addition to the PN-KDT. In the following 2 days, PN-KDT was progressively reduced, and at the same time, the NG-tube feeding formula was increased to 45 g three and four times a day, respectively (**Table 1**). Solid food (cKD with a 2.6:1 ratio) was reintroduced after 9 days from surgery and was well tolerated. Close monitoring of serum electrolytes and metabolic profile was conducted without detecting clinically noteworthy changes.

No exacerbation of paroxysmal neurological symptoms was observed during PN, nor at the time of enteral feeding reintroduction. At subsequent follow-up 2 months and 6 months after surgery, the patient depicted good general health, no recurrence of paroxysmal neurological events, substantially normal EEG, normal blood tests, and blood glucose and ketonemia levels comparable to those before surgery.

3. Discussion

Up to now, PN-KDTs described in the literature were only administered in hospitals specialized in the management of KDTs, when the diet needed to be initiated and an enteral approach was not feasible due to an acute condition as, for example, refractory seizures or in patients with drug-resistant epilepsies, already on KDT, in whom an acute condition impaired enteral feeding (3). Some attempts to systematize the application and management of PN-KDTs have been proposed in recent years by van der Louw et al. (4) and by Dressler et al. (5). However, in Italy, only a few Centers are equipped for the chronic and acute management of KDTs. In a recent Italian web-based survey from our group aiming at investigating caregivers' perceptions about the management of a patient undergoing the cKD in the acute settings, it was highlighted that in a surgical context, the fasting duration, support therapies, and feeding after gastrointestinal surgical procedures should be considered as unsolved themes to be addressed by experts (6).

To the best of our actual knowledge, we reported the first GLUT1Ds patient receiving short-term parenteral nutrition in the immediate post-surgical period, in a setting where PN-KDTs products were unavailable. What we have done was quite different from the recommendations, reported in **Table 2**, due to the urgency of the condition and the unfavorable circumstances, but it was associated to prompt recovery of ketonemia at the typical pre-surgery values.

It is generally accepted that the introduction of parenteral nutrition should be considered when the pediatric patient cannot receive enteral feeding for more than 48 h (7). In such a context, particularly when the patient is already receiving KDTs as the only treatment, as in many GLUT1DS patients, early contact with the patient's referring keto-team is essential to establish an effective shared and coordinated management. If the patient had been admitted to a Reference Center for the ketogenic diet, individualized feeding and hydration plans would have been conceived considering his caloric and fluid needs. In this scenario, a theoretical plan for managing our patient is presented in **Table 2**. However, theoretical assumptions often clash with contingent reality in critical conditions, as underlined by our case. In particular, although theoretically all fluids containing dextrose should ideally be avoided, from a practical standpoint, management of our case supports the hypothesis that

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TABLE 1 Timeline of Practical plan of Parenteral and Enteral Nutrition (PEN) management.

	Type of feeding			Macronutrients				Total calories	Ketogenic ratio
		Velocity	Quantity	Total lipids (g)	Total AA (g)	Nitrogen (g)	Total glucose (g)		
1st day	Fasting								
2nd day	PN-Olimel N4	30 ml/h	800 ml/day	24	2,026	3.2	60	560	0.30
3rd day	PN-Olimel N4	40 ml/h	960 ml/day	288	2,432	3.84	72	672	0.30
4th day	PN-Olimel N4	50 ml/h	1,200 ml/day	36	304	4.8	90	840	0.30
5th day	PN-Olimel N4	60 ml/h	1,440 ml/day	432	3,648	5.76	108	1,008	0.30
6th day	PN-Olimel N4	80 ml/h	1,500 ml/day	45	38	6	11,25	16,899	1.67
	NG-tube	3 times/day	30 g	10,674	5,186		11,898		
7th day	PN-Olimel N4	60 ml/h	1,440 ml/day			5.76		19,6785	1.67
	NG-tube	3 times/day	45 g	13,581	5,727		11,772		
8th day	PN-Olimel N4	40 ml/h	960 ml/day			3.84		19,518	1.67
	NG-tube	4 times/day	45 g	15,228	5,204		8,496		
9th day	Solid food *per os*			2,088	456		341	2,230	2.62

TABLE 2 Timeline of Theoretical plan for Parenteral and Enteral Nutrition (PEN) management.

	PN-Products to be used			Macronutrients				Total calories	Ketogenic ratio
		Velocity	Quantity	Lipid (g)	Amino Acid (g)	Nitrogen	Glucose (g)		
1st day	Aminoplasmal 10%	10 ml/h	300 ml		30			160	
	Glucose 5%	12 ml/h	200 ml				10		
	Fluids		as required						
2nd day	Aminoplasmal 10%	10 ml/h	300 ml		30			1,150	2.00
	Glucose 5%	25 ml/h	400 ml				20		
	Lipofundin 20%	32 ml/h	500 ml	100					
	Fluids		As required						
3rd day	Aminoplasmal 10%	22 ml/h	350 ml		35			1,427	2.08
	Glucose 5%	31 ml/h	500 ml				25		
	Lipofundin 20%	39 ml/h	625 ml	125					
	Fluids		As required						
4th day	Aminoplasmal 10%	26 ml/h	400 ml		40			1,685	2.31
	Glucose 5%	31 ml/h	500 ml				25		
	Lipofundin 20%	47 ml/h	750 ml	150					
	Fluids		As required						
5th day	Aminoplasmal 10%	28 ml/h	450 ml		45			1,943	2.50
	Glucose 5%	31 ml/h	500 ml				25		
	Lipofundin 20%	54 ml/h	875 ml	175					

in acute settings, when preparations for PN-KDTs are not available, standard intravenous solutions commercially available and promptly disposable at local hospitals might be at least an acceptable compromise.

As suggested by our case, their application should be limited to the very short period of the acute condition, and a change into enteral nutrition, with a slow increase in the amount of enteral feeding, should be promoted as soon as possible.

Notably, short-term parenteral nutrition with commercially available formulations was revealed to be safe and well-tolerated in our patient. In addition, control of paroxysmal symptoms (both epileptic seizures and paroxysmal moment disorder) was maintained during the transition from enteral to parenteral nutrition and when switching back to enteral nutrition.

Our patient remained free from paroxysmal symptoms despite reducing ketosis from baseline. Such a condition was observed even

in the case series reported by Armeno et al. (3), thus raising the question about the need to maintain a high ketogenic ratio during PN-KDT. Although suboptimal, we believe that the ketonemia values presented by our patient during PN may have contributed to the non-occurrence of side effects. The most commonly reported side effects of PN-KDTs include increased lipids profile, insufficient ketosis, or hypoglycemia, whereas less frequently altered liver, and pancreatic functions have been described; they are usually transient and reversible by switching to enteral feeding (8). Therefore, careful monitoring of glucose and ketones levels, along with electrolytes, lipid profile, and liver and pancreatic enzymes, is mandatory (4). As stated by Van der Louw et al. (4), adverse events can be avoided by limiting parenteral nutrition to a very short period of the acute condition and promoting a change into enteral nutrition, with a slow increase in the amount of enteral feeding as soon as possible. Our case seem to confirm this observation.

In conclusion, our experience supports the idea that PN may be considered an efficient temporary solution toward enteral KD in those GLUT1DS patients who are temporarily prevented from enteral feeding due to acute medical or surgical gastroenteric pathologies. However, for proper management and monitoring of dietary aspects in such tricky conditions, the close and continuous collaboration of a multidisciplinary team daily supporting and sharing expertise with colleagues operating in the frontline emergency setting is essential.

Data availability statement

The original contributions presented in this study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

Ethics statement

The studies involving human participants were reviewed and approved by Pavia Ethical Committee. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

Author contributions

CV, CF, AT, and VD contributed to the conception and design of the report. VG, MB, GF, MG, CM, LP, and CT contributed to the acquisition, analysis, and interpretation of the data. All authors drafted the manuscript, critically revised the manuscript, agreed to be fully accountable for ensuring the integrity and accuracy of the work, and read and approved the final manuscript.

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Conflict of interest

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The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Ketogenic therapies for glioblastoma: Understanding the limitations in transitioning from mice to patients

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Glioblastoma Multiforme is an aggressive brain cancer affecting children and adults frequently resulting in a short life expectancy. Current cancer therapies include surgery and radiation followed by chemotherapy, which due to their ineffectiveness, requires repeated exposure to the same therapies. Since the 1990s, researchers and doctors have explored other therapies, such as diet therapies, to aid in combating gliomas. The ketogenic diet has gained popularity due to Otto Warburg's theory that tumor cells prefer "aerobic glycolysis" and cannot metabolize ketones. The inability of gliomas to use ketones provides an excellent opportunity to weaken the tumor while protecting healthy cells during cancer treatments. This review will examine some of the current research using the ketogenic diet as a form of cancer therapy to determine if this intervention is manageable and effective in patients with glioblastoma. Peer-reviewed articles from 2009 to 2019 were used. The primary objective is to distinguish differences between pre-clinical and clinical research to determine if the ketogenic diet is reproducible from mouse models into humans to determine its effectiveness. The analysis revealed several limitations of the ketogenic diet as an intervention. The effectiveness is more robust in mice than in human studies. Furthermore, tolerability is marginally supported in human studies requiring more reproducible research to validate that the intervention is manageable and effective in patients with glioblastoma.

KEYWORDS

glioblastoma, ketogenic diet, calorie restriction, low carbohydrate diet, diet intervention

1. Introduction

Glioblastoma multiforme (GBM) is a fatal form of brain cancer that affects adults and children (1, 2). It is immensely invasive due to its ability to become vascularized (2). Nonetheless, what makes GBM so deadly is its unique ability to repair damaged DNA from radiation and chemotherapy. This unique ability enables GBM to become treatment resistant creating new challenges for doctors and patients (2, 3).

Treatment for patients with GBM is a burdensome process. Standard treatment requires partial or complete tumor removal then radiation and chemotherapy for six to 9 months (1, 3). Yet, the aggressive, vascular nature of GBM leads to disease progression, otherwise known as tumor recurrence, around 6 months post-surgery and treatment (3).

When progression occurs, the protocol is to repeat the standard therapy. However, repeated exposure to radiation and certain chemotherapies, such as temozolomide, leads to inflammation

and edema in the brain (2–4). These symptoms precede seizures and other neurological disorders that decrease survivability (2, 5). Therefore, researchers consider the current therapies for treating GBM antiquated and seek novel cancer treatments that prolong patient survival.

Scientists have been investigating cancer metabolism to understand its role in treatment resistance as early as the 1920s (6). In the 1950s, Nobel Prize winner Otto Warburg made a significant scientific discovery in cancer metabolism. Warburg discovered that cancer cells use what he termed "aerobic glycolysis" to generate energy from the conversion of glucose to lactate at high rates, even when oxygen is present (6, 7) (Figure 1). His discovery claims that cancer cells use metabolic pathways that are faster at producing energy, even though less, due to defective mitochondrial respiration—suggesting that cancer cells do not metabolize ketones (5). Warburg's findings are well-recognized and cited throughout cancer research, and his conclusions coined the research term the "Warburg Effect" (5, 7–11). This discovery is an immense contributor to new cancer therapies, including exploring diet interventions utilizing calorie restriction (CR) and the ketogenic diet (KD) (8).

The KD is a nutritional intervention that encourages ketone production. Ketones, specifically acetoacetate and β-hydroxybutyrate, are

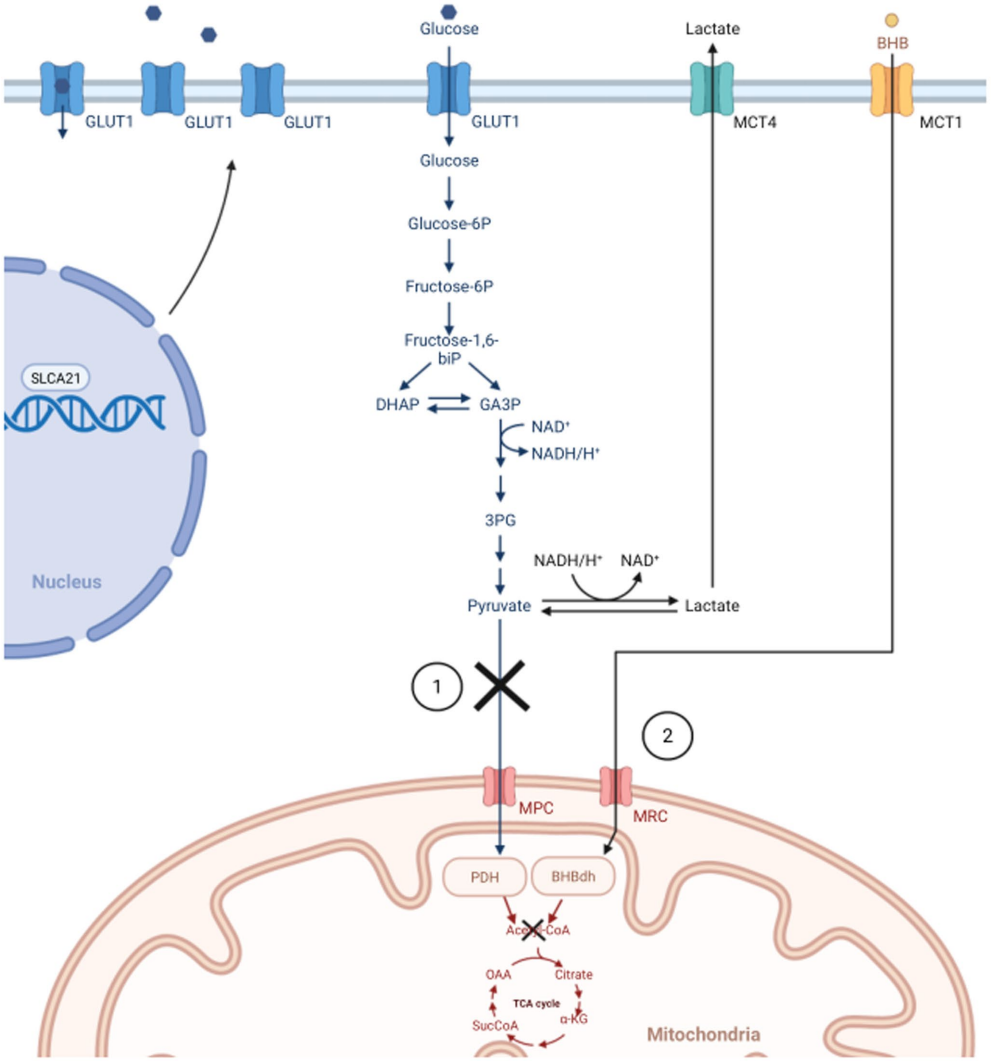

FIGURE 1
In normal cells, glycolysis only occurs in the cytoplasm under anaerobic conditions, which generates small amounts of energy. During aerobic conditions, normal cells prefer the TCA cycle and oxidative phosphorylation through the mitochondria. The Warburg Effect states that cancer cells prefer "aerobic glycolysis" for faster, safer energy production due to defective mitochondria. 1) In normal cells, pyruvate would be converted into Acetyl-CoA to start the TCA cycle. Due to dysfunctional mitochondrion (e.g., downregulated MPC, inhibition of PDH), cancer cells convert pyruvate to lactate to drive energy production. Subsequently, lactate is transported out of the cell *via* MCT, thus increasing the need for cancer cells to upregulate the SLCA21 gene to increase production of GLUT1 transporters allowing more glucose into the cell. 2) In healthy endothelial cells of the brain, BHB can be converted into Acetyl-CoA to facilitate the TCA cycle when glucose is low. Warburg states the process is not possible in cancer cells, and the reason for utilizing ketogenic diets in treating GBM. GLUT1, glucose transporter 1; DHAP, di-hydroxy acetone phosphate; GA3P, glyceraldehyde 3-phosphate; CoA, Coenzyme A; TCA, tricarboxylic acid; MPC, mitochondrial pyruvate carrier; PDH, pyruvate dehydrogenase; SLCA21, Solute Carrier Family 2 Member 1; MCT, Monocarboxylate transporter; BHB, β-hydroxybutyrate; MRC, mitochondrial respiratory complex; BHBdh, β-hydroxybutyrate dehydrogenase. Created with BioRender.com.

synthesized in the liver through a process called ketogenesis. During ketogenesis, fatty acids are broken down into ketones on an as-needed basis to generate energy (12, 13). This process usually accelerates during extended periods of fasting or CR when glucose is not readily available. In addition, insulin will be reduced creating a surge of fatty acids and ultimately activating ketogenesis (14). Since the brain relies heavily on glucose for energy, it will quickly resort to ketogenesis before any other organ in the body (15). Gliomas, however, are not so quick to convert to this process as they are considered "metabolically inflexible" due to impaired mitochondrial function (5, 10).

The first reported clinical research trial testing the Warburg Effect in patients with GBM was by Nebeling et al. in 1995 (16). The study used the KD to treat two children with advanced-stage brain tumors. The results cumulated from the study showed that a KD might be a possible adjuvant diet intervention with standard cancer therapy and increase patient survival.

The outcomes of this study spurred additional diet intervention research across the globe. Most of the research on the effect of the KD on brain tumors is in pre-clinical research (mouse models). Scientists perform in-depth examinations of the high-fat, low-carbohydrate diet to understand better its effect on brain tumors (gliomas) and survival in mice. However, there are fewer human clinical studies focusing on the KD in treating gliomas, specifically GBM. It is speculated that the few number of clinical trials is due to concerns over quality of life and well-being of terminally ill patients, not just due to diet tolerability and ketosis (5).

As there is a strong need for *de novo* cancer therapies in treating patients with GBM, this review aims to explore whether the KD combined with GBM cancer therapies is manageable and effective. This review will examine pre-clinical and clinical research that used a KD in treating GBM. The outcomes being measured to determine manageability and effectiveness include diet tolerability, tumor response, disease progression, and overall survival.

2. Methods

2.1. Search strategy

A literature search was performed through the Carlyle Campbell Library at Meredith College in Raleigh, North Carolina. A list of relevant key words was curated for both ketogenic diet and glioblastoma. The search included the following key terms: "ketogenic diet" and "glioblastoma," "ketogenic diet" and "gliomas," "calorie restriction" and "glioblastoma," "calorie restriction" and gliomas," "diet intervention" and "glioblastoma," "diet intervention" and "gliomas," and finally, "low-carbohydrate diet" and "glioblastoma," "low-carbohydrate diet" and "gliomas."

2.2. Study selection and data extraction

All initial studies identified from the searches were saved and uploaded into Covidence (RRID:SCR_016484) for title and abstract screening. Duplicate references were removed through the screening process. All eligible studies were reviewed in full by the author. A meta-analysis performed by Klement et al. provided additional screening of selected articles to confirm eligibility (17).

Inclusion criteria for this review included only peer-reviewed, scholarly articles published in English between 2009 and 2019. Study designs were *in vivo* pre-clinical research, patient case studies, randomized controlled trials, and retrospective studies focusing on GBM being treated with a KD. Exclusion criteria included non-peer reviewed articles published before 2009, literature reviews, systematic reviews, meta-analyses, and articles that did not measure glioblastoma, ketogenic diet or low-carbohydrate diet related to tumor response or disease progression.

3. Results

3.1. Summary of search results

A total of 142 articles were identified with 59 duplicates removed. Eight-three abstracts were screened for relevance to measures and outcomes of interest with 56 studies deemed irrelevant. Twenty-seven articles were full text reviewed to confirm eligibility. After further review, 16 articles were considered not eligible thus leaving 11 articles that qualified for this review (Figure 2). Of the 11 articles included for this review, four were animal studies and six were human studies with one study combining both animal and human trial data. The total number of GBM patients treated with a KD, or low-carbohydrate diet, was 76 and the total number of mice was 190 (Table 1).

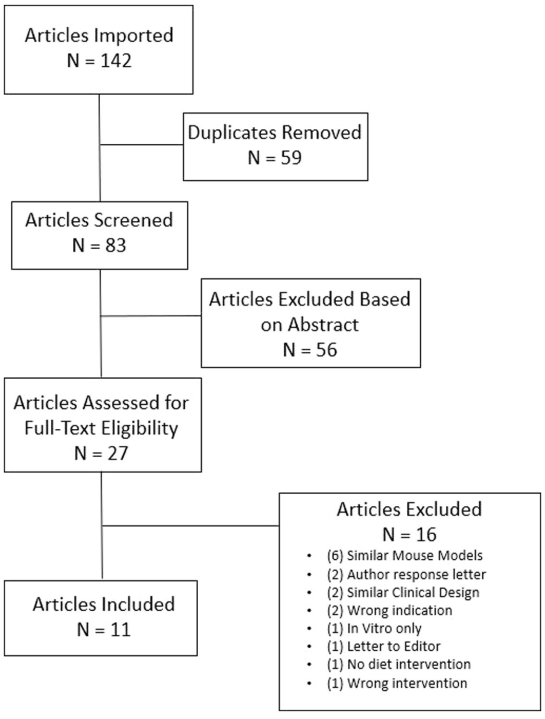

FIGURE 2
Flow diagram of search process. *N* is an abbreviation for number. Searches include: "ketogenic diet AND glioblastoma," "ketogenic diet AND gliomas," "calorie restriction AND glioblastoma," "calorie restriction AND gliomas," "diet intervention AND glioblastoma," "diet intervention AND gliomas," "low-carbohydrate diet AND glioblastoma," "low-carbohydrate diet AND gliomas." All initial studies retrieved from the search were uploaded to Covidence for title and abstract screening and reviewed for eligibility per outcomes being measured for the review.

TABLE 1 Study characteristics of articles investigating the KD in treating GBM.

Author	Country	Study purpose	Study type	Study population	Cell line	Study design	Max duration	Macronutrient composition	Conclusion
Abdelwahab et al. (7)	United States	To investigate the efficacy of a KD using KetoCal® and radiation therapy to treat malignant gliomas.	Mouse	$n = 60$	GL261	Mice implanted with gliomas then randomized to SD or KetoCal® groups with or without radiation.	299 days	72% fat 15% protein 3% carbs	Mice fed KetoCal® alone had increased survival compared to those fed SD. KetoCal® plus radiation demonstrated absence of tumor growth in 80% of mice.
De Feyter et al. (18)	United States	Brain tumor tissue would show reduced oxidative ketone metabolism compared to non-tumorous brain tissue; brain tumor growth would be slower when mice are fed a KD and survival would be longer.	Mouse	$n = 40$	9 L and RG2	Mice implanted with gliomas and randomized to KD or SD; MRI and MRS were used to assess tumor growth.	50 days	91% fat 9% protein 0% carbs	Gliomas can metabolize ketone bodies. No difference observed in ketone oxidation between gliomas and normal brain cells. KD did not demonstrate a positive effect in survival.
Lussier et al. (20)	United States	To investigate an unrestricted KD in alleviating tumor immune suppression in a malignant glioma.	Mouse	$n = 34$	GL261	Mice implanted with gliomas and depleted of CD8 T cells; Randomized to 4:1 KetoCal® or SD; bioluminescence to measure tumor burden.	60 days	72% fat 15% protein 3% carbs	A KD may be feasible by reducing immune suppression and promoting immune mediated killing of the glioma. The use of a KD as adjuvant therapy with current and newer therapies for treating gliomas is supported by this data.
Mukherjee et al. (19)	United States	To determine if a KD paired with a glutamine antagonist could suppress tumor growth and prolong survival.	Mouse	$n = 24$	VM-M3 and CT-2A	Mice received KetoGEN with calorie restriction and glutamine antagonist.	15 days	89.2% fat 8.7% protein 2.1% carbs	The benefits of using a glutamine antagonist in combination with a restricted KD can stop glioma cell growth and promote survival in mice.
Rieger et al. (24)	Germany	To investigate if KD affected efficacy of bevacizumab.	Mouse	$n = 32$	U87MG	Mice randomized to KetoCal® or SD and given chemotherapy twice a week.	28 days	72% fat 15% protein 3% carbs	KD showed strong effect to chemotherapy and affecting ATP levels in the tumor.
Elsakka et al. (10)	Egypt	To demonstrate a KD with hyperbaric oxygen therapy and calorie-restriction enhance metabolic efficiency in normal brain cells while inhibiting tumor metabolism.	Human	$n = 1$ 38-year old male	GBM	Individual case study of calorie-restricted KD with hyperbaric oxygen therapy, radiation, and chemotherapy. MRI and MRS to measure tumor growth and assess metabolites.	20 months	71% fat 22% protein 7% carbs 900 kcals/day	KD reduced lactic acid fermentation that inhibited tumor growth as well as reduced inflammation and edema in the brain. This environment reduced tumor invasion after surgery. Increased ketone bodies fueled and protected the patient's normal cells from the oxidative stress of radiation/chemotherapy treatment.

(Continued)

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TABLE 1 (Continued)

Author	Country	Study purpose	Study type	Study population	Cell line	Study design	Max duration	Macronutrient composition	Conclusion
Woodhouse et al. (5)	United States	To assess the feasibility and safety of the MAD in attaining ketosis in patients with gliomas.	Human	*n* = 29 males = 17 females = 12 ages = 30–76	GBM grade II-IV	Retrospective study that assessed medical records for patients with documented ketone values who received the MAD.	24 months	<20 g carbs/day	MAD is feasible and safe during radiation and chemotherapy treatment for patients with gliomas. Ketones may be a radiation sensitizer especially in non-methylated GBMs.
Zuccoli et al. (1)	Italy	To determine if a restricted KD combined with standard cancer therapy is effective in managing tumor growth.	Human	*n* = 1 65-year-old female	GBM grade IV with MGMT gene	Individual case study of a patient who completed a calorie-restricted KD (KetoCal®) and chemoradiation therapy.	13 months	72% fat 15% protein 3% carbs 600 kcals/day	GBM treated with a restricted KD showed evidence of rapid regression of disease following surgery.
Artzi et al. (11)	Israel	To detect and characterize changes in brain metabolites in patients with high grade gliomas treated with a KD using ^{1}H-MRS.	Human	*n* = 9 male = 5 female = 4 ages = 27–69	Gliomatosis Cerebri and GBM	Patients with GBM treated with KetoCal® and chemotherapies, then scanned with ^{1}H-MRS every other month to detect ketone metabolism until off study.	31 months	72% fat 15% protein 3% carbs	Two out of 5 patients with KetoCal® had brain tissue ketone detection. Patient with gliomatosis cerebri had a clear shift from glucose to ketone metabolism with KD alone. Inconclusive findings in other three patients. One patient had ketone detection within the tumor. Diet compliance was intermittent.
Champ et al. (8)	United States	To assess the safety of a KD, and its effect on glucose levels, when used as adjuvant therapy with radiation and chemotherapy.	Human	*n* = 6 ages 34–62	GBM grade III-IV	Retrospective medical record review of patients with high grade gliomas with documented glucose levels and serum ketone levels who consulted the treating physician about the KD as a form of treatment.	12 months	77% fat 15% protein 8% carbs	KD is considered safe and well-tolerated as an adjuvant therapy to cancer therapies. Restriction of carbohydrates to reduce glucose levels may improve treatment response and overall survival. The average time on diet was 7 months. 2 out of 6 patients had tumor recurrence.
van der Louw et al. (23)	Netherlands	Assess the feasibility and safety of the KD as a treatment option for patients with recurrent GBM.	Human	*n* = 10 male = 9 females = 1 ages = 33–65	GBM	Open-label, non-randomized study of recurrent GBM after surgical resection and chemoradiation. Patients received liquid and solid KDs.	14 weeks	Liquid: 88.8% fat 8.2% protein 2% carbs Solid: 88.4% fat 7.6% protein 8% carbs	60% diet compliance. Ketosis was reached within a week with no major side effects. The use of a KD as adjuvant therapy to standard cancer treatment, is feasible and safe in patients with GBM.
Rieger et al. (24)	Germany	To investigate the safety and tolerability of a KD in patients with recurrent GBM.	Human	Human: *n* = 20 male = 7 females = 13 ages = 30–72	Human = GBM	Open-label, pilot study with recurrent GBM greater than 6 months post-surgery and greater than 3 months post-radiotherapy.	16 weeks	Human: <60 g carb/day	KD is safe and tolerable in treating patients with GBM. Ketosis is not achieved indicating a genetic or unknown factor affecting the patients from achieving this state.

n, number; KD, ketogenic diet; SD, standard diet; ^{1}H-MRS, proton magnetic resonance spectroscopy; MAD, modified Atkin's die

astoma multiforme; MRS, magnetic resonance spectroscopy.

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3.2. Analysis of the KD to treat mice with GBM

Since using the KD in treating patients with GBM is still exploratory, most diet intervention research is conducted in mouse models to understand how the diets affect gliomas. Specifically, to the KD, many variations have been tested in mice to determine efficacy. Of the five mouse studies examined, variations included a liquid KD, calorie-restricted KD (CR-KD), and an unrestricted KD. Even with the studies utilizing different percentages of fat, protein, and carbohydrates, the ratio stayed around 4:1 (4 grams of fat to 1 gram of protein and 1 gram of carbohydrates). The macronutrients averaged 80% fat, 15% protein, and 5% carbohydrates across the animal studies (7, 18–20).

Tumor response while treated with a KD is a significant outcome to measure in mice. Most of the animals treated with a KD showed noteworthy results regarding tumor response. For instance, Abdelwahab et al. and Lussier et al. reported increased tumor response in mice treated with the KD compared to a standard diet (SD) (7, 20). Abdelwahab et al. included radiation treatment with either the KD or SD, which simulates typical treatment in humans. Both researchers also used a liquid formula called KetoCal® that is generally provided to patients with epilepsy to help minimize seizures.

Nine out of 11 mice that Abdelwahab et al. treated with radiation and KetoCal® showed satisfactory tumor response with no evidence of tumor recurrence for 104 days. Tumor burden was measured using bioluminescence with injections of luciferase prior to imaging. One mouse in the KetoCal® without radiation group experienced no tumor burden for 200 days before sacrifice. The tumor response for all mice on the KD, with or without radiation, was more compelling than the SD groups with the same treatment. These results display a possible correlation that the KD could enhance tumor response for patients when receiving radiation treatment.

Lussier et al. investigated whether an unrestricted KD could enhance immunotherapy treatment in mice implanted with GL261-Luc2 tumor cells (20). The mice were depleted of CD8 + T lymphocytes before tumor implantation and diet intervention to decrease immune function. Researchers designed four treatment groups that received either a KD or an SD with or without CD8 + T cells. Both groups on the KD with or without CD8 + T cells had decreased tumor growth reported by bioluminescence compared to both SD groups. Of note, the mice in the KD group with CD8 + T cells exhibited increased CD8+ T cell production that infiltrated the tumor. These results support the notion that a KD can enhance immune function and tumor recognition when treated with immunotherapies.

