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No effect of oral ketone ester supplementation on exercise capacity in patients with McArdle disease and healthy controls: A randomized placebo-controlled cross-over study

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Abstract

Patients with glycogen storage disease type V (GSDV), also known as McArdle disease, have blocked glycogen breakdown due to myophosphorylase deficiency, leading to exercise intolerance, muscle pain, and risk of muscle damage. Blood-derived ketone bodies (KBs) constitute an alternative energy source that could fuel the muscle independent of glycogenolysis. However, except for long-time fasting or ketogenic dieting, KBs are present in low quantities. This led us to explore the effects of a drink containing exogenously produced KBs in the form of D-β-hydroxybutyrate esters (KE) on exercise capacity and metabolism in patients with GSDV. Eight GSDV patients and four healthy controls (HC) were included in this placebo-controlled, cross-over study where subjects were randomized to receive a KE drink with 395 mgKE/kg or placebo drink on two separate days 25 min before a submaximal cycle exercise test. The primary outcome was exercise capacity as indicated by heart rate response (HR) to exercise. Secondary outcomes included perceived exertion (PE) and measures of KB, carbohydrate, and fat metabolism during exercise. In GSDV, the KE drink vs. placebo increased plasma KBs and KB oxidation ($p \leq 0.0001$) but did not improve exercise capacity as judged from HR ($p = 0.120$) and PE ($p = 0.109$). In addition, the KE drink lowered plasma glucose, free fatty acids, and lowered lipolytic rate and glucose rate of appearance compared with placebo. Similar results were found in the HC group. The present study indicates that an increase in KB oxidation by oral KE supplementation does not improve exercise capacity in GSDV possibly because of KB-induced inhibition of lipolysis and liver glucose output. Thus, oral KE supplementation alone cannot be recommended as a treatment option for patients with GSDV.

KEYWORDS
exercise capacity, exogenous ketone bodies, glycogen storage disease type V, ketone ester, McArdle disease

1 | INTRODUCTION

Glycogen storage disease type V (GSDV), also known as McArdle disease, is a rare inborn error of carbohydrate (CHO) metabolism. Patients have blocked muscle glycogenolysis in the skeletal muscle due to absent/near-absent myophosphorylase activity. Patients suffer from exercise intolerance, and all activities that require energy provision can cause muscle pain, which can lead to muscle contractures, rhabdomyolysis, and in severe cases, renal failure.[1–3] Symptoms occur within the first minutes of activity where patients quickly deplete the available fuels for energy generation, as muscle glycogenolysis is the primary source of energy at this stage. Pathognomonic for the disorder is the "second-wind" phenomenon, which is characterized by a sudden relief in symptoms and a decrease in heart rate (HR) 7–9 min after initiation of physical activity. The relief occurs due to the availability of alternative fuel substrates in the form of liver-derived glucose and increased fat oxidation; however, the increase is not enough to fully compensate for the blocked glycogen breakdown during more intense exercise.[4]

Currently, there is no satisfactory treatment for the activity intolerance seen in GSDV. Given that access to fuel substrates is limited in the exercising muscle, strategies to potentiate the use of alternative fuel substrates are sensible. Until now, only supplementation of exogenous sugar has been shown to abolish the second wind (decreased HR during exercise) and alleviate muscle symptoms in GSDV, but the effect is short-lived and must be restricted because of the risk of weight gain and other negative side effects.[5,6] Strategies to increase fat oxidation is another reasonable approach. However, supplementation with C7-triheptanoin-oil failed to improve exercise tolerance in patients with GSDV.[7] The ketone bodies (KBs), hydroxybutyrate (HOB), and acetoacetate (AcAc) can easily be oxidized in many tissues, including the skeletal muscle.[8] We hypothesized that KBs could be particularly desirable for working muscle in patients with GSDV as KBs could provide immediate energy supply in the critical first minutes of exercise and could potentially reduce the second wind phenomenon.

In a normal physiological state, endogenous KB production is low. Physiological ketosis with production of KBs can be reached with strict CHO restriction by prolonged fasting or by adhering to a ketogenic diet.[9–11] A pilot study performed in our research group has shown promising early results in patients with GSDV, with indications of improved exercise tolerance after 2 weeks on a modified ketogenic diet, with, among other results, lowering of HR during submaximal exercise compared to baseline.[12] However, a ketogenic diet can be difficult to adhere to, hence alternative ways to elevate blood KBs are of interest.

Another way to reach high blood KB concentrations is by oral intake or intravenous infusion of exogenously produced KBs. Recently, ketone salts (KS, sodium plus potassium β-hydroxybutyrate) or esters (KE, (R)-3-hydroxybutyl (R)-3-hydroxybutyrate) have become available for human consumption, which has shown to be a safe way to elevate blood KBs to high levels comparable to those achieved after prolonged fasting.[13,14] Research with exogenous KB supplementation is still in its early stages but has shown promising early effects in different health aspects, e.g. attenuation of glycemic response[15,16] and as performance improving substance in athletes along with being glycogen sparing during exercise.[13]

Based on these considerations, we hypothesized that a single oral administration of a KE supplement prior to exercise could increase exercise capacity compared to placebo in patients with GSDV. Furthermore, we aimed to investigate the KE-induced metabolic changes in KB-, fat-, and CHO metabolism during exercise using stable-isotope technique. All results were compared to healthy controls (HC). As the energetic deficiency in GSDV manifests when the muscle energy metabolism is challenged, cycle exercise testing is a suitable method to detect potential effects of an intervention on metabolism.[4,17,18] HR during submaximal exercise was chosen as the primary outcome as it illustrates the pathognomonic second wind in GSDV and as HR changes have been shown to illustrate interventional effects in previous trials in GSDV.[5,6,12]

2 | METHODS

2.1 | Participants

Twelve patients with genetically verified GSDV were invited to participate. Eight accepted and were included in this randomized, placebo-controlled, double-blind, cross-over study. Four HC were included. Inclusion criteria were age of minimum 18 years and genetically confirmed GSDV or HC. Exclusion criteria were any medical conditions that, in the opinion of the investigators, would prevent the patient from safely participating or interfering with the interpretation of results, and pregnancy or breastfeeding. The participants were randomly assigned to one of two arms in a 1:1 ratio (arm 1: the KE drink first and the placebo drink second; arm 2: the opposite) (Figure 1). Patients were recruited from Copenhagen Neuromuscular Center (CNMC) at Rigshospitalet, Denmark, which is a national referral center for patients with GSDV as well as through our collaborating site, the

Department of Neurology, Nijmegen, the Netherlands. HC were recruited from a website (forsøgspersoner.dk). The study was conducted in accordance with the Declaration of Helsinki. Participants received both oral and written information about the study and all participants gave written consent before inclusion. The study was approved by the Ethics Committee of the Capital Region, Copenhagen (H-18033230), and listed on clinicaltrials. gov (NCT03945370) before inclusion.

2.2 | Outcome measures

The primary outcome was the immediate effects of oral supplementation of KE vs. placebo on exercise capacity as judged by a decrease in heart rate (HR) during constant workload exercise. Secondary outcomes were evaluation of KB, fat, and CHO oxidation measured via indirect calorimetry and stable isotope technique at rest and during exercise after KE supplementation vs. placebo. Other secondary outcomes were evaluation of perceived exertion (PE, Borg scale)[19] and changes in plasma concentrations of KBs (HOB, AcAc), insulin, glucose, free-fatty acids (FFA), ammonia, pyruvate, lactate, and amino acids.

