KU Leuven
Biomedical Sciences Group
Faculty of Movement and Rehabilitation Sciences
Department of Movement Sciences
Exercise Physiology Research Group

The role of ketone bodies in physiological and muscular adaptations to exercise and training

Chiel Poffé

Jury:

Promoter:	Prof. Peter Hespel
Chair:	Prof. Jeroen Scheerder
Jury members:	
	Prof. Thierry Troosters
	Prof. Lies Langouche
	Prof. Louise Deldicque
External jury:	Prof. Iñigo Mujika

Dissertation presented in partial fulfilment of the requirements for the degree of Doctor in Biomedical Sciences

May 2020

DANKWOORD

Dinsdag 15 oktober 2013 omstreeks 9u30, aula 200L – 00.07, les inspanningsfysiologie van professor Peter Hespel: “Tot slot kunnen onze spieren ook nog zogeheten ketonlichaampjes gebruiken om energie te leveren, maar aangezien er nauwelijks iets geweten is over hun rol tijdens inspanning dienen jullie dit niet dieper te kennen.” Peter, zij het omwille van het laatste gedeelte van je zin, of omwille van je legendarische kunde om het vak inspanningsfysiologie op een klare en duidelijke manier over te brengen, is deze zin me tot op de dag van vandaag bijgebleven. Op dat moment had ik er nog geen idee van hoe ketonen en jij, mijn promotor, mijn leven de komende jaren zouden domineren. Ik ben je ontzettend dankbaar voor alle kansen die je me hebt gegeven, je steun en vertrouwen, en ik leer nog dagelijks ontzettend veel van jou. Ik hoop dat we in de toekomst de rol van ketonen samen verder kunnen ontrafelen zodat er in je cursus toch minstens een volledig hoofdstuk aan gewijd kan worden.

Deze thesis had nooit kunnen worden wat hij vandaag is zonder de input van enkele experten. Daarom zou ik graag alle leden van mijn commissie willen bedanken. Professor Iñigo Mujika, my sincere thanks for accepting the invitation to be part of my examination committee and to provide such valuable and extensive feedback. Your ability to perfectly integrate science and elite sports is admirable, and I definitely hope to meet you in the near future. Professor Lies Langouche, bedankt voor uw inzichten over ketonen vanuit een meer klinisch gerichte setting. Ik ben ontzettend benieuwd naar uw toekomstige resultaten. Professor Thierry Troosters, mijn welgemeende dank voor uw klare kijk en inzichten in de respiratoire en metabole compensaties tijdens inspanning. Ondanks dat ik als student niet heb mogen genieten van uw deel van de cursus inspanningsfysiologie, heb ik door de jaarlijkse surveillanties toch al behoorlijk wat kunnen opsteken van uw cursus. Professor Louise Deldicque, bedankt om mijn commissie te willen vervolledigen en voor alle inzichten die u mij nu en tijdens mijn studentenjaren hebt bijgebracht. Daarnaast wil ik u ook bedanken voor de externe stage die ik dankzij u heb kunnen volbrengen tijdens mijn masteropleiding. Tot slot wil ik graag professor Jeroen Scheerder bedanken om als voorzitter de jaarlijkse meetings in goede banen te leiden en mij te doen beseffen dat ik beter geen overstap maak naar het wielrennen.

In deze tijden beseft men pas echt hoe waardevol het is om een hechte groep collega’s te hebben waarop je altijd kan terugvallen. Sebastiaan, man van’t Stad, ik kon me geen betere bureaugenoot wensen dan jij. Het was een plezier om samen met jou aan mijn doctoraat te kunnen starten. Onze verhelderende discussies hebben me zeer veel bijgebracht. Daarnaast

bewaakte je ook minutieus mijn dagelijkse voedings- en bewegingspatroon door de perfect getimede uitstapjes naar het koffie/chocomelk-apparaat en de “Lunch Runs” en was je ontzettend begripvol voor mijn suikerwafelverslaving. Heel veel succes met het verder afwerken van je doctoraat en je olympische curlingtoekomst. Katrien, jouw ervaring, levenswijsheid en empathie zijn een grote meerwaarde geweest voor mij de afgelopen jaren. Jouw deur stond/staat altijd open voor een (uitgebreide) babbel en een lach. Bedankt voor je engagement binnen de onderzoeksgroep en tot ver daarbuiten. Monique, de constante in iedere studie. Hou zou ik jou ooit kunnen bedanken voor het oneindige werk dat je voor mij hebt gedaan. Van 5u ’s ochtends tot ‘s avonds laat en zelfs in het weekend en op feestdagen stond je altijd paraat om me te helpen. En hoewel het aantal bloedafnames maar bleef toenemen bij iedere nieuwe studie, bleef jij steeds de rust zelve. Mathias, naast onze nauwe band buiten de werkmuren, was het ook zeer aangenaam om jou als collega te hebben. Bedankt voor je hulp bij de PCR en veel succes met jouw studies. Ana, thanks for our interesting conversations and your funny quotes. I really admire your adventurous attitude. Frank, thank you for your expertise in the biochemical lab and your advice. Stijn en Ruud bedankt voor het nemen van de spierbiopten en jullie medische begeleiding van de proefpersonen. Stefan en Tijs bedankt voor al jullie advies en om mij op weg te zetten aan het begin van mijn doctoraat.

Een grote dankuwel ook aan iedereen van de Bakala Academy (Christophe, Jens, Felix, Martijn, Willem, Hendrik) voor al jullie hulp bij het (de)monteren van de fietsen en de testen op Bakala. Dr. Philip Jansen, bedankt voor de mooie samenwerking tijdens de Tour de France. Ruben, bedankt voor al jouw werk als bachelor- en masterstudent en ook nu nog tijdens je vrijwillige stage. Hopelijk kunnen we ook in de toekomst blijven samenwerken. Daarnaast ook een grote dank aan de talrijke bachelor- en masterstudenten (Machteld, Tim, Lander, Michael, Maarten, Brecht, Simon, Ruben, Bram, Tom, Martijn, Joachim, Pieter, Klajd, Indy, Thomas, Willem, Fien, Katrijn, Katrijn, Alexander, Kevin, Elias, Thiebo en Jaro) waar ik op kon rekenen tijdens de veeleisende studies. En tot slot, zonder proefpersonen geen studies. Daarom bedankt aan alle proefpersonen en atleten die het uiterste van zichzelf hebben gegeven en die zich geheel vrijwillig onderworpen hebben aan de veeleisende onderzoeksprotocols.

Mama en papa, bedankt voor alle kansen die jullie mij hebben gegeven en voor jullie onvoorwaardelijke steun. Zij het voor een tekening voor P.O., een doolhof voor de muizen, of een perfect uitgekiend systeem om de bewegingen van muizen te kunnen registreren, jullie stonden steeds paraat om me uit de nood te helpen. Tiana, Elias, en de rest van de familie Poffé

en Van Roelen, het is zeer fijn om zo'n hechte en intense familieband te hebben waar je steeds op kan vertrouwen. Niels en Nathan, bedankt om al zo lang mijn beste kameraden te zijn en steeds klaar te staan voor wat welverdiende ontspanning. Trainer Paul en mijn looppartners van Halen, bedankt om mij minstens tweemaal per week te doen afzien en me te doen beseffen dat hard trainen je toch verder brengt dan een flesje ketonen.

Leen, nog vooraleer ik wist wat te studeren, kon ik al op jou rekenen. Toen mijn vriendin, ondertussen mijn vrouw. Bedankt om er altijd voor me te zijn en me te steunen in al mijn impulsieve dromen. Jij hebt me de afgelopen jaren heel veel dingen leren appreciëren en me waardevolle idealen bijgebracht. Ik ben echt dagelijks jaloers op al je enthousiasme, empathie en je streven naar een betere wereld. Bedankt om me af en toe een spiegel voor te houden en me te doen beseffen wat het echte geluk is.

Chiel Poffé **Herk-de-Stad, mei 2020**

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LIST OF ABBREVIATIONS

$\Delta G'_{ATP}$	Energy release from ATP hydrolysis
3HBD	3-hydroxybutyrate dehydrogenase
5-HT	Serotonin
90S	90 s all-out sprint
AcAc	Acetoacetate
AcAc-CoA	Acetoacetyl-coenzyme A
ACTH	Adrenocorticotropic hormone
ADP	Adenosine diphosphate
AF	Acute fatigue
AK	Adenylate kinase
AMP	Adenosine monophosphate
AMPK	AMP-activated protein kinase
ATP	Adenosine triphosphate
ßHB	D-ß-hydroxybutyrate
BIC	Bicarbonate
BMC	Bone mineral content
BMD	Bone mineral density
BW	Body weight
CC	Capillary contacts
CFPE	Capillary-to-fiber perimeter exchange index
CK	Creatine kinase
CON	Control drink
CPT	Carnitine palmitoyltransferase
CSA	Cross-sectional area
CWI	Cold water immersion
CycloA	Cyclophilin A
DXA	Dual-energy X-ray absorptiometry
ELISA	Enzyme linked immunosorbent assay
eNOS	Endothelial nitric oxide synthase
EPT_{120min}	120 min endurance performance test
ERRα	Estrogen-related receptor alpha
ETC	Electron transport chain
FA	Fatty acids
FOR	Functional overreaching
G-6-P	Glucose-6-phosphate
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GDF15	Growth differentiation factor 15
GLUT4	Glucose transporter type 4
GPR41	G protein-coupled receptor 41
HDACs	Histone deacetylases
HIF1α	Hypoxia-inducible factor 1-alpha
HIIT	High-intensity interval training

HMGCL	ß-hydroxy ß-methylglutaryl-CoA lyase	*OTS*	Overtraining syndrome
HMGCS2	ß-hydroxy ß-methylglutaryl-CoA synthase 2	*PCr*	Creatine phosphate
HPA axis	Hypothalamic-pituitary-adrenal axis	*PGC1a*	Peroxisome proliferator activated receptor gamma coactivator 1 alpha
HR	Heart rate	*PHOS*	Glycogen phosphorylase
HSL	Hormone sensitive lipase	*Pi*	Inorganic phosphate
IEK	Intermittent exogenous ketosis	*RACE*	~3 h 15 min simulated cycling race
IL-6	Interleukin-6	*RER*	Respiratory exchange ratio
IMT	Intermittent endurance training	*RESTQ-76*	Recovery-Stress Questionnaire for Athletes
$IMT_{180'}$	3 h intermittent cycling	*RPE*	Rating of perceived exertion
IMTG	Intramyocellular triacylglycerol	*SCOT*	Succinyl-CoA-3-oxaloacid CoA transferase
KB	Ketone bodies	*SNARK*	Sucrose nonfermenting AMPK-related kinase
KE	(R)-3-hydroxybutyl (R)-3-hydroxybutyrate ketone ester	*T2*	Acetoacetyl-CoA thiolase
		TCA cycle	Tricarboxylic acid cycle
KS	Ketone salts	*TG*	Triacylglycerol
LT	Lactate threshold	$TT_{15'}$	15-min time-trial
MAPK	Mitogen-activated protein kinase	TT_{30min}	30-min time-trial
		TTE	Time-to-exhaustion
MCT	Monocarboxylate transporter	*VAS*	Visual analogue scale
Mdx	X-linked muscular dystrophy	$\dot{V}CO_2$	Carbon dioxide production
NAD	Nicotinamide-adenine-dinucleotide	V_E	Minute ventilation
NFOR	Non-functional overreaching	*VEGF*	Vascular endothelial growth factor
NLRP3	NOD-like receptor protein 3	$\dot{V}O_2$	Oxygen consumption
ORO	Oil red O	*ww*	Wet weight

ENGLISH SUMMARY

Ketone bodies (KB) – D-ß-hydroxybutyrate (ßHB), acetoacetate (AcAc) and acetone – are naturally occurring metabolites produced from fatty acids in the liver under conditions of low blood glucose and insulin levels. Although continuously produced, blood ketone concentrations rarely exceed 0.1 mM under normal conditions. However, in response to energetic and metabolic stress (e.g. starvation or a low carbohydrate "ketogenic" diet) blood βHB and AcAc concentrations can increase up to 5-8 mM and 1-2 mM, respectively. Under such conditions, KB serve as a readily backup fuel for the brain, heart and muscles, thereby preserving glucose and gluconeogenic reserves. In addition to their ability to provide an alternative energy supply, KB have also been shown to increase the efficiency (work over oxygen consumption) of a perfused rat heart suggesting that KB increase muscle efficiency compared to glucose and lipids. Based on the ability of KB to spare glucose and gluconeogenic reserves and their ability to increase muscle thermodynamics, elevating blood KB has already for a long time been considered to improve endurance exercise performance.