Since several mouse models support the effectiveness of a KD as a treatment for gliomas, the replication of these outcomes continued to reinforce the theory of the Warburg Effect (5, 7–11). However, in a study by De Feyter et al. ketones were found in glioma cells wavering that gliomas can metabolize ketones (18).

De Feyter et al. tested whether gliomas could utilize ketone bodies for energy production *in vitro* and *in vivo*. In specific, 9 L and RG2 tumor cells were tested. Mice received either a KD with CR or an SD. The researchers incorporated CR to induce high levels of ketones in the brain.

In vitro, De Feyter et al. exposed RG2 tumors to high levels of ketones and low levels of glucose. Analysis showed the cells demonstrated β-hydroxybutyrate oxidation but at a low rate. This finding indicated that at least the RG2 cell line may be able to metabolize ketones. When the same *in vitro* technique was applied to the 9 L cell line, the tumor cells could not metabolize ketones.

On the contrary, the animal studies that De Feyter et al. performed showed different results from the *in vitro* experiments. Both 9 L and RG2 cell lines implanted into the animals in the KD with CR group showed a high presence of ketones when compared to the SD group. De Feyter et al. investigated MCT1 since this is a transporter that allows the passage of lactate, pyruvate, and ketones into a cell. The researchers acknowledged a "stronger MCT1 immunoreactivity was observed in the RG2 gliomas when animals were fed the KD" (18).

Due to the discrepancies between the animal studies and cell cultures, the *in vitro* testing was repeated, exposing cells to low glucose with ketones or just low glucose. The second analysis showed that neither group demonstrated defined β-hydroxybutyrate oxidation.

The animals were examined for the effectiveness of the intervention on tumor growth and prolonged survival. De Feyter et al. reported the diet intervention did not affect tumor growth or overall survival based on imaging results and immunostaining for Ki-67 (a tumor proliferation biomarker). This discovery may be a case to reject the Warburg Effect theory indicating that glioma cells could use glycolysis or β-hydroxybutyrate oxidation for energy production and proliferation.

Further exploration into other forms of cancer metabolism was examined by Mukherjee et al (19). Their testing expanded beyond glycolysis and β-hydroxybutyrate oxidation in gliomas. Several references noted by Mukherjee et al. mention that gliomas utilize glucose and glutamine for energy production. They further conclude that cancer therapies increase glutamine in tumor cells allowing the cells to utilize the glutamine-glutamate cell cycle to generate energy readily.

Mukherjee et al. experimented with VM-M3 and CT-2A cell lines, which differed from the cell lines De Feyter et al. examined. According to Mukherjee et al., these cell lines have higher incidences of spontaneous and rapid growth throughout the brain, and the reason for use in the experiment. The researchers also used 6-diazo-5-oxo-L-norleucine (DON), which inhibits several enzymes and metabolic pathways, including the enzyme required for glutaminolysis, in gliomas. Concurrently, the mice received a CR-KD to reduce glucose further (19).

After three consecutive experiments using an SD with or without DON or a CR-KD with or without DON on VM-M3 cells, the mice Mukherjee et al. treated with the intervention and DON had the highest tumor response and overall survival compared to both control groups (44% compared to 19%). These findings indicate that the KD assisted in decreasing glucose in combination with the glutamine antagonist to stop tumor growth. In the CT-2A cells, the groups that received the intervention with DON saw reductions in cell growth and increased cell arrest. These results support the Warburg Effect theory and suggest that glutamine is vital in glioma survival. In the case of De Feyter et al., this research could speculate that specific cell lines of gliomas are potentially susceptible to β-hydroxybutyrate oxidation or are better equipped to adapt to their changing environment to survive.

Even though tumor response is a primary outcome of many mouse models to determine the effectiveness of a treatment or

intervention, prolonged survival is just as crucial as a secondary objective. Once implanted with glioma cells, mice typically only survive 15 to 18 days without treatment or intervention (7). For a mouse to survive beyond this time is considered a significant finding. The mouse research discussed in this review demonstrated that after implantation with gliomas and exposure to a KD, with or without other interventions, survival beyond 18 days was observed to support prolonged survival with a KD as therapy.

Many mouse models provide enough support for the Warburg Effect theory and that the KD is an effective treatment for gliomas. The KD combined with radiation therapy may be an ideal adjuvant treatment for patients with GBM. With these favorable mouse results, research branched out into the clinical setting. Can these same favorable results be replicated in clinical trials and demonstrate that a KD is a beneficial and effective therapy for patients?

3.3. Analysis of the KD to treat patients with GBM

In the seven human trials examined for this review, tumor response, disease progression, and overall survival were the primary outcomes to determine efficacy. These outcomes were similar in the mouse models, but, the results varied and were less prominent in the human trials. One possible reason is that mice have no choice in which diet or treatment they are given, and their environment is easily controlled and manipulated for conformity to the intervention. Human trials are more complex than mouse studies because multiple variables exist, and human genetics may be an outlier in treatment intervention. But simply, safety and tolerability are the main factors when experimenting with diet therapy in terminally ill patients.

The first study using a KD as an intervention in an adult patient with GBM was presented by Zuccoli et al (1). The patient in the single-case study was administered a CR-KD and reported no issues or complaints. However, laboratory values indicated a safety concern. In this intervention, a patient underwent treatment for GBM that included surgery, radiation, and the drug temozolomide (TMZ). The patient was given a KD that consisted of 600 kcals/day before starting radiation therapy and chemotherapy.

After 35 days, the diet changed to CR only due to high uric acid levels posing a safety concern. Even with stopping the KD and only continuing the CR, the patient did not exhibit tumor recurrence until 3 months after stopping the CR (1). This study supports one patient experienced diet tolerability, but ketoacidosis is still a safety concern. Yet, many of the articles included in this review support high levels of ketones for optimal results with standard cancer therapies.

As clinical trials start to show favorable tolerability results, a small retrospective study conducted by Champ et al. evaluated the safety of the KD and its effect on glucose levels (8). The study looked at patients with grade III-IV GBM who were given a KD with chemoradiation between March 2010 to April 2013. The patients completed the KD before, during, and after chemoradiation treatment. In addition to MRI data to verify tumor response and disease progression, they used the Revised Assessment in Neuro-Oncology (RANO) scale to provide additional data on tumor response and overall survival. The RANO scale was developed around 2011 and uses qualitative and quantitative analysis of MRI results to measure tumor size in response to treatments (22).

Champ et al. indicated that six patients out of 53 qualified for the analysis as determined by verifiable blood glucose values and being on a KD. The average blood glucose value prior to starting the KD was 127.7 mg/dl and dropped to 92.3 mg/dl during treatment. Moreover, three out of six patients received steroids during the treatment that may have adversely affected glucose levels. The control included patients on an SD who received chemoradiation therapy during the same period. The SD group had an average blood glucose value of 122 mg/dl during radiation therapy.

Even with only minor decreases in glucose levels, Champ et al. reported no adverse events or diet intolerability. However, only four out of six patients had confirmed ketosis by urine tests. For ketosis to be verifiable, blood glucose values must be <100 mg/dl and urine ketones ≥2 mmol/L (11, 17). Furthermore, Champ et al. mentioned that one patient included in the analysis opted to do CR with a KD. This patient had confirmed disease progression after 1 month of the diet therapy, but the disease later resolved without further requirement of chemoradiation through 12 months post-intervention.

Champ et al. further concluded that the average survival rate of the patients observed was 14 months post-treatment and intervention, and the average time to progression was around 10 months. These results support the KD affecting glucose levels in gliomas, even without the patient reaching a state of ketosis, for improved tumor response to cancer treatments.

Another notable clinical trial by van der Louw et al. involved a cross-over study design examining patients with GBM on two types of KD (23). The diet intervention began with liquid KetoCal® (4:1 ratio) at 2,400 kcals/day for 8 weeks, followed by a solid KD (2:1 ratio, which is 2 grams of fat to 1 gram of protein and 1 gram of carbohydrates) at 2,835 kcal/day for an additional 6 weeks. The study focused on safety and tolerability of a liquid KD versus a solid KD with chemoradiation. Van der Louw et al. determined tolerability would be achieved if patients had 60% diet compliance over the 14 week-intervention by reviewing food diaries. Safety was monitored by levels of glucose and urine ketones. Coping questionnaires were provided to the patients to measure their quality of life while on the diet.

Van der Louw et al. enrolled 11 patients but only nine achieved full ketosis as determined by urine testing. Six of the nine patients maintained diet compliance while completing the study, and four continued the KD while receiving a second chemoradiation treatment. No adverse events were reported during the study. However, responses to the coping questionnaire indicated that patients, and their partners, found the solid KD intervention challenging to maintain. Patients also reported nutrition counseling during the study was needed for motivation to continue the diet therapy rather than for support to maintain defined calories and macronutrient percentages.

Even with low coping scores, van der Louw et al. reported that the average survival of patients on the diet intervention was 12.8 months. This data supports that a KD is safe and mildly tolerable but does improve life expectancy this data supports that a KD is safe, mildly tolerable, and that diet therapy may improve life expectancy (2, 5). If patients with GBM can overcome the difficulties of maintaining a KD, this may allow enough time for ketones to develop to suppress tumor growth and protect brain tissue from chemoradiation (5, 10). Better response rates to cancer treatments decrease disease progression and increase survival.

In 2007, a pilot study was conducted by Rieger et al. that focused on feasibility of a KD therapy for patients with GBM (24). The study concluded in 2011; therefore, is included in this review. Rieger et al. evaluated the safety and tolerability of using a KD without CR in GBM patients who had disease progression. This study was intriguing as it included multiple aims and multiple qualitative and quantitative measurements in the study design.

The patients included in the study had to have shown disease progression within 6 months of surgery and within 3 months of receiving radiation or relapse during or after chemotherapy. Patients were placed on a low-carbohydrate diet with meal planning and recipes to guide them. The patients were allowed to eat to satiety but could only consume at least 60 g/day of carbohydrates. In addition, patients had options to consume yogurt drinks with a 3:2:1 ratio of grams of fat to protein to carbohydrates as needed.

Of the 20 patients enrolled, Rieger et al. reported that three patients discontinued early due to diet intolerability. Eight patients stayed on the diet intervention while undergoing additional cancer therapy. The remaining patients stayed on the diet intervention through the efficacy period but later stopped the diet. At least 92% of the patients had confirmed urine ketosis at least once during the intervention. Moreover, two patients had stable disease (no progression) after 6 weeks on the diet, while one presented with a minor tumor response considered related to the diet.

Rieger et al. also evaluated overall survival; the average survival after therapy was 32 weeks. These results are slightly less encouraging than van der Louw et al., who reported an overall survival of about 12.8 months, and Champ et al., who reported survival through 14 months. As Rieger et al. indicated, some of the patients discontinued the intervention thus possibly contributing to the lower average in survival. This information may indicate that a low-carbohydrate diet induces ketosis for better tumor response to treatments.

Another cross-over study by Elsakka et al. investigated a CR-KD followed by an unrestricted KD in treating a patient with low-grade GBM (10). This study added hyperbaric oxygen therapy (HBOT) that generates oxidative stress on the glioma. The CR-KD was a 4:1 ratio with 900 kcal/day; at 9 months, the diet switched to a KD without CR of 1,500 kcal/day. In addition, the patient received a total of 20 sessions of HBOT post-surgery.

Elsakka et al. reported that the 38-year-old patient had a partial resection of a low-grade glioma after a 21-day CR-KD. The patient was further treated with chemoradiation followed by TMZ for 6 months. Afterward, the patient switched to the unrestricted KD for 10 months. The patient's ketone levels averaged 2 mmol/L, and glucose levels averaged 65 mg/dl. At 24 months, the MRI results showed that the residual tumor decreased in size by 1.5 cm, and the patient was still alive with no disease progression. These results suggest that a cross-over CR to unrestricted KD in combination with HBOT may enhance tumor response and increase survival. The downside is that low-grade gliomas are noted to have better treatment responses than higher-grade gliomas (11).

In 2019, a different diet intervention emerged in treating patients with GBM. As there were still concerns about patient tolerability and safety, Woodhouse et al. experimented with a modified Atkin's diet (MAD) (5). The study assessed if MAD could aid cancer therapy to reduce repeated exposure to chemoradiation. This cancer treatment is known for causing pseudoprogression. Pseudoprogression is swelling and inflammation in the brain and around the tumor due to repeated doses of chemoradiation that can lead to decreased survival (3).

Woodhouse et al. enrolled 29 patients between the ages of 30 and 77. The MAD was a 1:2 ratio of fat to protein with <20 g/day of carbohydrates. The purpose of the 1:2 ratio was to test a more manageable diet that patients could tolerate while allowing them to achieve ketosis to reduce inflammation.

Woodhouse et al. defined ketosis as the presence of ketones >1 mmol/L, which was lower than the requirements for the study conducted by van der Louw et al. Even with 79% of the patients achieving ketosis by this value, 17% had disease progression. However, overall survival showed that 26.7% of the patients were still alive 2 years post-treatment. This survival time was the longest reported in all clinical trials included in this review. These results support that dietary interventions with calorie or carbohydrate restriction can be tolerated and could increase overall survival in terminally ill patients.

4. Discussion

4.1. Limitations in determining the effectiveness of a KD in treating patients with GBM

The pre-clinical and clinical research articles compiled for this review demonstrate support for the KD in treating patients with GBM. Mice demonstrated positive tumor responses to the KD, with some mice experiencing prolonged survival. The handful of clinical trials or human research studies showed positive results toward tolerability, tumor response, and prolonged survival. However, the methods used in these studies were equivocal as definitive support for a KD as adjuvant therapy to cancer treatment. In the pre-clinical and clinical studies, the glioma cell lines, diet interventions, glucose levels, and ketone levels were not all consistent to draw a valid correlation (Table 2). Other factors, such as genetics and glioma grade, may affect treatment efficacy with diet and drug interventions.

Several cell lines of gliomas can be tested in cell culture and mouse models. However, animal research is expensive and cumbersome. Specific cell lines of gliomas are more expensive than others, especially the aggressive cell lines common in humans (25). Scientists may need more funding to acquire these aggressive cells for adequate research. For example, most of the cell lines used in the mouse studies were GL261, 9 L, and RG2. The most aggressive human glioma cell lines, not used in the mice, are LN229, SNB19, U87, and U251.

According to Burden-Gulley et al., the best comparable glioma cell lines are LN229 and CNS-1 or any other cell lines obtained from human cancer tissue (26). Without testing these more human-relatable cancer cells, the effectiveness of a KD in patients with GBM is not adequately represented for reproducibility. Researchers should consider using genetically engineered mice that may enhance results that are reproducible in humans (27).

Shelton et al. conducted research in mice using highly invasive VM-M3 and CNS-1 glioma cells that could represent more

TABLE 2 Summary of Limitations and Inconsistencies Between Pre-Clinical and Clinical Research Treating GBM.

Author	Country	Study type	Diet intervention	Limitations	Inconsistencies observed between studies	Advantages
Abdelwahab et al. (7)	United States	Mouse	KetoCal®	• High glucose levels • Low to moderate ketone levels • Non-aggressive cell line • Intervention started 4 days after implantation	• Different glioma cell line	• Used radiation representative of standard cancer treatment seen in clinical setting
De Feyter et al. (18)	United States	Mouse	91% fat diet with 0% carbs	• High glucose levels • Non-aggressive cell line	• Different glioma cell line • Different macronutrient diet • Gliomas were able to utilize ketones • No effect on survival	• Intervention started 7 days post-implantation that would better represent late-stage tumor growth as seen in humans • High ketone levels
Lussier et al. (20)	United States	Mouse	KetoCal®	• Intervention started 4 days after implantation • Glucose and ketone levels not reported	• Focused on immunotherapy • Used immunodeficient mice • Measured tumor response but not overall survival	• None
Rieger et al. (24)	Germany	Mouse	KetoCal®	• Glucose levels not reported • Low ketone levels	• Different glioma cell line	• Intervention started 7 days post-implantation that would better represent late-stage tumor growth as seen in humans • Used chemotherapy representative of standard cancer treatment seen in clinical setting
Mukherjee et al. (19)	United States	Mouse	KetoGEN	• Intervention started 4 days after implantation • High glucose levels • Low ketone levels	• Used DON • Different glioma cell line • Used calorie restriction with KD • Different macronutrient diet	• Glutamine antagonist helped stop tumor growth in combination with KD
Elsakka et al. (10)	Egypt	Human	Comparable to KetoCal® used in mice	• Single-case study • Added another therapy that may not be available in all clinical settings	• Used calorie restriction and water fast with KD • Used HBOT	• Intervention reduced inflammation and edema in the brain • Low glucose levels • High ketone levels
Woodhouse et al. (5)	United States	Human	MAD	• Glucose levels not reported • Retrospective study • Some patients had MGMT gene	• Different macronutrient diet • Intervention only used during chemotherapy • Some patients had low-grade GBM	• Moderate to high ketone levels • Reported diet as tolerable
Zuccoli et al. (1)	Italy	Human	KetoCal®	• Single-case study • Patient had MGMT gene • Patients received steroid treatment	• Used calorie restriction and water fast with KD • Switch patient to calorie restriction only	• Low glucose levels • Moderate to high ketone levels • Reported tumor response and survival

(Continued)

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TABLE 2 (Continued)

Author	Country	Study type	Diet intervention	Limitations	Inconsistencies observed between studies	Advantages
Artzi et al. (11)	Israel	Human	KetoCal®	• Small sample size • Patient with low-grade glioma demonstrated favorable response to treatment • Glucose levels not reported • Not all patients were consistent with intervention	• Patients started intervention at different times in relation to chemotherapy • Treated a patient without GBM and included in results	• High ketone levels
Champ et al. (8)	United States	Human	Comparable to KetoCal® used in mice	• Small sample size • Retrospective study • Moderate to high glucose levels • Low ketone levels • Patients received steroid treatment	• Not all patients had reported ketone levels • Patients started intervention at different times in relation to chemotherapy	• Diet reported as tolerable • Reported overall survival and tumor response with RANO
Rieger et al. (24)	Germany	Human	< 60 g carbs/day	• Pilot study • Patients received steroid treatment • Low ketone levels • Moderate to high glucose levels	• Different macronutrient diet • Reported genetics or unknown factors affected inability to achieve ketosis	• Diet reported as tolerable
van der Louw et al. (23)	Netherlands	Human	KetoCal® and 2:1 ratio KD	• Small sample size • Non-randomized • 60% diet compliance • Moderate to high glucose levels	• Used two different types of KD • Different macronutrient diets	• High ketone levels

KD, ketogenic diet; GBM, glioblastoma multiforme; HBOT, hyperbaric oxygen therapy; DON, 6-diazo-5-oxo-L-norleucine; MAD, modified Atkins diet; RANO, Revised Assessment in Neuro-Oncology; MGMT, O6-methylguanine-DNA methyltransferase.

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reproducible results for patients with GBM (28). The results of their research provided support for the genetic and metabolic characteristics of human gliomas.

Of the mouse models included in this review, Mukherjee et al. tested the VM-M3 and CT-2A cells in mice. These gliomas were targeted as comparable cell lines for reproducing diet interventions and cancer therapy in patients with GBM. In addition, Mukherjee et al. also started the KD seven days after implantation of the gliomas to provide a more realistic biomarker for human disease onset. Some of the mouse models started the KD before tumor implantation and others immediately after recovery from the implantation (17). The presence of high ketones and low glucose to a newly implanted tumor may have a significant effect on tumor growth in an *in vivo* environment. Early glioma development in a human with high ketones and low glucose is most likely a rare occurrence.

Just as in mice, there are many factors that can affect the effectiveness of a KD in treating patients with GBM. Of the articles reviewed, a handful indicated that some patients with GBM had a genetically modified strain. This variation is the presence of a promoter gene called O6-methylguanine-DNA methyltransferase (MGMT) that turns off transcription for DNA repair in tumors when exposed to radiation or chemotherapy (5, 29). If MGMT genes are present in patients, the glioma will respond to chemoradiation with or without a diet intervention (30). This modified version of GBM can elicit a false positive result in diet intervention therapies with cancer treatment.

Another inconsistency observed in the pre-clinical research was the lack of standard cancer therapy. All but two of the mouse models did not use any standard cancer treatment (radiation or chemotherapy) to treat the mice. The KD was the only intervention used to treat the gliomas. In patients, this will not be a likely scenario. Patients diagnosed with GBM will most likely undergo treatment with surgery, radiation, and chemotherapy (1, 3, 5, 8, 10, 23). As the mouse models mostly used non-human cell lines, and researchers can control an animal's diet and environment, this monotherapy intervention may be suitable for mice but is not an accurate representation of real-life events.

Only one study was found for this review that evaluated a patient who was solely treated with a KD. Artzi et al. assessed ketone metabolites in the brain of patients with gliomas when treated with a KD (11). Out of the nine patients in the experiment, only one did the KD alone without standard cancer therapy. This intervention may have been chosen or recommended because the patient had a low-grade glioma that cannot be treated with the same GBM cancer therapy. This patient did maintain stable disease throughout the diet intervention. However, the KD alone did not decrease the tumor size or prolong survival.

The primary outcome assessed by Artzi et al. was to determine if gliomas can metabolize ketones. As most clinical KD studies monitored ketone levels *via* urine tests, this study experimented whether urine ketones could accurately represent total cerebral ketones. The study used proton magnetic resonance spectroscopy (^{1}H-MRS) to take images of the brain to determine if ketones exist either in the brain or in the tumor. Artzi et al. showed three patients maintained high levels of ketones (at least 2 mmol/L) according to urine tests, but ^{1}H-MRS detected no ketones in the brain.

Mouse models show favorable results with average serum ketone levels of 1.9 mmol/L. The urine ketone levels in patients averaged 1.8 mmol/L. The inconsistent ketone testing between both types of research leads to a mixed review on this topic. Based on the compiled information between the pre-clinical and clinical interventions, the KD may be more effective in treating GBM if patients can safely achieve serum ketone levels of 3 mmol/L or higher.

Ketone levels were one of the inconsistent findings making the effectiveness of this intervention challenging to assess. Glucose values were also not consistent (Table 3). As the low-carbohydrate and high-fat diets were aimed to deplete circulating glucose levels available to gliomas, not all mouse models included these values. For the studies that did, the glucose levels were between 90 and 160 mg/dl. It was not discussed if the levels in mice were comparable to human levels.

When addressing glucose levels in patients with GBM, it should be discussed that some cancer therapies can increase glucose and glutamine levels in the brain. This increase can make it problematic to use the KD to deplete enough glucose while maintaining patient safety and diet tolerability. Patients with GBM may experience inflammation and swelling of the brain as part of radiation therapy (2–4). The increased swelling can lead to seizures and other neurological dysfunctions that can be detrimental to the patient (5). When this occurs, doctors prescribe steroids to control the swelling and inflammation (31). This is an unfortunate situation for the KD since steroid use increases glucose levels defeating the purpose of the KD (17).

Finally, many of the KDs implemented in these experiments used relatively low percentages of protein in addition to carbohydrates. Even though glucose is a primary driver of cancer metabolism, amino acids also have a strong presence in cancer cells to allow them to thrive. Many tumor cells rely on leucine, methionine, glutamine, and arginine to regulate growth and promote survival through cellular pathways such as mTOR and PI3K (32, 33). Since the average percentage of protein consumed in both the animal and human studies was around 14%, a decrease in amino acids may be a confounding factor for tumor response with the diets.

5. Conclusion

GBM continues to be one of the deadliest brain tumors affecting people of all ages across the world. The current treatment regimens are not proving to be effective in preventing disease progression or prolonging survival. As patients with GBM typically survive about 15 months after diagnosis, GBM is one of the most researched cancers to find better and safer treatments. With the current therapy consisting of surgery, radiation, and chemotherapy, this course of treatment exposes patients to further brain damage.

Since the 1920s, doctors and scientists have tested CR diets and KDs for treating many diseases and disorders. For example, the KD is well known for being studied in patients with epilepsy to help control seizures (7, 21). This phenomenon of ketones aiding in treatments is centuries old and continues to be explored in bounteous science disciplines. Cancer therapies and diet interventions display a defined connection to Otto Warburg and the Warburg Effect.

TABLE 3 Glucose, ketone, and insulin levels while using the KD in treating GBM.

Author	Country	Study type	Glucose levels	Ketone levels (BHB)	Insulin levels	KD methods
Abdelwahab et al. (7)	United States	Mouse	Post-implantation Day 6 KD = 160 mg/dL KD + Radiation = 140 mg/dL Day 13 KD = 150 mg/dL KD + Radiation = 160 mg/dL	Post-implantation Day 6 KD = 1.3 mmol/L KD + Radiation = 2.2 mmol/L Day 13 KD = 1.2 mmol/L KD + Radiation = 1.6 mmol/L	Not reported	• SD for 3 days post-implantation. • Randomized to stay on SD or start KD with or without radiation on day 4. • Radiation given to respective groups on days 3 and 5 post-implantation.
De Feyter et al. (18)	United States	Mouse	Post-implantation Day 11 90 mg/dL Day 15 90 mg/dL Day 18 115.2 mg/dL	Post-implantation Day 11 3.5 mmol/L Day 15 3.0 mmol/L Day 18 2.5 mmol/L	Not reported	• KD started 7 days post-implantation.
Lussier et al. (20)	United States	Mouse	Not reported	Not reported	Not reported	• SD for 3 days post-implantation. • Randomized to stay on SD or start KD on day 4.
Rieger et al. (24)	Germany	Mouse	Not reported	KD alone: 1.7 mmol/L KD + chemotherapy: 1.4 mmol/L	Not reported	• Randomized to KD or SD 7 days after implantation. • Chemotherapy with diets started 12 days after implantation and given twice per week.
Mukherjee et al. (19)	United States	Mouse	KD-R: 90 mg/dL KD-R + DON: 100 mg/dL	KD-R: 1.3 mmol/L KD-R + DON: 1.2 mmol/L	Not reported	• Restricted KD started 4 days after implantation. • DON given at day 6, 8, 10, 12
Elsakka et al. (10)	Egypt	Human	Pre KD: 89 mg/dL Post-Surgery with KD: 72 mg/dL 3 monthS = 64 mg/dL 9 months = 75 mg/dL 15 months = 71 mg/dL 20 months = 65 mg/dL	Pre KD: Not reported Post-Surgery with KD: 3 mmol/L 3 months = 3 mmol/L 9 months = 0.7 mmol/L 15 months = 0.5 mmol/L 20 months = 0.7 mmol/L	Pre KD: 13.10 uIU/mL Post KD: 6.50 uIU/mL 3 months = 5.00 uIU/mL 9 months = 4.10 uIU/mL 15 months = 3.80 uIU/mL 20 months = 2.11 uIU/mL	• Water fast for 3 days prior to surgery. • Restricted KD for 21 days post water-fast prior to surgery. • HBOT started 2 weeks post-surgery. • No steroids administered but anti-seizure medications prescribed.
Woodhouse et al. (5)	United States	Human	Not reported	3 patients = ≥3 mmol/L 4 patients = 2.5 mmol/L 6 patients = 1.5 mmol/L 5 patients = 0.8 mmol/L 11 patients = 0.3 mmol/L	Not reported	• MAD started before chemoradiation therapy. • 6 weeks of MAD during chemoradiation therapy.

(Continued)

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TABLE 3 (Continued)

Author	Country	Study type	Glucose levels	Ketone levels (BHB)	Insulin levels	KD methods
Zuccoli et al. (1)	Italy	Human	Before fasting/diet: 135 mg/dL At end of first fast: 90 mg/dL At end of second fast: 72 mg/dL At end of restricted KD: 63 mg/dL	Before fasting/diet: 0.0 mmol/L At end of first fast: 1.75 mmol/L At end of second fast: 2.5 mmol/L At end of restricted KD: 2.5 mmol/L	Not reported	• Steroids and anti-seizure medications started prior to surgery and discontinued 30-days post-surgery. • Patient did 2-day water fast post-surgery. • Started KD and fasted 3 more days. • Restricted KD for 14 days prior to chemoradiation therapy. • Changed to calorie restriction only diet.
Artzi et al. (11)	Israel	Human	Not reported	Patient #1 and #4= >3 mmol/L Patient #2 and #5 = 3 mmol/L Patient #3 = 2 mmol/L	Not reported	• Patients #2, #3, and #5 started KD at same time as chemotherapy. • Patient #4 started KD a few months after chemotherapy. • Patient #1 was only treated with KD for GBM. • Patients #2, #3, and #4 received steroid treatment during KD with chemotherapy. • Patients #2, #4 and #5 were intermittent with KD.
Champ et al. (8)	United States	Human	Time of Surgery: Patient #1 = 78 mg/dL Patient #2 = 146 mg/dL Patient #3 = 152 mg/dL Patient #4 = 135 mg/dL Other two patients not reported Max During Radiation: Patient #1 = 99 mg/dL Patient #2 = 90 mg/dL Patient #3 = 77 mg/dL Patient #4 = 103 mg/dL Other two patients not reported.	1 patient had 0.8 mmol/L 1 patient had 0.3 mmol/L All other patients not reported.	Not reported	• Patient #1 started KD before chemoradiation • Patient #2, #3 and #4 started KD during chemoradiation. • Patient #5 and #6 started KD after chemoradiation. • Patients #1, #2 and #4 received steroids during treatment.
Rieger et al. (24)	Germany	Human	Average Before KD: 99 mg/dL Average During KD: 92 mg/dL	7 patients = 1 mmol/L 5 patients = 0.8 mmol/L 2 patients = 0.4 mmol/L 1 patient had 0.3 mmol/L 1 patient was not reported	Not reported	• 8 patients started steroids before KD • 11 patients started steroids during the KD

(Continued)

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TABLE 3 (Continued)

Author	Country	Study type	Glucose levels	Ketone levels (BHB)	Insulin levels	KD methods
van der Louw et al. (23)	Netherlands	Human	Phase A (liquid diet): 5 patients 85 mg/dL 2 patients 80 mg/dL 2 patients 90 mg/dL Phase B (solid diet): 7 patients 90 mg/dL 1 patients 100 mg/dL 1 patient not reported	Phase A (liquid diet): 1 patient = 5 mmol/L 4 patients = 4 mmol/L 4 patients = 3 mmol/L Phase B (solid diet): 4 patients = 3 mmol/L 3 patients = 2 mmol/L 1 patient = 1 mmol/L 1 patient not reported	Not reported	• KD for 2 weeks prior to treatment to reach ketones >3 mmol/L • Chemoradiation administered for 6 weeks with KD • KD only for 6 weeks followed by 1 cycle of chemoradiation • End of study was regular diet with chemoradiation for 5 months

KD, ketogenic diet; SD, standard diet; BHB, β-hydroxybutyrate; GBM, glioblastoma multiforme; HBOT, hyperbaric oxygen therapy; DON, 6-diazo-5-oxo-L-norleucine.