2.3 | Supplement description

The oral KE supplement (HVMN®product) contained D-β-hydroxybutyrate esters ((R)-3-hydroxybutyl (R)-3-hydroxybutyrate) (25 g/65 ml), water, stevia, natural flavors, malic acid, potassium, sorbate, and potassium benzoate. The placebo supplement (taste-matched) contained ketone-placebo-flavor-mix (HVMN®), bitter flavor (denatonium benzoate), stevia, and water. The supplement was administered per body weight, with a target KE concentration of 395 $mg/kg_{bodyweight}$. The same amount of both the placebo and the KE drink was poured into identical plastic cups and administered 95 min postisotope infusion start/25 min pre-exercise (Figure 1). The administration time point was chosen based on a pilot trial in a HC showing peak plasma HOB-level 25 min post KE-drink administration (unpublished data). The drink content was blinded for both the participants and the investigator responsible for running the exercise test.
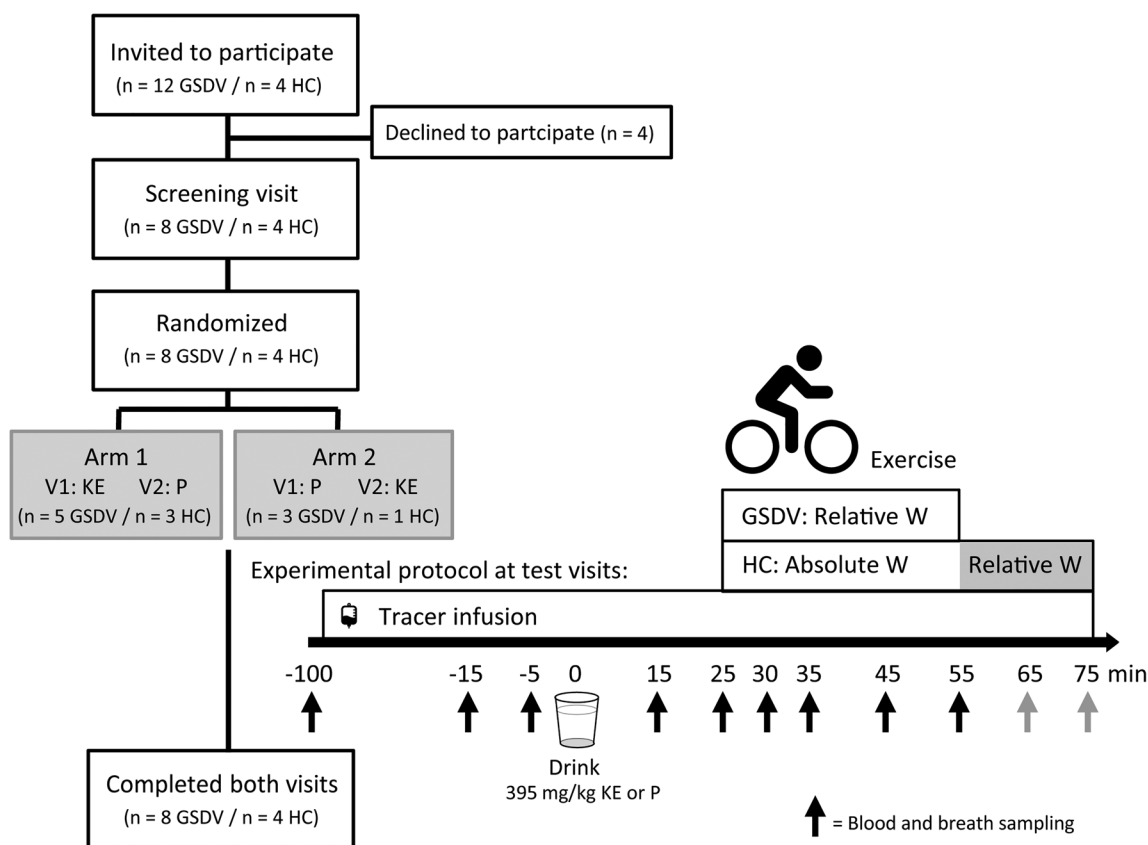

FIGURE 1 Flow chart over the participant flow and experimental protocol. GSDV, patients with glycogen storage disease type V; HC, healthy controls; KE, ketone supplement; min, minutes; n, number of participants; P, placebo supplement; V1, visit 1; V2, visit 2; W, workload; relative W: approximately 60%–70% of the maximal oxygen capacity of the participant; absolute W: same absolute workload as the matched GSDV patient. Arrows indicate time points of breath and blood sampling

2.4 | Experimental protocol

Participants were tested on three occasions at CNMC, which included a screening visit and two test visits (Figure 1). At the screening visit, participants performed an incremental exercise test to exhaustion to determine maximal oxygen uptake (VO_2 max) and maximal workload on a cycle ergometer (Corival recumbent; Lode). Blood safety parameters (ALAT, bilirubin, amylase, creatine kinase, myoglobin, creatine, GFR, potassium, sodium) were measured at the screening visit.

At test visits, participants arrived overnight fasted. Participants were instructed not to drink alcohol or caffeine and to refrain from physical activity 12 and 24 h prior to test, respectively. A venous catheter was inserted in a peripheral vein in both arms, one for blood extraction, and one for isotope infusion. Infusion of three stable isotope tracers [2,4-$^{13}C_2$]-D-β-hydroxybutyrate, [1,1,2,3,3-$^2H_5$]-glycerol and [6,6-$^2H_2$]-glucose (all from Cambridge Isotope Laboratories) were started 2 hours before the exercise test began and continued until the end of the test. The tracers were dissolved under sterile conditions on the day of examination and injected into a solution of 0.9% saline (NaCl) through a bacterial filter. The glucose and glycerol tracers were dissolved together, and the HOB tracer alone. Infusions were delivered by a Gemini PC2 pump (IMED). The glycerol-glucose tracer infusion rate was kept constant throughout the infusion, as was the infusion rate of the HOB tracer in the placebo trial. In the KE trial, the HOB tracer infusion rate was increased 5-fold after intake of the KE drink. The two test visits were separated by at least 1 week, ensuring washout of tracers and re-establishing of background tracer levels.

The exercise test was slightly different between participants with GSDV and HC, since patients with GSDV have much lower exercise capacity than that of HC, and because this alters absolute exercise-induced energy use and metabolism, HCs were tested at both a low absolute and a much higher relative workload. Patients with GSDV cycled for 30 min at a constant workload corresponding to approximately 60%–70% of their VO_2 max. The HC started with 30-min cycle-exercise at the same absolute workload corresponding to a matched GSDV patient, followed by 20 min at a relative workload corresponding to approximately 60%–70% of their VO_2 max. Pedaling speed was maintained between 60 and 75 rpm. HR and PE were noted every minute during the first 20 min of exercise and every other minute in the remaining test time.[19] Gas exchange rates (O_2 and CO_2) were measured (Cosmed Quark CPET) at four time points during rest and continuously during the exercise test only interrupted by air sampling (Figure 1). Samples of blood and expired air, were collected at baseline (before infusion start), and two times before and two times after drink administration, and four (GSDV)/six(HC) times during the exercise test (Figure 1).

2.5 | Blood and air samples

Collected blood was filled into EDTA-containing tubes and spun at 4°C for 10 min. Afterward, plasma was distributed to Eppendorf tubes and immediately frozen on dry ice and stored at −80°C until analysis. FFA were analyzed in duo using a Wako-NEFA-HR(2) kit (Fujifilm Wako Chemicals Europe GmbH) by spectrophotometry (Multiskan GO; Thermo Scientific, SkanIt™ Software; Thermo Fisher Scientific Inc.). A nondiffusible 15 L Douglas bag (Hans Rudolph Inc.) was used to collect the air samples, which were transferred to evacuate Exetainer Breath Vials (Labco Limited). Pyruvate, lactate, and AcAc were analyzed in plasma and HOB, glucose, and glycerol in plasma and infusates as well as the enrichment of the breath samples were measured at the Clinical Metabolomics Core Facility, Rigshospitalet with liquid- and gas-chromatography-tandem mass-spectrometry and gas chromatography isotope ratio mass-spectrometry (Thermo Finnigan MAT GmbH) as previously described.[8] The safety parameters were analyzed from freshly collected blood, and insulin from plasma at the Department of Clinical Biochemistry, Rigshospitalet (Cobas 8000; Roche).