Despite these beneficial effects of KB on muscle metabolism, traditional methods to increase circulating KB levels (e.g. fasting or a ketogenic diet) failed to enhance exercise performance due to their dependence on low-carbohydrate intake. However, recently with the development of the oral ingestible ketone ester (R)-3-hydroxybutyl (R)-3-hydroxybutyrate (KE) this potential could for the first time be investigated in the presence of adequate carbohydrate intake.

Study 1: Effect of KE on endurance exercise performance

KE may stimulate endurance performance by counteracting muscle glycogen breakdown during exercise. However, this glycogen sparing action may also negatively affect performance by impairing high-intensity glycolytic flux at the end of a race. Therefore, in a **first study**, we investigated whether KE provision during the initial phase of a simulated cycling race i) may increase glycogen availability in the final phase of the event, and ii) result in improved high-intensity performance at the end of the race.

In a cross-over design, twelve highly-trained male cyclists performed a simulated cycling race (RACE), which consisted of 3 h intermittent cycling ($IMT_{180'}$), a 15-min time-trial ($TT_{15'}$) and a maximal sprint of ~60 s (SPRINT). During RACE subjects received 60 g carbohydrates

per h combined with three boluses (25-20-20 g) KE or a control drink (CON) at 60 and 20 min before, and at 30 min during RACE. KE intake induced a stable ketosis ([ßHB]: ~3 mM) during the first half of the RACE, while ßHB levels were below 1 mM at the start of $TT_{15'}$ and SPRINT. However, KE ingestion did not counteract net muscle glycogen breakdown during $IMT_{180'}$ (KE: -78 ± 30 (SEM); CON: -60 ± 22 mmol · kg^{-1} ww, p = 0.08) or $TT_{15'}$ (KE: -9 ± 18; CON: -18 ± 18 mmol · kg^{-1} ww, p = 0.35), nor increased performance during the $TT_{15'}$ (mean power ouput: 273 ± 38 *vs.* 272 ± 37 W, for KE *vs.* CON, p = 0.83) or SPRINT (time-to-exhaustion: 59 ± 16 *vs.* 58 ± 17 s, for KE *vs.* CON, p = 0.66). In addition, the acidic properties of KB resulted in a mild, transient decrease in blood pH from ~7.42 to 7.36 together with a depletion of extracellular alkaline reserve from 26.0 to 21.6 mM (both $p < 0.001$ *vs.* CON). This dysregulated acid-base balance in KE was accompanied by increased fatigue perception halfway $IMT_{180'}$ (KE: 13.3 ± 0.6 *vs.* CON: 12.3 ± 0.3 out of 20, p = 0.06), and decreased the perception of hunger (KE: 3.6 ± 0.3 *vs.* CON: 4.9 ± 0.6 on a 10-point scale, p = 0.03) and desire to eat (KE: 5.1 ± 0.5 *vs.* CON: 6.5 ± 0.5 on a 10-point scale, $p < 0.01$) post-exercise. Therefore, based on our study and available literature, there is currently no evidence to indicate that acute KE ingestion may stimulate endurance exercise performance under conditions of adequate carbohydrate intake.

Study 2: Effect of KE and bicarbonate on endurance exercise performance

Given the negative implications of acidosis on exercise performance, we investigated in a **second study** whether counteracting ketoacidosis by concomitant ingestion of sodium bicarbonate (BIC) may unmask the ergogenic potential of KE. To investigate this hypothesis, we subjected nine-well trained male cyclists to a similar simulated cycling race as in study 1. In a randomized cross-over design subjects received either i) 65 g KE, ii) 300 mg · kg^{-1} body weight $NaHCO_3$ (BIC), iii) KE+BIC or iv) a control drink (CON) during RACE, together with 60 g carbohydrates per h.

In line with study 1, KE ingestion transiently elevated blood ßHB to ~2-4 mM during the initial 2 hours of RACE and resulted in a transient drop in pH from 7.43 to 7.35 without affecting exercise performance at the end of the RACE. Additional ingestion of BIC completely counteracted KE-induced acidosis (pH: 7.51 ± 0.01) and increased extracellular bicarbonate concentration from 25.5 to 31.1 mM (both $p < 0.05$ vs. CON). This resulted in a 5% higher mean power output during $TT_{15'}$ in KE+BIC (266 ± 4 W) compared to the other conditions (~255 W, $p < 0.05$), while time-to-exhaustion in SPRINT was similar between all conditions at ~60 s. In addition, concomitant ingestion of BIC also counteracted the suppressing effect of KE

on the 'perception of hunger' (KE: 3.8 ± 0.4 *vs.* CON: 5.6 ± 0.6 and KE+BIC: 5.9 ± 0.5, $p < 0.05$) and 'desire to eat' (KE: 4.0 ± 0.3 *vs.* CON: 5.8 ± 0.6 and KE+BIC: 5.8 ± 0.7, $p < 0.05$) post-exercise, without causing significant gastrointestinal distress. These data indicate that co-ingestion of KE and BIC is an effective, safe, and well-tolerated approach to enhance high-intensity performance at the end of an endurance exercise event.

Study 3: Effect of KE on the development of non-functional overreaching

Besides the effect of KE on exercise performance, previous studies also suggested that increasing blood KB may increase post-exercise recovery and modulate a range of putative factors implicated in the development of overreaching. Therefore, in a **third study**, we investigated whether KE supplementation may counteract the development of non-functional overreaching. Eighteen recreationally active male subjects performed a 3-week supramaximal cycling period (6 training days/week, 2 sessions/day) while receiving either KE (n = 9) or an isocaloric control drink (CON, n = 9) following each session.

The overload training period induced profound physiological dysregulations including a reduction of resting (-6 ± 2 bpm), submaximal (-15 ± 3 bpm) and maximal (-17 ± 2 bpm, all $p < 0.05$ *vs.* baseline) heart rate, together with increased nocturnal catecholamine excretion. In addition, subjects in CON were unable to increase their energy intake in proportion to the increased energetic demands of training. Conversely, other markers suggested to be involved in the development of overreaching (e.g. ACTH, cortisol, IL-6) were not affected by the overload training period. KE intake increased sustainable training load in the final week with 15% compared to CON (KE: 10266 ± 321 kJ *vs.* CON: 8962 ± 646 kJ, $p < 0.05$) and inhibited the training-induced increase in nocturnal catecholamine excretion, and also blunted the decrease in resting (KE: +2 ± 3 bpm), submaximal (KE: -7 ± 2 bpm) and maximal (KE: -10 ± 2 bpm, all $p < 0.05$ *vs.* CON) heart rate. In contrast to CON, subjects in KE were able to increase their energy intake during the overload training period, resulting in an energy balance. This indicates that KE is a potent strategy to counteract the development of non-functional overreaching and to stimulate endurance exercise performance during intensive training periods.

Study 4: Effect of KE on the muscular responses to overload training

Recent studies indicated that training overload not only evokes profound physiological dysregulations, but also impairs skeletal muscle adaptations. One of the primary muscular adaptations to endurance exercise training is an increase in muscle capillarization (*i.e.*

angiogenesis) to improve oxygen diffusion, nutrient uptake and metabolite clearance to and from skeletal muscle. Preliminary data indicates that increasing blood KB stimulates lymphangiogenesis in mice, and also increases the primary regulator of angiogenesis, *i.e.* vascular endothelial growth factor (VEGF), in-vitro. Therefore, in a **fourth study**, we assessed whether KE may stimulate muscle angiogenesis during endurance training overload. To investigate this hypothesis, we used the biopsies from *m. vastus lateralis* obtained in the context of study 3, before and after completion of the three-week overload training period.

Subjects in KE showed a 44% and 42% increase in capillary contacts (CC) (pre: 4.6 ± 0.2 *vs.* post: 6.6 ± 0.6 capillary contacts, $p < 0.01$) and capillary-to-fiber perimeter exchange index (CFPE) (pre: 8.9 ± 0.4 *vs.* post: 12.6 ± 0.8 capillaries per 1000 μm, $p < 0.001$). Conversely, these parameters remained unaffected in CON (CC: 5.1 ± 0.4 *vs.* 5.5 ± 0.2 capillary contacts; CFPE: 9.2 ± 0.7 *vs.* 10.3 ± 0.6 capillaries per 1000 μm for pre *vs.* post, respectively, $p = 0.99$ and $p = 0.33$). Concomitant with the augmented angiogenic response in KE, VEGF expression substantially increased both at the protein (+33 ± 17%) and mRNA (181 ± 71%, both $p < 0.05$) level, but not in CON. Conversely, neither muscle morphology nor fiber type distribution were altered in KE or CON. This indicates that KE ingestion is a promising intervention to stimulate beneficial training adaptations during endurance training overload.

In conclusion, the obtained data indicate that co-ingestion of KE and BIC may evolve as an attractive approach to acutely enhance endurance exercise performance in athletes, while intermittent KE administration is a potent strategy to enhance training adaptations and performance during endurance training overload.

NEDERLANDSE SAMENVATTING

Ketolichamen (KB) – D-ß-hydroxyboterzuur (ßHB), acetoacetaat (AcAc) en aceton – zijn natuurlijk voorkomende metabolieten die, bij lage concentraties van glucose en insuline in het bloed, geproduceerd worden door de lever uit de afbraak van vrije vetzuren. Hoewel ze continu worden geproduceerd, stijgen de ketonconcentraties in het bloed zelden boven 0.1 mM onder normale fysiologische omstandigheden. Tijdens perioden van energetische en/of metabole stress daarentegen, zoals tijdens vasten of het gebruik van een vetrijk, ketogeen dieet, zal de concentratie van ßHB en AcAc in het bloed toenemen tot respectievelijk 5-8 mM en 1-2 mM. Onder deze omstandigheden, zullen ketonen fungeren als een onmiddellijk beschikbare reservebrandstof voor onze hersenen, hart en spieren en zodoende glucose en gluconeogene reserves vrijwaren. Naast hun mogelijkheid om te fungeren als een alternatieve energiebron, leidt de toediening van KB aan een doorbloed rattenhart ook tot een verhoogde efficiëntie (geleverde arbeid over zuurstofverbruik) van de hartspier. Dit suggereert dat ketonen een meer efficiënte brandstof zijn voor spieren dan glucose en vetten. Aangezien KB glucose en gluconeogene reserves kunnen sparen en de metabole efficiëntie van spieren doet toenemen, wordt het verhogen van KB in het bloed reeds geruime tijd beschouwd als een potentieel middel om de uithoudingsprestatie te bevorderen.

Ondanks die voordelige effecten van KB op spiermetabolisme, slaagden traditionele methoden om in ketose te gaan (vasten of het gebruik van een ketogeen dieet) er niet in om de uithoudingsprestatie te verbeteren, omdat zulke methoden een (te) lage koolhydraatinname vereisen. Recent werd het echter, door de ontwikkeling van een zogeheten ketonester (R)-3-hydroxybutyl (R)-3-hydroxybutyrate (KE), mogelijk om het prestatiebevorderend potentieel van KB te onderzoeken in de aanwezigheid van een adequate koolhydraatinname.