The testing of the KD has been well studied and documented in mouse models in various forms. Human studies using the KD continue to be small and challenging to research. All the studies included in this review showed mixed results on the KD enhancing tumor response to treatments and prolonging survival. Studies in animals have stronger results in reduced tumor growth and increased survival. Despite these promising findings, the methods used need to be consistent. Glucose and ketone levels as well as macronutrients need to be comparable. And the glioma cell lines used in mice need to be closer to the aggressive, vascular human GBM cell lines to represent real-life events.

It is worth mentioning that many of the mouse models only used diet interventions to treat the gliomas, which will not be the same for patients. Patients undergo intense radiation and chemotherapy after surgeons remove all or part of the tumor. These treatments cause further damage to the brain, and medications prescribed to reduce edema and inflammation can increase glucose levels defeating the sole purpose of a KD. These are just some considerations to factor into a study design when using a KD as a diet therapy in mice implanted with gliomas.

Based on the findings from this review, a KD is encouraging to be an effective form of adjuvant therapy with standard cancer treatment in adult patients with GBM. However, more reproducible results between pre-clinical and clinical studies are needed for a concrete decision on manageability and effectiveness. Future clinical studies should consider utilizing a nutritionist to design adequate caloric intake and macronutrient amounts for safe and optimal ketosis while providing motivational support. Researchers should also consider starting the KD well before surgery to ensure high ketone and low glucose levels are achieved before chemoradiation. Lastly, future pre-clinical research should focus on validating *in vitro* and *in vivo* methods that clearly define optimal concentrations of serum glucose and serum ketone levels to inhibit or disrupt cell signaling to create ideal adjuvant diet interventions with cancer therapies.

Author contributions

The author confirms being the sole contributor of this work and has approved it for publication.

Conflict of interest

The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Ketogenic diet alleviates renal fibrosis in mice by enhancing fatty acid oxidation through the free fatty acid receptor 3 pathway

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Introduction: The ketogenic diet (KD), as a dietary intervention, has gained importance in the treatment of solid organ structural remodeling, but its role in renal fibrosis has not been explored.

Methods: Male C57BL/6 mice were fed a normal diet or a KD for 6 weeks prior to unilateral ureteral obstruction (UUO), a well-established *in vivo* model of renal fibrosis in rodents. Seven days after UUO, serum and kidney samples were collected. Serum β-hydroxybutyrate (β-OHB) concentrations and renal fibrosis were assessed. NRK52E cells were treated with TGFβ1, a fibrosis-inducing cytokine, and with or without β-OHB, a ketone body metabolized by KD, to investigate the mechanism underlying renal fibrosis.

Results: KD significantly enhanced serum β-OHB levels in mice. Histological analysis revealed that KD alleviated structural destruction and fibrosis in obstructed kidneys and reduced the expression of the fibrosis protein markers α-SMA, Col1a1, and Col3a1. Expression of the rate-limiting enzymes involved in fatty acid oxidation (FAO), Cpt1a and Acox1, significantly decreased after UUO and were upregulated by KD. However, the protective effect of KD was abolished by etomoxir (a Cpt1a inhibitor). Besides, our study observed that KD significantly suppressed UUO-induced macrophage infiltration and the expression of IL-6 in the obstructive kidneys. In NRK52E cells, fibrosis-related signaling was increased by TGFβ1 and reduced by β-OHB. β-OHB treatment restored the impaired expression of Cpt1a. The effect of β-OHB was blocked by siRNA targeting free fatty acid receptor 3 (FFAR3), suggesting that β-OHB might function through the FFAR3-dependent pathway.

Discussion: Our results highlight that KD attenuates UUO-induced renal fibrosis by enhancing FAO via the FFAR3-dependent pathway, which provides a promising dietary therapy for renal fibrosis.

KEYWORDS

ketogenic diet, renal fibrosis, β-hydroxybutyrate, fatty acid oxidation, free fatty acid receptor 3

Introduction

Over 10% of the world's population are suffering from chronic kidney disease (CKD) (1). As this disease progresses, CKD eventually develops into end-stage renal disease, requiring that patients receive dialysis or kidney transplantation. Renal fibrosis is a hallmark and common outcome in all types of progressive CKD, including chronic allograft nephropathy. Renal fibrosis is characterized by inflammation, myofibroblast activation and migration,

excess deposition of the extracellular matrix, and renal structural remodeling. Its typical pathological presentations indicate interstitial fibrosis and tubular atrophy (2–4). Because the intricate mechanisms of renal fibrosis remain unclear, there is still a lack of feasible targeted therapies that can alleviate or reverse fibrosis progression.

Recent studies report that insufficient energy supply from fatty acid oxidation (FAO) in cardiac myocytes and tubular epithelial cells is an important mechanism of myocardial/renal dysfunction or failure (5–8). With respect to what is known about this catabolic pathway, carnitine palmitoyltransferase 1a (Cpt1a) and acyl-coenzyme A oxidase 1 (Acox1) are its rate-limiting enzymes in FAO. Cluster of differentiation 36 (CD36) facilitates the uptake of long-chain fatty acids (9), and peroxisome proliferator-activated receptor-α (PPARα) and PPAR-γ coactivator-1a (PPARGC1a) are the key transcription factors that regulate the expression of target genes (Cpt1a and Acox1) involved in FAO (10, 11). Studies have found that ketogenic diet (KD) enhances myocardial FAO to prevent cardiac dysfunction and fibrosis in mice (5, 6).

Dietary intervention has become one of the most important non-drug therapies, especially for metabolic diseases. KD has been used as an approach to treat drug-resistant epilepsy for over 70 years (12). Recently, KD has generated a lot of interest due to its beneficial impact in various diseases, including Alzheimer's disease, obesity, cardiovascular diseases, cancer, and diabetes (13). KD is a high-fat, extremely low-glucose diet that enhances the metabolism of ketone bodies, including acetoacetate, β-hydroxybutyrate (β-OHB), and acetone. KD is considered a potential dietary intervention to treat solid organ structural remodeling, but its role in renal fibrosis has not been explored. Therefore, we examined the effects of KD on renal fibrosis induced by unilateral ureteral obstruction (UUO) in mice and elucidated the underlying mechanisms, which provides an acceptable dietary regimen for renal fibrosis.

Materials and methods

Animals

All procedures conformed to the Chinese Council on Animal Care guidelines. Our study was approved by the Institutional Animal Care and Use Committee of Tongji Medical College of Huazhong University of Science and Technology (Approval Number: TJH-202111009). Male C57BL/6 mice (8 weeks old, weighing 18–20 g) were purchased from Weitonglihua Laboratory Animal Technology (Beijing, China) and maintained under constant environmental and specific pathogen-free conditions. A portion of the C57BL/6 mice in this experiment underwent a UUO operation, a well-established *in vivo* model of disease progression in rodents, as previously reported (14). Briefly, UUO was conducted by double-knot ligation of the middle and upper segments of the left ureter after the mice were anesthetized. Serum and left kidney samples were obtained 7 d after surgery in the non-fasted mice. C57BL/6 mice were randomly divided into four groups: those fed a normal diet ("normal"), those fed KD ("KD sham"), those fed a normal diet who had undergone the UUO operation ("ND+UUO"), and those fed KD those who had undergone the UUO operation ("KD+UUO"). Six mice were used in each group. All mice were fed normal diet or KD ad libitum for 6 weeks prior to UUO. KD was purchased from Weitonglihua Laboratory Animal Technology and consisted of nearly 90% calories from fat and 10% calories from protein. Etomoxir (MCE, Shanghai, China, 60 mg/kg body weight for 6 d) was injected intraperitoneally 1 d before UUO.

Cell culture

NRK52E cells (rat kidney tubular epithelial cells [TECs]) were used *in vitro* and cultured in the Dulbecco's modified Eagle's medium (DMEM) (10% fetal bovine serum, Gibco, Invitrogen, Carlsbad, CA, USA) at 37°C in 5% CO2. The cells were digested with trypsin and seeded in six-well plates as required. After growing for 24 h, the cells were treated with recombinant transforming growth factor β1 (TGFβ1, 10 ng/mL, PeproTech, Rocky Hill, NJ, USA) with or without β-OHB (10 mM, MCE) for 48 h. A free fatty acid receptor 3 (FFAR3) agonist, AR420626 (5 μM; MCE), was used to verify the effect of FFAR3. Cells were co-stimulated with AR420626 and TGFβ1 for 48 h.

Gene knockdown of FFAR3 by small interfering RNA

Transfection with siRNA against rat FFAR3 (Target RefSeqID: NM_001108912.1, *FFAR3* gene of the Rattus norvegicus species; Sequences: Sence-GGAAUGUCCGAGCUAGAGATT; Antisense-UCUCUAGCUCGGACAUUCCTT) was purchased from AuGCT Biotechnology Company, Wuhan, China. NRK52E cells were transfected with negative control siRNA or siRNA targeting FFAR3 using Lipo6000 Transfection Reagent (Beyotime, Shanghai, China) 24 h before TGFβ1 stimulation. After 24 h of incubation, cells were treated with TGFβ1 and with or without β-OHB.

Measurement of serum β-OHB

The concentration of serum β-OHB 7 d after UUO was detected using a β-OHB detection kit (Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's instructions.

Histologic analysis and immunohistochemistry

Paraffin-embedded left kidney tissue sections were stained with hematoxylin and eosin (H&E) to evaluate the severity of glomerular and tubular interstitial injury 7 d after UUO. Masson's trichrome and picrosirius red staining were performed to measure interstitial fibrosis in the kidneys. Fluorescence microscopic examination (Nikon Eclipse, Tokyo, Japan) showed that collagen fibers were stained blue in Masson's trichrome staining and red in picrosirius red-stained kidney tissues. Fibrosis area calculation was conducted in a blinded manner using at least five randomly selected fields from each kidney section using Image-Pro Plus, version 6.0.

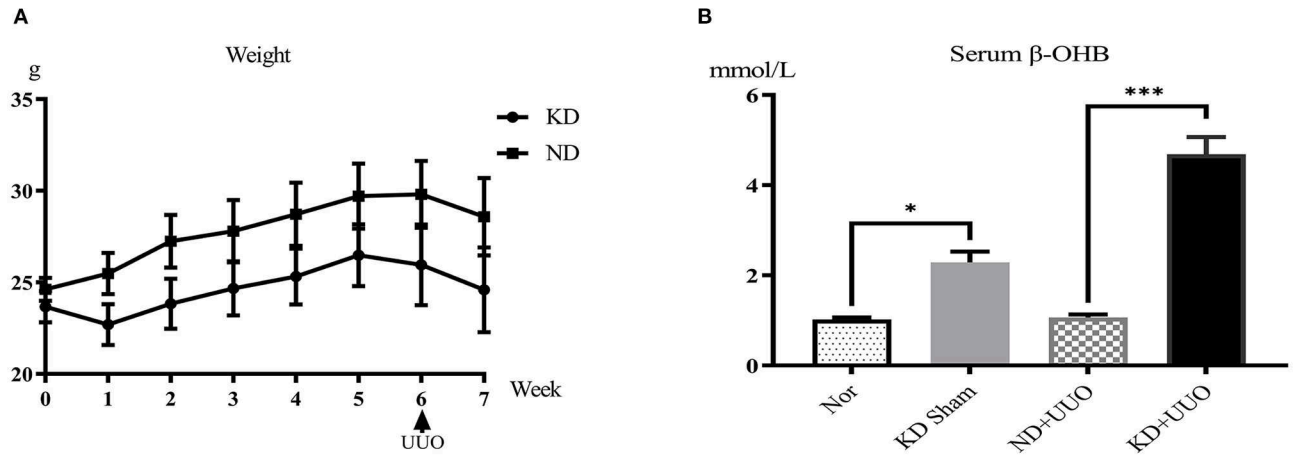

FIGURE 1
Ketogenic diet slowed down weight gain in mice and enhanced the β-OHB level in the serum. **(A)** The weight change of mice fed with ND or KD within 6 weeks before UUO and 1 week after UUO. **(B)** The serum β-OHB level (mmol/L) 7 d after UUO in all groups. Data are expressed as mean ± SEM. ***$P < 0.001$, *$P < 0.05$ as determined by one-way analysis of variance (ANOVA) followed by Tukey's post-hoc tests. Data shown are representative of 3 replicate experiments. KD, ketogenic diet; ND, normal diet; UUO, unilateral ureteral obstruction; β-OHB, β-hydroxybutyrate.

IHC was performed on paraffin-embedded kidney sections. Primary antibodies against α-smooth muscle actin (anti-α-SMA, 1:1000, Abcam, Shanghai, China), anti-collagen type I alpha 1 chain (anti-Col1a1, 1:800, Abcam), and anti-F4/80 (1:500, Cell Signaling Technology, Danvers, MA, USA) were used. The positive area of immunohistochemical staining was calculated using 10 randomly selected fields at ×400 magnification with Image-Pro Plus, version 6.0.

Immunofluorescence

For immunofluorescence analysis of NRK52E cells, cells on glass coverslips were fixed with ice-cold methanol for 10 min and then permeabilized with 0.1% Triton for 5 min at 25°C. The cells were blocked with 5% bovine serum albumin (BSA, Biosharp, Beijing, China), incubated overnight at 4°C with primary antibodies (anti-Cpt1a, 1:100, Abclonal, Wuhan, China), and then incubated in the dark with secondary antibodies (DyLight 488 goat anti-rabbit IgG, 1:500, Abbkine, Wuhan, China) for 1 h at 25°C. Finally, nuclei were stained with Hoechst (Beyotime) for 5 min.

Immunofluorescence analysis of the left kidney tissue was performed on paraffin-embedded sections mounted on glass slides. Primary antibody anti-F4/80 (1:1000, Cell Signaling Technology) and HRP-goat anti-rabbit IgG secondary antibody (1:4000, Abcam) were used. The slides were visualized under a fluorescence microscope (Nikon Eclipse, Tokyo, Japan).

Western blot analysis

Proteins from kidney tissues and cultured cells were extracted with RIPA lysis buffer (Beyotime). Protease and phosphatase inhibitor cocktails were added for the extraction of phosphorylated proteins. The total protein concentration was measured using a BCA protein assay kit (Beyotime). Proteins (40–80 μg) were separated using SDS-PAGE (10%) and transferred to a PVDF membrane. The membranes were blocked with 5% BSA for 1.5 h and incubated with primary antibodies overnight at 4°C, followed by secondary antibodies (goat anti-rabbit, 1:3000; anti-mouse, 1:5000; Servicebio, Wuhan, China) for 1.5 h. The following primary antibodies were used: anti-α-SMA (1:3000, Proteintech, Wuhan, China), anti-Col1a1 (1:1000, Cell Signaling Technology), anti-collagen type III alpha 1 chain (anti-Col3a1, 1:1000, Abclonal), anti-Cpt1a (1:1000, Abclonal), anti-Acox1 (1:1000, Abclonal), anti-phosphorylation-AMP-activated protein kinase (anti-p-AMPK, 1:1000, Abclonal), and anti-glyceraldehyde-phosphate dehydrogenase (anti-GAPDH, 1:50000, Abclonal). Images were visualized using a Gene Gnome XRQ system (Syngene, Cambridge, UK).

Real-time quantitative polymerase chain reaction

Total RNA was isolated from kidney tissues and cultured cells using an RNAfast200 reagent kit (Fastagen Biotech, Shanghai, China) and reverse-transcribed using a cDNA synthesis reagent kit (Yeasen, Wuhan, China). RT-qPCR was conducted using SYBR Green qPCR Master Mix (Vazyme, Wuhan, China). The primers used to amplify the specific gene fragments are listed in Supplementary Table S1. All samples were normalized to the housekeeping gene GAPDH and analyzed in triplicate using the ΔΔCT value method in StepOne Software v2.3.

Statistical analysis

All data are presented as mean ± standard error of the mean (SEM). Three or more group comparisons were made using one-way analysis of variance (ANOVA) followed by Tukey's post-hoc tests

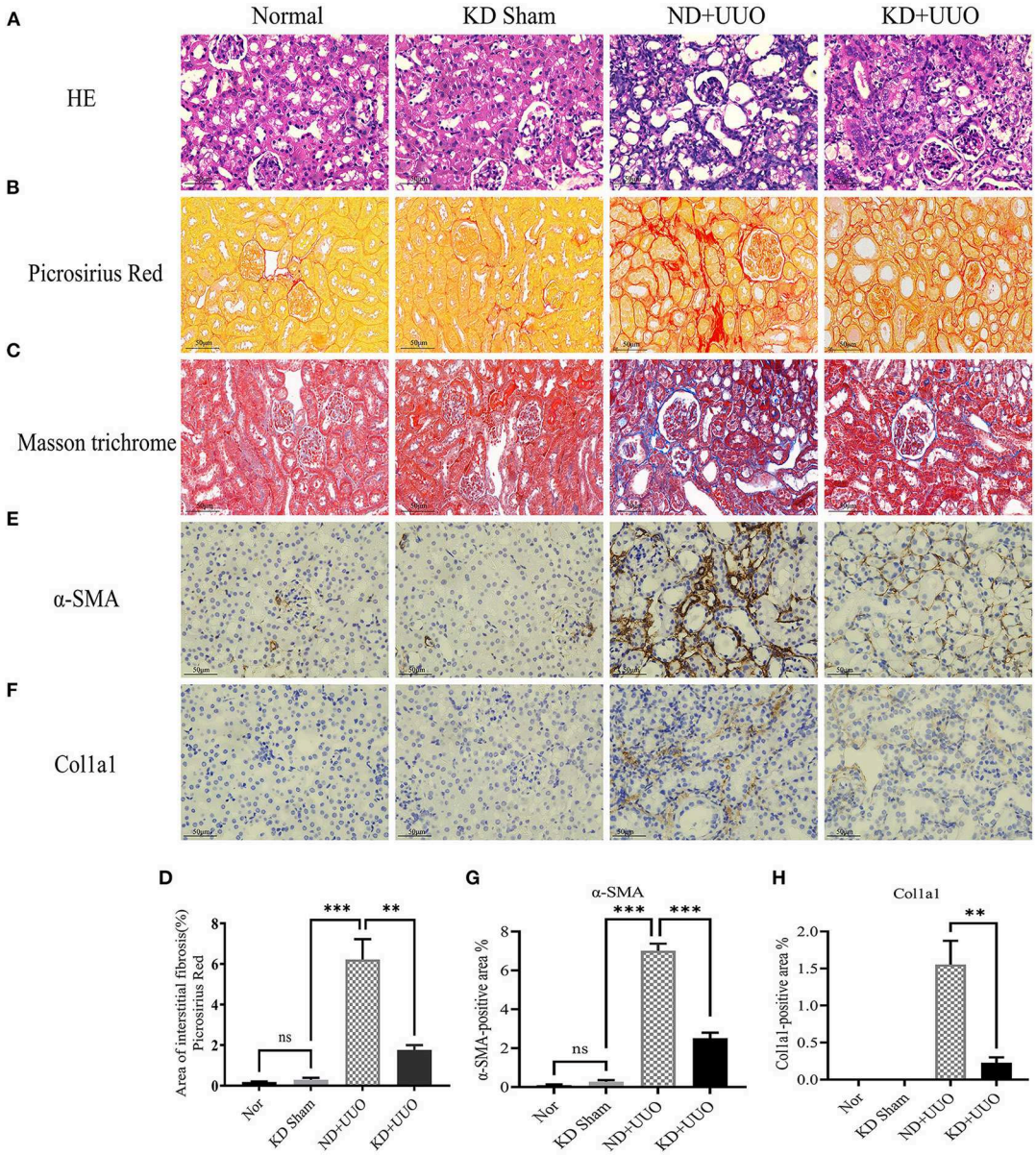

FIGURE 2
Ketogenic diet significantly alleviated structural destruction and excessive extracellular matrix deposition caused by UUO. **(A)** Histopathological examination (H&E) of kidney tissues in all groups showing structural destruction 7 d after UUO. **(B–D)** Masson's trichrome and picrosirius red staining of kidney sections and quantitative analysis of picrosirius red-stained sections. **(E, G)** Immunohistochemical staining images and quantitative analysis showing positive area of α-SMA 7 d after UUO in left kidney tissues from all groups. **(F, H)** Immunohistochemical staining images and quantitative analysis showing positive area of Col1a1 in kidney sections. Data are expressed as mean ± SEM. ***$P < 0.001$; **$P < 0.01$; ns, no significance as determined by one-way analysis of variance (ANOVA) followed by Tukey's post-hoc tests. Data shown are representative of 3 replicate experiments. KD, ketogenic diet; ND, normal diet; UUO, unilateral ureteral obstruction; α-SMA, α-smooth muscle actin; Col1a1, collagen type I alpha 1 chain.

in GraphPad Prism 9. Statistical significance was set at $p < 0.05$.

Results

KD significantly ameliorated renal fibrosis in the UUO mouse model

To investigate the effects of KD on kidney fibrosis, we fed mice either a KD or a normal diet for 6 weeks and monitored weekly weight changes in mice. The weight gain of mice fed the KD was slower than that of mice fed a normalv diet (Figure 1A). KD significantly enhanced β-OHB levels in the serum of mice (ND+UUO vs. KD+UUO: 1.068 ± 0.063 mol/L vs. 4.689 ± 0.377 mmol/L, $P < 0.001$, Figure 1B). H&E staining revealed that structural destruction caused by ureteral obstruction was significantly alleviated by KD (Figure 2A). Masson's trichrome and picrosirius red staining demonstrated that KD effectively attenuated excessive collagen deposition in the interstitium of obstructed kidneys (Figures 2B, C). Quantification of picrosirius

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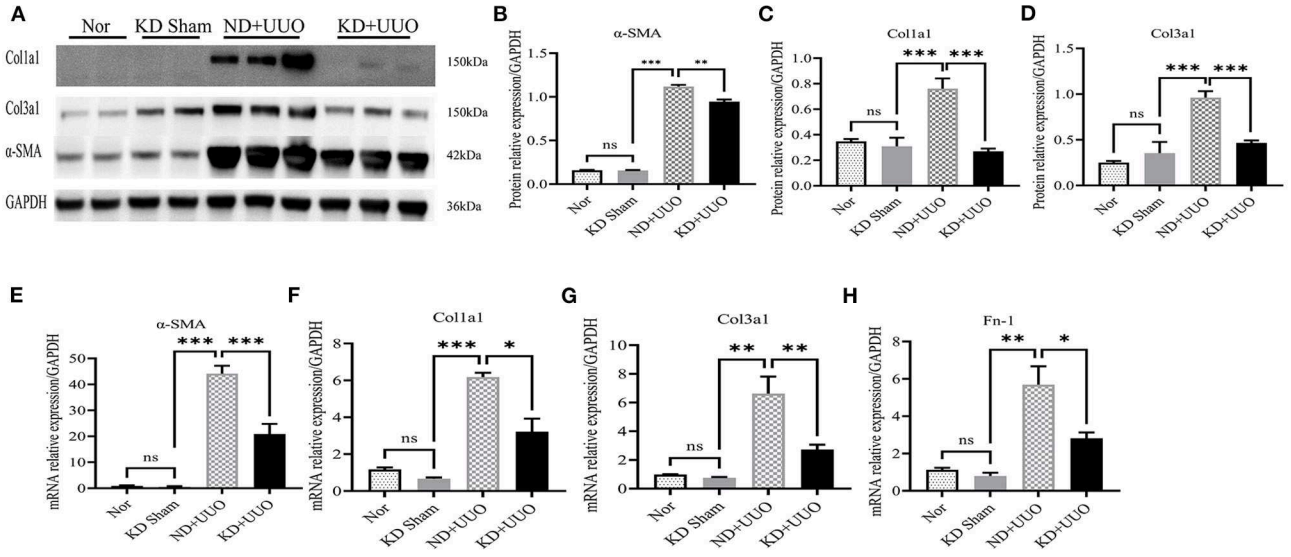

FIGURE 3
Ketogenic diet treatment alleviated UUO-induced renal fibrosis at the protein and mRNA expression level. **(A)** Western blotting analysis indicating the expression level of α-SMA, Col1a1 and Col3a1 7 d after UUO in kidney tissues from all groups. **(B–D)** Quantification of western blotting bands normalized to proteins bands of GAPDH. **(E–H)** Real-time quantitative polymerasechain reaction (RT-qPCR) indicating the mRNA expression level of α-SMA, Col1a1, Col3a1, and Fn-1 7 d after UUO in kidney tissue samples in the indicated groups. Data are expressed as mean ± SEM. ***$P < 0.001$; **$P < 0.01$; *$P < 0.05$; ns, no significance as determined by one-way analysis of variance (ANOVA) followed by Tukey's post-hoc tests. Data shown are representative of 3 replicate experiments. KD, ketogenic diet; ND, normal diet; UUO, unilateral ureteral obstruction; α-SMA, α-smooth muscle actin; Col1a1, collagen type I alpha 1 chain; Col3a1, collagen type III alpha 1 chain; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; Fn-1, fibronectin-1.

red staining showed an apparent decrease in the fibrotic area in the obstructed kidneys from KD feeding mice compared to those in the ND+UUO group (Figure 2D, from 6.226 ± 0.996% to 1.765 ± 0.229%). Using IHC and western blotting, we analyzed the protein markers of fibrosis, including α-SMA, Col1a1, and Col3a1, and further confirmed that the obstructed kidneys at 7 d after UUO were protected from the development of apparent fibrosis through KD. IHC showed that the positive areas of α-SMA and Col1a1 were drastically reduced in the KD+UUO group compared to the ND+UUO group (α-SMA: from 7.015 ± 0.362% to 2.514 ± 0.285%, $P < 0.001$; Col1a1: from 1.555 ± 0.319% to 0.230 ± 0.071%, $P < 0.01$; Figures 2E–H). Western blotting revealed that the protein expression levels of α-SMA, Col1a1, and Col3a1 were significantly upregulated by UUO ($P < 0.001$, Figures 3A–D). Upon intervention with KD, this increased fibrotic expression was attenuated (α-SMA, $P < 0.01$; Col1a1, $P < 0.001$; Col3a1, $P < 0.001$; Figures 3A–D). In accordance with protein expression, the mRNA expression levels of *α-SMA, Col1a1, Col3a1*, and fibronectin-1 (*Fn-1*) were attenuated in obstructed kidneys from the KD+UUO group, in contrast to those in the ND+UUO group (α-SMA, $P < 0.001$; Col3a1, $P < 0.01$; Col1a1 and Fn-1, $P < 0.05$; Figures 3E–H). These results suggested that KD treatment alleviated UUO-induced renal fibrosis *in vivo*.

KD enhanced FAO and mitigated renal fibrosis

Recent studies have shown that impaired FAO plays a vital role in the development of renal fibrosis (7, 8, 15). Therefore, we hypothesized that KD improved renal fibrosis by enhancing the activity of FAO pathway. First, we checked the expression levels of the rate-limiting enzymes in FAO, Cpt1a, and Acox1. Significantly decreased protein expression of Cpt1a and Acox1 was observed in the obstructed kidneys 7 d after UUO in mice fed with ND, compared to that in normal kidneys ($P < 0.001$, Figures 4A–C). KD upregulated the protein levels of Cpt1a and Acox1 and enhanced FAO pathway activity (Cpt1a, $P < 0.01$; Acox1, $P < 0.05$; Figures 4A–C). Changes in the expression of these proteins were further verified by RT-qPCR (Figures 4D, E). Next, we examined other genes involved in fatty acid uptake, oxidation, and synthesis. Consistent with the changes in Cpt1a and Acox1, the mRNA expression levels of *PPARα, PPARGC1a,* and *CD36* were dramatically reduced in obstructed kidneys ($P < 0.001$) and partially restored by KD ($P < 0.05$, Figures 4F–H).