2.6 | Calculations

Stable isotopes: Glucose, glycerol, and KB rate of appearance (R_a) and disappearance (R_d) were calculated as:

$$Ra = \frac{F - V[(C_1 + C_2)/2] \times [(E_2 - E_1)/(t_2 - t_1)]}{(E_1 + E_2)/2}$$

$$Rd = Ra - V \times \frac{C_2 - C_1}{t_2 - t_1}$$

Where F is the isotopic infusion rate (μmol/kg/min). V is the tracer distribution volume. C_1 and C_2 and E_1 and E_2 are the tracer concentrations and enrichments at times 1 (t_1) and 2 (t_2), respectively. The whole-body KB oxidation was estimated from the $^{13}CO_2$ release. Although [2,4-$^{13}C_2$]-D-β-HOB was infused, it gets rapidly converted to [2,4-$^{13}C_2$]AcAc; hence, the $^{13}CO_2$ release is from both HOB and AcAc oxidation. In the present study, KB oxidations rates were calculated without including an [2-^{13}C]acetate correction factor, resulting in an underestimation of the KB-oxidation rates. Despite this, during baseline and exercise at absolute workload, the oxidation rates in GSDV and

TABLE 1 Participant characteristics

ID	Sex (M/F)	Age (years)	Genotype	Height (cm)	Weight (kg)	BMI	VO$_2$ max (L/min/kg)/ Wmax (watts)	Relative W (60%–70% of VO$_2$ max) (watts)	Absolute W (watts)
GSDV01	F	28	c.148C > T, c.1477delC	174	93.0	30.7	17.7 / 76	42	
GSDV02	M	34	5′IVS14 + 1G > A c.280C > T	176	68.0	22.0	29.5 / 95	55–65	
GSDV03	F	41	c.148C > T (homo)	179	74.0	23.1	22.0 / 69	25–33	
GSDV04	M	70	c.148C > T (homo)	177	83.0	26.5	23.0 / 85	50–65	
GSDV05	F	48	c.148C > T 597-598delT	175	72.4	23.6	22.2 / 80	33	
GSDV06	M	54	c.148C > T (homo)	182	87.8	26.5	30.8 / 130	40–65	
GSDV07	F	42	c.148C > T (homo)	170	87.1	30.1	16.9 / 60	25–32	
GSDV08	M	36	c.482G > A (homo)	183	74.5	22.2	24.8 / 80	40–50	
Mean	M4:F4	44.1 ± 13		177 ± 4.3	80.0 ± 8.9	25.6 ± 3.4	23.4 ± 5.0 / 78 ± 11		
HC01	F	43		173	66.1	22.1	38.9 / 197	112	33
HC02	M	41		187	83.3	23.8	38.3 / 305	160	55
HC03	M	60		186	78.0	22.5	37.0 / 245	150	50
HC04	F	25		172	73.3	24.8	42.0 / 290	140	42
Mean	M2:F2	42.3 ± 14		179.5 ± 8.1	75.2 ± 7.3	23.3 ± 1.2	39.3 ± 2.5[a] / 259 ± 49[a]		

Abbreviations: F, female; GSDV, patients with glycogen storage disease type V; HC, healthy control; M, male; VO$_2$ max, maximal oxidative capacity; W, workload; W$_{max}$, maximal workload.

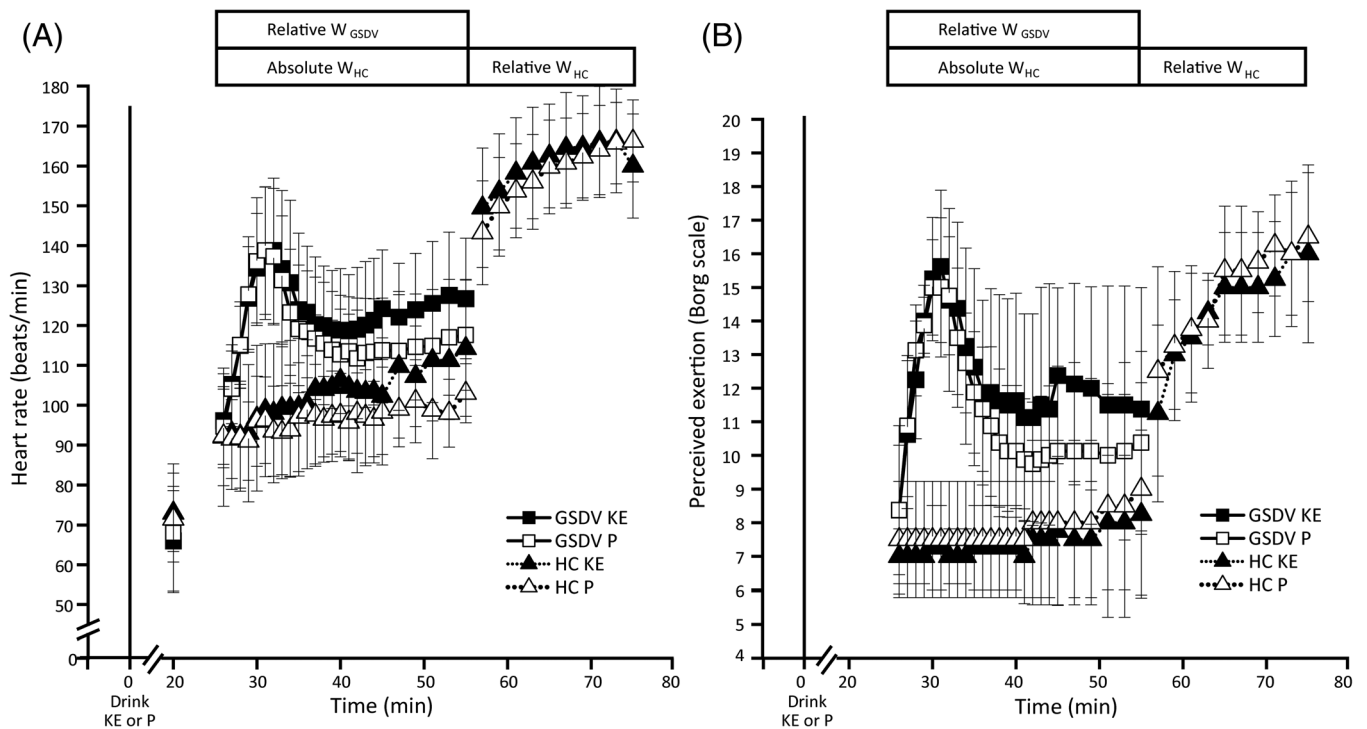[a]Indicates a significant difference between GSDV and HC, $p \leq 0.05$.