Studie 1: Effect van KE op uithoudingsprestatie

KE wordt beschouwd als een middel om de uithoudingsprestatie te verhogen doordat het de afbraak van spierglycogeen kan tegengaan. Dat effect kan echter ook nadelig zijn voor het prestatievermogen, aangezien de afbraak van spierglycogeen cruciaal is voor maximale prestaties aan het einde van een uithoudingswedstrijd. Daarom gaan we in een **eerste studie** na of inname van KE tijdens het eerste deel van een gesimuleerde wielerwedstrijd i) de glycogeenconcentratie naar het einde van de wedstrijd toe kan sparen, en ii) daardoor de maximale prestatie aan het slot van de wedstrijd kan verbeteren.

In een cross-over design voerden twaalf goed getrainde wielrenners een gesimuleerde wielerwedstrijd (RACE) uit die bestond uit 3 uur submaximaal fietsen ($IMT_{180'}$), gevolgd door een 15minuten tijdrit ($TT_{15'}$) en een maximale sprint van ~60 s (SPRINT). Tijdens RACE kregen de proefpersonen 60 g koolhydraten per uur met daarnaast drie dosissen (25-20-20 g) KE of een controledrank (CON) en dit 60 en 20 min voor, en na 30 min tijdens RACE. KE-inname resulteerde in een stabiele ketose ([ßHB]: ~3 mM) gedurende de eerste helft van RACE, terwijl de ßHB-concentraties in het bloed lager dan 1 mM waren bij de start van $TT_{15'}$ en SPRINT. KE-supplementatie leidde niet tot een verminderde netto spierglycogeenafbraak tijdens $IMT_{180'}$ (KE: -78 ± 30 (SEM); CON: -60 ± 22 mmol · kg^{-1} ww, p = 0.08), noch tijdens $TT_{15'}$ (KE: -9 ± 18; CON: -18 ± 18 mmol · kg^{-1} ww, p = 0.35) en zorgde ook niet voor een betere prestatie tijdens $TT_{15'}$ (gemiddeld vermogen: 273 ± 38 *vs.* 272 ± 37 W, voor KE *vs.* CON, p = 0.83) of SPRINT (tijd tot uitputting: 59 ± 16 *vs.* 58 ± 17 s, voor KE *vs.* CON, p = 0.66). Daarnaast zorgde het zuurgehalte van KB voor een tijdelijke, matige daling in bloed pH van ~7.42 naar 7.36 en een vermindering van de extracellulaire alkalische reserve van 26.0 naar 21.6 mM (beide $p < 0.001$ *vs.* CON). Het verstoorde zuur-base evenwicht in KE ging gepaard met een verhoogde perceptie van vermoeidheid halverwege $IMT_{180'}$ (KE: 13.3 ± 0.6 *vs.* CON: 12.3 ± 0.3 op een maximum van 20, p = 0.06) en zorgde voor een verminderd 'hongergevoel' (KE: 3.6 ± 0.3 *vs.* CON: 4.9 ± 0.6 op een 10-puntenschaal, p = 0.03) en 'eetlust' (KE: 5.1 ± 0.5 *vs.* CON: 6.5 ± 0.5 op een 10-puntenschaal, $p < 0.01$) na RACE. Op basis van deze studie en de beschikbare literatuur, is er tot op heden geen enkele evidentie dat acute KE-inname de uithoudingsprestatie zou kunnen bevorderen bij optimale koolhydraatinname.

Studie 2: Effect van KE en bicarbonaat op uithoudingsprestatie

Aangezien voorgaande studies aantoonden dat acidose de prestatie negatief beïnvloedt, hebben we in een **tweede studie** onderzocht of het tegengaan van ketoacidose, door het bijkomend innemen van natriumbicarbonaat (BIC), het prestatiebevorderend potentieel van KE tot zijn recht zou doen komen. Om dit na te gaan hebben we negen goed getrainde wielrenners onderworpen aan een gesimuleerde wielerwedstrijd zoals in studie 1. In een gerandomiseerd cross-over design kregen de proefpersonen een van de volgende condities i) 65 g KE, ii) 300 mg · kg^{-1} lichaamsgewicht $NaHCO_3$ (BIC), iii) KE+BIC of iv) een controledrank (CON) gedurende RACE, tezamen met 60 g koolhydraten per uur.

Evenals in studie 1 zorgde KE-inname voor een tijdelijke verhoging van de bloed ßHB-concentratie tot ~2-4 mM gedurende de eerste 2u van RACE en resulteerde dat in een tijdelijke daling in pH van 7.43 naar 7.35 zonder de prestatie aan het einde van de wedstrijd te

beïnvloeden. Bijkomende inname van BIC deed de ketoacidose volledig teniet (pH: 7.51 ± 0.01) en zorgde voor een verhoging van de extracellulaire bicarbonaatconcentraties van 25.5 naar 31.1 mM (beide $p < 0.05$ *vs.* CON). Daarnaast leidde dat tot een 5% hoger gemiddeld vermogen in TT_{15}, ten opzichte van de andere condities (KE+BIC: 266 ± 4 W *vs.* andere condities ~255 W, $p < 0.05$), terwijl de tijd tot uitputting in de sprint ~60 s was voor alle condities. Voorts verhinderde KE+BIC het onderdrukkende effect van KE op het 'hongergevoel' (KE: 3.8 ± 0.4 *vs.* CON: 5.6 ± 0.6 en KE+BIC: 5.9 ± 0.5 op een 10-puntenschaal, $p < 0.05$) en 'eetlust' (KE: 4.0 ± 0.3 *vs.* CON: 5.8 ± 0.6 en KE+BIC: 5.8 ± 0.7 op een 10-puntenschaal, $p < 0.05$) na de wedstrijd volledig zonder te leiden tot verhoogde gastro-intestinale klachten. Deze data tonen aan dat het innemen van KE met BIC een doeltreffende, veilige en goed verdraagbare methode is om de maximale prestatie aan het slot van een uithoudingswedstrijd te verbeteren.

Studie 3: Effect van KE op de ontwikkeling van niet-functionele overreaching

Naast de effecten van KE op de uithoudingsprestatie, suggereerden recente studies dat het verhogen van bloed KB concentraties het herstel na inspanning kan bevorderen, en eveneens een aantal factoren die vermoedelijk betrokken zijn bij de ontwikkeling van overreaching kan beïnvloeden. Daarom zijn we in een **derde studie** nagegaan of KE supplementatie de ontwikkeling van niet-functionele overreaching kan tegengaan. Achttien recreationeel actieve, mannelijke proefpersonen werden onderworpen aan een drie-weken-durende supramaximale trainingsperiode op de fiets (6 trainingsdagen/week, 2 trainingssessies/dag), terwijl ze ofwel KE ($n = 9$) of een isocalorische controledrank (CON, $n = 9$) kregen aan het einde van elke training.

De overtrainingsperiode resulteerde in uitgesproken fysiologische verstoringen zoals een daling in rust (-6 ± 2 bpm), submaximale (-15 ± 3 bpm) en maximale (-17 ± 2 bpm, alle parameters $p < 0.05$ *vs.* baseline) hartslag en een toename in catecholamine excretie gedurende de nacht. Daarnaast waren subjecten in CON niet in staat om hun energie-inname proportioneel te verhogen met het toegenomen energieverbruik door de overtrainingsperiode. Andere indicatoren waarvan vaak gesuggereerd wordt dat ze betrokken zijn bij de ontwikkeling van overtraining (bv. ACTH, cortisol, IL-6), werden niet beïnvloed door de intensieve trainingsperiode. KE-inname zorgde ervoor dat de proefpersonen een 15% hogere trainingsbelasting konden verdragen dan CON in de laatste week (KE: 10266 ± 321 kJ *vs.* CON: 8962 ± 646 kJ, $p < 0.05$), en ging de toename in nachtelijke catecholamine excretie, en daling in rust (KE: +2 ± 3 bpm), submaximale (KE: -7 ± 2 bpm) en maximale (KE: -10 ± 2 bpm, alle parameters $p < 0.05$ *vs.* CON) hartslag (gedeeltelijk) tegen. In tegenstelling tot CON slaagden

subjecten in KE er wel in om hun energie-inname proportioneel te doen toenemen met de verhoogde energievraag van de intensieve trainingen. Dit geeft aan dat KE een veelbelovende strategie is om de ontwikkeling van niet-functionele overreaching af te remmen en om de uithoudingsprestatie tijdens een intensieve trainingsperiode te verbeteren.

Studie 4: Effect van KE op spieradaptaties tijdens trainingsoverload

Recente studies gaven aan dat trainingsoverload niet enkel zorgt voor ernstige fysiologische verstoringen, maar ook spieradaptaties tenietdoet. Een van de primaire spieradaptaties aan uithoudingstraining is een toename in spiercapillarisatie (angiogenese) om zo de zuurstofdiffusie, nutriëntenopname en het verwijderen van metabolieten van en naar de skeletspier te verbeteren. Preliminaire data geven aan dat het verhogen van bloed KB de angiogenese van de lymfevaten in muizen stimuleert en ook de belangrijkste regulator van angiogenese, namelijk de vasculaire endotheel groeifactor (VEGF), in-vitro verhoogt. Daarom hebben we in een **vierde studie** onderzocht of KE de spierangiogenese tijdens trainingsoverload kan stimuleren. Om deze hypothese te onderzoeken hebben we de spierbiopten van de *m. vastus lateralis*, die verkregen werden voor en na de drie-weken-durende overtrainingsperiode in de context van studie 3, gebruikt.

Subjecten in KE vertoonden een toename van 44% en 42% in het aantal capillaire contacten (CC) (pre: 4.6 ± 0.2 *vs.* post: 6.6 ± 0.6 capillaire contacten, $p < 0.01$) en in capillairen/spiervezel-perimeter-exchange index (CFPE) (pre: 8.9 ± 0.4 *vs.* post: 12.6 ± 0.8 capillairen per 1000 µm, $p < 0.001$). Daarentegen wijzigden die parameters niet in CON (CC: 5.1 ± 0.4 *vs.* 5.5 ± 0.2 capillaire contacten; CFPE: 9.2 ± 0.7 *vs.* 10.3 ± 0.6 capillairen per 1000 µm voor pre *vs.* post, respectievelijk, $p = 0.99$ en $p = 0.33$). Tezamen met het angiogene effect in KE, verhoogde de VEGF-expressie substantieel in KE, zowel op proteïne- (+33 ± 17%) als mRNA-niveau (+181 ± 71%, beide $p < 0.05$), terwijl dat niet het geval was voor CON. Spiermorfologie en spiervezeldistributie wijzigden niet in beide groepen. Dat geeft aan dat KE inname een veelbelovende interventie is om positieve trainingsadaptaties te stimuleren tijdens trainingsoverload.

Samengevat, tonen de verkregen resultaten aan dat het innemen van KE met BIC kan uitgroeien tot een doeltreffende manier om acuut de uithoudingsprestatie te verhogen bij atleten, terwijl het langdurig innemen van KE een effectieve manier is om trainingsadaptaties en uithoudingsprestatie te verhogen tijdens intensieve trainings- of wedstrijdperioden.

CHAPTER I

GENERAL INTRODUCTION

1. Ketone bodies

The term ketone bodies (KB) refers to three water-soluble molecules, namely ß-hydroxybutyrate (ßHB), its dehydrogenated counterpart acetoacetate (AcAc), and their spontaneous breakdown product acetone (43, 130, 273). These compounds are called ketone bodies because they either contain a ketone group [RC(=O)R'] or, in case of ßHB, a hydroxyl group that can be easily converted into a ketone group (**Figure 1**) (222).