To further explore the role of FAO in kidney fibrosis, we used a Cpt1a inhibitor, etomoxir, in the UUO mouse model. Picrosirius red and H&E staining showed more collagen deposition and more severe tubular damage 7 d after UUO in the obstructed kidneys of mice treated with etomoxir and KD, compared with those fed with KD alone (Area of fibrosis, ND+UUO vs. KD+UUO vs. KD+UUO+Eto: 4.088 ± 0.599% vs. 0.924 ± 0.041% vs. 2.650 ± 0.201%, $P < 0.01$; Figures 5A–C). IHC analyses revealed that the α-SMA-positive and Col1a1-positive areas significantly increased with etomoxir treatment in the kidneys (ND+UUO vs. KD+UUO vs. KD+UUO+Eto, α-SMA: 7.015 ± 0.362% vs. 2.514 ± 0.285% vs. 6.476 ± 0.364%, $P < 0.001$; Col1a1: 1.555 ± 0.319% vs. 0.230 ± 0.071% vs. 1.580 ± 0.353%, $P < 0.05$; Figures 5D–G). These changes were further determined based on the expression of fibrosis-associated proteins. Compared with the kidneys from the mice fed

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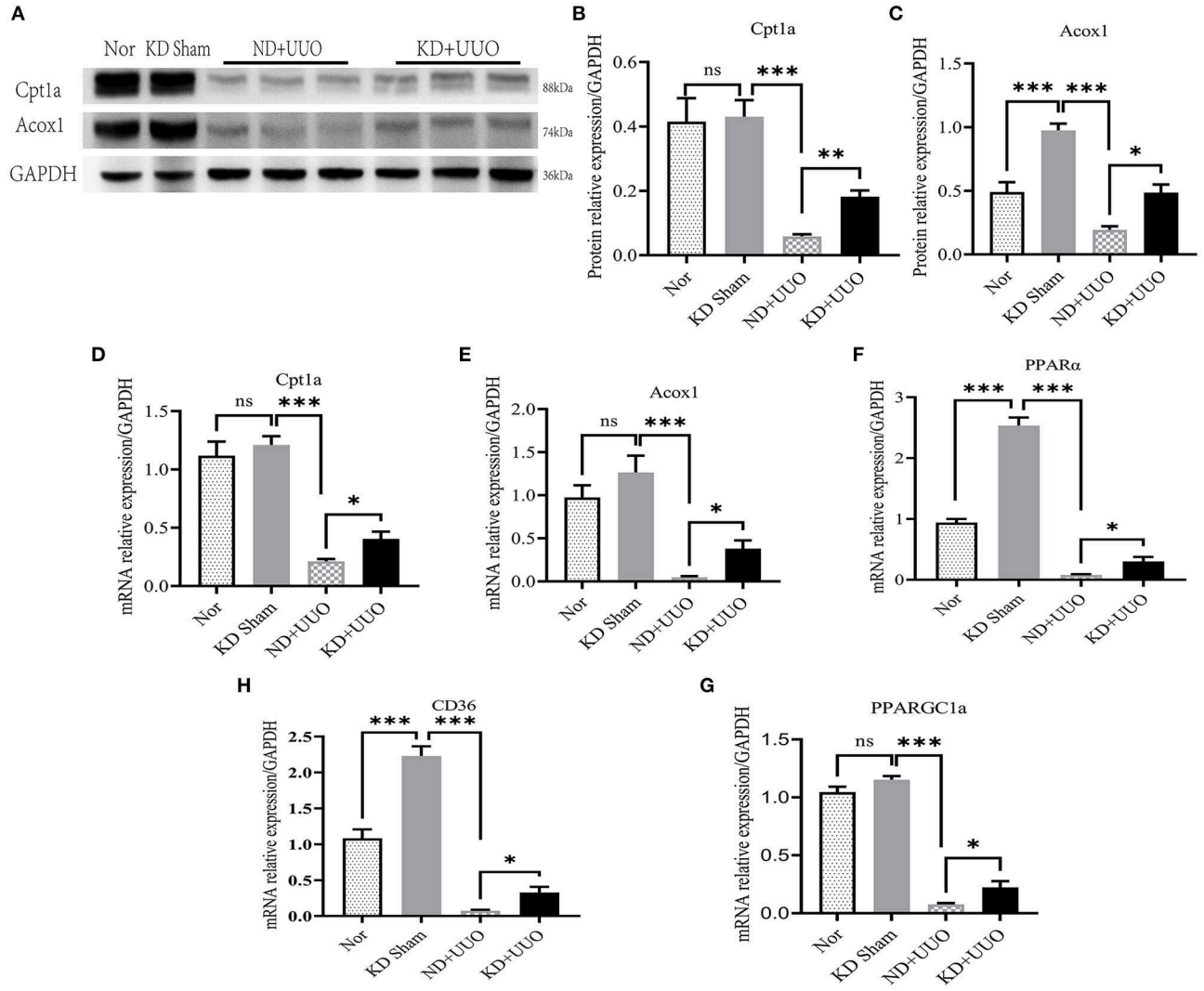

FIGURE 4
Ketogenic diet significantly restored the impaired expression levels of Cpt1a and Acox1 caused by UUO and enhanced the FAO pathway *in vivo*. **(A)** Western blotting analysis indicating the protein expression level of Cpt1a and Acox1 7 d after UUO in kidney tissues of mice. **(B, C)** Quantification of western blotting bands normalized to proteins bands of GAPDH. **(D–H)** Real-time quantitative polymerasechain reaction (RT-qPCR) indicating the mRNA expression level of Cpt1a, Acox1, PPARα, PPARGC1a and CD36 7 d after UUO in kidney tissues. Data are expressed as mean ± SEM. ***$P < 0.001$; **$P < 0.01$; *$P < 0.05$; ns, no significance as determined by one-way analysis of variance (ANOVA) followed by Tukey's post-hoc tests. Data shown are representative of 3 replicate experiments. KD, ketogenic diet; ND, normal diet; UUO, unilateral ureteral obstruction; Cpt1a, carnitine palmitoyltransferase 1a; Acox1, acyl-coenzyme A oxidase 1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PPARα, peroxisome proliferator-activated receptor-α; PPARGC1a, PPAR-γ coactivator-1a; CD36, cluster of differentiation 36.

with KD alone, the obstructed kidneys treated with both etomoxir and KD showed higher expression levels of α-SMA and Col1a1 7 d after UUO, but still less than those from the mice in the ND+UUO group (α-SMA, ND+UUO vs. KD+UUO: $P < 0.001$, KD+UUO vs. KD+UUO+Eto: $P < 0.05$; Col1a1, ND+UUO vs. KD+UUO: $P < 0.001$, KD+UUO vs. KD+UUO+Eto: $P < 0.01$; Figures 6A–C). Collectively, the obstructed kidneys treated with etomoxir suffered more severe renal injuries and fibrosis, although these mice were fed KD. The effect of KD on renal fibrosis was attenuated when FAO was inhibited. In brief, these data indicated that FAO played an important role in renal fibrosis, and we concluded that KD might effectively alleviate renal fibrosis induced by UUO through improving FAO.

KD reduced macrophage infiltration in the UUO mouse model

Inflammation is involved in the progression of fibrosis and plays an important role in tissue damage and repair (16). Previous studies have shown that macrophages contribute to the development of fibrosis (16–18). We further detected the expression of inflammatory indices in obstructed kidneys. The UUO-induced pro-inflammatory cytokines/chemokines, such as interleukin-1β (*IL-1β*), interleukin-6 (*IL-6*), and tumor necrosis factor-α (*TNF-α*), were increased 7 d after UUO ($P < 0.001$, Figures 7A–C). The expression of *IL-6* was downregulated in the kidneys of the KD + UUO group ($P < 0.01$, Figure 7B).

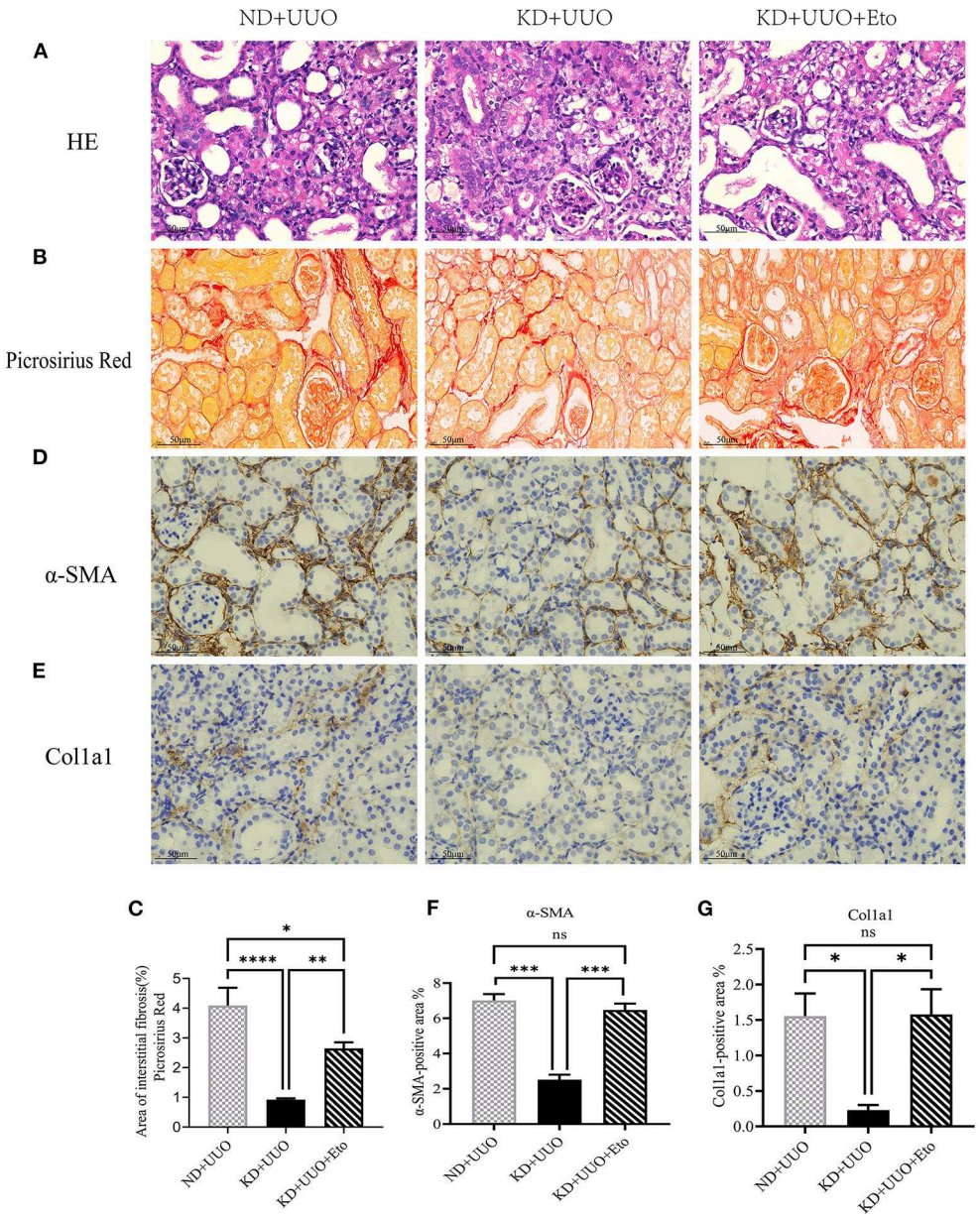

FIGURE 5
The protective effect of ketogenic diet on renal structural destruction and excessive extracellular matrix deposition was abolished by etomoxir. **(A)** Histopathological examination (H&E) of kidney tissues in all groups showing more severe tubular damage 7 d after UUO in the mice treated with etomoxir. **(B, C)** Picrosirius red staining of kidney sections and quantitative analysis of picrosirius red-stained sections. **(D, F)** Immunohistochemical staining images and quantitative analysis showing positive area of α-SMA 7 d after UUO in kidney tissues. **(E, G)** Immunohistochemical staining images and quantitative analysis showing positive area of Col1a1 7 d after UUO in kidney sections. Data are expressed as mean ± SEM. ***$P < 0.001$; **$P < 0.01$; *$P < 0.05$; ns, no significance as determined by one-way analysis of variance (ANOVA) followed by Tukey's post-hoc tests. Data shown are representative of 3 replicate experiments. KD, ketogenic diet; ND, normal diet; UUO, unilateral ureteral obstruction; Eto, etomoxir; α-SMA, α-smooth muscle actin; Col1a1, collagen type I alpha 1 chain.

However, our data demonstrated no significant reduction in the expression of *IL-1β* and *TNF-α* in the KD+UUO group (Figures 7A, C). In our study, KD significantly suppressed UUO-induced infiltration of macrophages identified by the surface marker F4/80 in obstructed kidneys (ND+UUO vs. KD+UUO: 29.38 ± 1.625 vs. 20.83 ± 1.621 cells/HPF, $P < 0.01$; Figures 7D, E). However, this protective effect was blocked by etomoxir (Figure 7E).

β-OHB enhanced FAO activity and improved fibrosis *via* the FFAR3-dependent pathway in TECs

Next, we explored the mechanism of renal fibrosis improvement by KD in renal TECs. β-OHB is not only a simple energy intermediate metabolite but also plays an important role as a signaling molecule in different physiological contexts (19, 20).

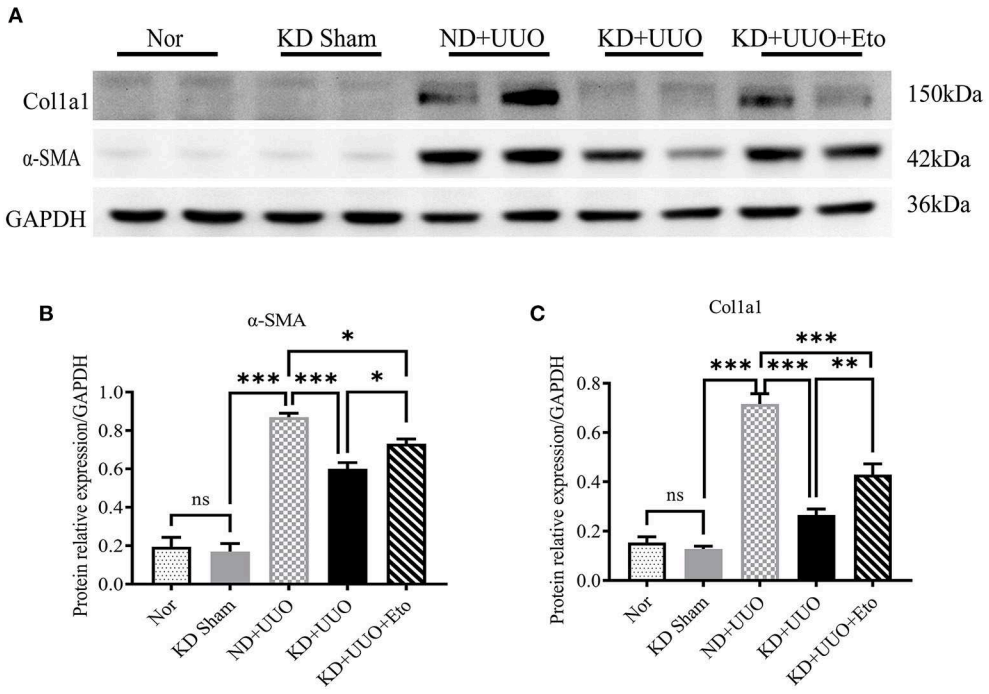

FIGURE 6
Etomoxir inhibited the FAO pathway and aggravated renal fibrosis 7 d after UUO. **(A)** Western blotting analysis indicating the protein expression level of α-SMA and Col1a1in kidney tissues in all groups. **(B, C)** Quantification of western blotting bands normalized to proteins bands of GAPDH. Data are expressed as mean ± SEM. ***$P < 0.001$; **$P < 0.01$; *$P < 0.05$; ns, no significance as determined by one-way analysis of variance (ANOVA) followed by Tukey's post-hoc tests. Data shown are representative of 3 replicate experiments. KD, ketogenic diet; ND, normal diet; UUO, unilateral ureteral obstruction; Eto, etomoxir; α-SMA, α-smooth muscle actin; Col1a1, collagen type I alpha 1 chain; GAPDH, glyceralde-hyde-3-phosphate dehydrogenase.

NRK52E cells were used to examine how β-OHB, as the most abundant form of ketone bodies, modified FAO pathway activity.

The mRNA expression levels of fibrosis-related signaling molecules, including *α-SMA, Col1a1, Fn-1*, and vimentin (*Vim*), were elevated by TGFβ1 and markedly downregulated by β-OHB after stimulation for 48 h ($P < 0.01$, Figure 8A). Next, we measured FAO-associated gene expression and determined that Cpt1a was significantly expressed in the cytoplasm of rat TECs (Figure 8B). Treatment with β-OHB for 48 h restored the impaired expression of Cpt1a and *PPARα*, consistent with our *in vivo* experiments ($P < 0.05$, Figures 8A–D). In addition, β-OHB is known to activate AMPK to ameliorate inflammasomes (21). We observed remarkably increased phosphorylation of AMPK by β-OHB ($P < 0.001$, Figures 8C, D). We then investigated the binding mechanism of β-OHB to the cell surface. β-OHB is currently known to bind to two classes of G-protein-coupled receptors: hydroxycarboxylic acid receptor 2 (HCAR2) and FFAR3 (19, 20). We detected changes in the expression of the two receptors using PCR and western blotting. Although the mRNA level of *HCAR2* was markedly elevated by TGFβ1 ($P < 0.001$), β-OHB did not alter its expression in TECs (Figure 8A). Conversely, β-OHB significantly reverted the reduced expression of FFAR3 induced by TGFβ1 ($P < 0.05$, Figures 8C, D). Consistent with the changes *in vitro*, the protein expression level of FFAR3 dramatically decreased in obstructed kidneys and was restored by KD in mice (Figure 8E).

To further demonstrate the role of FFAR3 in fibrosis, a FFAR3 agonist, AR420626, was used to mimic the β-OHB activation pathway. Similarly, AR420626 significantly mitigated fibrosis induced by TGFβ1 and greatly increased FAO after co-stimulation with TGFβ1 for 48 h (Figures 8F–H), suggesting that activation of FFAR3 might increase the FAO activity and eventually mitigate fibrosis. Next, we knocked down the expression of FFAR3 using siRNA to examine whether β-OHB functioned through the FFAR3-dependent pathway in TECs. The expression of FFAR3 was inhibited immensely by siRNA (Figure 8I). The protective effect of β-OHB on fibrosis was abolished by siRNA in TECs (Figure 8J). In summary, our results indicated that β-OHB mitigated fibrosis by enhancing cellular FAO via the FFAR3-dependent pathway.

Discussion

In this study, we observed for the first time that KD attenuates UUO-induced renal fibrosis and protects renal structure from obstructive destruction *in vivo*. Moreover, KD significantly reduces macrophage infiltration in renal tissue. The effects of KD on renal fibrosis depend mainly on its enhancement of FAO activity. Additionally, our work *in vitro* revealed that β-OHB enhances FAO *via* FFAR3, which improves fibrosis in TECs. These findings demonstrate that KD represents a promising dietary therapeutic strategy for treating renal fibrosis and CKD.

The UUO in rodents is conducted to mimic human obstructive nephropathy. The UUO-induced renal fibrosis is characterized by severe structural damage, including a decreased number of

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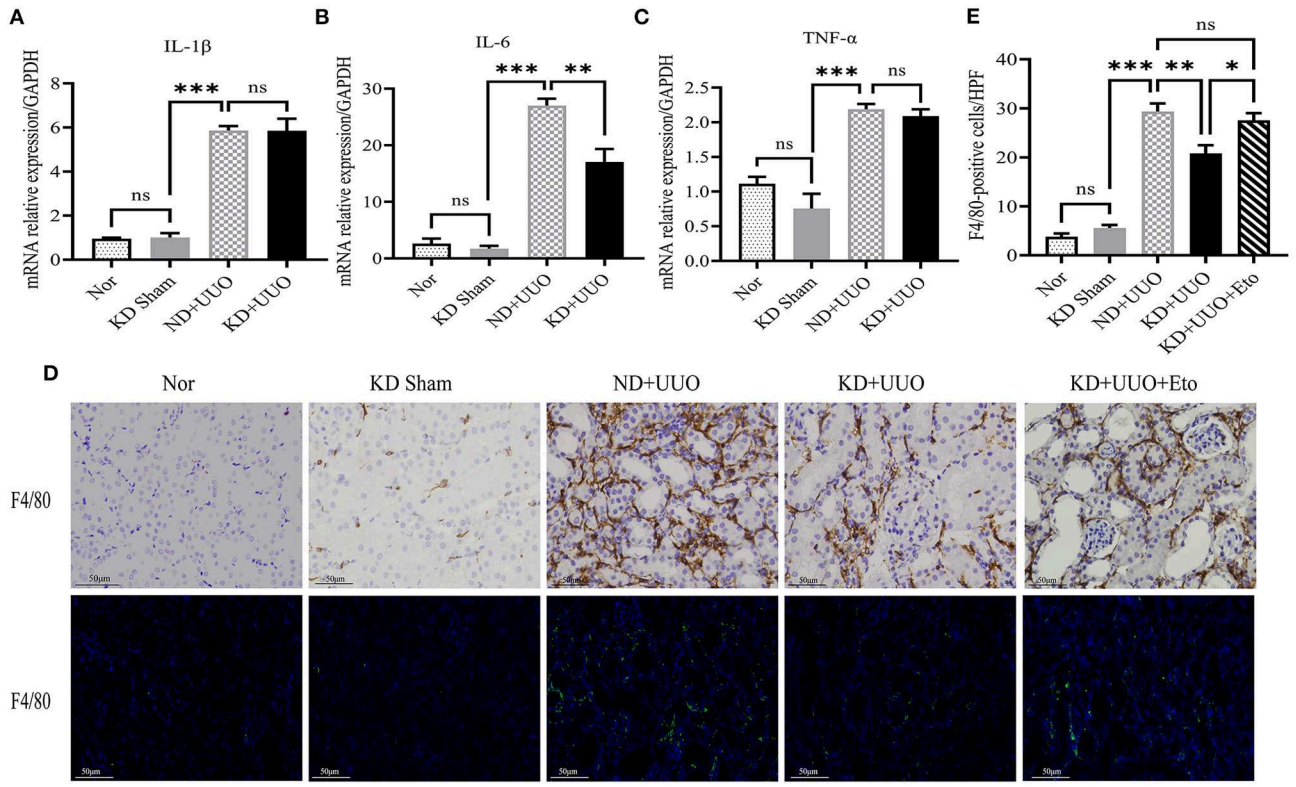

FIGURE 7
Ketogenic diet reduced the release of the proinflammatory cytokine and macrophage infiltration in the obstructed kidney. **(A–C)** Real-time quantitative polymerasechain reaction (RT-qPCR) indicating the mRNA expression level of IL-1β, IL-6 and TNF-α 7 d after UUO in kidney tissues. **(D)** Immunohistochemical and immunofluorescence images of stained kidney sections with F4/80-specific antibody indicating the number of macrophages 7 d after UUO. **(E)** Quantitative analysis of the number of F4/80-positive cells/HPF. Data are expressed as mean ± SEM. ***$P < 0.001$; **$P < 0.01$; *$P < 0.05$; ns, no significance as determined by one-way analysis of variance (ANOVA) followed by Tukey's post-hoc tests. Data shown are representative of 3 replicate experiments. KD, ketogenic diet; ND, normal diet; UUO, unilateral ureteral obstruction; Eto, etomoxir; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IL, interleukin; TNF-α, tumor necrosis factor-α; HPF, high power field.

tubules, tubular atrophy, dilated tubules, and excess deposition of the extracellular matrix, eventually leading to the common pathological manifestations of kidney fibrosis (14). In recent years, deficient FAO in renal TECs has been found to be crucial for renal fibrosis (7, 8, 15, 22). As highly metabolic cells, TECs prefer FAO and maintain high expression level of FAO- related enzymes, which generates more energy than the oxidation of glucose (7, 8, 15, 23). Impairment of FAO in TECs induces ATP depletion, intracellular lipid deposition, cell death, and dedifferentiation, eventually leading to fibrosis (7, 8). Our results also confirmed severely impaired FAO in the UUO-induced fibrosis model. Previous studies (7, 8, 15) and our results indicate that restoration of impaired FAO effectively prevents the progression of renal fibrosis. Thus, targeted therapies for FAO in the early stages of fibrosis provide new insights into the prevention of renal fibrosis.

Dietary interventions for the treatment of various diseases are drawing widespread attention and have gained increasing importance. KD, as a typical dietary intervention, is characterized by the supply of a large mass of calories from fat, a small proportion derived from protein, and very few calories from glucose. In the context of constant total calorie intake, KD enhances ketogenesis, suppresses oxidative stress, increases insulin sensitivity, and significantly increases FAO in the body (13, 24–26). Two recent heart studies have found that KD reversed cardiac fibrosis by enhancing myocardial FAO metabolism in mice (5, 6). Similar results were found in our study on the kidneys. In this study, we clearly indicated that KD promoted the absorption and oxidation of fatty acids, improved energy metabolism dysfunction, and protected against renal fibrosis in the UUO-induced fibrosis model. KD alone could not significantly enhance FAO in the sham mice consuming KD probably because of the relatively high level of FAO in normal kidneys. In addition, KD alleviates pulmonary fibrosis and effectively slows polycystic kidney disease progression through the inhibition of mTOR signaling (27, 28). However, another study reports that KD inhibits mitochondrial biogenesis and promotes cardiac fibrosis (29). The reason for this difference might lie in the animal model, highlighting the detrimental effects of long-term KD in normal rats. Furthermore, macrophages exert a pro-inflammatory and pro-fibrotic role in the process of kidney fibrosis (16, 18). We found that the macrophage infiltration in obstructed kidneys was reduced significantly, which was beneficial for alleviating fibrosis.

β-OHB, which accounts for approximately 70% of the total ketone bodies, is produced by the liver and transported to the heart and kidney (19, 20, 30). As well as being an energy carrier, β-OHB has been certified to have anti-oxidative, anti-pyroptosis,

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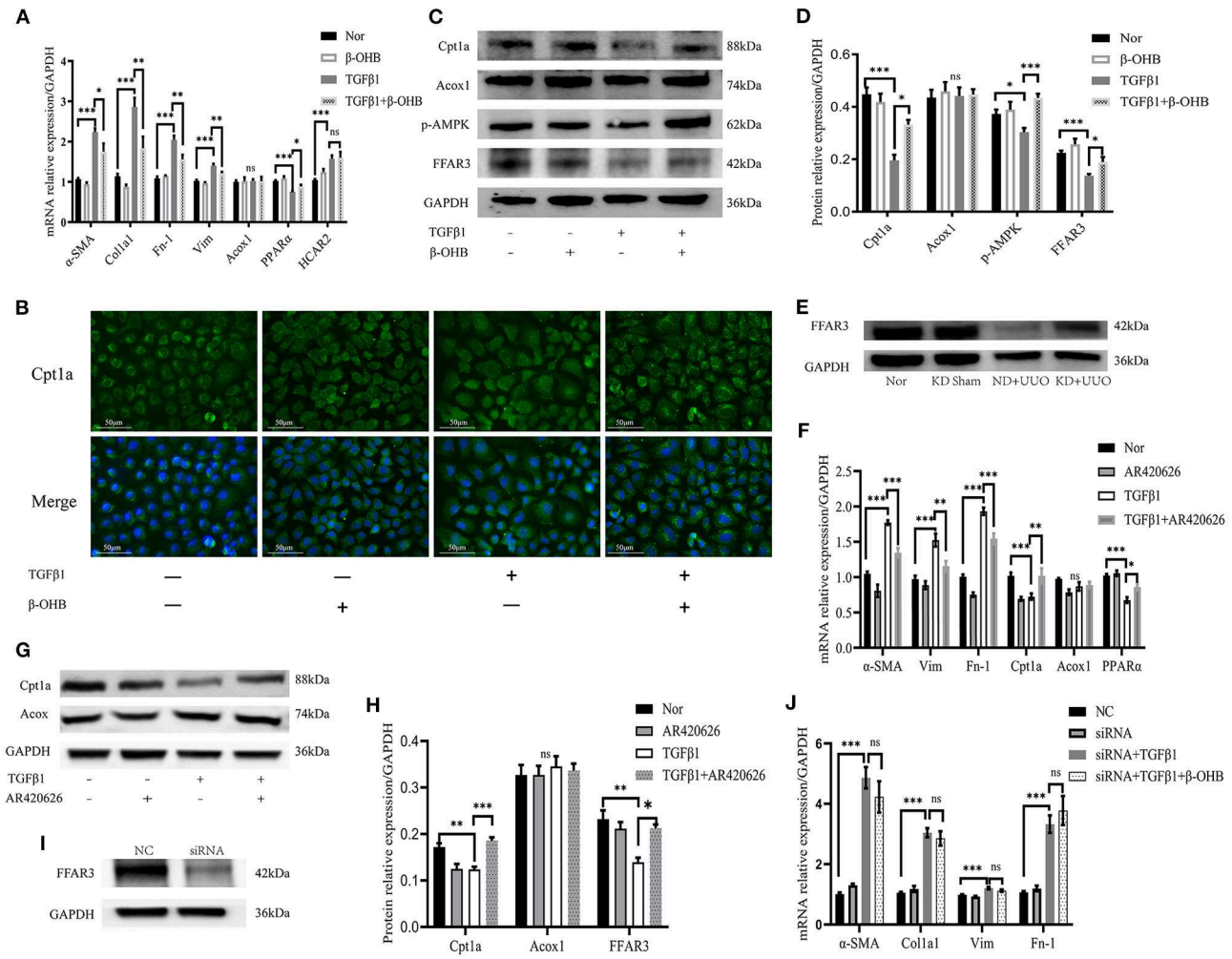

FIGURE 8
β-OHB alleviated fibrosis by enhancing FAO via the FFAR3-dependent pathway in NRK52E. **(A)** Real-time quantitative polymerasechain reaction (RT-qPCR) indicating the mRNA expression level of α-SMA, Col1a1, Fn-1, Vim, Acox1, PPARα and HCAR2 in TECs. After incubation for 24h, the cells were treated with TGFβ1 (10 ng/mL) with or without β-OHB (10 mM) for 48h. **(B)** Immunofluorescence images of cultured cells indicating cellular localization of Cpt1a (green). The nuclear region was stained with Hoechst (blue). Cell culture and stimulation was performed as above. **(C, D)** Quantification analysis of western blotting indicating the protein expression level of Cpt1a, Acox1, p-AMPK and FFAR3 in cultured cells. **(E)** Western blotting analysis indicating the protein expression level of FFAR3 7 d after UUO in kidney tissues of mice. **(F)** RT-qPCR showing the mRNA expression level of α-SMA, Fn-1, Vim, Cpt1a, Acox1, and PPARα in TECs. The cells were co-stimulated with TGFβ1 (10 ng/mL) and AR420626 (5 μM) for 48h. **(G, H)** Quantification analysis of western blotting indicating that AR420626 reverted significantly the inhibition of Cpt1a and FFAR3 induced by TGFβ1 in NRK52E cells. **(I)** Western blotting analysis indicating the decreased protein expression level of FFAR3 by siRNA. **(J)** The mRNA expression level of α-SMA, Col1a1, Fn-1, and Vim in cultured cells determined by RT-qPCR. The NRK52E cells were transfected with control siRNA or siRNA against FFAR3 at 24h before TGFβ1 stimulation. After 24h incubation, the cells were further treated with TGFβ1 with or without β-OHB. Data are expressed as mean ± SEM. ***$P < 0.001$; **$P < 0.01$; *$P < 0.05$; ns, no significance as determined by one-way analysis of variance (ANOVA) followed by Tukey's post-hoc tests. Data shown are representative of 3 replicate experiments. β-OHB, β-hydroxybutyrate; FAO, fatty acid oxidation; TECs, kidney tubular epithelium cells; TGFβ1, transforming growth factor β1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; α-SMA, α-smooth muscle actin; Col1a1, collagen type I alpha 1 chain; Fn-1, fibronectin-1; Vim, vimentin; Acox1, acyl-coenzyme A oxidase 1; PPARα, peroxisome proliferator-activated receptor-α; HCAR2, hydroxycarboxylic acid receptor 2; Cpt1a, carnitine palmitoyltransferase 1a; p-AMPK, phosphorylation-AMP-activated protein kinase; FFAR3, free fatty acid receptor 3; KD, ketogenic diet; ND, normal diet; UUO, unilateral ureteral obstruction; NC, negative control; siRNA, small interfering RNA.

and anti-inflammatory effects and associates with multitudinous signaling pathways, including the NLRP3 inflammasome, NF-κB, FOXO3, endoplasmic reticulum stress, histone deacetylases, and autophagy signaling pathways (20, 21, 31–35). Furthermore, β-OHB inhibits tumor growth and protects from ischemia-reperfusion injuries in the heart, kidney, and liver (34–37). We first observed that β-OHB restores Cpt1a and PPARα expression and, thus, mitigated fibrosis induced by TGFβ1 in TECs. PPARα, as a nuclear receptor transcription factor, participates in the regulation of FAO and oxidant production in mitochondria and peroxisomes (7, 8). PPARGC1a cooperates with PPARα, which modulates energy metabolism (7, 10). Proximal tubule PPARα and PPARGC1a attenuates renal fibrosis and inflammation induced by UUO and other injuries (38–41). In our study, KD and β-OHB significantly increased the expression of PPARα and PPARGC1a in obstructed kidneys and TECs,

which activated the downstream genes of FAO. The network of interactions among AMPK, PPARα, and PPARGC1a, is involved in regulating cellular energy homeostasis. Most notably, AMPK stimulates mitochondrial biogenesis through the activation of PPARGC1a and alleviates the inhibition of Cpt1a (40, 42–44). Based on our results, β-OHB increases the phosphorylation of AMPK and indirectly enhances the expression of Cpt1a in TECs.