FIGURE 2 Heart rate (A) and perceived exertion (B) in GSDV and HC during exercise with ketone supplementation (KE) or placebo (P). Values are mean ± SD. GSDV: patients with glycogen storage disease type V (squares); HC: healthy controls (triangles). Patients with GSDV exercised at a constant workload corresponding to 60%–70% of their VO$_2$ max for 30 min. The HC exercised at the same absolute workload as the matched GSDV patient for 30 min; thereafter, the workload was increased to the HC relative workload corresponding to 60%–70% of their VO$_2$ max for 20 min

HC can be compared due to a similar loss of ^{13}C non-appearing as ^{13}CO$_2$ in the *tricarboxylic acid cycle* (TCA cycle).[8,20]

2.7 | Statistical considerations

The statistical software SPSS (IBM; SPSS statistics v25) was used. Power calculation for the GSDV group was performed based on assumptions of expected variance in the primary outcome HR (5 bpm) and the minimal relevant difference between KE and placebo supplements (5 bpm). The accepted risk of type 1- and type 2-error was set at 5% and 80%, respectively, giving a sample size of 8 should ensure the feasibility of the work. Results are presented as means ± standard deviations (SD). A two-way repeated-measures ANOVA was performed to compare outcomes with repeated measurements, both to compare main effects between placebo and KE supplementation and to compare GSDV vs. HC. Bonferroni adjustment was used to adjust for multiple comparisons. A Mann–Whitney U-test was used to compare means of nonrepeated outcomes. A p-value of ≤0.05 was set as significant.

3 | RESULTS

3.1 | Participants

All included participants completed both study visits. Both drinks were well tolerated by all participants without any adverse events. Participant characteristics are presented in Table 1. Participants with GSDV had significantly lower VO$_2$ max ($p = 0.004$) and Watt$_{max}$ ($p = 0.004$) at screening compared to healthy controls. HC and GSDV were comparable with regards to BMI ($p = 0.461$) and age ($p = 0.933$). Sex was equally distributed in the two groups.

3.2 | Primary outcome

3.2.1 | Heart rate

Within-group differences: there was no difference in HR (bpm) during the submaximal exercise test between placebo (P) and KE supplementation, neither in the GSDV (P: 117.9 ± 14.3 vs. KE: 123.0 ± 15.2, $p = 0.120$) or the HC group (P: 114.0 ± 11.2 vs. KE: 115.2 ± 15.9, $p = 0.567$) (Figure 2A). In the GSDV group, the HR data were completely overlapping in the first critical period of exercise. In the
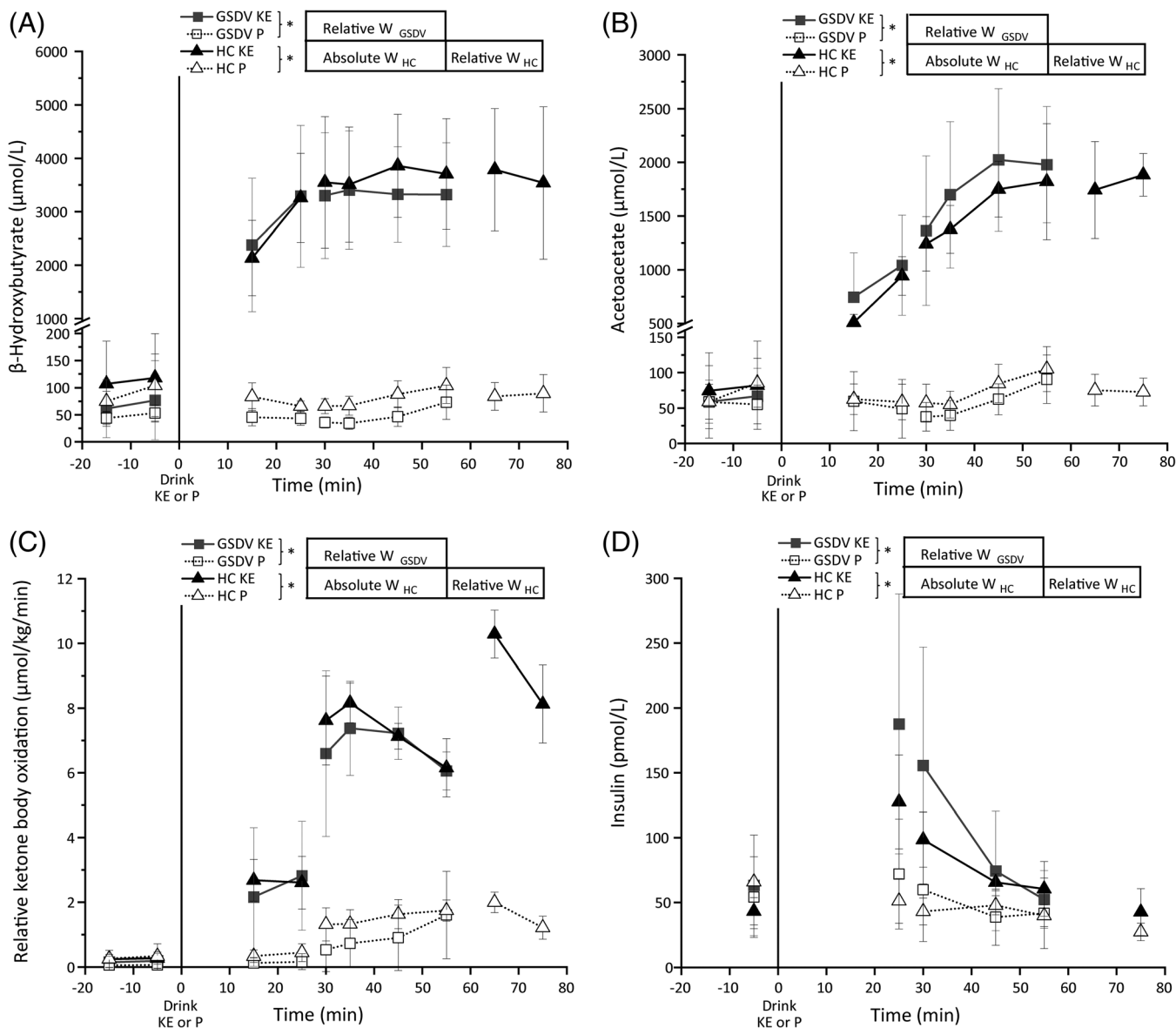
FIGURE 3 Ketone body metabolism and insulin response in GSDV and HC during exercise with ketone ester supplementation (KE) (black) or placebo (P) (white). (A) Plasma HOB concentration, (B) plasma AcAc concentration, (C) relative KB oxidation rates, and (D) plasma insulin concentration. Values are mean ± SD. *Indicates statistical significance $p \leq 0.05$. GSDV: patients with glycogen storage disease type V (squares); HC: healthy controls (triangles). Patients with GSDV exercised at a constant workload corresponding to 60%–70% of their VO_2 max for 30 min. The HC exercised at the same absolute workload as the matched GSDV patient for 30 min, thereafter the workload was increased to the HC relative workload corresponding to 60%–70% of their VO_2 max for 20 min

prolonged exercise phase after the 2nd wind had kicked in, there was a nonsignificant tendency toward higher HR after the KE drink compared to placebo from the 11th- to the 30th-min time point (P: 114.7 ± 14.3 vs. KE: 122.1 ± 15.2, $p = 0.110$). In the HC group, there was no significant difference in the HR response between KE and placebo drink, when looking at the absolute (P: 96.5 ± 11.0 vs. KE: 102.5 ± 16.8, $p = 0.274$) and the relative workload exercise period (P: 158.2 ± 11.5 vs. KE: 160.6 ± 13.7, $p = 0.284$).

Between-group differences: There was an expected significant difference in HR during the first 30-min

between GSDV and HC ($p = 0.044$, KE-trial data), where the groups exercised at the same absolute workload.