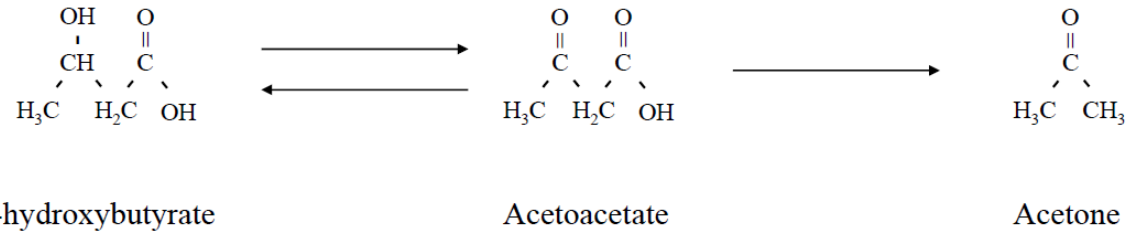


Figure 1. Chemical structure of the three ketone bodies.

1.1 Historical perspective

In the mid-19th century, KB for the first time came to the attention due to their abundant presence in the urine of patients afflicted by diabetic coma (118, 162). Owing to their association with this severe condition, KB were considered detrimental for health and were thought to be produced as a byproduct of the incomplete combustion of fat due to insufficient carbohydrate availability (300, 377). However, in the 1930s, the dramatic increase of KB during diabetic coma was identified to arise from insulin and/or glucose deficiency causing hepatic conversion of free fatty acids to KB, a condition now known as ketoacidosis (39, 53, 54, 194). From then on, KB were considered as normal components of the blood and even appeared to have curative effects in children with drug-resistant epilepsy (109, 330, 337).

1.2 Ketone bodies as an energy substrate

During the next years, it was identified that KB can be oxidized by most extra-hepatic tissues and act as an alternative metabolic fuel (319). In a series of seminal experiments, Cahill and Owen demonstrated that the brain, which was believed to rely exclusively on carbohydrate resources (199, 342), derives more than 60% of its energy needs from KB during prolonged starvation (38, 42, 287). The concomitant reduction in glucose utilization did not only preserve gluconeogenic reserves (101), including muscle glycogen and vital muscle proteins, but also extended survival time for more than two months (42). Besides the ability of KB to serve as an

alternative metabolic fuel for the brain, KB can also be utilized by skeletal muscles during exercise, even in the presence of sufficient carbohydrate resources (63, 103, 104). These observations support the concept of the glucose-free fatty acid cycle, that fat-derived products like KB are hierarchically dominant over carbohydrates for oxidative phosphorylation (62, 312).

In addition to the hierarchical dominance of KB over carbohydrates, ßHB and AcAc were identified in 1945 to increase bovine sperm motility while decreasing oxygen consumption, suggesting an improved metabolic efficiency (225, 226). Later on, it was observed that perfusing an isolated rat heart with 4 mM ßHB and 1 mM AcAc resulted in a 24% increase in cardiac efficiency (hydraulic work over oxygen consumption), which was mediated by a reduction *versus* oxidation of the mitochondrial NAD and co-enzyme Q couple, respectively (328). As a result, the redox span between site I and site II of the electron transport chain increases, causing greater energy release from ATP hydrolysis ($\Delta G'_{ATP}$). Although KB have been shown to increase cardiac efficiency, it should be emphasized that the ATP yield per oxygen atom and per carbon atom, is lower in KB compared to glucose and fatty acids, respectively (142, 197, 286).

1.2.1 Ketone body metabolism

The concentration of KB in the blood is determined by the balance between hepatic production (i.e. ketogenesis) and extra-hepatic utilization and breakdown (i.e. ketolysis) of KB. In the postprandial state, blood KB rarely exceed 0.5 mM in healthy subjects (377). However, hyperketonemia, defined as blood ßHB above 1 mM (222, 273), often occurs as a transient response to physiological conditions of carbohydrate restriction or depletion, including fasting or very prolonged exercise (39, 208). Nonetheless, prolonged hyperketonemia can also be induced by various pathological conditions, including diabetes mellitus, some inborn metabolic errors, as well as by binge drinking (213, 256, 327).

1.2.1.1 Ketogenesis

Ketogenesis **(Figure 2, left panel)** occurs in the mitochondria of hepatocytes and primarily utilizes free fatty acids liberated from adipose tissue as a substrate. In addition, a small fraction of ketogenesis (< 5%) is derived from the degradation of the ketogenic amino acids leucine, lysine, phenylalanine, isoleucine, tryptophan, tyrosine and potentially also threonine (273, 367). The production of KB is stimulated by reduced circulating levels of glucose and insulin as well

as by depletion of glycogen stores, which stimulates lipolysis resulting in increased free fatty acids flux to the liver (253, 319). Fatty acids so formed subsequently undergo ß-oxidation to produce acetyl-CoA, which under conditions of sufficient glucose availability directly combines with oxaloacetate to be oxidized by the citric acid cycle and electron transport chain to generate ATP (222). However, if glucose levels decrease, oxaloacetate is utilized for gluconeogenesis and no longer available to condense with acetyl-CoA, resulting in an accumulation of acetyl-CoA in hepatic mitochondria. Subsequently, the enzyme acetoacetyl-CoA thiolase (T2) catalyzes the condensation of two acetyl-CoA molecules to from acetoacetyl-CoA (AcAc-CoA). AcAc-CoA then combines with acetyl-CoA to form ß-hydroxy ß-methylglutaryl-CoA (HMG-CoA) in a reaction catalyzed by HMG-CoA synthase. In the next step, HMG-CoA dissociates into AcAc and acetyl-CoA by HMG-CoA lyase (HMGCL).

A minor fraction of the AcAc produced is directly exported to the blood, while the remaining AcAc is reduced to ßHB by 3-hydroxybutyrate dehydrogenase (3HBD) in a step involving oxidation of NADH to NAD^+. Therefore, the ratio of ßHB to AcAc ultimately depends on the redox potential ($NADH/NAD^+$) within hepatic mitochondria, and generally increases from 1 in the postprandial state to approximately 6 following fasting (41, 222, 273, 288). ßHB is subsequently transported by the monocarboxylate transporter 7 (MCT 7) across the mitochondrial and sarcolemmal membranes of the liver towards the circulation, however also other MCT's may be involved (175).

1.2.1.2 Ketolysis

Upon passing to the circulation, KB are transported into the mitochondrial matrix of extra-hepatic tissues such as skeletal muscle, brain and heart by the MCT1 and MCT2 transporters (95, 133) **(Figure 2, right panel)**. Following re-oxidation of ßHB to AcAc by 3HBD, AcAC is converted into AcAc-CoA by succinyl-CoA-3-oxaloacid CoA transferase (SCOT). In the subsequent step, AcAc-CoA dissociates into two molecules acetyl-CoA to enter the tricarboxylic acid (TCA) cycle for terminal oxidation and production of ATP. Due to the absence of SCOT in hepatocytes, KB cannot undergo ketolysis within the liver thereby preventing futile cycling between AcAc and HMG-CoA (128).

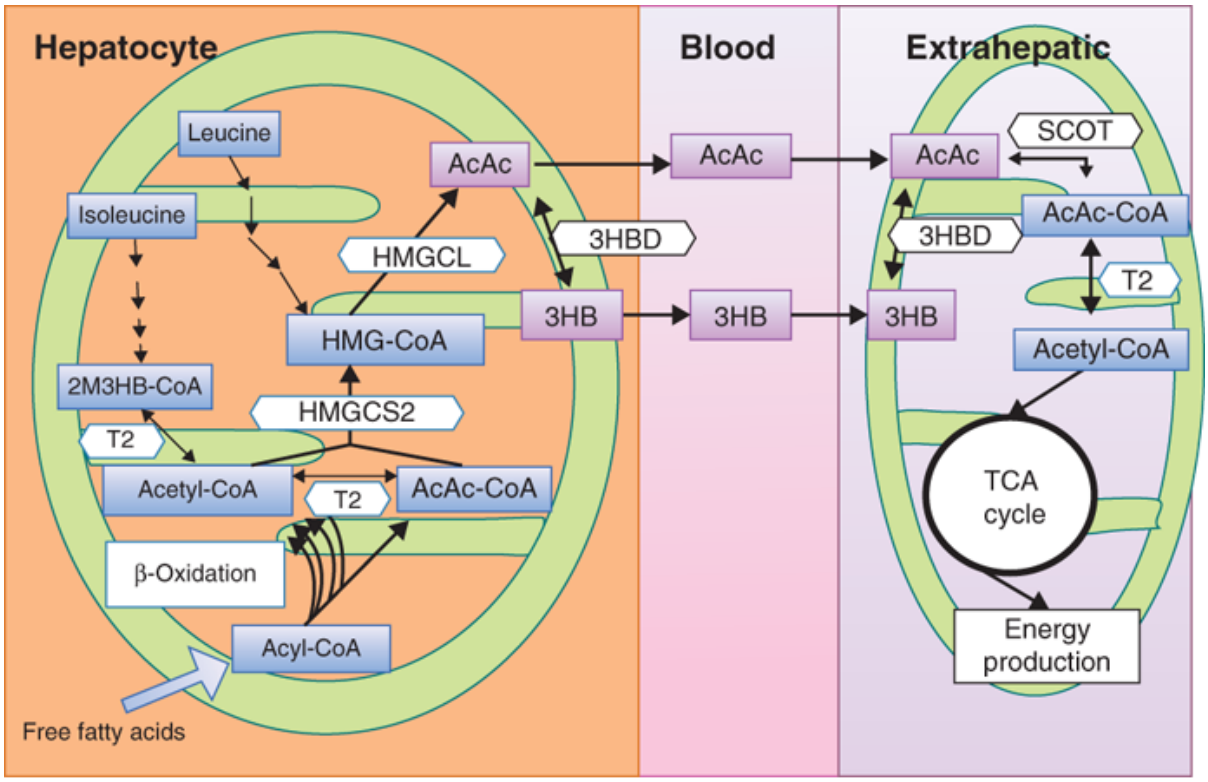


Figure 2. Metabolic pathways of KB metabolism.
AcAc-CoA, acetoacetyl-CoA; HMG-CoA, ß-hydroxy ß-methylglutaryl-CoA; 2M3HB-CoA, 2-methyl-3-hydroxybutyryl-CoA; T2, acetoacetyl-CoA thiolase; HMGCS2, HMG-CoA synthase 2; HMGCL, HMG-CoA lyase; 3HBD, 3-hydroxybutyrate dehydrogenase; SCOT, succinyl-CoA-3-oxaloacid CoA transferase (SCOT). Reproduced from Saraflogou *et al.*, 2017 (326), with permission.

1.2.1.3 Regulation of ketone body metabolism

The rate of KB production is regulated by three enzymes: hormone-sensitive lipase in adipocytes and acetyl CoA carboxylase and HMGCS2 in hepatocytes (222). The first two enzymes are controlled by circulating insulin (252) *versus* glucagon and noradrenaline (46, 131, 254, 350) levels, which respectively inhibit and promote ketogenesis. The activity of HMGCS2, which induces ketogenesis, increases during conditions of low glucose availability such as starvation and following a high-fat diet, while it is inhibited by elevated blood insulin levels (309). In contrast, the utilization of KB by extra-hepatic tissues is only controlled by the rate-limiting enzyme SCOT (114). Besides the regulation of ketone metabolism by hormonal factors such as insulin and noradrenaline, high circulating levels of KB also inhibit their own production by agonizing the nicotinic acid receptor PUMA-G in adipose tissue, thereby reducing hepatic free fatty acid availability (361).