β-OHB binds to two G protein-coupled receptors, HCAR2 and FFAR3. HCAR2 (also known as Gpr109A) is expressed in various cell types such as immune cells, adipocytes, and colonic epithelial cells. Its activation inhibits adipocyte lipolysis and induces anti-inflammatory effects (19, 20, 45, 46). We observed that treatment with β-OHB did not change the expression of HCAR2 in TECs. FFAR3 (also known as Gpr41) is highly expressed in sympathetic ganglions. β-OHB antagonizes FFAR3 and suppresses sympathetic nervous system activity (47). However, another study reported that β-OHB, as an agonist for FFAR3, modulates sympathetic neurons (48). Whether agonism or antagonism of FFAR3 by β-OHB may depend on the specific tissue and disease contexts. Furthermore, FFAR3 signaling mediates glucose-stimulated insulin secretion and maintains energy homeostasis (49–51). In this study, the expression of FFAR3 was significantly decreased in TECs stimulated by TGFβ1, while β-OHB significantly enhanced its expression, suggesting that activation of FFAR3 may be controlled by β-OHB. We further confirmed that β-OHB functions via the FFAR3-dependent pathway by the agonist activating and siRNA blocking FFAR3.

Some limitations in our work should be noted. First, this study necessitates further exploration of the molecular mechanisms in knockout mice. Second, recent studies showed a more remarkable protective effect of KD than exogenous supplementation of ketone bodies, which suggests that KD is incompletely dependent on ketone bodies to fulfill its functions (5, 6). Accumulating evidence suggests that KD reduces intestinal pro-inflammatory Th17 cells and alleviates colitis by altering intestinal microbiota (52–54). Therefore, exogenous supplementation of β-OHB does not completely supersede KD, which induces more extensive systematic metabolic changes. Our work of β-OHB *in vitro* cannot fully elucidate the mechanisms of KD on renal fibrosis *in vivo*. Lastly, this study focused on HCAR2 and FFAR3 regulated by β-OHB. It has been reported that there are many G protein-coupled receptors, including HCAR1/3 and FFAR1/2, which regulate cellular and physiological functions in the body (55). Further research is required to understand how other receptors are involved in FAO.

Conclusion

In conclusion, our current study elucidated the mechanism underlying the protective effect of KD on renal fibrosis through the enhancement of FAO and reduction in macrophage infiltration. These results shed new light on the role of energy metabolism disturbances in the progression of renal fibrosis and provide a potential therapeutic approach for CKD and renal fibrosis.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/genbank/, NM_001108912.1.

Ethics statement

The animal study was reviewed and approved by the Institutional Animal Care and Use Committee of Tongji Medical College of Huazhong University of Science and Technology.

Author contributions

YQ contributed to research design, performance of the research, data analysis, and the writing of the paper. XH contributed to the experimental design and assisted with data analysis. CX and CL participated in establishment of model and sample collection. RC and YX participated in data analysis and revised the article. JY conceived the study, designed the experiments, and supervised the research. All authors contributed to manuscript revision and read and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2023.1127845/full#supplementary-material

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Case report: Resolution of malignant canine mast cell tumor using ketogenic metabolic therapy alone

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Background: Mast cell tumors (MCT) are common neoplasms in dogs and are similar to most other malignant cancers in requiring glucose for growth, regardless of histological grade. Ketogenic metabolic therapy (KMT) is emerging as a non-toxic nutritional intervention for cancer management in animals and humans alike. We report the case of a 7 years-old Pit Bull terrier that presented in 2011 with a cutaneous mast cell tumor under the right nostril.

Methods: The patient's parent refused standard of care (SOC) and steroid medication after initial tumor diagnosis due to the unacceptable adverse effects of these treatments. Following tumor diagnosis, the patient's diet was switched from Ol'Roy dog food to raw vegetables with cooked fish. The tumor continued to grow on this diet until July, 2013 when the diet was switched to a carbohydrate free, raw calorie restricted ketogenic diet consisting mostly of chicken and oils. A dog food calculator was used to reduce calories to 60% (40% calorie restriction) of that consumed on the original diet. A total of 444 kilocalories were given twice/day at 12 h intervals with one medium-sized raw radish given as a treat between each meal.

Results: The tumor grew to about 3–4 cm and invaded surrounding tissues while the patient was on the raw vegetable, cooked fish diet. The tumor gradually disappeared over a period of several months when the patient was switched to the carbohydrate free calorie restricted ketogenic diet. The patient lost 2.5 kg during the course of the calorie restriction and maintained an attentive and active behavior. The patient passed away without pain on June 4, 2019 (age 15 years) from failure to thrive due to an enlarged heart with no evidence of mast cell tumor recurrence.

Conclusion: This is the first report of a malignant cutaneous mast cell tumor in a dog treated with KMT alone. The resolution of the tumor in this canine patient could have been due to the diet-induced energy stress and the restriction of glucose-driven aerobic fermentation that is essential for the growth of most malignant tumors. Further studies are needed to determine if this non-toxic dietary therapeutic strategy could be effective in managing other canine patients with malignant mast cell tumors.

KEYWORDS

ketogenic diet, canine, glucose, aerobic fermentation, nutrition, calorie restriction

Introduction

Canine cutaneous mast cell tumor (MCT) is a common malignant cancer in a range of dog breeds (1–4). Emerging evidence indicates that cancer is a mitochondrial metabolic disease (5, 6). Aerobic glucose fermentation (Warburg effect), linked to defective oxidative phosphorylation (OxPhos), has been documented in canine MCT as it has been in the majority of human cancers regardless of histological or genetic heterogeneity (7, 8). Unlike normal cells, cancer cells can grow in the absence of oxygen using glucose and glutamine as fermentable fuels (8). Ketogenic metabolic therapy (KMT) is a non-toxic therapeutic strategy for cancer management that restricts the availability of fermentable fuels while elevating levels of non-fermentable fatty acids and ketone bodies (9, 10). Metabolism of ketone bodies in normal cells increases the redox span between mitochondrial Complexes I and III, thus increasing the delta G' of ATP hydrolysis while, at the same time, reducing the formation of reactive oxygen species (ROS) through the Complex II coenzyme Q couple (10–12). It is for these reasons that ketone bodies are considered *good medicine* for enhancing mitochondrial energy efficiency and general physiological health (13, 14). We therefore proposed that KMT might be helpful in managing canine MCT.

Calorie reduction and calorie restricted diets can also target multiple hallmarks of cancer including angiogenesis, inflammation, edema, and tumor cell viability (15–17). In contrast to cells with normal mitochondrial OxPhos capacity, cancer cells lack metabolic flexibility and cannot efficiently use fatty acids, ketone bodies, or other respiratory fuels for ATP synthesis (10). The well-documented abnormalities in mitochondrial number, structure, and function compromise energy synthesis through OxPhos (8). KMT used either alone or in combination with standard of care has therapeutic benefit for managing a broad range of animal and human cancers (9, 18–20). In this report, we present evidence showing that KMT used alone was able to resolve a malignant cutaneous MCT in a dog.

Case report

A 7 years-old, 60 pound female Pit Bull (DOB, January 3, 2004) presented on July 28, 2011 at the Banfield Pet Hospital, Conyers, GA, USA with a cutaneous mass under the right nostril. Microscopic analysis of tissue smears revealed high cellularity with loose sheets of round to oval cells. The cells had indistinct borders and the cytoplasm contained large numbers of prominent metachromatic cytoplasmic granules. The nuclei varied mildly to moderately in size and nucleoli were rarely visible. Eosinophils were scattered frequently throughout the smear preparations. The cutaneous mass was diagnosed as mast cell tumor, but was not graded at that time (see lab report in **Supplementary material**). Based on clinical behavior, this tumor was likely a progressive low-grade neoplasm at the time of diagnosis (2–4). A complete blood work analysis revealed no deviations from normal ranges at the time of initial diagnosis (**Supplementary material**).

Although steroid medication (prednisone) and standard of care (SOC) including surgery, chemotherapy, and radiation therapy were discussed as therapeutic options, the patient's parent refused any of these treatments due to their unacceptable adverse effects. Significant adverse effects have been reported in a dog with cutaneous MCT treated with SOC (21, 22). Two weeks after tumor diagnosis, the patient's diet was switched from Ol'Roy canned dog food to a raw vegetable diet with cooked fish and lentils. The tumor continued to grow on this diet invading local tissues and reaching a size of about 4 cm (**Figure 1A**). Local invasion is a hallmark of malignancy and the first step of the metastatic cascade (23, 24). Two weeks prior to the July 2013 image shown in **Figure 1A**, the patient's diet was switched to a calorie restricted raw ketogenic diet. A full image of the patient's face is shown in the **Supplementary material**. As no further diagnostic analysis was done on the tumor in 2013, it is not known if the grading of the tumor would have increased over the 2 years period from initial diagnosis in 2011 to that shown in **Figure 1A** and in the **Supplementary material**. The decision to switch the diet from raw vegetables to a raw calorie restricted KD was based on the on the view that dogs evolved from wolves, which are largely carnivores, and on the general information presented in the following YouTube video.[1] Using the following dog food calculator, http://www.dogfoodadvisor.com/dog-feeding-tips/dog-food-calculator, the patient's parent estimated that a 60 lb (27.3 kg), light-duty working dog should consume about 1,500 kilocalories (Kcal)/day. Consequently, a 40% restriction of this value was used to estimate a daily caloric intake for this patient of about 900 Kcal/day. The new diet was formulated to contain the following ingredients:

1. One organic raw chicken leg with bone (150 Kcal); 26 g fat: 36 g protein.
2. One organic raw chicken egg (54 Kcal); 1.5 g fat: 6.0 g protein.
3. One tablespoon (14.3 g) of pure LouAna coconut oil (120 calories); 14.3 g fat: 0 g protein.
4. Three teaspoons (12.6 g) of grizzly pollock oil for dogs (120 calories); 12.6 g fat: 0 g protein.

This carbohydrate-free dietary formulation produced about 444 Kcal/meal with a fat: protein ketogenic ratio of about 1.3:1 and was fed to the patient twice/day in the morning and in the evening at 12 h intervals. There were no issues of compliance. As the patient enjoyed treats, one medium-sized raw radish (0 protein and 0 fat) was given between each meal. The tumor gradually disappeared over a period of several months when the patient was switched to the raw calorie restricted ketogenic diet (**Figures 1B–D**). A image of the patient's face taken in 2016 is shown in the **Supplementary material**. The patient lost 2.5 kg (about 8% body weight) during the course of the calorie restriction and maintained an attentive and active behavior according to the parent. The patient was fed this calorie restricted ketogenic diet until 2019. Once the tumor was no longer apparent, the patient was occasionally given cooked chicken as an alternative to raw chicken. The patient passed away at the upper age of longevity for this breed (age 15 years) in the arms of the parent without pain on June 4, 2019 from failure to thrive due to an enlarged heart. No evidence of mast cell tumor recurrence was observed on the nose or anywhere else on the patient's body. It is

Abbreviations: OxPhos, oxidative phosphorylation; KMT, ketogenic metabolic therapy; SOC, standard of care; KD, ketogenic diet; KGI, glucose ketone index.

1 https://youtu.be/sBjnWfT8HbQ

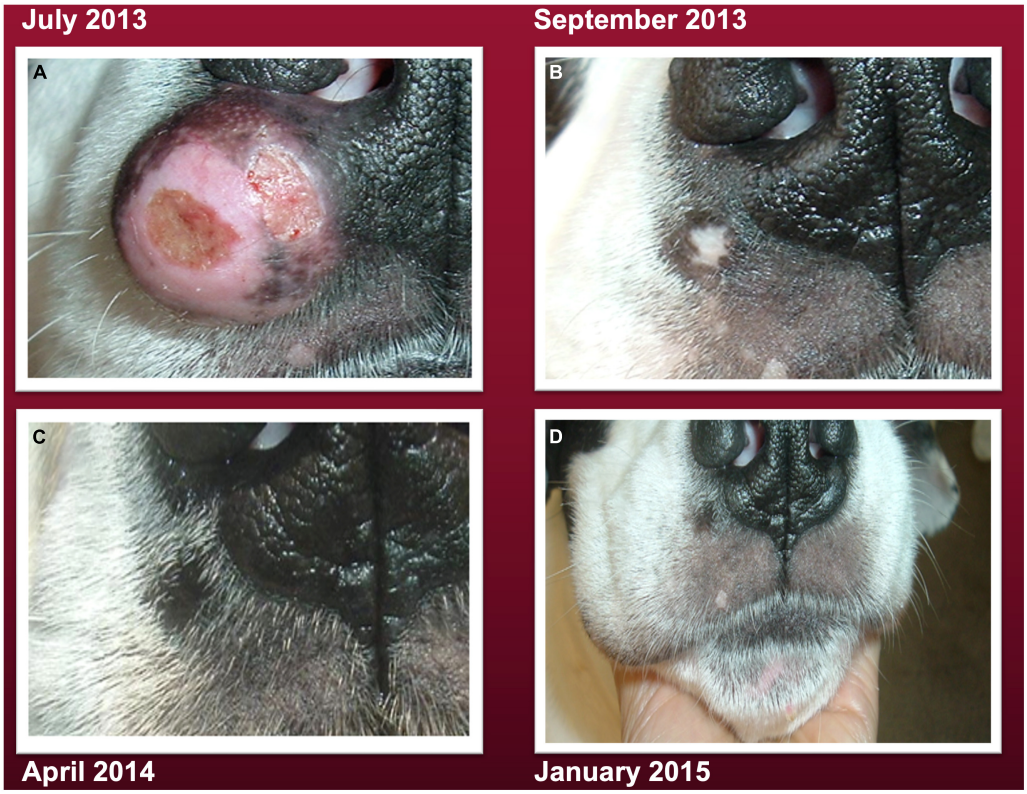

FIGURE 1
A large cutaneous mast cell tumors (MCT) (photographed on July 18, 2013) is seen under the right nostril and invasion to the nasal planum, consistent with malignancy (23). **(A)** The tumor gradually resolved over several months after the patient's parent initiated the carbohydrate-free, calorie restricted ketogenic diet **(B–D)**, as described in methods. The small bare patch on the lip below the tumor can serve as a reference point to assess the degree of the diet-linked tumor shrinkage. A facial image from October 2016 is also shown in the **Supplementary material**.

known that overall survival for grade II MCT is about 21.5 months and for grade III MCT is only about 9.2 months (2, 25). This patient survived for 63 months living a normal life span after resolution of the cancer. A timeline of the case is shown in **Figure 2**.

Discussion

This is the first report to our knowledge of a malignant cutaneous MCT in a dog treated with KMT alone. It is clear from our observations that the KMT protocol used for our patient did not cause any adverse effects. No adverse effects or safety concerns were reported previously in canines treated with KD for managing epilepsy (26, 27). The diet-linked resolution of the tumor in this canine patient could have been due in part to the restriction of glucose and to an inhibition of aerobic fermentation (Warburg effect) that is essential for the growth of most malignant tumors (8, 28, 29). A limitation of our report, however, is the absence of data collected on blood glucose, blood ketone bodies, and glucose ketone index values (GKI) in a manner similar to those collected previously in our case report of a human brain tumor patient (30, 31).

It is recognized that the genome of most cancer cells, including those in MCT, contain numerous types of pathological somatic mutations (32–34). Abnormalities in mitochondrial DNA and biochemistry have also been reported previously in canine cutaneous MCT (7). These genetic abnormalities will prevent metabolic flexibility and adaptability to nutritional stress (35). Adaptability to abrupt environmental change is a property of the normal genome, which was selected for in order to ensure survival under environmental stress (35). According to established evolutionary concepts, only cells possessing flexibility in nutrient utilization will be able to survive under nutrient stress (6, 36). Environmental forcing over eons has selected for genomes that are capable of adapting to abrupt change in order to maintain metabolic homeostasis (37–39). Previous studies showed that normal dogs are remarkably adaptable to food restriction and physiological stress (40). The genomic defects that occur in MCT, together with mitochondrial dysfunction, will prevent the metabolic flexibility needed for rapid adaption to nutrient stress thus leading to tumor cell elimination through a combination of autophagy and autolytic cannibalism (36). The metabolically flexible normal canine cells will outcompete the mutated inflexible MCT cells for the availability of restricted nutrients thus leading to the elimination of the tumor cells (41, 42). It is therefore tempting to speculate that in contrast to the normal body cells, the neoplastic cells in the patient's MCT were unable to adapt to the nutritional stress produced from the carbohydrate-free, calorie restricted ketogenic diet thus causing rapid tumor resolution.

Calorie restriction and restricted ketogenic diets have had success in reducing growth and metastasis in a range of malignant tumors in mice and humans (9, 19, 41, 43, 44). In none of these cases, however, was resolution of the tumor achieved with diet alone. Although a human glioblastoma patient has remained alive for over 8 years using KMT alone (no steroids, no radiation,

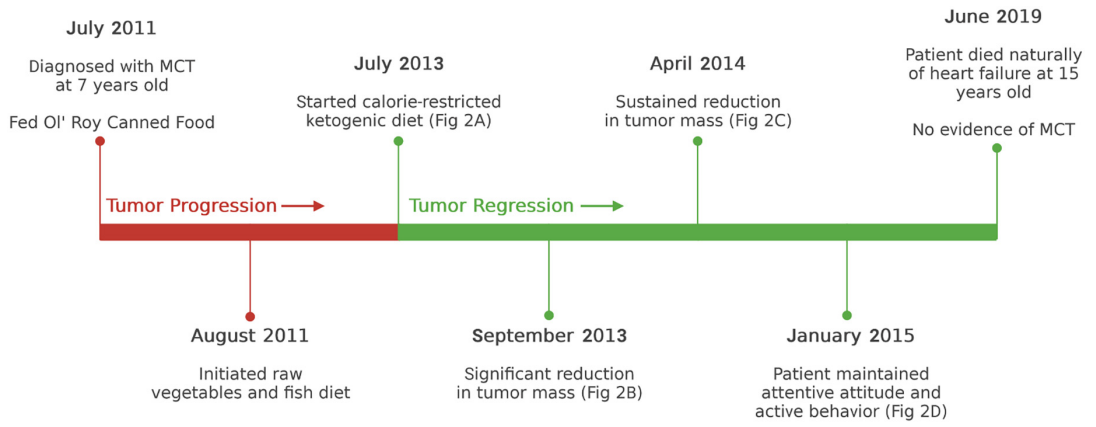

FIGURE 2
Timeline of clinical course with dates of dietary treatments.

no chemotherapy), the tumor in this patient was not resolved, but continues to grow slowly requiring periodic debulking for continued management (31). Synergy between restricted KD and glutamine targeting drugs could also facilitate resolution of more aggressive tumors especially for those that involve systemic metastasis and growth in the nervous system (16, 45). The resolution of the MCT in this canine patient should be viewed as anecdotal until further studies are conducted in other canine patients using a therapeutic strategy that is the same or similar to that used on our canine patient.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

Ethical review and approval was not required for the animal study because there was no risk of toxicity or adverse events of the treatment recommended for the patient. Treatment was conducted in the patient's home environment. Written informed consent was obtained from the owners for the participation of their animals in this study.

Author contributions

TS wrote most of the report. PM, LT, and DL assisted with development of figures and report editing. LN edited and validated the accuracy of the conclusion. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2023.1157517/full#supplementary-material

1. Original lab report on the patient from the Banfield Pet Hospital, Conyers, GA.
2. Before and after ketogenic metabolic therapy (KMT) treatment images of the patient.

SUPPLEMENTARY FIGURE 1
Additional images depicting the patient before and after ketogenic metabolic therapy (KMT). **(A)** Large mast cell tumors (MCT) under the right nostril in July 2013. **(B)** Image of the patient with sustained resolution in October 2016.

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Long-term follow-up of nutritional status in children with GLUT1 Deficiency Syndrome treated with classic ketogenic diet: a 5-year prospective study

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Introduction: The classic ketogenic diet (cKD) is an isocaloric, high fat, low-carbohydrate diet that induces the production of ketone bodies. High consumption of dietary fatty acids, particularly long-chain saturated fatty acids, could impair nutritional status and increase cardiovascular risk. The purpose of this study was to evaluate the long-term effects of a 5-year cKD on body composition, resting energy expenditure, and biochemical parameters in children affected by Glucose Transporter 1 Deficiency Syndrome (GLUT1DS).

Methods: This was a prospective, multicenter, 5-year longitudinal study of children with GLUT1DS treated with a cKD. The primary outcome was to assess the change in nutritional status compared with pre-intervention, considering anthropometric measurements, body composition, resting energy expenditure, and biochemical parameters such as glucose and lipid profiles, liver enzymes, uric acid, creatinine, and ketonemia. Assessments were conducted at pre-intervention and every 12months of cKD interventions.

Results: Ketone bodies increased significantly in children and adolescents, and remained stable at 5years, depending on the diet. No significant differences were reported in anthropometric and body composition standards, as well as in resting energy expenditure and biochemical parameters. Bone mineral density increased significantly over time according to increasing age. Body fat percentage significantly and gradually decreased in line with the increase in body weight and the consequent growth in lean mass. As expected, we observed a negative trend in respiratory quotient, while fasting insulin and insulin resistance were found to decrease significantly after cKD initiation.

Conclusion: Long-term adherence to cKD showed a good safety profile on anthropometric measurements, body composition, resting energy expenditure,

and biochemical parameters, and we found no evidence of potential adverse effects on the nutritional status of children and adolescents.

KEYWORDS

GLUT1-Deficiency Syndrome, ketogenic diet, long-term effect, nutritional status, body composition, energy expenditure

1. Introduction

Glucose Transporter 1 Deficiency Syndrome (GLUT1-DS; OMIM #606777) is a rare neurometabolic disorder resulting from an autosomal dominant mutation in SLCA1 (solute carrier family 2 member 1), a gene that encodes GLUT-1, the main transporter of glucose across the blood–brain barrier (BBB) and the plasma membrane of astrocytes. This genetic defect of GLUT-1 compromises the glucose uptake into the brain leading to an energy crisis. Patients with GLUT1-DS typically present with seizures, complex motor disorders, and impaired neurodevelopment (1, 2).

Since the initial description of GLUT1-DS in 1991 (3), the only known medical therapy for GLUT1-DS is the classic ketogenic diet (cKD), a normocaloric, hyperlipidic, normoprotein and low-carbohydrate diet, whose main purpose is to induce the constant production of ketone bodies, mainly acetoacetate (ACA) and β-hydroxybutyrate (BHB). This diet simulates the effects of long-term fasting, but does not deprive the body of the necessary calories for growth and development (1, 4). In GLUT1-DS, cKD is effective because the transport-mechanism for carbohydrates is inadequate and ketone bodies replaces carbohydrates as a source of energy for the brain (1). Moreover, other results suggest that this diet has an antiepileptic effect due to ketone bodies, that are involved in the alteration of mitochondrial function (e.g., promoting ATP synthesis and increasing mitochondrial biogenesis), in a decrease in glutamate release and its concentration in the synaptic cleft, in the activation of γ-aminobutyric acid (GABA) synthesis, and in the protection of the neurons against oxidative stress through various cellular mechanisms, such as the increase of reduced glutathione (GSH) and the increase of uncoupling protein (UCP) expression (5–10).

In the cKD, the intake of each macronutrient is designed according to the ketogenic ratio (KR), which is the ratio of the amount of fat to the sum of the amounts of carbohydrate and protein (both expressed in grams). The most common ratios used in clinical protocols are 4:1 and 3:1, i.e., 4–3 g of fat versus 1 g of protein and carbohydrate. The choice of KR depends on age, individual production, and brain sensitivity to ketone bodies (2–4): in clinical practice the KR may differ from 2,5–4:1 since the treatment with cKD in patients with GLUT1-DS is lifelong, so clinicians aim to reduce the amount of fat to a level without symptoms (seizures, paroxymal exercise-induced dyskinesia, lack of energy) and a level of ketosis within the recommended range. Given the high lipid content and the reduction in other nutrients (carbohydrates, fiber, vitamins, and minerals), short-term effects could include acidosis, hypoglycemia, dehydration, lethargy, and gastrointestinal symptoms such as constipation, nausea, and abdominal pain (11, 12). One study reported that only insulin resistance and insulin sensitivity indexes changed after 12 weeks of cKD, suggesting that KD seems to have no effect on inflammatory cytokines production and abdominal fat distribution in the short term (2). Tagliabue et al. found no statistically significant differences in the gut microbiota at 3 months, except for a bacterial group suggested to be involved in the exacerbation of the inflammatory state of the intestinal mucosa associated with animal fat consumption (13). Administering a 6-month cKD to patients with medically refractory epilepsy, Tagliabue et al. found an increase in fat oxidation and a decrease in respiratory quotient, without appreciable changes in resting energy expenditure (REE) (14). In addition, the possible effects of high fat consumption might include vitamin deficiencies, hyperlipidemia, kidney stones, reduced bone mineral density, weight loss, and reduced height gain (15). However, only a few studies have investigated the long-term effects of cKD (16, 17). A ten-year study of a sample of 10 children showed that initial dyslipidemia normalized and no significant difference was observed in Body Mass Index (BMI), systolic and diastolic blood pressure, as well as carotid intima-media thickness (17). Regarding nutritional status, which includes growth pattern, body composition, bone mineral density, biochemical parameters, and energy expenditure (18), one study revealed that most GLUT1-DS patients (80%) maintained or even improved their growth pattern at 12-month follow-up (19). Other studies have shown that after 12 months of cKD there were no significant changes in ghrelin and leptin, nor in body fat, glucose and lipid profile (16, 20). Only in one study was cKD administered for more than 5 years. This study, however, was exclusively focused on body composition, bone mineral content, and bone mineral density, and it was conducted on a case series of GLUT1-DS adults, suggesting that maintenance of a cKD does not result in major adverse effects (21). However, no study investigated the long-term effects of a cKD on all nutritional status components in children.

Therefore, the aim of our study was to evaluate the long-term effects of a 5-year cKD on body composition, resting energy expenditure, and biochemical parameters in a sample of children affected by GLUT1-DS.

2. Methods

2.1. Ethics statement

The study protocol was approved by the ethics committee of the Fondazione IRCCS Policlinico San Matteo di Pavia (reference number 20180083746) and complied with all principles of the Declaration of Helsinki. All caregivers provided written informed consent before the beginning of the study.

2.2. Study design and inclusion criteria

This was a prospective, multicenter, 5-year longitudinal study of children with GLUT1DS treated with a cKD. The primary outcome was to evaluate the change from pre-intervention in nutritional status, including anthropometric measurements, body composition, resting energy expenditure, and biochemical parameters, such as glucose, insulin, HbA1c, HOMA-IR, lipid profile (triglycerides [TGs], total cholesterol [TC], low-density lipoprotein cholesterol [LDL-C], and high-density lipoprotein cholesterol [HDL-C]), liver enzymes (alanine aminotransferase[ALT], aspartate aminotransferase[AST], gamma-glutamyl transferase [GGT]), uric acid, creatinine, and ketonemia.

We conducted assessments prior to the initiation of cKD intervention and then every 12 months or 5 years during the cKD interventions.

2.3. Participants

Patients were recruited from the Department of Child Neuropsychiatry of the Casimiro Mondino IRCCS Foundation (Pavia, Italy) and the Pediatric Neurology Unit of the Vittore Buzzi Hospital (Milan, Italy) starting in October 2010 and followed until March 2019.

Patients were required to have no contraindications as defined by the latest Consensus (4), such as β-oxidation defects, carnitine deficiency, pyruvate carboxylase deficiency, porphyria and other specific disorders involving fatty acid transport and oxidation. Moreover, we excluded children with parents or caregivers that were noncompliant or unable to maintain adequate nutrition (4).

Nutritional measurements were performed at the International Center for the Assessment of Nutritional Status (ICANS) of the University of Milan. cKDs were implemented at the Center for Research on Human Nutrition and Eating Disorders in Pavia and at ICANS of the University of Milan according to similar guidelines (4).

Fifteen children and adolescents (11 females and 4 males, mean age 8.6 ± 3.0 years), all diagnosed with GLUT1-DS, were prospectively enrolled. Specifically, patients with GLUT1- DS underwent fasting lumbar puncture (at least 5–6 h of fasting), where a cerebrospinal fluidglucose level less than 0.6 was considered suspicious for the presence of GLUT1-DS (22). In addition, for final confirmation, all patients were subjected to mutation analysis of the SLC2A1 gene.

2.4. Ketogenic diet setting

Patients started the dietary protocol at home, with a gradual increase in the ketogenic ratio and with no fasting required, according to the previously published protocol (2).

At pre-intervention, each patient filled out a dietary history with a registered dietician to assess their habitual caloric intake, intolerances, and food preferences. The cKD was tailored to the patient, considering REE-related energy expenditure measured by indirect calorimetry and physical activity level, as well as the appropriate ketogenic ratio to be achieved. Where necessary, appropriate modifications were made to the caloric prescriptions during follow-up. All participants received a normocaloric diet, with a minimum protein intake of 0.7 g/kg (23) and a sugar-free, multivitamin, multimineral, and potassium citrate supplementation (depending on gender and age). Each patient's allergies, intolerances, and food preferences were also taken into account. Before the beginning of the dietary protocol, patients and caregivers received preliminary counseling to clarify any doubts and facilitate understanding of the ketogenic diet, the attention and time required for meal preparation, food costs, and possible side effects of the diet.