3.3 | Secondary outcomes

3.3.1 | Perceived exertion

Changes in PE during exercise duration followed the HR changes, with no within-group difference between the KE and placebo drink (GSDV: P:11.3 ± 2.0 vs. KE: 12.2 ± 2.8,
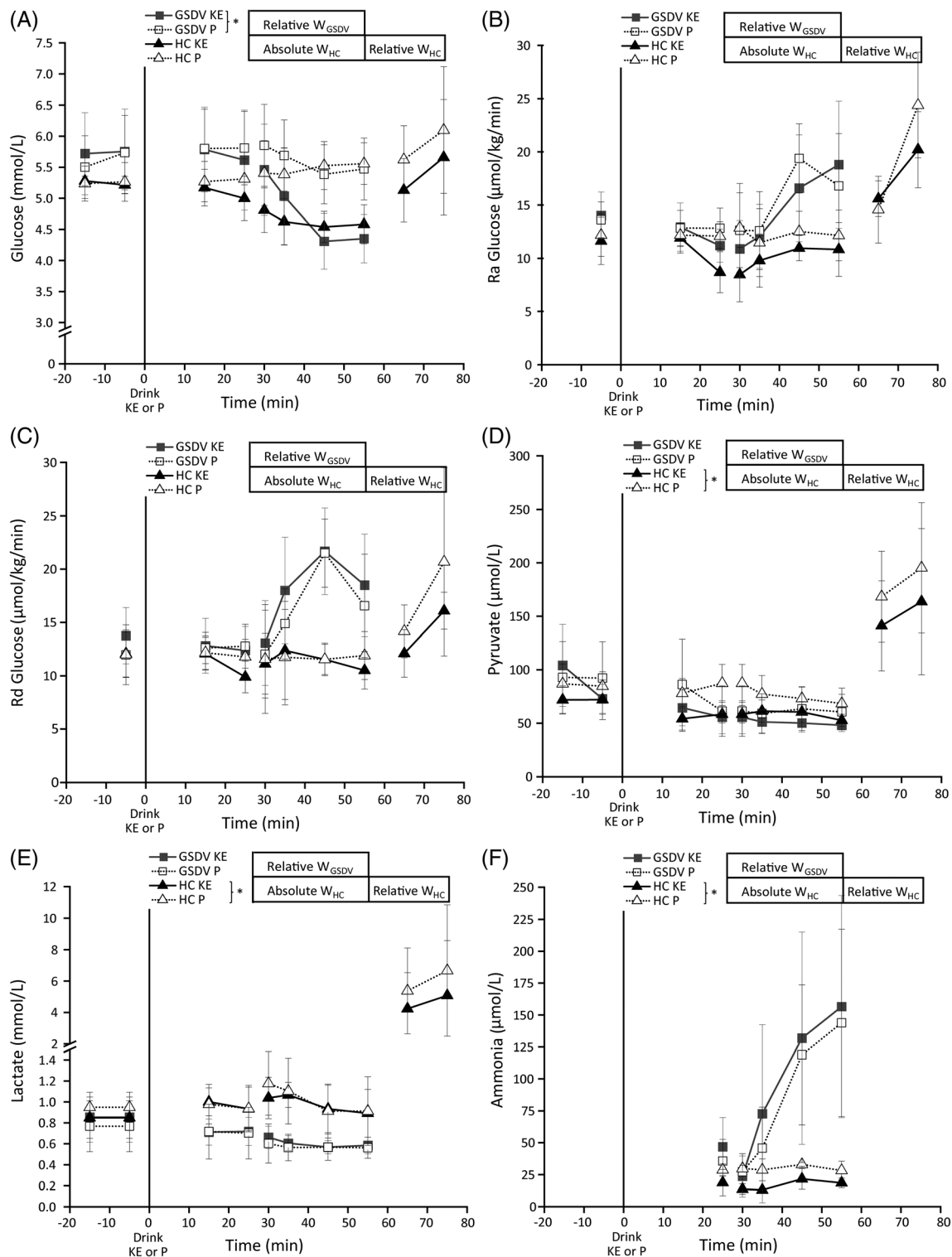
FIGURE 4 Carbohydrate metabolism and ammonia response in GSDV and HC during rest and exercise with ketone supplementation (KE) (black) or placebo (P) (white). Values are mean $\pm$ SD. *Indicates statistical significance $p \leq 0.05$. (A) Plasma glucose, (B) glucose rate of appearance (R_a), (C) glucose rate of disappearance (R_d), (D) plasma pyruvate, (E) plasma lactate and (F) plasma ammonia. GSDV: patients with glycogen storage disease type V (squares); HC: healthy controls (triangles). Patients with GSDV exercised at a constant workload corresponding to 60%–70% of their VO_2 max for 30 min. The HC exercised at the same absolute workload as the matched GSDV patient for 30 min, thereafter the workload was increased to the HC relative workload corresponding to 60%–70% of their VO_2 max for 20 min
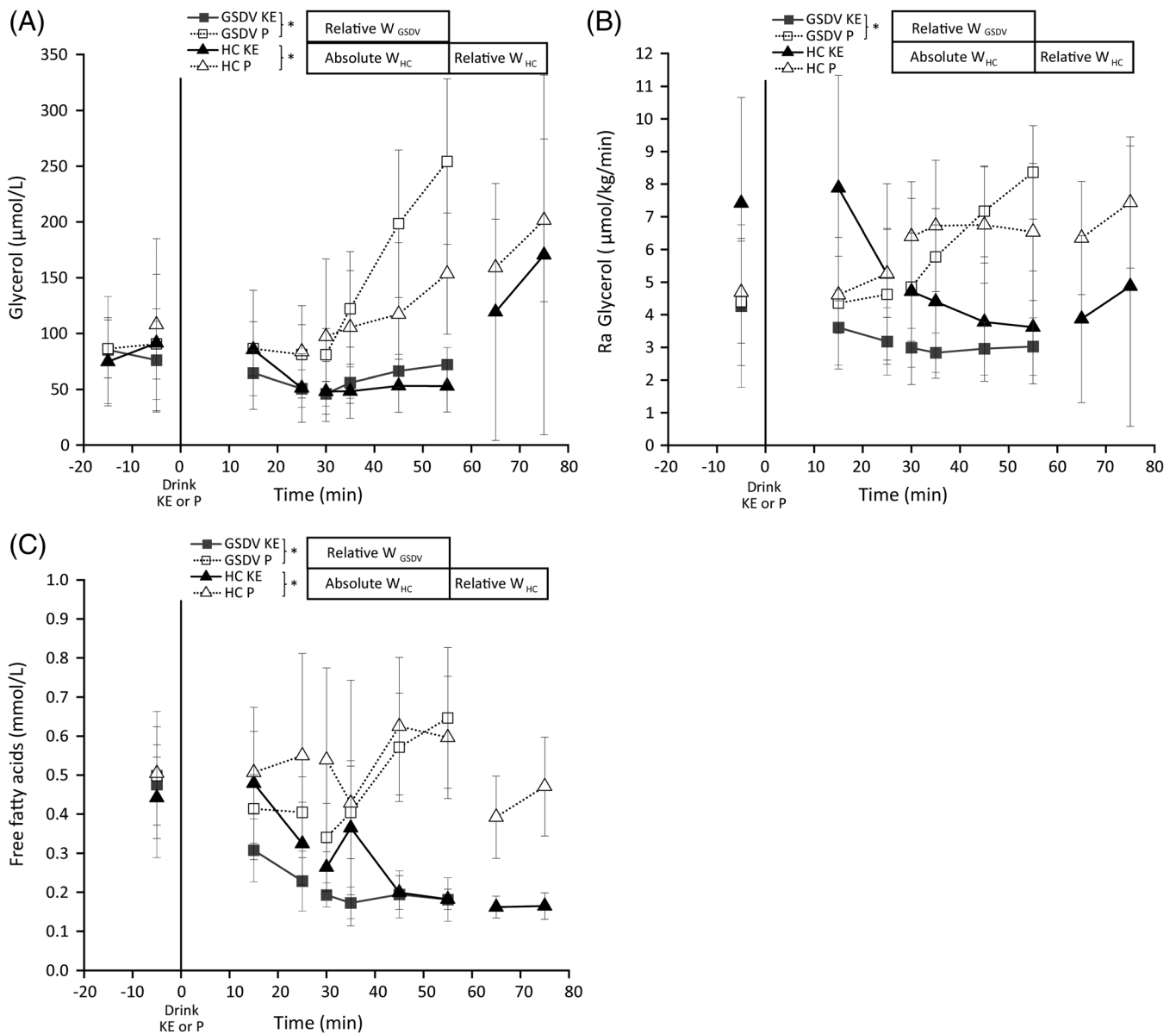
(A)