1.3 Ketone bodies as signaling metabolites

In addition to the canonical roles of KB in energy metabolism, recent studies have identified that AcAc and ßHB also play a signaling role in cellular homeostasis (277, 322, 333, 409). Zou et al. observed that AcAc stimulates muscle satellite cell activation and proliferation in mice, thereby counteracting muscular dystrophy in X-linked muscular dystrophy (mdx) mice, while stimulating regeneration of *m. tibialis anterior* following cardiotoxin-induced injury (409). ßHB on the other hand, possesses numerous signaling roles (278) of which the most relevant are its ability to inhibit histone deacetylases (HDACs) (333), the NOD-like receptor protein 3 (NLRP3) inflammasome (405), and the G protein-coupled receptor 41 (GPR41) (203), all at physiologically relevant concentrations. An inhibition of HDACs by ßHB counteracts histone de-acetylation and thereby stimulates gene expression which in turn restricts oxidative stress (333). Moreover, as the activity of class IIa HDACs in skeletal muscle is responsive to endurance exercise, ßHB may also affect the adaptations to exercise and training (86, 255). Besides restricting oxidative stress, ßHB has also been shown to counteract inflammation by decreasing the activity of the NLRP3 inflammasome (405), and to suppress sympathetic nervous system activity, energy expenditure and heart rate through inhibition of GPR41 (203) in animal models.

1.4 Ketone bodies as modulators of appetite and satiety

Although it is unclear whether due to a signaling effect or via metabolic oxidation (220), a recent meta-analysis indicated that KB decrease appetite while increasing satiety (120). The precise mechanisms whereby KB affect appetite sensations remains to be determined, however ßHB has been shown to modulate both central actions in the brain (221), as well as peripheral secretion of hormones (352), implicated in appetite regulation. In support of a central effect, intracerebral infusions of ßHB have been shown to decrease food intake and body weight in rats (7), while ßHB also increased hypothalamic expression of orexigenic agouti-related peptides *in vitro* (221).

At the peripheral level, appetite is primarily regulated by the hormones leptin and ghrelin, which respectively suppress and stimulate appetite by acting on the hypothalamus and vagus nerve (18, 207, 231). Leptin is mainly produced by adipose tissue and enterocytes in the small intestine and its concentration increases in proportion to body fat stores, insulin and glucocorticoids (110). Ghrelin on the other hand, is produced by oxyntic cells of the gastrointestinal tract in response to energy unavailability (276). Previous studies indicated that

KB both may increase circulating leptin levels (349, 365) as well as may reduce the concentration of ghrelin in the blood (352). Besides ghrelin and leptin, recent studies also identified other hormones, including growth differentiation factor 15 (GDF15) to be involved in the regulation of appetite (189, 242, 384). GDF15 is a stress-induced hormone that is mainly produced by the liver and adipose tissue, and possibly also skeletal muscle, and lowers food intake (121, 205, 227, 295). Evidence for a potential involvement of GDF15 in the appetite suppressing effect of KB comes from two recent studies showing a positive association between blood KB and circulating GDF15 levels (295, 406).

2. Methods to induce ketosis

2.1 Endogenous ketosis

As already outlined above, starvation results in a progressive increase of KB in the blood: < 0.5 mM following an overnight fast, ~1-2 mM after 24 h fasting and up to 4-5 mM after 6 days of fasting (40) **(Figure 3)**. But outside prolonged fasting or starvation, ketosis can also be induced by adopting a high-fat (~80% of energy intake), low-carbohydrate (< 20 g·day^{-1}), low-protein (~15% of energy intake), a so-called 'ketogenic' diet (292, 305). The extent of ketonemia following a ketogenic diet largely depends on the amount of carbohydrate intake, with typical ßHB values observed around 3 mM. However they can increase up to ~7 – 8 mM following severe, long-term carbohydrate restriction (293). Besides dietary modifications, prolonged exercise, especially when performed in the fasted state, also stimulates KB production and results in hyperketonemia both during (ßHB: 0.5 – 1.0 mM), and after (ßHB: 1 – 4 mM) exercise (12, 192, 208). The magnitude of ketosis during and after exercise is severely reduced by high-carbohydrate ingestion (208), as well as by increased training status (190–192). The reduced ketogenic response to exercise in trained *versus* untrained individuals has been attributed to a decrease in post-exercise free fatty acids concentrations (190, 192) and/or to elevated activity of ketolytic enzymes (393, 394).

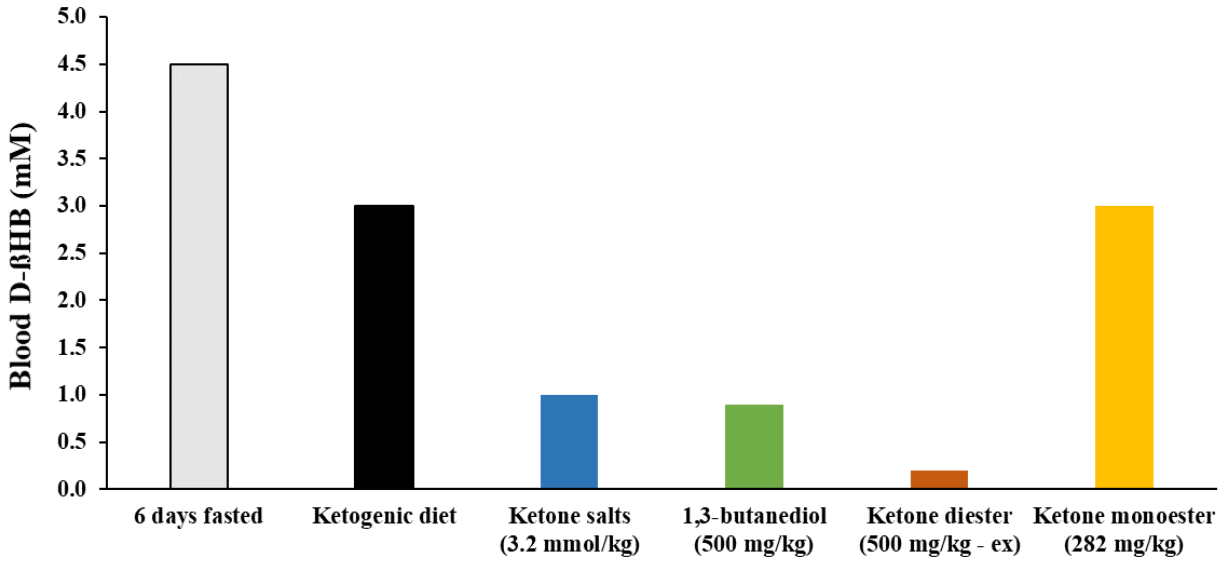


Figure 3. Blood D-ßHB concentrations in response to various endogenous and exogenous conditions of hyperketonemia.
D-ßHB concentrations in response to ketone diester were assessed during exercise (ex), while all other conditions reported D-ßHB values at rest.

2.2 Exogenous ketosis

Despite the widespread use of fasting or ketogenic diets to study the role of KB, both methods are associated with profound metabolic alterations (e.g. increased circulating free fatty acids (298)) that impede isolation of the metabolic effects specific to KB. Therefore, starting in the 1960s, studies started to utilize exogenously provided KB in the form of ketone body salts (KS) to be administered intravenously (10, 11, 267). Also oral ingestion of KS and ketone precursors (1,3-butanediol) has been explored but only resulted in a small increase of blood KB (ßHB: 0.6 – 1.0 mM) and in the presence of substantial gastrointestinal distress (282, 320, 329, 332, 353). Moreover, the high amount of salt (sodium, potassium or calcium) present in KS as well as its racemic composition (D-ßHB and L-ßHB) further limits its applicability (75, 282, 353).

The recent development of ketone esters may provide an alternative and safe approach to induce ketosis in the absence of high inorganic ion ingestion (51, 198, 344). These ketone esters include the R,S-1,3-butanediol acetoacetate diester (79, 232) and the (R)-3-hydroxybuyl (R)-3-hydroxybutyrate ketone monoester (51, 63). The ketone diester was synthetized by transesterification of t-butylacetoacetate and R,S-1,3,butanediol, and dissociates into two AcAc equivalents and one racemic ßHB equivalent upon oral ingestion (232, 355). However, ingestion of two 250 mg·kg^{-1} body weight (BW) doses of this ketone diester at 50 and 30 min

before a 31.2 km cycling time trial only marginally increased blood KB (ßHB: 0.2 mM, AcAc: 0.4 mM) while resulting in severe gastrointestinal distress (232).

In contrast, the (R)-3-hydroxybutyl (R)-3-hydroxybutyrate ketone monoester (KE) is synthetized by transesterification of ethyl (R)-3-hydroxybutyrate and (R)-1,3-butanediol (63). Ingested KE is cleaved into D-ß-hydroxybutyrate and (R)-1,3-butanediol by carboxylesterases and nonspecific esterases in the gut and small intestine, whereafter both metabolites enter the portal circulation **(Figure 4)** (51, 160, 343). Within the liver, (R)-1,3,butanediol is further metabolized to D-ßHB by dehydrogenases (79), resulting in a total delivery of two D-ßHB equivalents by KE. Both equivalents are subsequently transported to the circulation, followed by incorporation in extra-hepatic mitochondria to generate ATP (cfr. paragraph 1.2.1.2). Ingestion of 282 $mg \cdot kg^{-1}$ BW KE results in ketosis for up to 3 hours, with peak ßHB concentrations at ~3 mM observed between 30 – 60 min following ingestion (353). In addition, this KE is generally also well-tolerated (344, 354), making it an ideal candidate to investigate the unique role of KB, predominantly D-ßHB, in human metabolism.

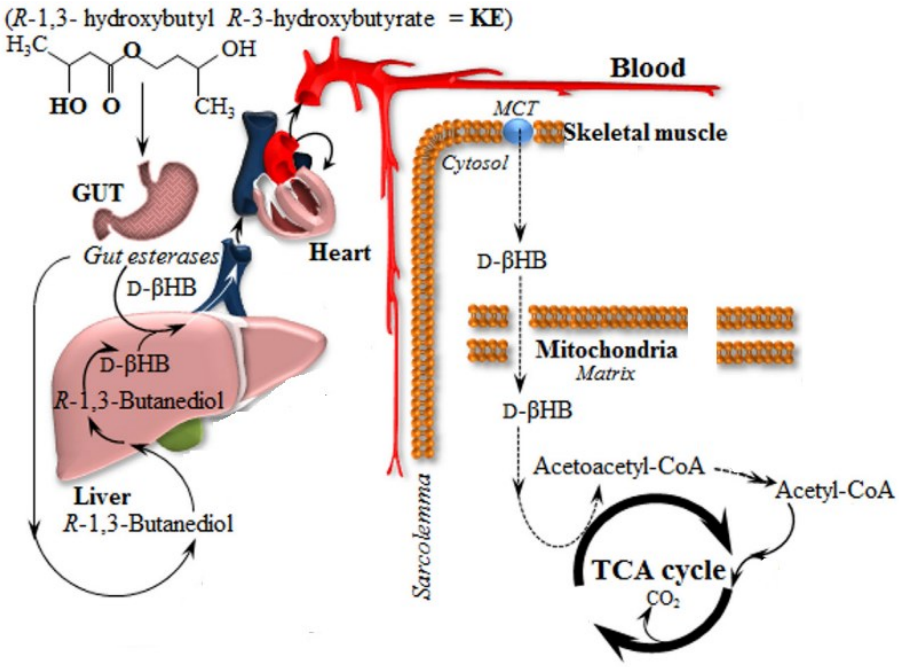


Figure 4. Metabolism of the ketone ester (R)-3-hydroxybutyl (R)-3-hydroxybutyrate.
Figure adapted from Cox *et al.,* 2016 (63), with permission.