TABLE 1 Composition of the prescribed cKDs.

	Mean	SD
Energy intake (kcal/day)	1,532	535
Energy intake/BW (kcal/kg)	46.1	23.0
Protein (g/day)	29.9	13.5
Protein/BW (g/kg)	0.9	0.6
Fat (g/day)	146.6	52.4
Fat (%)	86.3	10.1
SFA (g/day)	53.9	20.0
SFA (%)	36.1	11.7
Carbohydrate (g/day)	22.3	10.5
Carbohydrate (%)	5.8	2.7
Ketogenic ratio	2.9	0.6

BW, body weight; SFA, Saturated Fatty acids.

Table 1 shows macronutrient composition and ketogenic ratio at the beginning of the cKD after the first week of ketosis induction.

Patients started the diet therapy at home with an initial ratio of 1:1, then gradually increased to a ratio of 2:1, and finally 3:1 or 4:1. The final ketogenic ratio was determined based on patient tolerance and ketonemia trends to ensure stable blood BHB values >2.0 mmol/L.

To monitor patients during follow-up, caregivers were instructed to check and note capillary ketonemia and ketonuria daily.

2.5. Neurological assessment

Neurological evaluations and electroencephalography (EEG) were performed at pre-intervention and once a year thereafter, at the Department of Neurology and Child Psychiatry, Fondazione IRCCS Istituto Neurologico Casimiro Mondino in Pavia and at the Pediatric Neurology Unit, "V. Buzzi" Hospital in Milan, according to the 2011 Italian consensus on ketogenic therapy based on the WHO guidelines (22).

The following neurological symptoms were monitored: seizure types and their frequency, paroxysmal dyskinesia, spasticity, ataxia, dystonia, dysarthria, and muscle strength. Also, caregivers were asked to note alertness, activity, and seizure episodes on a daily basis.

2.6. Main outcomes: assessment of nutritional status

Nutritional status assessment is the result of anthropometric measurements, body composition in terms of fat and fat free mass, resting energy expenditure, and biochemical parameters (24).

Anthropometric measurements were performed by the same trained dietitian according to conventional measurement criteria and procedures (24).

Body weight (BW, kg) and body height (BH, cm) were measured with an accuracy of 100 g and 0.5 cm, respectively. Body mass index (BMI) was calculated using the formula Body Weight (kg) / Body Height^2 (m^2).

Sex-specific BMI-for-age percentiles and Z scores were calculated based on the 2000 Centers for Disease Control and Prevention (CDC) growth charts (25). According to CDC guidelines, a z-score of ≤ -2 was considered severely underweight, a score between -2 and -1 was considered underweight, between -1 and $+1$ was considered normal weight, between +1 and +2 was considered overweight, and a score ≥ 2 was considered obese.

Waist circumference (WC) was measured to the nearest 0.1 cm with a non-elastic tape. With the patient standing, and following normal exhalation, the measurement was taken at the midpoint between the last rib and the iliac crest in a horizontal plane. WC was quantified according to reference tables for age and sex (26).

Skinfold thickness was measured on the non-dominant side of the body using a Holtain LTD caliper at the triceps skinfold landmark. All measurements were taken in triplicate for all sites, and the mean of the three values was calculated. The intra-observer variation for skinfold measurements ranged from 2.5 to 2.9%.

Arm Muscular Area (AMA) and Arm Fat Area (AFA), indicators of nutritional status degree, muscle and fat mass amount, respectively, were calculated according to the following formulas:

AMA = [Arm circumference (cm) - (Tricipital Skinfold (mm) * 3.14)]^2/ (4 * 3.14).

AFA = [Arm circumference (cm) / (4 * 3.14)] - AMA^2.

AMA and AFA have been interpreted according to the CDC percentiles (25).

Body composition assessment was performed by Dual Energy X-Ray Absorptiometry (DEXA) at the ICANS center, using a GE Lunar iDXA, Boston, United States.

DEXA is a body composition analysis technique that allows the identification of three compartments: Bone Mineral Content (BMC), Lean Body Mass (LM), and Fat Mass (FM). Total body scans were performed by a single operator on all subjects in the supine position. The whole body of each subject was scanned in an average exposure time of 15 min. Coefficient of Variation were less than 1% for all measurements (27). Bone Mineral Density (BMD) can be obtained from this examination: a z-score < -2 indicates a value below the expected range for age, thus detecting presence of osteoporosis (27). We calculated fat mass index (FMI, kg/m2) in children and adolescents by dividing FM by the square of height. Total FM, Fat Mass Index, and Lean Mass Index were interpreted according to the body composition of reference children (28).

Resting energy expenditure (REE) was measured by means of an open-circuit ventilated hood system (Sensor Medics 29, Anaheim, CA, United States). All measurements were taken in the fasting state (minimum 12–14 h of fasting), for at least 30 min according to a detailed previously published protocol (14). REE was calculated using the abbreviated Weir equation (29).

Regarding *biochemical parameters*, fasting blood samples were collected by venipuncture of the antecubital vein in a sitting or reclining position, using vacuum-sealed test tubes. After centrifugation (800 g for 10 min at 5°C), aliquots of serum sample were stored at 80°C until further analysis. An autoanalyzer (Cobas Integra 400 plus, Roche Diagnostics, Mannheim) was used to determine serum concentrations of glucose, TC, HDL-C, LDL-C, TG, AST, ALT, GGT, creatinine, and uric acid. Circulating insulin was measured in duplicate by an autoanalyzer (Cobas e411 Hitachi, Roche Diagnostics). The homeostatic model of insulin resistance assessment (HOMA-IR) was calculated as [fasting glucose (mg/dL) × fasting insulin (mU/L)/405] (30). Glycated hemoglobin (HbA1c) was determined by turbidimetric inhibition immunoassay for hemolyzed whole blood using an autoanalyzer. The % HbA1c was obtained by the ratio of HbA1c concentration to total blood hemoglobin concentration. The following values were defined as high: TC $\geq$ 200 mg/dL, LDL-C > 130 mg/dL, TG > 150 mg/dL, HOMA-IR $\geq$ 3.16 for children, blood glucose $\geq$100 mg/dL, HbA1c $\geq$ 6%, and insulin >23 μU/mL, while the following values were considered low: HDL < 40 mg/dL for males and < 50 mg/dL for females (30–32). As for liver enzymes, AST $\geq$ 30 U/L, ALT $\geq$35 U/L, and GGT $\geq$ 18 U/L were considered high in females, while AST $\geq$ 45 U/L, ALT $\geq$40 U/L, and GGT $\geq$ 28 U/L were regarded as high in males according to the of the ICANS laboratory normal upper limit. Capillary ketonemia was measured with an *in vitro* β-ketone self-testing medical diagnostic device (GlucoMen LX PLUS, Menarini Diagnostics, test range 0.1 mmol/L-8.0 mmol/L). Ketonuria was measured using a urine ketone test (Ketostix®, Bayer Diabetes, Berkshire, United Kingdom).

2.7. Statistical analysis

Continuous variables are presented as mean ± standard deviation. An independent t-test was used to compare the means of nutritional and biochemical variables among GLUT1-DS children. Levene's test was performed to assess the equality of variances for a variable calculated for the groups. A one-way repeated measures ANOVA with post-hoc Bonferroni comparison test was run to determine if there were differences in the variables of interest during the 5 years of dietary treatment.

3. Results

3.1. Pre-intervention

Table 2 shows the clinical characteristics of all patients, with mutations' types, phenotypes and pharmacological treatments at diagnosis.

Fifteen patients were recruited. All patients resulted positive to the SLC2 A1 test. The main phenotypes were generalized epilepsy and intellectual disability. Ten patients (67%) were not following any drug therapy. Three patients started cKD being younger than 6 years old.

Table 3 shows the nutritional characteristics at baseline. Only two patients were underweight and one was obese, as shown also by high waist circumference, high body fat and high AFA. The remaining reported normal nutritional status and body composition. All 15 were included in the study.

3.2. Intervention

All patients completed the 5-year protocol. The range of the prescribed KRs was 2–3:1 with carbohydrates intake lower than 10% and fat percentage amounting to minimum 80%. Energy and protein

TABLE 2 Clinical characteristics of recruited patients.

Patient	Age at diagnosis	SLC2 A1 testing	Mutations	Phenotype	Pharmacological treatments	Kind of diet and KR	Age at the diet-start
1	6 years	Yes	Protein mutation c.26C > T, gene p.Thr9Met, p.T9M	Generalized epilepsy (absence seizures)	No	cKD - 3:1	6 years
2	9 years	Yes	Protein mutation R223W, gene p.Arg223Trp	Focal and generalized (absence epilepsy),PED (chorea, dystonia)	Oxcarbazepine	cKD - 2,5:1	9 years
3	5 years	Yes	Protein mutation R153C, gene p.Arg153Cys	Generalized epilepsy (absence epilepsy and tonic clonic sezures), chorea, ataxia, stroke like episodes	No	cKD - 2.5:1	5 years
4	9 years	Yes	Protein mutation R458W, gene p.Pro485Leu	Generalized epilepsy (tonic clonic sezures), PED (chorea and dystonia), migraine, weakness	Valproate	cKD - 2.5:1	9 years
5	10 years	Yes	Protein mutation g.33411C > T, gene p. Arg126Cys	Generalized epilepsy (tonic clonic sezures, myclonic absence sezures), PED (dystonia), intellectual disability	Levetiracetam	cKD - 2.5:1	11 years
6	9 years	Yes	Protein mutation V165I, gene p.Val165Ile	PED (chorea, dystonia), intellectual disability	No	cKD - 3:1	9 years
7	NA	Yes	Protein mutation R249Afs131X, gene p. Arg249Ala fs*131	Generalized epilepsy (myclonic absence seizures), intellectual disabilityi	No	cKD - 3:1	NA
8	15 years	Yes	Protein mutation R400H, gene p.Arg400His	Intellectual disability, PED (dystonia, myoclonias), ocular movement disorder	Valproate	cKD - 2.5:1	15 years
9	NA	Yes	Protein mutation c.370delC, gene p.Leu124Trpfsx12	Intellectual disability,generalized epilepsy (myclonic seizures), chorea and ataxia, ocular movement disorder	No	cKD - 3:1	NA
10	7 years	Yes	Protein mutation p.N34S, gene p.Asn34Ser	Intellectual disability, generalized epilepsy (absence seizures)	No	cKD - 3:1	7 years
11	7 years	Yes	Protein mutation C.1198 > T, gene p. Arg400Cys	Intellectual disability, generalized epilepsy (absence and myoclonic seizures), PED (dystonia) ocular movement disorder	No	cKD - 3:1	7 years
12	NA	Yes	Protein mutation c.884C > T, gene Thr295Met	Generalized epilepsy (absence seizures), PED (dystonia)	No	cKD - 2,5:1	3 years
13	NA	Yes	Protein mutation p.R223W	Generalized epilepsy (myoclonic seizures), ocular movement disorder	No	cKD - 2,5:1	4 years
14	NA	Yes	Protein mutation R458W, gene p.Pro485Leu	Generalized epilepsy (tonic clonic sezures), PED (chorea and dystonia), weakness	Valproate	cKD - 3:1	8 years
15	11 years	Yes	Protein mutation c.26C > T, gene p.Thr9Met, p.T9M	Generalized epilepsy (absence seizures)	No	cKD - 3:1	11 years

cKD, classic Ketogenic Diet; NA, not applicable; KR, ketogenic ratio; PED, Paroxysmal Exercise-induced Dyskinesia.

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TABLE 3 Nutritional characteristics at baseline.

	N (%)
Weight	
< −2 z-score	2 (13,3%)
> +2 z-score	1 (6,7%)
Height	
< −2 z-score	2 (13,3%)
> +2 z-score	0 (0%)
BMI	
< −2 z-score	2 (13,3%)
> +2 z-score	1 (6,7%)
Waist circumference	
< −2 z-score	0 (0%)
> +2 z-score	1 (6,7%)
Arm Circumference z-score	
< −2 z-score	1 (6,7%)
> +2 z-score	1 (6,7%)
Arm Muscle Area z-score	
< −2 z-score	1 (6,7%)
> +2 z-score	0 (0%)
Arm Fat Area z-score	
< −2 z-score	0 (0%)
> +2 z-score	1 (6,7%)

BMI, Body Mass Index, kg/m^2.

intake was adjusted according to body weight (23), maintaining the same ketogenic ratio.

3.3. Post-intervention

All children reached the therapeutic range of KBs (beta-hydroxybutyrate >2.0 mmL/l) and tolerated the diet well.

Table 4 and Figure 1 show the annual changes from the beginning of the cKD.

KBs increased significantly in children and adolescents with values greater than 2 mmol/L, and remained stable at 5-y.

Concerning growth, we found a significant increase in weight and height during the time course but according to the baseline percentiles. BMI z-score did not change during the 5 years, as body composition in terms of waist circumference, AMA and AFA.

Specifically, one patient who was underweight at baseline remained underweight, while the obese patient attained normal weight.

BMC and BMD increased significantly over time according to increasing age. FM% decreased significantly and gradually, in line with the increase in BW and the consequent growth in LM.

As expected, we found a negative trend in respiratory quotient going from indicative of a balanced diet (RQ > 0.80) to a hyperlipidic diet (RQ < 0.7), while fasting insulin and HOMA index decreased significantly after cKD initiation. No patient showed biochemical parameters above the cutoff; only two children reported a high HOMA-IR index value (> 3.16) and both TC and LDL-C levels above the cutoff, which returned to normalafter 1 year.

Overall, all biochemical parameters related to protein, lipid, glycaemic, and liver metabolism remained stable during the course of the dietary intervention.

4. Discussion

To our knowledge, this is the first study to investigate the long-term effects of cKD on nutritional status, in terms of anthropometric measurements, body composition, resting energy expenditure, and biochemical parameters, during a 5-year follow-up in a cohort of GLUT1-DS children and adolescents.

The reported negative RQ trend confirmed stable metabolic adaptation due to low carbohydrate intake and increased use of fat as an energy substrate and, thus, adherence to cKD (33, 34).

With regard to growth curves in children and adolescents, a slight yet non-significant decline in the z-scores of BMI-for-age, height-for-age, and weight-for-age can be observed, confirming the results reported by Tagliabue et al. who showed that after 6 months of KD, height, weight, and BMI remained approximately constant compared to pre-intervention values (14). Similarly, Ferraris et al. found that in 34 children with both GLUT1-DS and refractory epilepsy, 80% exhibited no growth retardation after 12 months of KD. Among all 15 children and adolescents in our study, only one female child remained underweight during the 5-year follow-up, and she was affected by both GLUT1-DS and cerebral palsy; the remaining children and adolescents maintained their growth trend or even improved their initial BMI z-score.

As for body composition, no significant changes in fat and lean mass standards were observed, probably due to the strict follow-up period and the closely monitored total daily calorie intake, which allows complete lipid oxidation without impairing body composition. A significant increase was found only in lean, bone mass, and not in body fat mass. Only in one female child who remained underweight and suffered from both GLUT1-DS and cerebral palsy did we observe a progressive reduction in lean mass, probably due to disease worsening. The remaining children all maintained their growth z-scores in both lean and body fat mass, except for the child who was obese at baseline. During the 5-year follow-up, in fact, he had an FM z-score in the normal range, in contrast to baseline where it was higher than normal range. These conflicting results might either be explained by reduced insulin leading to altered GH-pathways, or by the strictly controlled caloric intake, which reduces fat accumulation (35).

Our results also show that there was no significant worsening of bone health following a cKD, evidencing a significant increase in BMD as a function of increasing age. Bertoli et al. seem to support this hypothesis with a case series of adults affected by GLUT1-DS and other ultra-rare diseases (20, 21). In contrast, Bergqvist et al., in their 15-month longitudinal study of 25 children with refractory epilepsy, showed a decrease in BMC z-scores according to age and height (36). However, it should be considered that subjects already exhibited poor bone mineralization at pre-intervention, while in our study, patients had normal z-scores since pre-intervention (21). Moreover, in that study (36), patients were suffering from epilepsy, and therefore undergoing pharmacological treatments known to affect bone mineralization. In contrast, none of our patients were taking

TABLE 4 Timecourse of the nutritional changes.

	Baseline		1year		2years		3years		4years		5years		*p*-value
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	
Anthropometric measurements													
Weight (kg)	33,2a	23.2	32,1a	19.3	33,9a	18.9	*38,6b*	19.4	*40,4b*	21.0	*40,9b*	19.1	**0.026**
Weight *z*-score	−0.5	2.0	−0.8	2.0	−0.9	2.1	−0.7	2.0	−0.9	2.0	−0.8	2.1	0.975
Height (cm)	124,9a	28.7	*128,6b*	26.2	*132,4b*	24.8	*137,7b*	26.3	*140,8b*	25.0	*142,0b*	22.0	**0.002**
Height *z*-score	−0.7	1.3	−0.8	1.1	−0.7	1.1	−0.6	1.1	−0.6	1.1	−0.7	1.1	0.994
BMI (kg/m^2)	18.6	6.0	17.6	4.4	17.7	3.9	18.8	3.6	18.8	4.5	19.2	4.9	0.582
BMI z-score	−0.1	1.9	−0.6	2.1	−0.8	2.4	−0.8	3.1	−0.8	2.3	−0.8	2.4	0.978
Waist Circumference (cm)	61.9	19.2	60.9	14.6	62.7	13.4	66.2	13.0	66.5	13.5	66.8	13.2	0.546
Waist Circumference *z*-score	1.8	2.5	1.2	1.9	0.8	1.6	0.8	1.6	0.9	1.8	0.6	2.0	0.466
Arm Circumference (cm)	21.9	6.5	20.7	5.6	20.9	4.9	23.0	4.9	21.4	5.0	23.1	5.6	0.258
Arm Circumference *z*-score	0.1	0.6	−0.7	1.6	−0.7	1.2	−0.6	1.2	−0.7	1.1	−0.6	1.3	0.316
Triceps_skf (mm)	14.2	7.1	13.3	5.0	13.3	5.8	13.1	5.2	14.5	7.8	14.2	7.5	0.848
Arm Muscle Area (cm^2)	25.5	14.6	23.3	13.5	23.1	9.9	28.6	11.2	27.8	13.5	30.1	15.7	0.216
Arm Muscle Area *z*-score	0.2	1.5	−0.7	1.6	−0.6	1.1	−0.5	1.0	−0.6	1.6	−0.7	1.5	0.516
Arm Fat Area (cm^2)	15.8	10.4	13.1	7.7	13.6	8.4	15.3	8.0	10.5	6.6	14.6	10.7	0.313
Arm Fat Area *z*-score	0.4	1.4	0.0	0.7	−0.4	0.7	0.6	0.8	−0.3	0.8	−0.1	1.4	0.412
Body composition													
BMC (g)	1,056,7a	709.7	945,4a	474.2	1,186,6a	739.8	*1,567,4b*	844.2	*1,340,8b*	635.0	*1,470,1b*	898.6	**0.031**
BMD	0,780a	0.218	0,732a	0.151	0,810a	0.186	*0,898b*	0.205	*0,846b*	0.172	*0,925b*	0.211	**0.001**
BMD *z*-scores	0.1	0.7	0.1	1.0	−0.2	1.1	0.1	0.1	−0.1	1.1	−0.1	1.6	0.427
FM (kg)	10.8	8.8	8.1	5.5	10.2	7.4	13.3	6.2	10.7	6.7	12.2	9.1	0.284
FMI	5.9	3.0	4.9	1.8	5.4	2.3	6.1	1.8	5.1	2.2	5.4	3.5	0.100
FMI *z*-score	0.1	1.6	−0.5	1.1	−0.2	1.4	0.4	0.6	−0.6	1.2	−0.4	1.3	0.616
FM (%)	31.1	8.1	29.9	5.8	29.7	6.7	30.4	6.0	27,74b	5.9	27,43b	8.7	**0.013**
FM *z*-score	−0.1	1.7	−0.5	1.2	−0.4	1.6	0.3	0.6	−0.5	1.5	−0.3	1.3	0.077
LM (kg)	21,2a	14.5	20,9a	12.3	22,3a	12.7	24,6a	12.3	27,4b	14.0	30,7b	13.9	**0.001**
LMI	11,6a	2.0	11,3a	1.1	11,7a	2.1	13,1a	2.0	14,0b	2.3	14,3b	3.3	**0.032**
LMI *z*-score	0.2	1.2	0.1	1.2	−0.1	1.1	0.4	0.5	−0.1	1.2	−0.1	1.6	0.637
Resting energy expenditure													
VO_2	0.1	0.1	0.2	0.1	0.1	0.1	0.2	0.1	0.2	0.0	0.2	0.0	0.543
VCO_2	0.1	0.1	0.1	0.0	0.1	0.0	0.1	0.0	0.1	0.0	0.1	0.0	0.686
RQ	0,81a	0.1	0,77a	0.1	0,77a	0.0	*0,76b*	0.1	*0,76b*	0.0	*0,76b*	0.0	**0.041**

(Continued)

TABLE 4 (Continued)

	Baseline		1year		2years		3years		4years		5years		*p*-value
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	
REE (kcal/die)	844a	473	896a	387	915a	392	*1059b*	399	*1153b*	333	*1146b*	374	**0.030**
REE/weight (kcal/kg weight)	36.0	10.7	39.1	10.9	39.7	11.3	33.1	13.1	34.9	7.6	31.7	9.7	0.260
Biochemical parameters													
Urea (mg/dl)	19	9	19	5	19	6	21	5	18	5	18	5	0.908
Creatinine (mg/dl)	0.3	0.1	0.3	0.1	0.3	0.1	0.4	0.1	0.4	0.1	0.4	0.1	0.146
Uric acid (mg/dl)	4.7	1.3	5.6	1.6	5.6	1.2	5.3	1.1	5.3	1.0	5.4	1.0	0.154
TC (mg/dl)	168	42	168	39	163	39	157	30	160	32	163	26	0.446
HDL-C (mg/dl)	56	15	59	18	57	16	56	16	52	14	51	11	0.978
LDL-C (mg/dl)	101	30	100	26	100	28	96	22	98	25	104	20	0.419
TC/HDL	3.1	0.7	3.0	0.7	3.0	0.7	2.9	0.6	3.3	1.0	3.3	0.6	0.549
LDL/HDL	1.9	0.6	1.8	0.5	1.9	0.7	1.8	0.5	2.0	0.8	2.1	0.5	0.997
TG (mg/dl)	66	24	58	18	57	17	64	24	66	25	64	29	0.525
Glucose (mg/dl)	87	5	79	10	79	8	86	7	86	9	87	11	0.108
Insulin (mU/mL)	10,9a	12.0	*4,5b*	2.9	*5,5b*	3.2	*5,8b*	6.5	*5,1b*	3.2	*5,7b*	2.9	**0.047**
HbA1c (%)	4.8	0.4	4.5	0.3	4.5	0.6	4.7	0.3	4.7	0.3	4.5	0.6	0.216
HOMA index	2,3a	2.6	*0,9b*	0.6	*1,0b*	0.8	*1,7b*	1.5	*1,3b*	0.7	*1,3b*	0.6	**0.048**
AST	29.1	15.4	26.4	7.0	25.3	8.0	21.2	6.5	22.4	9.7	20.0	6.3	0.290
ALT	20.5	23.9	22.2	10.1	19.2	12.8	13.9	4.1	16.5	5.6	14.9	7.8	0.649
GGT	14.5	8.1	12.9	6.0	11.2	5.2	11.6	4.5	11.3	3.6	10.7	4.2	0.340
KBs (mmol/L)	0.1	0.1	2.8	0.7	2.5	1.8	2.4	1.9	2.5	1.8	2.6	1.4	**0.041**

In bold, $p < 0{,}05$. a,b.

BMI, Body Mass Index; BMC, Bone Mineral Content; BMD, Bone Mineral Density; FM, Fat Mass; FMI, Fat Mass Index; LM, Lean Mass; LMI, Lean Mass Index, VO_2, oxygen volume; VCO_2, Carbon Dioxide Volume; RQ, Respiratory Quotient; REE, Resting Energy Expenditure; TC, Total Cholesterol; HDL, High Density Lipoprotein; LDL, Low Density Lipoprotein; TG, Tryglicerides; HBA1C, Glycosilated Hemoglobin; HOMA, Homeostasis model assessment; ALT, Alanine Amino Transferase; AST, Aspartate Amino Transferase; GGT, γ-glutamyl transferase; KB, Ketone Bodies.

anti-epileptic drugs. The metabolic acidosis produced by cKD could play a role in the decrease in BMC, and studies in this regard with increased vitamin D and calcium supplementation or with alkalizing agents would be interesting (37). All of our patients take calcium and vitamin D supplements, as well as an alkalinizer, and this could be the other reason why we observed no worsening of bone. No studies have evaluated 24-h calciuria in GLUT1-DS patients, but our data suggest that chronic ketosis due to controlled cKD does not affect bone health. The role played by the Growt Hormone-stimulating hormone ghrelinmight also be of interest. However, in a previous study, we found no changes in ghrelin levels in 30 GLUT1-DS and refractory epilepsy patients on cKD, nor a correlation between cKDand nutritional status or body composition (16). In conclusion, these data suggest that a well-structured cKD provides adequate energy intake to maintain patients' growth percentiles during childhood and adolescence (19), although GLUT1-DS disease has been found to be associated with nutrition and growth impairment at least until puberty (38). In addition to monitoring growth parameters, it is also recommended to periodically check body composition status in cKD subjects at risk for malnutrition (37).

Concerning biochemical parameters, no statistically significant changes were observed in lipid profile: only two children reported elevated TC and LDL-C levels above upper limits at baseline, which returned to normal as early as the first year of KD and remained in the normal range during the 5-year follow-up. These results are in contrast to those of the study by Reza Zamani et al. who reported increased TG, TC, and LDL, and decreased HDL in 33 children (39). In line with our results, a 10-year follow-up study by Heussinger et al. concluded that the lipid profile parameters reflected the pre-intervention status and showed no statistically significant changes in BMI, diastolic and systolic blood pressure, and carotid intima-media thickness as well (17), validating the hypothesis of fat utilization as an energy substrate that prevents body fat accumulation in children and adolescents. In addition, the absence of detrimental effects of fat intake on body fat accumulation and lipid profile could support the findings of Dehghan M et al. (40) that dietary fats, including saturated and unsaturated fatty acids, were associated with lower risk of total mortality and stroke when compared to high carbohydrate intake. Moreover, the quality of fats typical of Mediterranean countries (favoring foods rich in mono- and polyunsaturated fatty acids at the expense of saturated ones, such

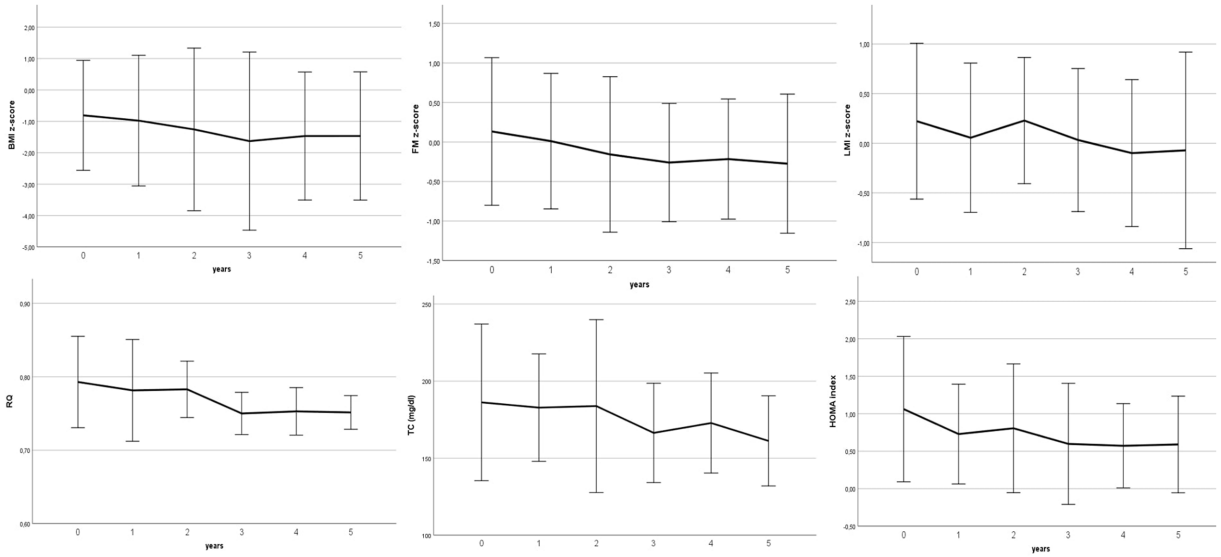

FIGURE 1
Timecourse of principal nutritional outcomes.

as oily fish, extra virgin olive oil, and nuts) has probably played a role in maintaining a stable lipid profile. On the other hand, insulin profile decreased significantly in the short term, as observed in short-term studies with follow-ups of 3, 6, or 12 months (2, 14, 16) and remained low even after 5 years. When a decrease in insulin is noted, it is reasonable to assume that this results in reduced stimulation of the GH hormone and, consequently, reduced IGF-1 production. Spulber et al. confirm this hypothesis by showing a negative correlation between β-hydroxybutyrate and growth rate and IGF-1 levels. Thus, even moderate calorie restriction could worsen growth rate in height, a hypothesis that should be considered when establishing a subject's energy requirements (41). Constant monitoring of the REE and energy intake in line with measured needs allowed us to maintain physiological height growth. Regarding liver and kidney function, none of the patients reported abnormal values and significant changes in AST, ALT, GGT, urea, creatinine, and uric acid values.

The quality of the data collected is one of the strengths of our study, as all measurements and biochemical assays were mainly collected at the same center, thus ensuring less variability. It should also be noted that this is a multicenter study. In addition, it is the only study in the literature that has comprehensively assessed the nutritional status of GLUT-DS patients over such a long follow-up and has considered both body composition and REE, in addition to growth and biochemical parameters. REE and body composition were assessed by indirect calorimetry and DEXA, the gold standard methods for measuring energy needs and BMD, FM and LM, respectively. Finally, all of our patients were following the same diet, that is the cKD: although not traditionally recommended, patients with GLUT1-DS, especially during the adolescence, could be treated with a more moderate ketogenic, lower-fat diet, such as the Atkins diet, because it is hard to adhere to a strict cKD (5). It would be interesting to know the long-term effects on nutritional status of this other dietary treatment.