(B)

(C)

FIGURE 5 Fat metabolism in GSDV and HC during rest and exercise with ketone supplementation (KE) (black) or placebo (P) (white). Values are mean ± SD. *Indicates statistical significance $p \leq 0.05$ (A) Plasma glycerol, (B) glycerol rate of appearance (R_a) and (C) plasma free-fatty acids. GSDV: patients with glycogen storage disease type V (squares), HC: healthy controls (triangles). Patients with GSDV exercised at a constant relative workload corresponding to 60%–70% of their VO_2 max for 30 min. The HC exercised at the same absolute workload as the matched GSDV patient for 30 min, thereafter the workload was increased to the HC relative workload corresponding to 60%–70% of their VO_2 max for 20 min

$p = 0.109$; HC: P: 9.8 ± 2.0 vs. KE: 9.4 ± 1.5, $p = 0.678$) (Figure 2B). In line with the HR data, there was a between-group difference in PE in the first 30 min of exercise at absolute workload ($p = 0.002$).

3.3.2 | Ketone-body metabolism

Ingestion of the KE drink resulted in a rapid significant increase in both circulating HOB ($p = 0.00008$) and AcAc

($p = 0.0001$) in patients with GSDV from overnight-fasted levels of HOB concentrations from 78.6 ± 55.8 to 2378.6 ± 1248.0 μmol/L 15-min after ingestion, and to 3289.9 ± 1327.0 μmol/L at beginning of exercise, where the levels plateaued (Figure 3A). A similar significant increase was found in the HC group (Figure 3A). AcAc, in contrast to HOB, did not plateau at the beginning of exercise but continued to increase (Figure 3B). Both AcAc and HOB levels remained high throughout the exercise period. In line with the elevation of plasma KBs,

TABLE 2 Amino acids

AA (μmol/L)	KE supplement				Placebo supplement			
	Predrink	Exercise 5 min	Exercise 30 min	Exercise 50 min	Pre drink	Exercise 5 min	Exercise 30 min	Exercise 50 min
Glutamate								
GSDV	114.0 ± 56.9	132.6 ± 70.6	160.0 ± 89.6		119.2 ± 37.7	124.0 ± 53.8	124.7 ± 47.0	
HC	89.9 ± 53.7	86.8 ± 20.6	129.1 ± 73.0	129.0 ± 88.4	109.6 ± 51.9	96.4 ± 55.1	108.8 ± 48.1	111.3 ± 53.6
Serine								
GSDV	98.4 ± 15.8	89.7[a] ± 14.5	86.8[a] ± 11.8		104.1 ± 20.9	102.6[a] ± 18.2	101.1[a] ± 17.5	
HC	101.6 ± 21.2	91.3 ± 19.5	85.5 ± 15.6	86.4 ± 21.9	109.7 ± 8.9	101.8 ± 8.8	101.9 ± 9.0	102.9 ± 10.5
Glycine								
GSDV	228.7 ± 83.5	214.2[a] ± 76.4	201.7[a] ± 67.3		234.9 ± 79.8	239.5[a] ± 74.9	226.6[a] ± 69.7	
HC	208.6 ± 75.3	194.2 ± 67.2	184.5 ± 55.8	191.0 ± 62.4	220.1 ± 39.5	217.2 ± 39.5	212.9 ± 41.1	230.3 ± 42.8
Glutamine								
GSDV	570.5 ± 41.5	618.8 ± 48.1	619.6 ± 80.5		602.9 ± 82.1	608.3 ± 76.6	586.3 ± 86.8	
HC	519.6 ± 30.9	580.2 ± 38.2	547.0 ± 54.5	559.3 ± 70.7	496.4 ± 102.3	510.1 ± 110.9	503.1 ± 88.0	564.7 ± 95.7
Threonine								
GSDV	109.7 ± 21.5	105.6[a] ± 23.2	101.9[a] ± 22.3		121.1 ± 27.8	123.6[a] ± 25.2	118.8[a] ± 27.8	
HC	104.6 ± 24.1	98.2 ± 21.3	92.0 ± 17.3	90.6 ± 21.1	129.8 ± 15.1	126.9 ± 16.0	123.0 ± 11.6	123.8 ± 14.8
Alanine								
GSDV	324.0 ± 63.7	294.2[a] ± 50.2	228.5[a] ± 45.3		331.9 ± 56.0	335.4[a] ± 63.3	263.6[a] ± 51.2	
HC	314.7 ± 112.1	304.0 ± 87.4	273.9 ± 70.3	401.5 ± 96.2	335.0 ± 61.0	346.0 ± 31.6	323.1 ± 22.1	495.9 ± 41.0
Tyrosine								
GSDV	56.1 ± 14.2	56.4 ± 15.5	56.1 ± 17.3		54.3 ± 14.1	56.3 ± 13.7	55.6 ± 14.4	
HC	48.7 ± 8.9	48.2 ± 9.8	45.8 ± 10.1	48.8 ± 11.5	50.1 ± 10.5	50.1 ± 9.0	47.8 ± 9.6	54.0 ± 10.2
Methionine								
GSDV	23.5 ± 4.1	23.8[a] ± 3.9	22.3[a] ± 4.2		24.5 ± 3.1	26.1 ± 3.1	25.7 ± 4.7	
HC	21.8 ± 4.8	21.9 ± 5.5	21.0 ± 4.4	22.1 ± 6.3	22.6 ± 2.3	23.3 ± 1.8	23.0 ± 2.2	26.3 ± 2.7
Leucine								
GSDV	119.6 ± 26.1	124.6 ± 34.9	124.2 ± 31.9		121.0 ± 23.5	125.9 ± 28.0	125.9 ± 34.5	
HC	116.6 ± 20.3	117.7 ± 19.5	117.4 ± 11.6	118.0 ± 16.2	120.3 ± 20.8	121.7 ± 17.7	119.6 ± 14.8	125.1 ± 18.6
Phenylalanine								
GSDV	57.4 ± 8.1	57.7 ± 7.3	56.6 ± 8.8		55.1 ± 6.3	58.9 ± 8.3	58.3 ± 8.7	
HC	51.4 ± 4.9	51.7 ± 3.9	50.9 ± 2.8	52.3 ± 5.6	51.6 ± 3.2	52.6 ± 1.3	51.1 ± 3.5	57.2 ± 4.2

(Continues)

TABLE 2 (Continued)