3. Endurance exercise performance

3.1 Exercise metabolism

Maintaining contractile activity (i.e. actin-myosin crossbridge cycling) is essential to power exercise and is critically dependent on the delivery of ATP to the myosin, Na^+-K^+, and sarcoplasmatic reticulum ATPases (141). Consequently, the utilization of ATP increases more than 100-fold above resting values during exercise (~ 0.4 mmol ATP · kg^{-1} · s^{-1}) (116, 294) and poses an immense energetic challenge to the contracting myofibers. Given the limited availability of ATP in skeletal muscle (~5 mmol · kg^{-1} wet weight) (345), ATP must be immediately resynthesized to meet the high rate of ATP utilization during exercise **(Figure 5)**. During short-term (< 60 s) maximal exercise, ATP resynthesis is primarily effected through phosphorylation of ADP by adenylate kinase (myokinase), degradation of creatine phosphate and by the anaerobic breakdown of muscle glycogen-derived glycosyl units (116, 146, 294). In contrast, during endurance exercise oxidative phosphorylation of carbohydrates and lipids delivers the major fraction of ATP. The primary substrates for oxidative phosphorylation include free fatty acids resulting from the breakdown of peripheral adipose tissue and intramyocellular triglycerides, as well as blood glucose derived from muscle and liver glycogenolysis and gluconeogenesis (26, 141, 146). Although amino acids and KB can also be oxidized by skeletal muscle during exercise, they contribute only minimally to energy metabolism (~5%) under normal physiological conditions (26, 95, 370).

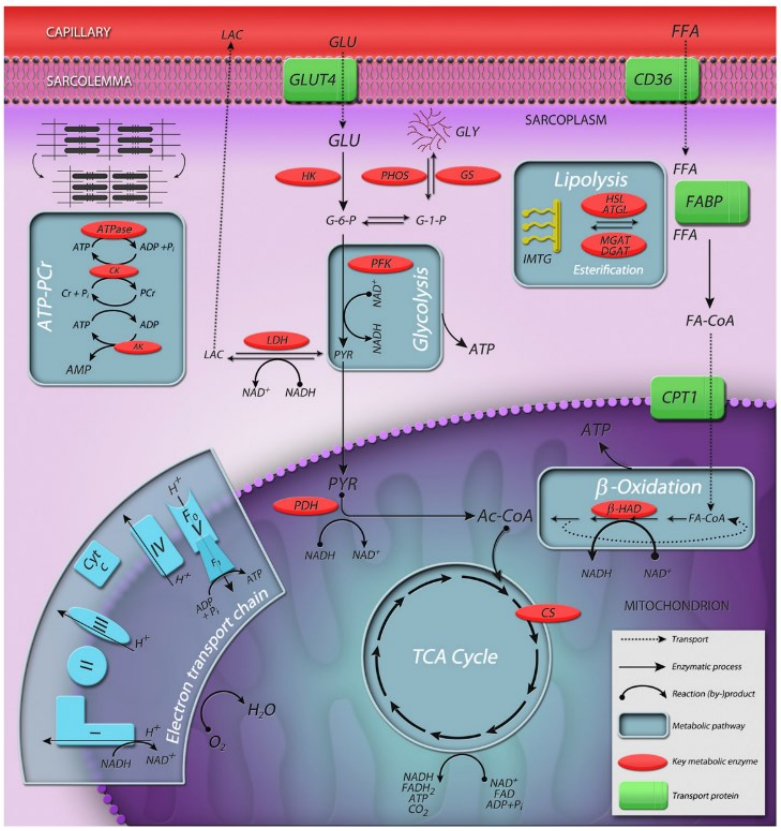


Figure 5. Metabolic pathways of ATP hydrolysis and regeneration in skeletal muscle.
Figure reproduced from Egan and Zierath, 2013 (88), with permission. ATP is hydrolized by myosin ATPase to power actin-myosin crossbridge cycling. ATP regeneration is accomplished by multiple pathways. (1) Degradation of creatine phosphate (PCr) by creatine kinase (CK) generates free creatine and inorganic phosphate (P_i), which reacts with ADP to form ATP. (2) Adenylate kinase (AK) converts two ADP molecules to form ATP and AMP. (3) Anaerobic glycolysis, where either muscle glycogen (GLY) or circulating blood glucose (GLU) is catalyzed by respectively glycogen phosphorylase (PHOS) and hexokinase (HK) to glucose-6-phosphate (G-6-P). G-6-P is then degraded to pyruvate (PYR) and ultimately lactate (LAC) by lactate dehydrogenase (LDH), and generates ATP by substrate phosphorylation. (4) Aerobic respiration, acetyl-CoA (Ac-CoA) produced from glucose (aerobic glycolysis) or lipids (ß-oxidation) enters the mitochondrial tricarboxylic acid (TCA) cycle and produces among others NADH and $FADH_2$, which are subsequently oxidized by the electron transport chain (ETC) to synthetize ATP.

3.2 Substrate selection during exercise

3.2.1 Effect of exercise intensity and duration

The relative contribution of the various metabolic pathways to generate ATP is determined by the intensity **(Figure 6)** and duration **(Figure 7)** of exercise (323, 372). At low intensities (< 25% $\dot{V}O_2$ max), almost the entire energy turnover is covered by the oxidation of free fatty acids liberated from peripheral adipose tissue via lipolysis. In addition, a minor extent is delivered by blood glucose derived from liver glycogen breakdown and gluconeogenesis. As exercise

intensity increases (~ 65% $\dot{V}O_2$ max), the absolute contribution of blood free fatty acids and blood glucose remains similar, while the additional energy required is evenly derived from the breakdown of muscle glycogen and intramyocellular triglycerides. The additional ATP required for exercise at even higher intensities is almost completely provided by muscle glycogen degradation, making it the predominant fuel during moderate to intense exercise (140, 167). Performing exercise either at a fixed intensity or at fluctuating intensities resulting in an identical average exercise load, does not significantly alter substrate metabolism (289).

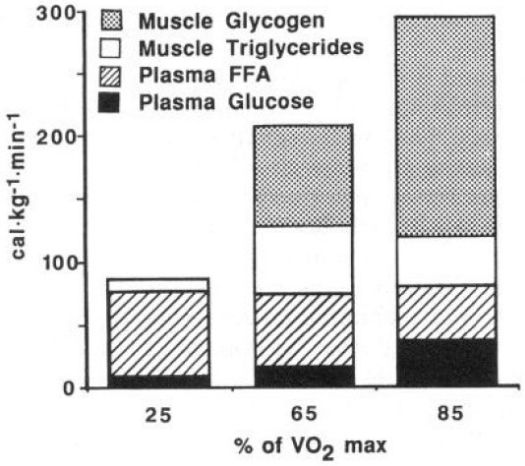


Figure 6. Effect of exercise intensity on energy substrate use.
Contribution of primary energy substrates after 30 min of exercise at 25, 65 and 85% of $\dot{V}O_2$ max. Adapted from Romijn *et al.,* 1993 (323), with permission.

During exercise at low intensities (~25% $\dot{V}O_2$ max), the metabolic profile remains constant with increasing durations. However, at higher intensities (~65% $\dot{V}O_2$ max), the proportion of energy derived from muscle glycogenolysis decreases as a function of time and is replaced by a progressive increase of blood free fatty acid oxidation, and a small increase in the contribution of blood glucose (323).

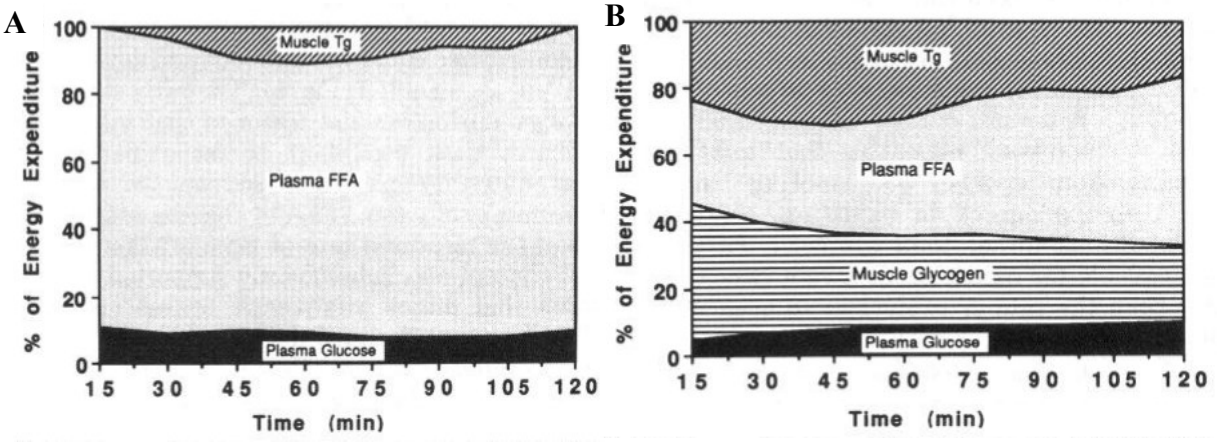


Figure 7. Effect of exercise duration on energy substrate use.
Contribution of primary energy substrates during 2 h cycling at 25% (panel A) and 65% (panel B) of $\dot{V}O_2$ max. Figure reproduced from Romijn *et al.,* 1993 (323), with permission.

3.2.2 Effect of metabolic factors

3.2.2.1 Regulation of carbohydrate metabolism

Glucose metabolism during exercise is regulated at three levels: (i) delivery to the working muscle, (ii) transport across the muscle membrane and (iii) intracellular oxidation through glycolysis or glucose oxidation **(Figure 8)** (183, 359). Glucose supply to skeletal muscle primarily occurs by a marked increase in blood flow (~0.4 $L \cdot min^{-1}$ at rest (316) *versus* up to 10 $L \cdot min^{-1}$ during exercise (45)), proportional to exercise intensity. Although the precise mechanisms that couple the exercise-induced increases in metabolism and muscle perfusion has to be determined, suggested candidates include adenosine, ADP, ATP, and nitric oxide (154, 195). Besides enhanced blood flow, glucose delivery to skeletal muscle can also be increased by elevating blood glucose concentrations through carbohydrate ingestion (145, 249).

Glucose transport across the plasma membrane during exercise depends on the insulin-independent translocation of GLUT4 to the cell surface (184, 408), and might additionally be regulated by alterations in GLUT4 intrinsic activity (206). The signaling pathways regulating GLUT4 translocation have been studied extensively, but the precise controlling mechanisms remain to be fully elucidated. Traditionally, the increased glucose uptake by skeletal muscle during exercise has been attributed to increased activity of skeletal muscle AMP-activated protein kinase (AMPK) based on in-vivo studies using AICAR, a pharmacological activator of AMPK (265). However, there is only limited evidence that AMPK regulates contraction-induced glucose uptake (25, 248, 250). In addition, previous studies reported that Ca^{2+} release from the ryanodine receptors in the sarcoplasmic reticulum stimulates contraction-induced glucose transport ex-vivo (325, 400). However, this seems to be insufficient to enhance glucose passage in vivo (184). Mechanical stress may also be a potent stimulus to enhance the glucose uptake by skeletal muscle through stimulation of stretch-activated pathways including Rac1 (358, 360), mitogen-activated protein kinase (MAPK) (48), and sucrose nonfermenting AMPK-related kinase (SNARK) (209). Recent studies also suggested other potential regulators of glucose uptake including nitric oxide (251, 315) and reactive oxygen species (156, 315),

however their in-vivo contributions to contraction-stimulated glucose uptake remains to be determined.