We are aware that there are a number of potential limitations: a control group was not included in the study, as GLUT1-DS management guidelines require the use of cKD from day 1 of diagnosis. Another limitation is the small number of patients recruited. However, it should be noted that GLUT1-DS is a very rare disease, so it is difficult to find a large representative sample: multicenter studies should be conducted to study larger samples. Furthermore, gender differences within the selected sample were not taken into account, with women representing the majority of the subjects included. It should also be considered that children with GLUT1-DS may have disease-related growth patterns. Therefore, an even more precise estimate of growth would require growth curves, which do not exist today, and should be designed *ad hoc* for this condition. As for BMD assessment, it should be noted that, although the subjects were adequately supplemented, no statistical analysis of changes in serum calcium and vitamin D values was performed. Finally, it is worth highlighting that the age of diagnosis and consequently the age of cKD initiation were different, which probably caused a different impact on nutritional status.

Here we present the 5-year effects of cKD. Long-term adherence to cKD had a good safety profile on anthropometric measurements, as well as body composition, resting energy expenditure, and biochemical parameters, and we found no evidence of potential adverse effects on the nutritional status of children and adolescents.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The studies involving human participants were reviewed and approved by Fondazione IRCCS Policlinico San Matteo di Pavia (reference number 20180083746). Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

Author contributions

SB and RD: conceptualization and methodology. RD, AL, and AF: formal analysis. SB, RD, CL, MP, CF, AT, PV, VD, OS, and RP: investigation. RD, AF, SO, and RP: data curation. RD and MP: writing–original draft preparation. SB, AL, AB, CF, VD, AT, SO, PV and RP: writing–review and editing. SB: supervision. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Low glycemic index therapy in children with sub-acute sclerosing panencephalitis (SSPE): an experience from a measles-endemic country

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Introduction: Sub-acute sclerosing panencephalitis (SSPE) is a chronic, progressive neurodegenerative disorder, commonly seen in measles-endemic countries leading to progressive neuronal loss and death. Currently, there is no proven cure for this devastating disease. We started a low glycemic index therapy (LGIT) in children with SSPE using the same principle as per its role in intractable epilepsy.

Methodology: Low glycemic index diet was started in children with a confirmed diagnosis of SSPE based on Dyken's criteria. All children were then classified into four stages according to disease progression. The response to diet was evaluated by improvement in their myoclonic jerks, motor activities, and changes in their stage of the disease.

Results: A total of 12 children were enrolled. The mean age was 6.65 years (range 3.3–10 years), with a male-to-female ratio of 2:1. Five children were at stage IV, five were at stage III, and two were at stage II at the start of the diet. Nine (75%) children showed improvement in their stage of illness. Of three children who were at stage IV at the initiation of the diet, one improved to stage II and two to stage III. Four children at stage III reverted to stage II. Two children initiated at stage II went into total remission. Seven (58.3%) children showed a >50% reduction in myoclonic jerks with three (25%) having a 100% reduction. Three (25%) children died due to pneumonia.

Conclusion: LGIT may play an effective role in the management of SSPE and gives hope to families having children with this potentially life-threatening disease.

KEYWORDS

SSPE, LGIT, children, neurodegenerative, myoclonic jerks

What this article adds:

A novel approach for a potentially life-threatening disease.
LGIT is a more adaptable and acceptable approach in LMICs with poor educational status.

Introduction

Sub-acute sclerosing panencephalitis (SSPE) is a chronic, progressive neurodegenerative disorder that leads to progressive neuronal loss and death (1).

The Jabbour classification characterizes SSPE by progressive cognitive decline and behavior changes followed by focal or generalized seizures as well as myoclonus, ataxia, and visual disturbance, eventually leading to the final stage of a vegetative state (1–4). SSPE is considered diagnostic if the patient fulfills at least two major and one minor Dyken's criteria (4, 5). The prognosis of SSPE is very poor, and most patients die within 1–3 years of diagnosis (4, 6). Various therapies have been attempted for treatment, but none have been proven to be effective (7).

SSPE is caused by a persistent infection or mutant measles (2). Although no prevalence data for SSPE exist, every epidemic of measles shows a rise in the cases of SSPE in Pakistan (8). Measles remains endemic in Pakistan with epidemics occurring every 2–3 years, resulting in the deaths of approximately 20,000 children annually (2, 8). While the measles conjugate vaccine (MCV1 and MCV2) coverage has improved in recent years, it is still below the recommended WHO level (9, 10). In addition to vigorous efforts for measles elimination through vaccination, it is important that we consider additional therapies for children who are already suffering from the fatal consequences of SSPE (8, 9).

A ketogenic diet (KD) has been shown to be effective in the control of intractable epilepsies (11, 12) and certain neurodegenerative disorders, i.e., Alzheimer's and Parkinson's disease (13). KD works as a neuroprotective agent by slowing or stopping neurodegeneration (13, 14). The proposed mechanisms underlying this process include the inhibition of glycolysis, resulting in the increased formation and concentration of ketone bodies, increased ATP (adenosine triphosphate) production providing more energy for the brain, decrease in free radicals, antioxidant actions (12, 13), and halting apoptosis, and stabilizing nerve-cell synapses (11, 15).

KD is a high-fat, low-carbohydrate, and moderate-protein diet which emulates the effect of fasting in producing ketosis. The classic ketogenic diet (Classic KD) comprises 90% fat and 10% carbohydrate and protein. Variation in KD distributions include modified Atkins diet (MAD), low glycemic index therapy (LGIT), and medium chain triglyceride (MCT) (12).

LGIT comprises approximately 60% fat, 30% protein, and 10% carbohydrate. The greater allowance for carbohydrates is due to the fact that the CHO foods chosen must have a glycemic index lower than 55. As foods with low glycemic index cause a lower and slower rise in blood glucose and insulin levels and lower chances of hypoglycemia, LGIT requires less strict monitoring (12, 14).

Considering the use of KD therapy as a new dimension in the management of SSPE (7, 16), we decided to start LGIT in a group of patients diagnosed with SSPE. Our main aim was to help decrease seizure frequency in these patients and assess improvement in their overall functions.

Methodology

Children coming to the Aga Khan University and National Institute of Child Health with complaints of progressive neurodegenerative regression and myoclonic seizures were evaluated for SSPE. EEGs were conducted for each patient and were considered diagnostic if they showed classic periodic, quasiperiodic, and high-voltage slow wave complexes (Radermecker complexes) with slow background activity (17). CSF was evaluated by liquor assay using an antibody index specific for measles IgG in CSF. Values of 1.5 U/ml were considered diagnostic. Those fulfilling the above criteria were then counseled on the role of LGIT in intractable seizures.

TABLE 1 General characteristics of cases.

	Minimum	Maximum	Mean
Age	3 years 3 months	10 years	6.65
Gender	8 boys	4 girls	–
Ethnicity	Varied		
Duration of treatment	2 months	24 months	13 months
Medicines	2	5	3.5

Children were then classified into one of four stages of the disease based on the Jabbour criteria: (4, 13) "Stage I, characterized by behavioral change and cognitive decline; Stage II, which is the start of the myoclonus, gradually becoming more frequent and severe; Stage III, where various combinations of pyramidal and extrapyramidal features develop, such as rigidity, dystonia, tremor, spasticity, and hemiparesis; and Stage IV, characterized by an akinetic-mute state with episodes of drenching sweat, blood pressure fluctuation, and respiratory rate abnormalities" (4, 18).

The response to diet was evaluated with the improvement in patients' myoclonic jerks per day, improvement in motor activity, and overall responsiveness leading to a change in their stage of the disease.

After thorough counseling of parents/caregivers and a formal consent procedure, the children were started on a carbohydrate washout (CHOW) diet to help the team and family understand parental compliance with the complexity of changes required. After 2 weeks on the CHOW, the children were started on the LGIT.

Diet was calculated based on the patient's ideal body weight. The type of food was chosen after taking a dietary history from the parents on food items available to them. Calculations and recipes were then formulated accordingly. A detailed written dietary prescription was given to the families in their language of understanding. Parents with no formal education and those with a mother tongue other than Urdu had their diet explained verbally, with a translator who was preferably a close relative and understood Urdu and lived with the family. All medications were converted into tablet form. All children were started on micronutrient supplements. Families were told to strictly follow the given guidelines on sugar-free daily use substances for personal hygiene such as shampoo, soap, and toothpaste. Parents were required to maintain a record sheet (Annex A in Supplementary material) about seizure frequency, feed volume and frequency, dietary intolerance, blood sugars, and urine ketones and side effects. Parents were also requested to make home videos of the child where possible.

Investigations were conducted as per pre-established protocol; however, children from extremely poor families only underwent basic testing of a complete blood count, ALT, serum electrolytes, and uric acid. The first EEG was carried out as part of the initial diagnosis.

TABLE 2 Summary table indicating individualized serology of patients with SSPE.

S. no	Serum IgG measles U/ml	CSF IgG measles U/ml (antibody index)	Oligoclonal bands and type	Electroencephalogram (EEG) at baseline
1.	>5,000	2.13	+ve Type 2	Intermittent high-voltage generalized delta bursts intermixed with bilateral fronto-central dominant sharp activity followed by 0.5–3 s of suppression period. Background diffuse delta activity.
2.	>5,000	1.5	+ve Type 2	Periodic burst with high amplitude with suppression, background showing diffuse delta theta activity.
3.	>5,000	1.5	+ve TYPE 2	Frequent quasi periodic high-voltage delta burst, followed by relatively low voltage activity with an interval of 2.5–4 s. Background showing diffuse theta and delta slowing.
4.	3646.1	1.67	+ve Type 3	Quasiperiodic bursts of high amplitude delta waves at intervals of 4–8 s with suppression of 0.5–2 s. Background showing diffuse delta theta activity.
5.	>5,000	1.5	+ve Type 2	Periodic bursts of high amplitude slow waves intermixed with spike and sharp waves are seen at intervals of 3–5 s, followed by 0.5–2 s of suppression period. Background showing diffuse delta theta slowing 4529476.
6.	>5,000	2.06	+ve Type 2	Frequent quasiperiodic generalized high-voltage delta slow waves interval of 3–12 s with myoclonic jerks Background showing diffuse delta theta slowing.
7.	>5,000	1.59	+ve Type 2	Periodic generalized high-voltage delta waves, burst with suppression of 0.5–1 s. Background showing diffuse theta and delta slowing.
8.	>5,000	1.5	+ve Type 2	Intermittent generalized high-voltage sharp and slow waves followed by a 2.0–4.0 s of suppression period. Background showing diffuse theta and delta slowing.
9.	>5,000	1.3	+ve Type 2	Intermittent to frequent bilateral fronto-central quasiperiodic independent (right predominant) at the time seems to be generalized high-voltage spike, sharps, and slow waves, followed by 0.5–3 s of electro decremental response without any clinical correlation. Background showing delta theta slowing.
10.	>5,000	1.68	+ve Type 3	Periodic generalized high-voltage sharp and slow waves followed by 2.0–4.0 s of suppression period. Diffuse theta and delta slowing.
11.	>5,000	1.5	+ve Type 2	A quasiperiodic build-up of rhythmic activity over the right hemisphere, followed by a sudden generalized suppression for approximately 1–1.5 s than there is a high-voltage generalized delta wave, which gradually wanes off in the next few seconds. Intermittently, right hemispheric epileptiform discharges are seen. Diffuse theta and delta slowing.
12.	>5,000	1.48 (Annex B in Supplementary material)	+ve Type 2 (Annex C in Supplementary material)	Intermittent delta theta burst intermixed with spike and wave discharges with suppression of 0.5 s. Background diffuse delta theta slowing (Supplementary Figure 2).

Follow-ups were conducted monthly either physically or through phone calls. EEGS were carried out only after considering the affordability of the patients. A change denoting improvement was considered if the high-voltage slow wave complexes became infrequent, or there were no ictal discharges associated with jerks, or the background returned to normal.

Results

This study included 12 patients. Of these, 11 patients were confirmed for SSPE-based CSF/serum MV-specific IgG index. One child was diagnosed as probable SSPE based on the borderline-raised CSF antibody index but fulfilled all other criteria. The general characteristics of the patients are shown in Table 1.

Oligoclonal bands were positive in all children, and measles IgG in CSF and serum was measured in all cases, with very high CSF values in all except one patient and high serum values in all patients. EEG was diagnostic of SSPE in all patients upon initial diagnosis (Table 2).

Parents of all children agreed to start the diet after formal consent. Two children continued CHOW after they showed improvement and parents refused to change to LGIT.

Patients' ages ranged from 3 years 3 months to 10 years at the time of dietary therapy initiation. Patients belonged to various

ethnic backgrounds. This included three families that were Sindhi-speaking, three were Balochi-speaking, two were Pashto-speaking, two were Urdu-speaking, one was Punjabi-speaking, and one was Balti-speaking.

All children were malnourished at the start of the diet. In total, 75% of children were in the <5th centile for their weight for age, while the remaining 25% were in the <25th centile. The history of measles was observed in all children, with >90% having measles at or before 2 years of age and five patients developing it younger than 1 year of age. Six children were at stages III and IV and had a rapidly progressive disease, which had reached their current stage between 1 and 6 months.

One child was previously diagnosed with HIV and was under treatment for HIV before developing SSPE. MRIs were conducted for five children before initiating the diet. The findings were normal in three cases; one case showed multifocal hyperintense signals in T2 and flare in the cortical and subcortical areas, and the other case showed a large left temporal arachnoid cyst.

The total duration of LGIT ranged from 2 months to a maximum of 2 years with a mean of 13 months. Children, who were started on LGIT, were on a minimum of two and a maximum of five anti-seizure medications (ASM). Seven children received Isoprinosine prior to starting LGIT with no change noted (Table 1).

Five children were at stage IV, five were at stage III, and two were at stage II at the start of the diet. Nine (75%) children improved in their stages. Two of these children were at stage IV on the initiation of the diet; one improved to stage II and two improved to stage III. Four of the improved children were at stage III at the start of the diet and reverted to stage II. Two children, initially only on CHOW at stage II, went into complete remission, with no seizures or jerks and total physical independence. Two children at stage IV and one at stage III at the start of the diet showed no change in their state (Table 3).

Continuous myoclonic jerks and significant head drops were initially observed in all children. Seven (58%) children showed a >50% reduction in myoclonic jerks. Three (42.8%) of these seven children were in complete remission of myoclonic jerks, two of whom had completely remitted on the disease and one improved from stage IV to stage III.

Based on the number of jerks noted in motor activity and change in stage, the average duration of response to the diet was 1 month with a range of 15 days to 3 months. Blood sugar ranged between 50 and 100 mg/dl in all cases. Urinary ketones were recorded minimally with an average of +1 to +2 ketones noted.

Five children, two without and three with support, started ambulating. Two relapsed again, one after breaking the diet and the other after developing fatal pneumonia.

Three children died due to pneumonia. One of them became ambulant as mentioned above, but the other two died very early after starting treatment with no change seen during the early stages. Nine children are alive, six of whom are still on treatment, whereas three are lost to follow-up; these families were called to inquire about their status, and the children are still alive on the last call (Figure 1).

Case #2 (Table 3) is the longest survivor on the diet, whereas the shortest duration of the diet was 2 months (lost to follow-up).

Six children required admission, three due to pneumonia and two due to dehydration, requiring intravenous hydration.

TABLE 3 Change in stages of SSPE.

	Stage (baseline)	SSPE stage after 3 months	SSPE stage after 6 months
Patient 01	4	3	Died
Patient 02	4	3	3
Patient 03	4	2	3
Patient 04	3	2	Lost to follow-up
Patient 05	3	3	Lost to follow-up
Patient 06	3	2	3
Patient 07	4	4	4
Patient 08	4	4	4
Patient 09	3	2	4
Patient 10	2	0	0
Patient 11	3	3	2
Patient 12	2	0	0

One child was admitted for monitoring dietary compliance and reinforcement.

A total of eight children were on an oral diet, including three children who had their NG tubes removed to start the diet. The most common complications encountered during the therapy were chest congestion and constipation. The four children on NG feeding showed better compliance as compared to those on the oral diet. In one child, the mother broke the diet by giving carbohydrates and the child regressed.

Follow-up EEGs were conducted in six children between 3 and 6 months after initiating the diet. No change was seen in five of the six children. One child went into remission and the EEG normalized (Supplementary Figure 1).

A summary of the individualized results of LGIT in children suffering from SSPE is shown in Table 4.

Discussion

Early onset of measles is strongly associated with the development of SSPE (15), the most common neurodegenerative disorder in Pakistan. While, in recent years, the experience of developed countries with SSPE mortality characterizes it as a "vanishing disease," (6) measles-endemic countries experience SSPE as the most common post-infectious neurodegenerative cause of mortality in children (19).

SSPE is entirely preventable by vaccination but is still prevalent in Pakistan due to low acceptance and coverage of the vaccine. Unpublished data from a large pediatric public sector hospital in Karachi with a specific pediatric neurology section report the average number of SSPE cases to be approximately 8–10 per month. A 1988 study from Pakistan reported that SSPE represented approximately 10% of inflammatory afflictions of the cerebral parenchyma, and its incidence rate was approximately 100 times more than that observed in developed countries (19). A 2014 study

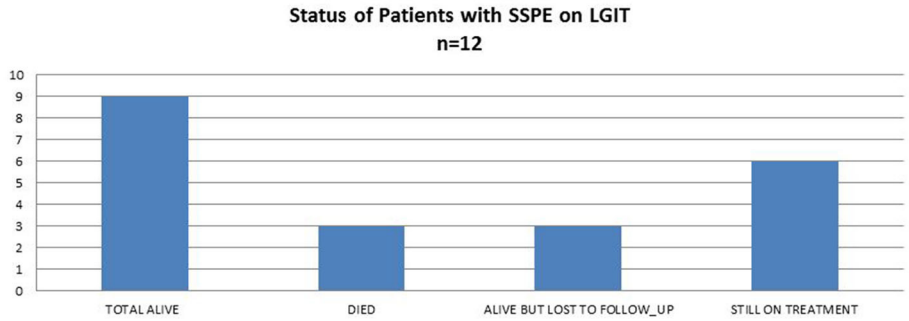

FIGURE 1
Current status of patients with SSPE on LGIT.

by Ibrahim et al. also showed an increase in the cases reported with every epidemic of measles (8, 9).

This study details the preliminary data of 12 children who, after the initiation of LGIT, showed a difference in both the control of myoclonic jerks and overall improvement in the stage of SSPE.

A systematic review conducted in India on treatment options for SSPE has indicated that KD or its various types hold the potential to be incorporated into future SSPE treatment plans (20). Another multicenter review also mentions KD as a possible option in the treatment of SSPE (7). Two case reports from India and USA have used classic KD in children with SSPE. One study reported almost complete remission of the disease in a child with stage IIIa on classic KD with improvement in cognitive as well as physical activity in 3 months (15). The other reported the case of a child showing initial improvement in myoclonic jerks after classic KD but regressing again after a few months (16).

However, a practical issue for LMICs is that classic KD is associated with higher non-compliance (21). Our own previous experience (unpublished) with KD in untraceable epilepsies also showed poor compliance with the classic KD. The cost of the classic KD often becomes devastating to most families (21). This is why, we chose to start the LGIT type of KD in our patients.

All children in our study had a history of measles before or at 2 years; however, this did not alter the response to treatment. Overall, eight (63.6%) children showed >50% reduction in myoclonic jerks, and nine (75%) children improved in their stages of disease. However, the ones not showing a significant change were the ones lost to follow-up, and we assume that, despite starting LGIT, they were also non-compliant. It is interesting to note that one patient who was in stage IV for 1 year before the start of the diet showed a response of almost 100% control in myoclonic jerks and improvement in stage III and is currently the longest survivor, giving hope that this diet may be effective, despite the stage and duration of the disease.

Two children in our study who were in stage II showed complete remission only with the CHOW. This suggests that the earlier the diet is started, the better the chances of complete remission. Both these families refused to change to LGIT despite improvement. In poor, uneducated families, compliance with a strict diet is difficult (21). CHOW seemed more acceptable and easier to manage for these families.

Mortality rates for SSPE are generally high, with death occurring within 1–3 years of the onset (2). The longest reported remission is an individual who was diagnosed at 17 years and, after initially regressing, stabilized and went into spontaneous remission with no clear explanation (22). In comparison, mortality was 25% in our study. Although the time frame of our study was only 2 years, 75% of children are still surviving, giving some hope in an endemic society like Pakistan.

Pneumonia and recurrent chest congestion were the most common complications seen in our patients. The three children who died also had severe pneumonia and eventually respiratory failure. One of these children was ventilated, and the LGIT was changed to an intravenous formulation with protein and fat calculated as per the LGIT. He showed improvement initially, however, after 1 month, he had another bout of pneumonia at home and eventually died. KD is associated with recurrent pneumonia and aspiration, which has been related to high lipid content or lower immunity (23, 24). However, the commonest complication with SSPE is also reported to be pneumonia, and at present, it is unclear whether this could be due to the primary condition or a complication of the diet (25). Children in stages III and IV are either on NG feeding or are being fed by parents forcibly. This may result in aspiration pneumonia, causing regression in the achieved improvement. A study from Lahore, Pakistan, states that malnutrition and poor socioeconomic status may be the predictors of a higher risk of complications for children with SSPE (26).

The results seen in this case series are promising and suggest that LGIT can play a beneficial role in the management of SSPE. LGIT is specifically a more practical option in lower- and middle-income countries (LMICs), where the affordability, adaptability, and acceptability of LGIT are comparatively higher than classic KD.

Conclusion

Vaccination remains the only way that the potentially fatal SSPE can be eradicated. However, for pediatric neurologists, treating children with this devastating disease is extremely challenging. The findings of these cases may serve as hope for the improvement in the quality of life of patients suffering from SSPE.

TABLE 4 Summary table showing individualized results of LGIT in children suffering from SSPE.

Pt. code	Current status of child (dead/alive)	Age (at the time of LGIT initiation)	Gender	Duration of the diet	Status (baseline)	3 months follow-up	6 months follow-up	Complications /issues encountered	Mode of feeding
1.	Dead	5.8 years	M	5 months	**Stage:** Stage IV **Jerks:** Whole body stiffness and continuous myoclonic jerks. **Motor activity**: Bed bound. **Speech and recognition:** No response to any vocal or physical stimulus, Bed bound. **EEG:** Intermittent high-voltage generalized delta bursts intermixed with bilateral fronto-central dominant sharp activity followed by 0.5–3 s of suppression period. Background diffuse delta activity.	**Stage:** Stage III **Jerks**: Myoclonic jerks improved by 20%. **Motor activity:** Bed bound. **Speech and recognition**: Eye-opening and movement improved on command, recognizes smiles but still bed bound. **EEG:** Semi periodic rhythmic monomorphic build-up of delta activity in a bilateral posterior quadrant, lasting for 2-3 s, followed by a brief low voltage of <0.2 s.	Died at 5 months	Oral ulcers, constipation, pneumonia, and vomiting.	NG
2.	Alive	7 years	F	2 years	**Stage:** Stage IV **Jerks:** Continuous myoclonic jerks, stiffness, head drops. **Motor activity:** Bed bound since 1 year. **Speech and recognition:** No speech, no response. **EEG**: Periodic burst with high amplitude with suppression, background showing diffuse delta theta activity.	**Stage:** Stage III **Jerks:** Myoclonic jerks stopped within 1 month of initiating the diet. **Motor activity:** Full support sitting on a customized chair. **Speech and recognition**: No speech, responds to pain and name calling by opening eyes and turning toward the examiner. **EEG:** Not done.	**Stage:** Stage III **Jerks:** No jerks. **Motor activity:** Full support sitting on a customized chair. **Speech and recognition:** No speech, respond to pain and name calling. **EEG** Periodic burst suppression intermittent spike and waves no ictal record noted.	Bedsore grade IV Pneumonia and repeated Chest congestion, vomiting, dehydration. Admitted to the hospital for rehydration.	NG feeding
3.	Alive (on Treatment for HIV)	4 years	M	1 year 11 months	**Stage:** Stage IV **Jerks:** Continuous head drops, full body jerks (100/day), eye up rolling, no neck holding. **Motor activity:** Bed bound. **Speech and recognition**: No speech. **EEG:** Frequent quasi periodic high-voltage delta burst, followed by relatively low voltage activity with an interval of 2.5–4 s. Background showing diffuse theta and delta slowing.	**Stage:** Stage II **Jerks:** 3–4 head drops/day. Whole body jerks decreased by 50%. **Motor activity:** Start support sitting, standing with support. **Speech and recognition:** Started talking and responding to questions could give his place of residence when asked nasogastric tube removed with full oral intake. EEG not done.	**Stage:** Stage III (relapsed) **Jerks:** Head drops 1–2/min **Motor activity:** Support sitting, standing. **Speech and recognition:** Stopped talking again after breaking the diet. On restarting, jerks have stopped and can only sit with support and no speech. **EEG:** Slow background. Intermittent to infrequent burst of high amplitude burst followed by a slow period of 0.5 s intermixed with spikes. No ictal discharges/.	Relapse due to non-compliance pneumonia and recurrent chest congestion, fever, admitted for non-compliance management. Non-compliance with the diet by the mother (mother gave roti after 2 months of diet, mother language barrier, lack of mother's understanding)	Initially on NG feeding, After 2 months Ng removed now on oral diet.

(Continued)

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TABLE 4 (Continued)

Pt. code	Current status of child (dead/alive)	Age (at the time of LGIT initiation)	Gender	Duration of the diet	Status (baseline)	3 months follow-up	6 months follow-up	Complications /issues encountered	Mode of feeding
4.	Alive	5 years	M	5 Months of therapy then lost to follow-up	**Stage:** Stage III **Jerks:** Uncontrolled whole body jerks continuous head drops, abnormal eye movement. **Motor activity:** No neck holding, bed bound. **Speech and recognition:** No speech, no response. **EEG:** Quasiperiodic bursts of high amplitude delta waves at intervals of 4–8 s with suppression of 0.5–2 s. Background showing diffuse delta theta activity.	**Stage:** Stage II **Jerks:** Whole body jerks improved by 25%. After 3 months, no jerks during sleep, only brief in the awake state. **Motor activity:** Support sitting, head control improved. **Speech and recognition**:, More alert, smiles, focusing. No EEG done.	Lost to follow-up after 5 months of diet. No EEG done.	Lost to Follow-up. Chest congestion, vomiting. Language barrier.	Oral
5.	Alive	3.3 years	M	2 Months of therapy then lost to follow-up	**Stage:** Stage III **Jerks:** Continuous head drops, continuous whole body jerks while awake. **Motor activity:** No neck holding, bed bound. **Speech and recognition:** No speech, no response. **EEG:** Periodic bursts of high amplitude slow waves intermixed with spike and sharp waves are seen at intervals of 3–5 s, followed by 0.5–2 s of suppression period. Background showing diffuse delta theta slowing 4529476.	**Stage:** Stage III **Jerks:** 10 % decrease in myoclonic jerks **Motor activity:** Neck holding improved, support sitting. **Speech and recognition**: No speech, response to pain only. **EEG:** Not done.	Lost to follow-up after 2 months of therapy.	Vomiting, loose stools. Lost to follow-up after 2 months. Language barrier, Lack of mother's understanding, family not supportive.	On Ng feed then Ng removed.
6.	Dead	8 years	M	1 year 2 months	**Stage:** Stage III **Jerks**: Whole body stiffness, continuous myoclonic jerks, > 150/day **Motor activity:** Support sitting and standing. **Speech and recognition:** Delayed verbal response. **EEG:** Frequent quasiperiodic generalized high-voltage delta slow waves interval of 3–12 s with myoclonic jerks. Background showing diffuse delta theta slowing.	**Stage:** Stage II Then, regressed after pneumonia in 1 month. **Jerks:** Myoclonic jerks decreased by 40 % in 1 month. Relapsed again after a month. **Motor activity**: Bed bound, support sitting. **Speech and recognition:** Started talking after 1 week of diet but after relapse speech lost, no response, increased crying with a loud voice. **EEG:** Not done.	**Stage**: Stage III (died after 1 year) **Jerks:** Uncontrolled continuous whole body jerks. **Motor activity:** Bed bound **Speech and recognition:** No speech, respond to pain only. **EEG:** Not done.	Admitted in Peshawar with pneumonia, long distance management repeated chest congestion, constipation.	On oral feed then NG placed.