AA (µmol/L)	KE supplement				Placebo supplement			
	Predrink	Exercise 5 min	Exercise 30 min	Exercise 50 min	Pre drink	Exercise 5 min	Exercise 30 min	Exercise 50 min
Valine								
GSDV	208.7 ± 50.0	204.9 ± 52.6	202.1 ± 52.1		203.1 ± 30.0	205.5 ± 35.5	200.0 ± 32.1	
HC	208.3 ± 32.3	203.8[a] ± 27.0	202.7[a] ± 27.4	203.8[a] ± 30.7	227.0 ± 28.7	222.1[a] ± 30.0	216.3[a] ± 30.6	219.8[a] ± 31.7
Lysine								
GSDV	169.5 ± 38.2	168.0 ± 42.2	167.2 ± 43.5		173.7 ± 39.2	179.3 ± 41.0	173.8 ± 41.6	
HC	162.8 ± 29.6	163.7 ± 34.3	155.5 ± 31.9	162.5 ± 37.1	170.6 ± 21.1	174.1 ± 18.2	165.6 ± 20.8	179.0 ± 28.7
Isoleucine								
GSDV	55.5 ± 13.5	58.2 ± 18.2	58.6 ± 16.9		56.9 ± 10.8	62.0 ± 11.6	60.0 ± 12.0	
HC	62.0 ± 15.3	63.9 ± 12.2	61.5 ± 14.2	63.8 ± 16.1	58.3 ± 8.0	59.0 ± 8.5	57.9 ± 7.5	65.2 ± 5.1
BCAA								
GSDV	383.7 ± 87.2	387.8 ± 102.3	384.8 ± 98.2		381.0 ± 63.2	393.5 ± 73.7	385.9 ± 76.8	
HC	386.9 ± 64.0	385.4 ± 57.0	381.6 ± 48.3	385.5 ± 59.7	405.5 ± 56.2	402.7 ± 55.4	393.8 ± 52.0	410.1 ± 44.6
Ess AA								
GSDV	486.8 ± 53.1	484.9 ± 67.2	475.2 ± 72.2		497.4 ± 50.8	521.5 ± 52.2	499.3 ± 60.5	
HC	476.3 ± 65.8	474.3 ± 67.1	454.7 ± 57.0	465.1 ± 72.8	508.7 ± 21.8	513.9 ± 23.0	494.4 ± 21.2	529.0 ± 34.6

Note: Values are presented as means ± standard deviation.

Abbreviations: AA, amino acids; GSDV, glycogen storage disease type V; HC, healthy controls; KE, ketone supplement; min, minutes; P, placebo supplement.

[a] indicates significant difference between between KE and P supplementation, $p \leq 0.05$.

an increase was found in relative KB-oxidation rates (μmol/kg/min) with KE vs. placebo in both groups (GSDV: P: 0.6 ± 0.5 vs. KE: 4.3 ± 1.3, $p = 0.0001$; HC: P:1.1 ± 0.4 vs. KE: 5.3 ± 0.7, $p = 0.00006$) (Figure 3C). There were no between-group differences in the ketone response.

3.3.3 | Plasma insulin

Plasma insulin (pmol/L) increased after ingestion of the KE drink and peaked 25 min post ingestion (at start of exercise), compared to placebo in both groups (GSDV: P: 53.0 ± 32.9 vs. KE: 117.5 ± 65.0, $p = 0.016$; HC: P: 45.4 ± 10.9 vs. KE: 88.0 ± 21.2, $p = 0.013$). In both groups, insulin dropped during exercise (Figure 3D).

3.3.4 | Carbohydrate metabolism and plasma ammonia

Plasma glucose concentrations in the GSDV group were lower after ingestions of KE vs. placebo drink ($p = 0.020$), with predrink values of 5.75 ± 0.68 to end-exercise value of 4.35 ± 0.39 mmol/L (Figure 4A). In the HC-group a similar trend was found ($p = 0.087$). Glucose R_a (μmol/kg/min) did not differ significantly between supplements (GSDV: P: 14.4 ± 2.9 vs. KE: 14.0 ± 3.2, $p = 0.628$; HC: P: 13.8 ± 3.5 vs. KE: 12.0 ± 2.2, $p = 0.265$) but tended to be lower with the KE drink (Figure 4B). Glucose R_d (μmol/kg/min) did not differ between supplements (GSDV: P: 14.6 ± 3.1 vs. KE: 15.7 ± 3.6, $p = 0.199$; HC: P: 13.1 ± 3.3 vs. KE: 12.0 ± 2.2, $p = 0.512$) (Figure 4C). There was no difference in plasma lactate (mmol/L) or pyruvate (μmol/L) between supplements in the GSDV group (lactate: P: 0.64 ± 0.2 vs. KE: 0.67 ± 0.1, $p = 0.561$; pyruvate: P: 73.7 ± 27.0 vs. KE: 63.8 ± 15.7, $p = 0.215$) (Figure 4D-E). In the HC group, both plasma lactate and pyruvate were lower (especially during the relative workload period) after the KE drink vs. placebo (lactate: P: 2.11 ± 1.0 vs. KE: 1.78 ± 0.79, $p = 0.035$; pyruvate: P: 102.1 ± 23.3 vs. KE: 81.7 ± 20.9, $p = 0.007$). There was no difference in ammonia response (μmol/L) between placebo and the KE drink in GSDV (P: 74.3 ± 38.3 vs. KE: 86.3 ± 55.8, $p = 0.337$). In the HC group, subjects had significantly lower ammonia response with the KE drink (P: 34.9 ± 5.9 vs. KE: 23.0 ± 6.2, $p = 0.01$) (Figure 4F).

3.3.5 | Fat metabolism

Plasma glycerol concentrations were lower after the KE drink compared to placebo (GSDV: P: 130.5 ± 40.1 vs. KE: 61.7 ± 18.1, $p = 0.00009$; HC: P: 143.4 ± 64.7

vs. KE: 80.1 ± 60.0, $p = 0.001$) (Figure 5A). Likewise, R_a of glycerol was lower after KE drink vs. placebo in GSDV (P: 5.6 ± 1.7 vs. KE: 3.3 ± 1.1, $p = 0.007$), a similar nonsignificant trend was seen in HC (P: 6.1 ± 1.2 vs. KE: 5.1 ± 3.2, $p = 0.548$) (Figure 5B). Glycerol R_d was lower after the KE drink vs. placebo in the GSDV group (P: 5.3 ± 1.7 vs. KE: 3.3 ± 1.1, $p = 0.017$) but not significantly in HC (P: 6.0 ± 1.8 vs. KE: 4.9 ± 3.1, $p = 0.513$) (data not shown). The plasma FFA concentration was lower with the KE vs. placebo drink in both groups (HC: P: 0.51 ± 0.17 vs. KE: 0.29 ± 0.09, $p = 0.046$; GSDV: P: 0.47 ± 0.12 vs. KE: 0.25 ± 0.08, $p = 0.0003$) (Figure 5C).

3.3.6 | Amino acids

We found significantly lower concentrations of the amino acids (AA): threonine, alanine, methionine, serine, and glycine after the KE drink compared to placebo in the GSDV group ($p < 0.05$) (Table 2). The same nonsignificant tendency was seen in the HC group. In the HC group, only the AA valine showed a significant increase with KE drink compared to placebo ($p = 0.001$). There was no difference between placebo and KE drink for the rest of the AA presented.

4 | DISCUSSION

Our results demonstrate that an acute oral KE supplementation (395 mg/kg): 1) Significantly increases plasma KB levels and KB oxidation but 2) does not improve exercise capacity assessed by HR (primary outcome) and PE (secondary outcome) during submaximal exercise in patients with GSDV and HC.

Exogenous KBs consumed through drinks containing either KE or KS is an emerging trend in research, as it constitutes an easy way to elevate blood KBs without dieting or fasting. Most studies today have been carried out in healthy individuals with the primary focus to explore the metabolic effects of the supplement and to investigate the potential as a performance-enhancing drink.[13,14,16,21–23] Our study is the first investigation of the potential effects of KE supplementation in a cohort of patients with an inborn error of CHO metabolism. The present study found that participants with GSDV had a similar increase in plasma KB concentrations compared to HC, which was comparable with levels reached in previous trials investigating an oral KE supplementation in healthy, indicating a comparable uptake and bioavailability in patients with GSDV.[13,14,24]

Similar to findings in health, our results show a shift in fuel-oxidation away from CHO oxidation and hepatic

glycogenolysis (decrease R_a and plasma glucose) toward HOB oxidation, which increased significantly in both the HC and the GSDV group.[8,13,14] In the HC group, we observed a significant lowering of plasma lactate and pyruvate and numeric lowering of plasma glucose with the KE supplementation reflecting the reduced glycolysis, which is in line with previous studies.[13] KE supplementation also impaired fat metabolism likely caused by a reduced FFA availability since plasma FFA concentration decreased in both the HC and GSDV participants. Furthermore, a significantly lower lipolytic rate (R_a glycerol) was found in GSDV, which was particularly pronounced during exercise. These findings are in line with previous studies that have also demonstrated an exogenous KB inhibition of lipolysis.[14]

Even though patients with GSDV have compromised CHO metabolism, they still rely heavily on the metabolism of liver-derived glucose, which along with increased fat oxidation, is the key to the relieving second wind. Thus, further inhibition of the CHO metabolism axis along with inhibition of lipolysis is not desirable in this cohort and might be one of the explanations for the failure of KE supplementation to improve exercise capacity in patients with GSDV.