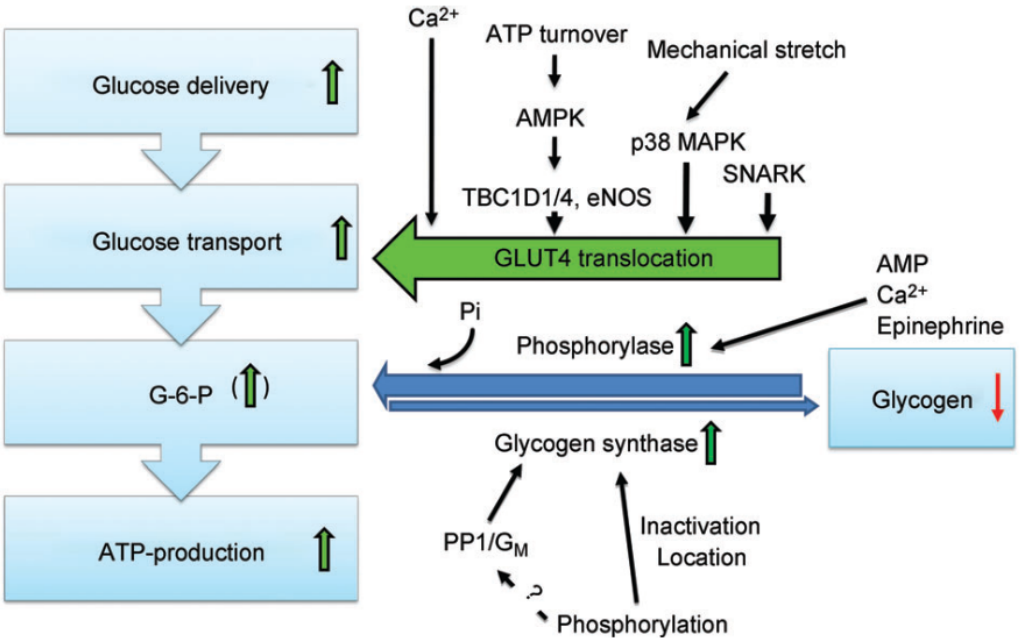


Figure 8. Mechanisms by which exercise increases glucose oxidation in skeletal muscle.
Figure adapted from Jensen & Richter, 2012 (183), with permission.

During exercise, skeletal muscle preferably utilizes muscle glycogen as an energy substrate, resulting in an inverse relation between myocellular glucose uptake from the blood and muscle glycogen content (321, 397). Therefore, high muscle glycogen concentrations stimulate muscle glycogenolysis via glycogen-induced activation of glycogen phosphorylase (159), while it inhibits glucose phosphorylation by increasing the amount of glucose 6-phosphate (G-6-P) and subsequent inhibition of hexokinase (359). Moreover, glycogen may also inhibit blood glucose oxidation by counteracting GLUT4 translocation (78, 158). The exercise-induced degradation of muscle glycogen will alleviate the glycogen-induced downregulation of hexokinase, causing a progressive increase in glucose oxidation during continued exercise (323).

3.2.2.2 Regulation of lipid metabolism

Our understanding of the regulation of exercise-induced lipid metabolism lags far behind that of carbohydrate metabolism. However, the myocellular oxidation of lipids also depends on free fatty acids delivery, transport and oxidation. Fatty acids (FA) are mainly delivered from the lipolysis of triacylglycerol (TG) liberated from adipose tissue and from the breakdown of intramyocellular triacylglycerol (IMTG). Lipolysis is regulated by the rate-limiting enzyme

hormone sensitive lipase (HSL), the activity of which progressively increases during prolonged exercise through an increase in plasma adrenalin versus a decrease in insulin (224, 386). A small amount of FA can diffuse freely across the lipid bilayer of the muscle membrane. However, the majority of FA enter the muscle cell by carrier proteins of which the fatty acid translocase FAT/CD36 has been the most investigated (122, 185, 193). The delivery of FA by breakdown of IMTG is under control of HSL, adipose triglyceride lipase, and monoglyceride lipase (185, 388), and seems to increase/decrease in response to low/high plasma free fatty acid availability (200, 387). The precise mechanisms by which exercise regulates IMTG breakdown remain to be fully elucidated. However, it is clear today that neither IMTG breakdown nor adipose tissue lipolysis nor membrane fatty acid transport is responsible for the inability of lipid metabolism to provide the required energy during high-intensity exercise (201).

Following entrance of long chain FA in skeletal muscle, they react with CoA in the cytosol to generate long chain fatty acyl-CoA. In the next step, long chain fatty acyl-CoA and carnitine are converted to fatty acid-acylcarnitine by carnitine palmitoyltransferase 1 (CPT1) in the outer mitochondrial membrane. Subsequently, CPT2 located at the inner mitochondrial membrane removes the carnitine and adds coenzyme A to produce fatty acid acyl-CoA for the ß-oxidation (185).

3.2.2.3 Integration of carbohydrate and lipid metabolism

The concept of a reciprocal relationship between the oxidation of carbohydrates and lipids was introduced in the 1960s by Randle *et al.* in their “glucose-fatty acid cycle“ (312–314). These studies indicated that higher lipid availability, by means of plasma FAs, increases fat oxidation and downregulates carbohydrate oxidation in skeletal muscle by changes in the intracellular environment (e.g. increased acetyl-CoA, citrate and G-6-P). However, as these experiments were performed in rodents, the energy metabolism of which depends much less on the oxidation of intramyocellular substrates (e.g. IMTG and muscle glycogen) compared to humans, subsequent studies investigated whether the glucose-fatty acid cycle would also be applicable in human skeletal muscle during exercise (346). Recent studies confirmed that increasing the availability of FA to exercising muscle in humans indeed increases the reliance on lipids (83, 84, 153, 324), while increasing carbohydrate delivery before and during exercise enhances carbohydrate oxidation and decreases fat oxidation (66, 67, 171, 334).

Although the principle of the glucose-fatty acid cycle remained intact, recent studies identified novel mechanisms controlling the balance between the utilization of carbohydrates *versus* lipids during exercise **(Figure 9)** (174). In contrast to the original concept, it is now recognized that increased availability of plasma FA does not alter acetyl-CoA or G-6-P. However, increasing the amount of FA during exercise at 40 – 80% of $\dot{V}O_2$ max, reduces the exercise-induced accumulation of ADP, AMP and inorganic phosphate (P_i), thereby decreasing the activity of pyruvate dehydrogenase and glycogen phosphorylase resulting in an inhibition of muscle glycogenolysis (84, 324). At lower intensities (40 – 65% of $\dot{V}O_2$ max), elevated FA levels also induce an increase in the production of citrate and NADH, while lowering the activity of pyruvate dehydrogenase (283). In contrast to the detailed understanding of the regulatory sites by which alterations in FA effect carbohydrate metabolism, the mechanism by which changes in carbohydrate availability affect lipid oxidation remain to be determined.

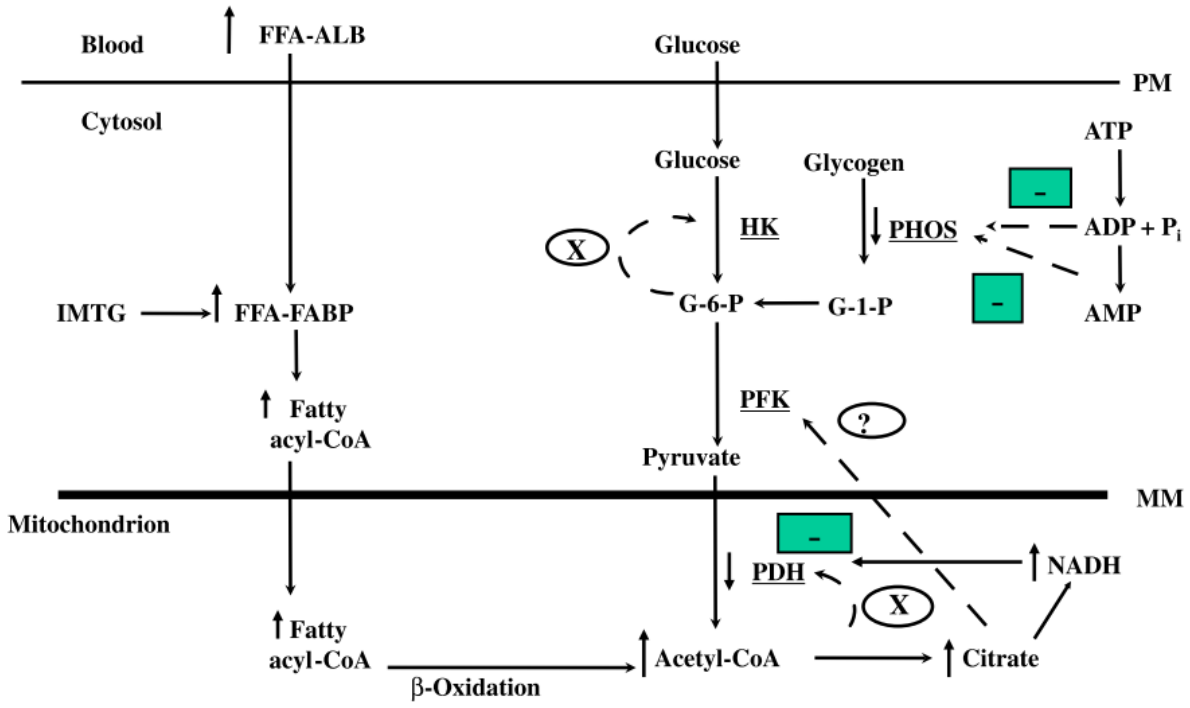


Figure 9. Mechanisms by which increased free fatty acid availability downregulates carbohydrate metabolism.
X, no effect; dashes, reduced; question mark, unclear. Figure adapted from Spriet, 2014 (346). With permission.

3.2.3 Effect of nutrition

Given the ability to modulate energy metabolism by altering substrate availability, numerous studies have explored the potential of changing substrate availability by dietary

manipulation to enhance endurance exercise performance. The association between muscle glycogen depletion and the development of fatigue during endurance exercise (157, 297), resulted in the development of dietary interventions aimed to increase muscle glycogen content. Therefore, a high carbohydrate (60-65% of energy intake), moderate protein (15-20%) and low fat (20%) diet evolved as the classical recommendation to optimize endurance performance (64).

Another perspective to optimize exercise performance is to spare muscle glycogen by enhancing the contribution of fat by adopting a high fat, low carbohydrate diet (75-80% fat, 15-20% protein, < 50 g·day^{-1} carbohydrates) (33, 303, 382). This approach indeed results in an upregulation of fat utilization and oxidation (29, 30, 125, 144, 223, 308) and maintains submaximal (< 60% $\dot{V}O_2$ max) exercise capacity and efficiency (331). However, this approach reduces exercise economy and performance at higher intensities that are relevant to real-life endurance competitions (33, 331).

Despite these negative implications, there has been a resurgence of interest in the utilization of a high fat diet with extreme low carbohydrate intake (< 20 g·day^{-1}), termed 'ketogenic diet' (292, 305), to stimulate endurance performance. The intended benefit of such diet is twofold: (i) increasing the contribution of fat as an energy substrate, and (ii) to expose skeletal muscle to high levels of circulating KB to benefit from the metabolic advantages of ketosis (cfr. paragraph 1.2) (281, 380, 381). Although a ketogenic diet also increases maximal fat oxidation rates more than twofold (~1.5 *versus* ~0.7 g·min^{-1}) (380), there is no evidence to suggest that such an approach stimulates endurance performance (33, 152). In contrast, the low carbohydrate intake required by the ketogenic diet even decreases high-intensity exercise performance by downregulating glycolysis via inhibition of pyruvate dehydrogenase (28).

Therefore, current nutritional guidelines for optimal endurance performance include the utilization of a high-carbohydrate diet in preparation of an endurance competition (up to 10-12 g carbohydrates·kg^{-1}·day^{-1}), as well as high carbohydrate intake during exercise (60 – 90 g·h^{-1}) (31). This dietary practice is often combined with other nutritional training strategies including exercising in the fasted state (373), performing multiple exercise bouts per day with no carbohydrate intake in between sessions (119), and carbohydrate periodization (33).