(Continued)

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TABLE 4 (Continued)

Pt. code	Current status of child (dead/alive)	Age (at the time of LGIT initiation)	Gender	Duration of the diet	Status (baseline)	3 months follow-up	6 months follow-up	Complications /issues encountered	Mode of feeding
7.	Alive	5 years	M	7 months therapy, lost to follow-up	**Stage:** Stage IV **Jerks**: Myoclonic jerks every 2–3 sec. No neck holding, uncountable head drops. **Motor activity:** Bed bound. **Speech and recognition:** No verbal response. **EEG:** Periodic generalized high-voltage delta waves, burst with suppression of 0.5–1 s, background showing diffuse theta and delta slowing.	**Stage:** Stage IV **Jerks:** After 2 months of diet, 50% improvement in jerks and head drops. **Motor activity:** No neck holding, bed bound. **Speech and recognition:** No speech, no response. **EEG:** Not done.	**Stage:** Stage IV (lost to follow-up after 7 months). **Jerks:** Remains at 50% improvement in jerks. **Motor activity:** Bed bound. **Speech and recognition:** No speech, no response. **EEG:** Not done.	Lost to follow-up after 7 months pneumonia, low socioeconomic level, poor literacy level, loss of interest in the diet, parents became exhausted. Constipation, chest congestion.	NG
8.	Alive	10 years	F	11 months	**Stage:** Stage IV **Jerks:** Whole body stiffness, tremors, continuous myoclonic jerks, uncontrolled arm movements. **Motor activity**: Bed bound. **Speech and recognition:** Not responsive. Severely malnourished **EEG:** Intermittent generalized high-voltage sharp and slow waves followed by a 2.0–4.0 s of suppression period. Background showing diffuse theta and delta slowing.	**Stage:** Stage IV **Jerks:** After 1 month of diet, body stiffness improved, tremors decreased but were not recorded, decreased myoclonic jerks by 20%. **Motor activity:** Bed bound. **Speech and recognition:** Eye opening on pain. **EEG:** Not done.	**Stage:** Stage IV **Jerks:** Remains at 20% improvement in myoclonic jerks. **Motor activity:** Bed bound **Speech and recognition:** No speech, respond to pain only. **EEG:** Not done.	Weight loss and dehydration admitted for rehydration and dietary monitoring, constipation, pneumonia, weight loss low socioeconomic level, lost to follow-up after 5 months because of cost and reverted after being called.	NG
9.	Dead	4.8 years	M	7 months	**Stage:** Stage III **Jerks**: Head drops, whole body myoclonic jerks 100 per day. **Motor activity:** Support sitting. **Speech and recognition:** No speech **EEG:** Intermittent to frequent bilateral fronto-central quasiperiodic independent (right predominant) at time seems to be generalized high-voltage spike, sharp and slow waves, followed by 0.5–3 s of electro decremented response without any clinical correlation. Background showing delta theta slowing.	**Stage:** Stage II **Jerks**: Decreased jerks by 50%. **Motor activity:** Started support walking after 1.5 months of diet. **Speech and recognition:** No speech but responds to pain and name calling. **EEG:** Not done.	**Stage:** Stage IV (relapsed) Diet at 7 months. **Jerks:** Remains at 50 % improvement in jerks. **Motor activity:** Bed bound. **Speech and recognition:** No speech. **EEG:** Not done.	Pneumonia, dehydration, chest congestion, and vomiting.	Oral

(Continued)

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TABLE 4 (Continued)

Pt. code	Current status of child (dead/alive)	Age (at the time of LGIT initiation)	Gender	Duration of the diet	Status (baseline)	3 months follow-up	6 months follow-up	Complications /issues encountered	Mode of feeding
10.	Alive	9 years	F	1 year 4 months	**Stage:** Stage II **Jerks:** Uncontrolled head drops, jerks, mild eye twitching. **Motor activity:** Support standing and walking. **Speech and recognition:** Talking but with a delayed response. **EEG:** Periodic generalized high-voltage sharp and slow waves followed by a 2.0–4.0 s of suppression period. Diffuse theta and delta slowing.	**Stage:** Stage 0 **Jerks**: After 15 days of CHOW 50% decrease in head drops, after a month, no head drops, myoclonic jerks stopped 100%. fMotor activity: Walking without support. **Speech and recognition:** Talking and more responsive. **EEG:** Not done.	**Stage:** Stage 0 **Jerks:** No jerks. **Motor activity:** Walking without support, looking after own bodily needs but with assistance. **Speech and recognition:** Full speech and communication. **EEG:** Not done.	Issues with oral intake, food choices, difficulty to start LGIT.	Oral (CHOW)
11.	Alive	5 years	M	1 year 5 months	**Stage:** Stage III **Jerks:** Continuous jerks, whole body stiffness. **Motor activity:** No neck holding, bed bound. **Speech and recognition:** No speech. **EEG:** Quasiperiodic build-up of rhythmic activity over the right hemisphere, followed by a sudden generalized suppression for approximately 1–1.5 s than there is a high-voltage generalized delta waves, which gradually wane off in next few seconds. Intermittently, right hemispheric epileptiform discharges seen. Diffuse theta and delta slowing.	**Stage**: Stage III **Jerks:** Myoclonic jerks decreased by 60%. **Motor activity:** Support sitting. **Speech and recognition:** Response improved. After 1 month of diet and after 4 months of diet:	**Stage:** Stage II **Jerks:** Remains at 60 % decrease. **Motor activity:** Support standing and neck holding improved. **Speech and recognition:** Following commands. **EEG:** Continuous quasiperiodic high-voltage generalized delta bursts intermixed with spike, polyspike, sharp and slow waves, followed by 1.5–3.5 s of suppression period, clinically associated with myoclonic jerks.	Constipation, pneumonia, vomiting, fatty stools.	Initially oral, now on NG feed after pneumonia.
12.	Alive	10 years	F	4 Months	**Stage:** Stage II **Jerks:** Whole body jerks, uncontrolled head drops. **Motor activity:** Walking with support but frequent episodes of fall. **Speech and recognition:** Delayed verbal response. **EEG:** (Supplementary Figure 2). Intermittent delta theta burst intermixed with spike and wave discharges with suppression of 0.5 s. Background diffuse delta theta slowing.	**Stage**: Stage 0 **Jerks:** Decreased head drops 10–12 per h after 15 days of CHOW. Myoclonic jerks stopped by 100 % after 2 months. **Motor activity:** No episode of fall, started walking without support. **Speech and recognition:** Full conversation.	**Stage:** Stage 0 **Jerks:** No jerks. **Motor activity:** Waking without support, looking after own bodily needs but with assistance. **Speech and recognition:** Full conversation. **EEG:** (Supplementary Figure 1). Normal awake EEG showing a background of 8–9 Hz and no burst of delta theta waves. No spike and wave discharges seen.	Issues with oral intake, food choices, difficulty to start LGIT low socioeconomic level.	Oral (CHOW)

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A controlled trial with better follow-ups comparing both LGIT and the classic KD would be helpful to reach a conclusion as to which specific diet would be more beneficial.

Limitations

Due to limited resources, we were unable to conduct strict follow-ups and repeat investigations as required. This is the first study of its kind, and even though there was a limited number of patients, there was a demonstrated improvement. However, a full trial with strict control would be more advantageous.

Being a potentially fatal condition, it was not possible to do a randomized controlled trial for this study.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary material, further inquiries can be directed to the corresponding author.

Ethics statement

The studies involving human participants were reviewed and approved by 2023-8550-24416. SI: Low Glycemic Index Therapy in Sub Acute Sclerosing Panencephalitis (SSPE): An Experience from a Measles endemic country. ERC Aga Khan University. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

Author contributions

SI did the conception of the study and edited the manuscript. SI and HF contributed equally to the design, collection of the literature, table preparation, and writing of the manuscript. Both authors contributed substantially to the writing and revision of the manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2023.1203144/full#supplementary-material

SUPPLEMENTARY FIGURE 1
EEG after CHOW patient #12.

SUPPLEMENTARY FIGURE 2
EEG at start of therapy patient #12.

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Ketonemia variability through menstrual cycle in patients undergoing classic ketogenic diet

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Introduction: Ketogenic dietary therapies (KDT) are well-established, safe, non-pharmacologic treatments used for children and adults with drug-resistant epilepsy and other neurological disorders. Ketone bodies (KBs) levels are recognized as helpful to check compliance to the KDT and to attempt titration of the diet according to the individualized needs. KBs might undergo inter-individual and intra-individual variability and can be affected by several factors. Possible variations in glycemia and ketone bodies blood levels according to the menstrual cycle have not been systematically assessed yet, but this time window deserves special attention because of hormonal and metabolic related changes.

Methods: This study aims at searching for subtle changes in KBs blood level during menstrual cycle in female patients undergoing a stable ketogenic diet, by analyzing 3-months daily measurement of ketone bodies blood levels and glucose blood levels throughout the menstrual cycle.

Results: We report the preliminary results on six female patients affected by GLUT1DS or drug resistant epilepsy, undergoing a stable classic ketogenic diet. A significant increase in glucose blood levels during menstruation was found in the entire cohort. As far as the ketone bodies blood levels, an inversely proportional trend compared to glycemia was noted.

Conclusion: Exploring whether ketonemia variations might occur according to the menstrual cycle is relevant to determine the feasibility of transient preventive diet adjustments to assure a continuative treatment efficacy and to enhance dietary behavior support.

Clinical trial registration: clinicaltrials.gov, identifier NCT05234411.

KEYWORDS

ketonemia variability, classic ketogenic diet, menstrual cycle, ketogenic dietary therapies efficacy, drug resistant epilepsy, epilepsy, GLUT1-DS

1. Introduction

Ketogenic dietary therapies (KDTs) are well-established, safe, non-pharmacologic treatments used for children and adults with drug-resistant epilepsy and other metabolic disorders (1–3). There are currently four major KDTs: the classic ketogenic diet (CKD), the modified Atkins diet (MAD), the medium chain triglyceride diet (MCT), and the low glycemic index treatment (LGIT). There have been 4 randomized controlled trials to date (3 with class III evidence, and one with class II evidence) focusing on efficacy of KDTs compared to medications or a placebo arm, which have led to recognition of KDTs as valid and safe treatments (4). Diet quality plays a vital role in the achievement and maintenance of optimal ketosis, thus, an individualized approach, constant monitoring and the assurance of a prompt interface with keto-team are fundamental (2, 5–8).

According to international guidelines, for patients with drug resistant epilepsy KDT should be continued for at least 3 months to evaluate its efficacy, and, if well tolerated and effective, it can be continued for years and even lifelong (4). For GLUT1 deficiency syndrome (GLUT1-DS), a treatable metabolic encephalopathy characterized by complex movement disorders, drug-resistant epilepsy, and cognitive impairment, KDT is recognized as the gold standard treatment (9) and patients undergoing KDT are likely to achieve an optimal control of seizures (10).

Classic ketogenic diet is a high fat, adequate protein and low carbohydrate normo-caloric diet (1). The high-fat regimen provides about 87%–90% of daily energy intake from lipids, which are processed into free fatty acids in the liver, then oxidized in mitochondria, producing high levels of acetyl-Coenzyme A (acetyl-CoA), which cannot be oxidized in the Krebs cycle. The excess acetyl-CoA is converted to ketone bodies (KBs): acetoacetate and subsequently to acetone and beta-hydroxybutyrate (BHB) (11). The KBs can cross the blood–brain barrier and are transported by monocarboxylic acid transporters to brain interstitial space, the glia and the neurons. In these tissues, KBs act as substrates in the Krebs cycle and respiratory chain, contributing to brain energy metabolism. Clinical magnetic resonance spectroscopy in pediatric patients on the ketogenic diet demonstrated measurable beta-hydroxybutyrate, with a strong correlation to beta-hydroxybutyrate blood levels (11). Although the mechanism by which KDTs exerts its anticonvulsant effects is unclear, steady-state blood levels of BHB have been shown to correlate with the degree of seizure control (12, 13). The importance of maintaining stable KBs is relevant as they constitute an alternative fuel for cerebral metabolism instead of glucose in GLUT1-DS patients (2, 14). The maximum levels of blood ketones are obtainable with use of a 4:1 or 3:1 CKD and eventually addiction of MCTs. However, KBs blood levels undergo a significant inter-individual and intra-individual variability due to several factors beyond diet composition and ketogenic ratio, such as hydration, infection, steroidal therapy, and physical activities (15). The persistence of catamenial seizures has been reported in epileptic women on Modified Atkins Diet (16) and a feasibility trial aimed at stabilizing ketone levels by increasing MCT fat intake though menstrual cycle has been performed (16). This evidence suggests the need for personalized monitoring of individuals for optimization of their diet, especially at the beginning of the treatment but during whole follow-up. Indeed, international guidelines recommend that KBs should be checked at home by parents several times per week, preferably at different times of the day (4). There are currently no data on possible variations in glucose blood levels and ketone bodies blood levels according to different phases of the menstrual cycle in patients undergoing CKD. And, conversely, there is no data in literature about possible variations of the menstrual cycle induced by CKD. Whether a variability could actually occur should be worthy of investigation since reduced KBs levels could lead to increased seizure presentation in patients with drug resistant epilepsy and even movement disorder manifestation or increased fatigue and reduced attention in patients with GLUT1-DS. Thus, we believe it might be clinically relevant to assess possible patterns of variation of ketone bodies and glucose blood levels during menstrual cycle in patients undergoing KDT, considering a protective approach aimed at avoiding seizure exacerbation or overall clinical picture modifications whether fluctuations of ketone bodies and glucose blood levels in specific intervals of menstrual cycle will be demonstrated.

Abbreviations: KD, Ketogenic Diet; KDTs, Ketogenic Dietary Therapies; KBs, Ketone Bodies; GLUT1DS, GLUT1 Deficiency Syndrome; MCT, Medium Chain Triglycerides; MAD, Modified Atkins Diet; LGIT, Low Glycemic Index Treatment; MR, Magnetic Resonance.

2. Materials and methods

This is a longitudinal multicenter study aimed at investigating the ketone bodies and glucose blood levels during menstrual cycle in female patients with a diagnosis of GLUT1-DS or drug resistant epilepsy undergoing CKD. Patients' recruitment began in September 2021 and was performed at Mondino Foundation in Pavia, Ospedale dei Bambini V. Buzzi in Milan, and "Prof. Dr. Juan P. Garrahan" Hospital in Buenos Aires. The study protocol complied with the tenets of the Helsinki Declaration and was approved by the ethical committee of the IRCCS Policlinico San Matteo of Pavia on 12 June 2020 (code number: 20200047779). The protocol has been registered in clinicaltrials.gov (ID number NCT05234411 Name: KETOMENS, Ketonemia through the menstrual cycle).

2.1. Data collection

For each patient, baseline demographic and anthropometric data (age, height, weight, BMI circumferences, and body composition), clinical data (epilepsy etiology, epilepsy features and other neurological symptoms' semiology and frequency, comorbidities, and general medical history), biochemical data (glycemia and BHB plasma level obtained from capillary blood and results of routine blood exams scheduled per follow-up), and therapeutic regimens (concomitant drug therapy, KD protocol, ketogenic ratio, compliance with diet prescription) were gathered. During the study, patients or caregivers were asked to compile a diary made up of 2 distinct sections: the clinical diary and the nutritional diary. The first one included information about the menstrual cycle (date of the menstrual period and its duration, possible symptoms, i.e., headache or stomach ache), ketone bodies and glucose blood levels, neurological symptoms (seizures, movement disorders, fatigue and/or a worsening of concentration skills) and physical activity (at rest/normal daily

activities/physical activity). Ketone bodies and glucose blood levels were measured in fasting conditions (i.e., before meals) twice a day, both in the morning and in the evening, through a reflectometer, for 3 months. The nutritional diary serves to verify whether the patient is correctly following the dietary prescription and to rule out any possible bias influencing glucose and ketone bodies blood levels.

2.2. Participants

The study recruited patients diagnosed with drug-resistant epilepsy and GLUT1 deficiency syndrome undergoing a stable ketogenic dietary therapies from at least 3 months, who started KDT after the conventional metabolic screening (4). Participants were enrolled during their regular follow-up visits and were instructed to complete the clinical and nutritional diary at home. Eligible participants were female patients aged 13 years or older, who had been undergoing a Classic Ketogenic Diet (CKD) for at least 3 months and thus previously diagnosed with Drug-resistant epilepsy, according to ILAE definition (17) or GLUT1-DS. Included patients must have a regular menstrual cycle to correctly estimate the menstrual cycle phases during the study observation and to exclude potential causes of hormonal abnormal profiles. A detailed participant inclusion and exclusion criteria are listed in Table 1.

2.3. Data analyses

The quantitative variables were described as mean ± standard deviation (or median and quartiles when appropriate), categorical variables were described as row count and percentage. Subject-wise comparisons between two measures (value in menstrual phase vs. basal value) were performed unpaired Wilcoxon rank tests. The global analysis was performed with mixed effects models to take into account the repeated measures for each subject. The day and the status (menstrual phase vs. basal value) was considered as covariates.

3. Results

Six patients were recruited among the three participating Centers. Three patients were affected by GLUT1-DS and the others had drug-resistant epilepsy. Age range was 13–18 years. All were in the normal weight range and maintained it for the duration of the study. All of them were following a stable CKD for a long time interval (time range 1–8 years) when included in the study. The compliance to the ketogenic dietary therapy was high in the entire cohort according to the food diaries provided. See Table 2 for demographics and clinical data.

TABLE 1 Inclusion and exclusion criteria.

Inclusion criteria	Exclusion criteria
Patients with drug resistant epilepsy or GLUT1-DS undergoing KD from at least 3 months before inclusion to the study	Patients who experienced secondary amenorrhea
Patients who had regular menarche at least 3 months before inclusion to the study	Patients who have irregular menstrual cycle
Absence of recognized endocrinologic problems/ disease	Pregnant patients

Two out of six patients (33,3%), both with a diagnosis of GLUT1-DS, were found to have an increased fatigue during days of menstruation. No changes in seizures or movement disorder manifestations, when present, were found in patients with GLUT1-DS according to menstrual period. In 1 out of 3 patients with DRE, an increase in seizure frequency was found during menstruations.

In the overall cohort, glycemia levels were found to be significantly higher (value of p 0.003) during menstruations compared to the remaining days. In the 7 days immediately before menstruations, the glycemia levels were found to be lower than in the remaining non menstrual days (value of p 0.0019) (see Figure 1).

Ketone bodies blood levels were found to be lower during menstruations in 4/6 patients, even if not statistically significant. See Table 3 for individual patients' ketone bodies and glucose blood values.

Physical activity, albeit mild, remained stable over time and therefore did not affect glycemia.

As far as ketone bodies blood levels, even if statistically significant variations were not observed, an inversely proportional trend compared to glycemia levels was documented (see Figure 2).

4. Discussion

To the best of our knowledge, the present study is the first one aimed at observing the course of KBs blood levels during the menstrual cycle in patients with GLUT1-DS and drug-resistant epilepsy undergoing KDT. Longitudinal studies serve in identifying changes in one or more variables between different periods, describing participants' intra-individual and inter-individual changes over time and monitoring the degree and pattern of those changes (18). This is relevant for the proposed research since whether considered a reliable biomarker of KDT intervention, detecting whether KBs blood level changes are likely to occur in specific conditions, might lead to consideration of the suitability of transient preventive diet adjustments. Understanding this will also help to enhance dietary behavior support to assist patients in improving their diet quality.

Monitoring of urine and blood ketosis is recognized as helpful to check compliance to the KDT and to attempt titration of the diet according to the individualized needs. Nevertheless, it is not well understood how important ketosis is in achieving seizure and other possible disease symptoms control, since KBs may act via different mechanisms and blood KBs levels were not found to always correlate with seizure outcome (19).

There is a double rationale for investigating changes in ketone bodies and glucose blood level during the menstrual cycle. Firstly, during the luteal phase a reduction in glucose uptake related to the action of progesterone and increased insulin resistance have been documented (20–25). Secondly, interactions between seizures and menstrual cycle are possible, as suggested by variations in seizure frequency according to the day, phase and ovulatory status of the menstrual cycle, configuring "catamenial epilepsy" (26). The cyclic hormonal changes at the basis of catamenial seizure exacerbations are consistent with the neurophysiologic activity of estrogen and

TABLE 2 Participants clinical records.

	Age	Diagnosis	Duration of KDT at time of evaluation	KD ratio	Symptoms during menstrual cycle
Patient 1	15 years	GLUT1DS	6 years	2.3:1	Fatigue
Patient 2	18 years	GLUT1DS	8 years	3:1	–
Patient 3	14 years	GLUT1DS	5 years	3:1	Fatigue
Patient 4	14 years	Drug resistant epilepsy Lennox like	4 years	4:1	Increased seizure frequency
Patient 5	13 years	Drug resistant epilepsy	1 year	4:1	–
Patient 6	14 years	Drug resistant epilepsy Lennox like	2 years 8 month	3:1	–

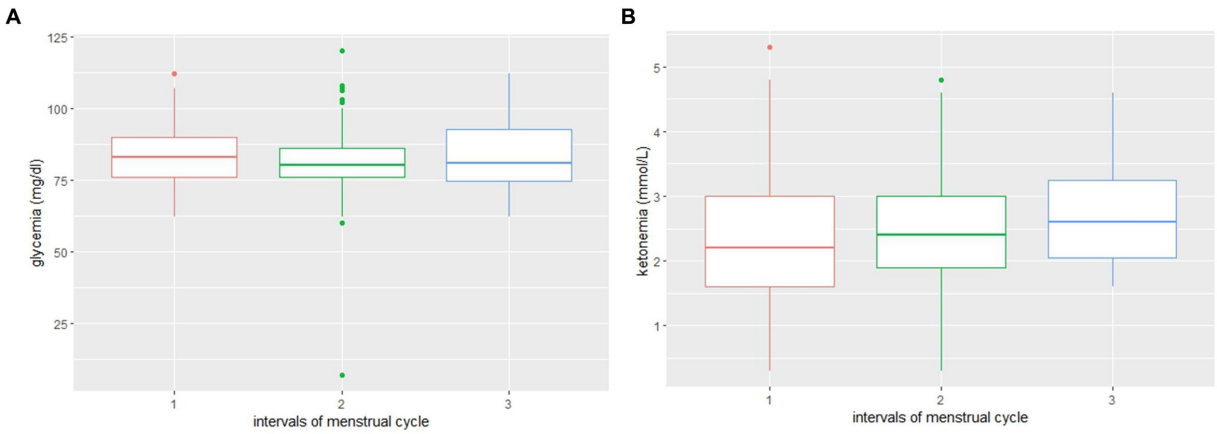

FIGURE 1
(A) Distribution of glycemia values during three phases: 1. Menstruations; 2. Non-menstruation days; and 3. Week before menstruations. **(B)** Distribution of ketone bodies blood level values during three phases: 1. Menstruations; 2. Non-menstruation days; and 3. Week before menstruations.

progesterone; indeed, for women with catamenial epilepsy who have regular menses, intermittent treatment approaches are thought to increase anti-seizure intervention during established phases of the menstrual cycle (27).

A prolonged CKD might have a reductive effect on glucose plasma level through a partial suppression of pancreatic action (28). A subject undergoing CKD, due to the chronic metabolic shift, in the presence of an increased energy requirement such as the one occurring during luteal phase, would utilize KBs as a preferential substrate, possibly decreasing their serum concentration. The increased requirement of fatty acids and cholesterol due to the yellow body could reduce KBs plasma level as well (20, 29). Other minor influencing factors could lead to KBs level fluctuations, such as a reduced food intake due to pre-menstruation discomfort or a minor compliance to the diet through the luteal phase, which implies increased carbohydrates consumption, especially in adolescents.

In the small cohort analyzed, significantly higher glycemic levels were found in the overall population during menstruation period. Even though not significantly lower in the menstruation period, ketone bodies blood levels were found to be lower during menstruations in the majority of patients analyzed. These findings support the hypothesis that blood ketone bodies levels and blood glucose levels might undergo inversely proportional subtle changes during the menstrual cycle. No evident clinical correlation was found with the biochemical data, except for seizure worsening during menstruation in one patient.

Whether patients with DRE undergoing KDT and likely to have catamenial exacerbations might benefit from an individualized treatment approach aimed at increasing ketogenic ratio in the time window of menstruation period remains to be investigated. The proposed study, for which preliminary results in a small cohort of six patients are presented, was designed to identify whether blood glucose and ketone bodies level variations are likely to undergo quantitative changes, considering that dietary therapies should be individualized. Broader population data are needed to assess variability rate and clinical implications.

This study has some limitations: first, the small size of the group of the participants was due to the difficulties encountered during subject recruitment. Besides the rarity of the pathologies considered, most of the patients would not have been able to collect the detailed data required, therefore they were not eligible for the study. Second, there is the absence of endocrinological and biochemical profiling (e.g., hormonal levels) but these observations might be scheduled in a different study, whether significant glucose and ketone bodies blood level variations according to menstrual cycle phases will be demonstrated. Third, the observation period is relatively short and data are auto-reported by the caregivers. Last, ketone bodies and glucose blood levels were measured through a reflectometer, which may be less precise in measuring these values but it is the mostly used tool for self-monitoring in clinical practice.

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TABLE 3 Individual ketone bodies and glucose blood level measurements.

	Glucose blood values during menstruations	Ketone bodies blood values during menstruations	Glucose blood values during non menstruations days	Ketone bodies blood values during non menstruations days
	Min-Max	Min-Max	Min-Max	Min-Max
	Mean	Mean	Mean	Mean
	Median	Median	Median	Median
	SD	SD	SD	SD
Patient 1	64–85 mg/dL	1.6–4.5 mmol/L	60–94 mg/dL	1.8–4.2 mmol/L
	76.07 mg/dL	2.7 mmol/L	74.77 mg/dL	2.94 mmol/L
	76 mg/dL	2.6 mmol/L	76 mg/dL	2.9 mmol/L
	5.96 mg/dL	0.72 mmol/L	6.91 mg/dL	0.54 mmol/L
Patient 2	62–107 mg/dL	0.3–2.2 mmol/L	67–98 mg/dL	0.3–1.9 mmol/L
	80.7 mg/dL	1.00 mmol/L	75.85 mg/dL	1.17 mmol/L
	80 mg/dL	1.00 mmol/L	76.5 mg/dL	1.30 mmol/L
	9.66 mg/dL	0.41 mmol/L	14.68 mg/dL	0.49 mmol/L
Patient 3	84–97 mg/dL	0.9–1.1 mmol/L	84–117 mg/dL	0.7–2.7 mmol/L
	90.6 mg/dL	1.13 mmol/L	93 mg/dL	1.5 mmol/L
	91 mg/dL	1 mmol/L	89 mg/dL	1.3 mmol/L
	5.3 mg/dL	0.4 mmol/L	7.2 mg/dL	0.5 mmol/L
Patient 4	75–112 mg/dL	1.6–5.3 mmol/L	62–120 mg/dL	1.3–4.6 mmol/L
	92.65 mg/dL	3.03 mmol/L	90.5 mg/dL	2.71 mmol/L
	92 mg/dL	3.05 mmol/L	91 mg/dL	2.70 mmol/L
	7.9 mg/dL	0.86 mmol/L	10.08 mg/dL	0.79 mmol/L
Patient 5	75–98 mg/dL	1.3–3.5 mmol/L	60–98 mg/dL	1.4–3.9 mmol/L
	86.41 mg/dL	2.31 mmol/L	82.55 mg/dL	2.45 mmol/L
	86.5 mg/dL	2.1 mmol/L	82 mg/dL	2.4 mmol/L
	6.65 mg/dL	0.63 mmol/L	6.96 mg/dL	0.56 mmol/L
Patient 6	70–83 mg/dL	1.5–4.7 mmol/L	62–80 mg/dL	1.4–4.8 mmol/L
	75.8 mg/dL	2.74 mmol/L	74 mg/dL	2.80 mmol/L
	75 mg/dL	2.1 mmol/L	74 mg/dL	2.5 mmol/L
	4.9 mg/dL	1.19 mmol/L	6.36 mg/dL	0.88 mmol/L

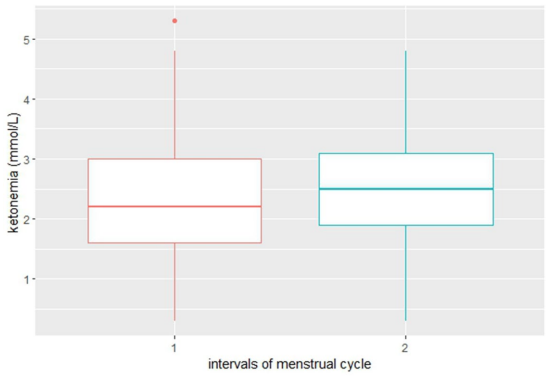

FIGURE 2
Distribution of ketone bodies blood level values during 1. Menstruations and 2. Non-menstruation days.

The present study has also some strengths: it is the first one in literature that evaluates the possible ketone bodies and glucose blood levels variations during the menstrual cycle in female patients undergoing KDTs. Second, it is a multicenter study, allowing to gather data from different countries and hospitals, despite the small sample size. Third, we included patients with a minimum CKD duration of 1 year, reducing the risk of ketone bodies fluctuations derived from other factors (i.e., the common variation that occurs in the first months of dietary therapy).

In conclusion, preliminary results showed a significant increase in glycemia levels during menstruation in the entire cohort and an inversely proportional trend of KB levels compared to glycemia. These data can be explained by several factors such as progesterone action and increased insulin resistance during menstruation, increase of energy requirement and thus KBs consumption during lethal phase, a reduction of KBs due to an increase of fatty acids utilization by yellow body. Importantly, not only a worsening of seizures might be a consequence of a reduction of ketone bodies blood level, but also other

disease symptoms otherwise controlled by KDTs such as movement disorder, fatigue, concentration, and cognitive performance. Further research is needed to understand the role of ketosis in seizure and other disease symptoms control and thus the best ways to reach ketosis with an optimum balance of disease symptoms control and side effects.

Data availability statement

The original contributions presented in the study are included in the article/supplementary files, further inquiries can be directed to the corresponding author.

Ethics statement

The studies involving human participants were reviewed and approved by Ethical committee of the IRCCS Policlinico San Matteo of Pavia on 12 June 2020 (code number: 20200047779). Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

Author contributions

CF and LP: conceptualization, methodology, and data curation. LP, CF, MG, MA, CT, CM, and CV: investigation. LP, CF, MG, CV, MT, and IB: writing—original draft preparation. LP, CF, MG, CV, MT, IB, EB, MA, GV, RC, PV, AT, and VDG: writing—review and editing. VDG and AT: supervision. VDG: project administration.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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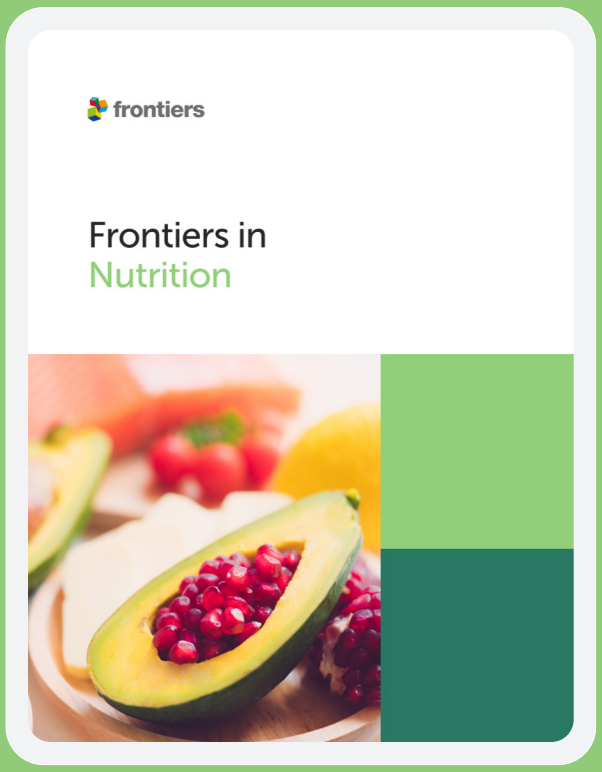

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