The mechanism behind the fuel shift has been suggested to be both a direct effect of the circulating KBs and a secondary hormonal effect.[8,25] Previous studies testing oral KE supplementation have in line with the present study showed an increase in plasma insulin and inhibition of glycolysis/glycogenolysis and inhibition of lipolysis.[14,16] By contrast, insulin secretion is decreased after intravenous HOB infusion in healthy men, however, decreased blood glucose was also described, indicating a direct affection by the circulating KB′s on liver glucose output and hence blood glucose levels.[8] The opposite insulin response is most likely due to the administration route since oral administration stimulates the incretin effect. The inhibition of CHO metabolism and lipolysis in the present study can be caused by both insulin secretion and/or direct enzymatic inhibition by circulating KBs. Whether the one or the other mechanism is the most significant remains to be investigated.

Results from our previous study in patients with GSDV pointed to an improved exercise capacity after 2 weeks on a modified ketogenic diet regime.[12] Based on the present study, there seem to be different effects of adhering to a ketogenic diet and supplying exogenously produced KBs. Firstly, a ketogenic diet decreases insulin—as opposed to an increase occurring with ingestion of the KE drink. Secondly, the diet showed indications of improved fat oxidation—whereas the KE drink results in an impairment. Thirdly, it has been speculated that the body becomes keto adapted when adhering to the diet for a longer time and thus better at metabolizing KBs. For these reasons, a ketogenic diet might be preferable to oral KE supplementation. This, however, remains to be tested in future trials comparing the two.

Ketone bodies are desirable fuel substrates for working muscle.[8,13] In the present study, we succeeded to significantly increase KB oxidation, but the negative results on exercise capacity show that the KB oxidation increase is not enough to fully compensate for the inhibited CHO and fat oxidation in both patients with GSDV and the HC. Reaching high enough KB concentrations, with a minimum increase in plasma KBs to >2 mM, has been suggested as a prerequisite for gaining an ergogenic effect of KB supplementation.[13,26] In the present study, we succeeded to increase plasma HOB concentrations to a mean level above 3 mM, which is much higher than the suggested limit and much higher than levels normally reached on a ketogenic diet.[26]

For muscle to oxidize HOB, it is first converted to AcAc, which is converted to acetoacetyl-CoA by using/draining the TCA cycle from succinyl-CoA. The acetoacetyl-CoA form is then converted to succinate and thus reintroduced in the TCA cycle, and into two acetyl-CoA for oxidation as usually from glycolysis and β-oxidation. Thus, the availability of TCA cycle intermediates is a prerequisite for the TCA cycle to run on KBs. It seems that KBs "burn in the flame of CHOs" or alternatively AA, thus a CHO supply is needed to sustain anaplerosis and thus TCA flux.[13,27] Inhibition of glycolysis leads to reduced TCA intermediates to fuel the cycle, which is likely the case in this study, and therefore, we would not expect higher plasma KB concentrations to have beneficial effects. An interesting perspective is the combination of CHOs and KB supplements, to ensure TCA intermediates.

The few previous trials that have investigated exogenous KB supplementation and exercise outcomes in healthy have found conflicting results, with only one study reporting actual improvement of exercise capacity after supplementation of a combination of KE and CHO in healthy athletes.[13] The remaining trials in healthy had negative results,[21–23] and this despite, that the participants, in the studies by Leckey et al.[21] and Evans et al.,[23] were served a standardized breakfast before testing ensuring optimal nutritional state (CHO fed).

Amino acids are, besides being protein building blocks, also an important energy source and contribute, next to glucose/glycogen, to increase TCA cycle intermediates in the skeletal muscle.[28] This is shown in GSDV with their known excessive increase in ammonia caused by the breakdown of AA during muscle contraction.[28–30] Glutamate is the key AA in that process, albeit it is produced in large quantities from other AA like glutamine, alanine, and the BCAA (valine, leucine, and isoleucine).[29,30] In the

present study, there was no significant change in the concentrations of the above-mentioned AA with KE drink compared to placebo in both groups, but if any a tendency toward lower concentrations of glutamate and glutamine with the KE drink compared to placebo, indicating greater consumption. Both methionine and threonine dropped significantly with the KE drink compared to placebo, which could be explained by the increased demand of succinyl-CoA used in the conversion of acetoacetyl-CoA to acetyl-CoA.

The present study cannot exclude positive effects on exercise capacity and endurance of KE + CHO or KE + CHO + AA supplementation in a cohort of patients with GSDV. However, we know that oral supplementation of BCAA does not improve exercise tolerance in patients with GSDV.[31] Furthermore, AA are poor suppliers of TCA cycle intermediates compared to glucose/glycogen. For these reasons, we doubt that the combination KE + CHO + AA would be more beneficial than KE + CHO alone.

This study investigated if administration of an oral KE supplementation, inducing acute hyperketosis, could improve exercise capacity to the same extent and in the same easy manner for the patients as we have seen in the oral sucrose studies done by Haller and Vissing.[5,6] We cannot exclude that chronic lower quantities of the KE supplementation may induce a different response. This would be a different treatment for the patients—as a diet supplementation, and not an acute energizer, and remains to be investigated further.

The small sample size is a limitation. A larger cohort would make a stronger statement. However, the sample size chosen was based on a power calculation, which should ensure feasibility of the work with regards to the primary outcome. GSDV is a rare disorder, and stable isotopes are very costly, and for these reasons, the smallest sample size possible was chosen.

In conclusion, the present study indicates that an increase in KB oxidation by acute oral KE supplementation failed to improve exercise capacity in patients with GSDV as well as in HC. This, likely because of the KE-induced inhibition of lipolysis and glycolysis/liver glucose output, driven by insulin stimulation and/or direct enzymatic inhibition of the circulating KBs, leading to reduced FFA and glucose availability in the muscle. Based on the present study, oral KE supplementation alone cannot be recommended as a treatment option for patients with GSDV.

AUTHOR CONTRIBUTIONS
Nicoline Løkken: Conceptualization; methodology; investigation; funding-acquisition; and writing original draft. Jesper H. Storgaard: Investigation and critical review/editing. Karoline L. Revsbech: Investigation and critical review/editing. Nicol C. Voermans: Resources and critical review/editing; Gerrit Van Hall: Conceptualization; methodology; resources; supervision; and review/editing. John Vissing: Conceptualization; methodology; resources; supervision; funding-acquisition; and critical review/editing. Mette C. Ørngreen: Conceptualization; Methodology; investigation; supervision; and critical review/editing.

ANIMAL RIGHTS
This article does not contain any studies with animal subjects.

ETHICAL APPROVAL AND PATIENT CONSENT STATEMENT
All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2000. Informed consent was obtained from all subjects for being included in the study. The study was approved by the Ethics Committee of the Capital Region, Copenhagen (H-18033230) and listed on clinicaltrials.gov (NCT03945370) prior to inclusion.

DATA AVAILABILITY STATEMENT
Data archiving is not mandated but data will be made available on reasonable request.

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