3.3 Ketone ester intake to increase endurance exercise performance

3.3.1 Rationale

The concept that increasing circulating ketone levels may evoke an ergogenic effect in endurance exercise is based on the ability of KB to provide an alternative and energetically efficient oxidative fuel source for skeletal muscle and the brain during starvation (38, 42, 287, 305). By providing an alternative energy supply, elevated blood KB may reduce glucose oxidation and potentially delay muscle glycogen depletion and concomitant fatigue during exercise. In addition, the thermodynamic advantages of KB compared to carbohydrates and lipids (225, 226) may increase the metabolic efficiency during exercise. Furthermore, as KB are the dominant energy substrate for the brain under ketotic conditions (287), and are associated with increased cognitive function in some patient groups (epilepsy, Alzheimer's and Parkinson's disease) (375, 376, 378, 379), it is believed that exogenous ketosis may delay central fatigue as well as improve decision making during prolonged exercise (381).

Despite these beneficial pleiotropic effects of KB, this potential has not been shown to translate into increased endurance exercise performance mainly due to the limited carbohydrate intake required by conventional nutritional approaches to induce endogenous ketosis (e.g. fasting or ketogenic diet). However, exogenous ketosis produced by oral ingestion of KE substantially increases blood KB even in the presence of high carbohydrate intake (63), and as such might unlock the ergogenic effect of KB during endurance exercise.

3.3.2 State of the art

The effect of KE on exercise performance and energy metabolism was only recently explored for the first time in a series of studies by Cox and his co-workers (63). In a first experiment it was demonstrated that following the ingestion of 573 $mg \cdot kg^{-1}$ BW KE, blood ßHB concentrations were lower after 45 min cycling at moderate intensity (40% Wmax) compared to 45 min of rest (rest: ~6 mM *versus* ~4 mM during exercise). Even higher exercise intensity (75% Wmax) further decreased blood [ßHB] to approximately 3 mM. This intensity-dependent decrease of [ßHB] during exercise, turned out to result from elevated ßHB oxidation during exercise and thereby significant contribution (16-18%) of ßHB to total oxygen consumption **(Figure 10)**.

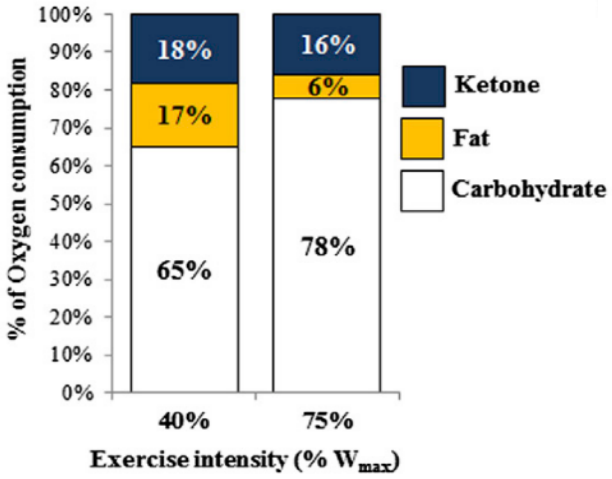


Figure 10. Contribution of ßHB oxidation to total oxygen consumption during 45 min cycling at 40% and 75% of Wmax following ingestion of 573 mg·kg^{-1} BW KE.
Figure reproduced from Cox *et al.*, 2016 (63). With permission.

In a subsequent experiment, the authors identified that co-ingestion of ~40 g KE and ~70 g carbohydrates, compared to carbohydrate (~120 g) alone, slightly improved mean power output (~2%) in a 30 min simulated time-trial following a 1 h constant-load submaximal cycling bout. The ergogenic effect was attributed to a decrease in net muscle glycogen breakdown due to direct inhibition of glycolytic flux together with promotion of IMTG oxidation and depressed adipolysis **(Figure 11)** (63). However, from the perspective of performance enhancement, this experiment was performed under inadequate nutritional conditions as it was performed in the fasted state and with lower rate of carbohydrate intake in the KE than in the control condition, which conceivably shifts substrate oxidation towards increased fat metabolism (71, 187, 373). Therefore, it is unclear whether the metabolic effects of KE observed by Cox *et al.*, were directly related to KE *per se*, or were rather caused by different exogenous carbohydrate availability between the experimental conditions.

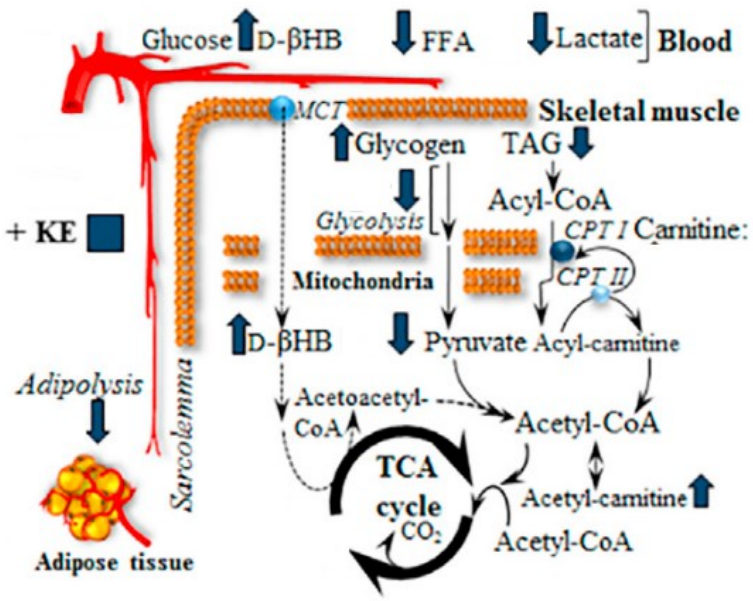


Figure 11. Effect of additional ingestion of KE on substrate metabolism during endurance exercise.
Blue arrows indicate the alterations in major energy metabolism pathways during exercise following ingestion of ~40 g KE and ~70 g carbohydrates compared to carbohydrates (~120 g) alone. Figure reproduced from Cox *et al.*, 2016 (63). With permission.

A follow-up study using high-carbohydrate intake pre-exercise and isocaloric carbohydrate ingestion during exercise, observed no effect of KE on a 10 km run preceded by 1 h of submaximal exercise (97). Although this study did not assess substrate metabolism, the authors speculated that reduced glycolytic flux by KE may impair performance in high-intensity efforts demanding high-rate glycol(geno)lytic ATP production (96, 97, 147).

Given the requirement of high-rate glycolytic ATP production in search of success in the final stage of endurance competitions, it is reasonable to postulate that KE supplementation should rather be used during the initial phase of an endurance event to spare muscle glycogen, and thereby possibly preserve the capacity for high glycolytic flux in the final. However, to date, no studies have investigated whether such approach can increase endurance exercise performance, indeed.

3.3.3 Potential drawbacks

3.3.3.1 Appetite suppression

Although KE has the clear potential to increase endurance exercise performance, the appetite suppressing effect of KB may hinder optimal carbohydrate intake during exercise and as such may limit performance. A recent study in humans indeed indicated that ingestion of ~29 g KE at rest, suppressed circulating ghrelin levels and also the perception of hunger and desire to eat compared to isocaloric glucose ingestion (352). However, it remains to be determined whether this appetite suppressing effect of KE is also present during exercise.

3.3.3.2 Acidosis

In addition to a potential appetite suppressing effect, the acid load of ßHB and AcAc may also negatively impact performance by dysregulating blood acid-base balance. Ingestion of ~20 and ~23 g KE, respectively at rest (353) and during submaximal exercise (76) decreased blood pH from 7.42 to 7.31 and 7.37, respectively, for up to 1 h. Although it has been argued that acidosis has little influence on the development of muscular fatigue (389, 390), others indicated that increased [H^+] inhibits excitation-contraction coupling and reduces glycolytic and glycogenolytic rate, which in turn blunts rate of ATP production (77, 166, 348). In addition, the associated alterations in intra- and extracellular distributions of other ions (e.g. Ca^{2+}, K^+, Cl^-) might also negatively impact contractile function (44, 336). Therefore, it is clear that extracellular acidosis, even at moderately decreased pH values (318), as observed following KE ingestion, could negatively impact exercise performance (47).

The primary defense mechanism against perturbations in extracellular pH and electrolyte gradients between the intra- and extracellular environment is provided by the bicarbonate buffering system. Excess H^+ production during exercise stimulates chemoreceptors to increase the ventilatory drive and causes binding of H^+ with bicarbonate (HCO_3^-) to generate carbonic acid (H_2CO_3). This subsequently stimulates the enzyme carbonic anhydrase to force the dissociation of H_2CO_3 into H_2O and CO_2. The increased respiratory drive subsequently promotes CO_2 exhalation to repair normal blood pH. As the capacity of this extracellular buffering system is dependent on the availability of HCO_3^-, the so-called ‘alkaline reserve’, supplementation of oral sodium bicarbonate ($NaHCO_3$) to increase the extracellular pool of HCO_3^- has evolved as an attractive strategy to counteract exercise-induced decreases in blood pH. A recent meta-analysis indicated that ingestion of 300 mg·kg^{-1} BW $NaHCO_3$ increases 1-

min sprint performance in male athletes on average by 1.7% (47). In addition, experiments in our laboratory recently also showed that a similar dose of $NaHCO_3$ improved mean power output in a 90 s all-out exercise bout at the end of a 3 h simulated cycling race (Dalle *et al.,* in revision). The ergogenic effect of the additional buffering capacity generated by oral $NaHCO_3$ ingestion has been attributed to increases in H^+ and La^- efflux from skeletal muscle (47, 68, 257, 347). Hence, additional intake of $NaHCO_3$ to counteract KE-induced acidosis may facilitate an ergogenic effect of KE in endurance exercise events.

4. Overload training

4.1 Classification

In order to increase endurance exercise capacity and performance, athletes intermittently participate in overload training or competition (e.g. training camps and multi-stage cycling races). As a result, the athlete may experience acute fatigue (AF) resulting in short-term decrements in performance. But, if this increased training load is compensated for by adequate rest and recovery, it generally results in beneficial training adaptations and improved performance within days. In contrast, increased training stress that is not timely interspersed by appropriate recovery results in an abnormal training response, known as overtraining, which has been estimated to occur at least once in the career of 30 to 60% of all elite athletes (214, 216, 246, 274, 311). Besides increased training load being the primary factor responsible for the development of overtraining, other stress factors such as sleep deprivation, competition, training monotony, negative energy balance or mental fatigue may further promote this maladaptive state by increasing the internal load (*i.e.* physiological and psychological stress) for a given external training load (*i.e.* intensity and duration) (107, 134, 217, 258).

The possible outcomes of overtraining include a continuum from functional overreaching (FOR), over non-functional overreaching (NFOR) to the overtraining syndrome (OTS), depending on the time course of the decrement in performance and the subsequent restoration phase **(Figure 12)** (16, 258). FOR is characterized by a short-term decrement in performance, in which restoration or supercompensation may occur following a short recovery period (days to weeks) (9, 60, 137, 217, 230, 259). As such, FOR is often considered as an essential component of efficient training (138) and has even been suggested to elicit greater performance benefits compared to consistent training below the FOR threshold (61, 230, 366). However,

FOR is associated with an increased susceptibility to illness and sleep disturbance (143), and precedes the more severe state of NFOR which results in a stagnation or decrease in performance for several weeks up to months and may ultimately end up in the development of OTS requiring months to years to recover (258). Therefore, prevention of non-functional overreaching is pivotal in training management.

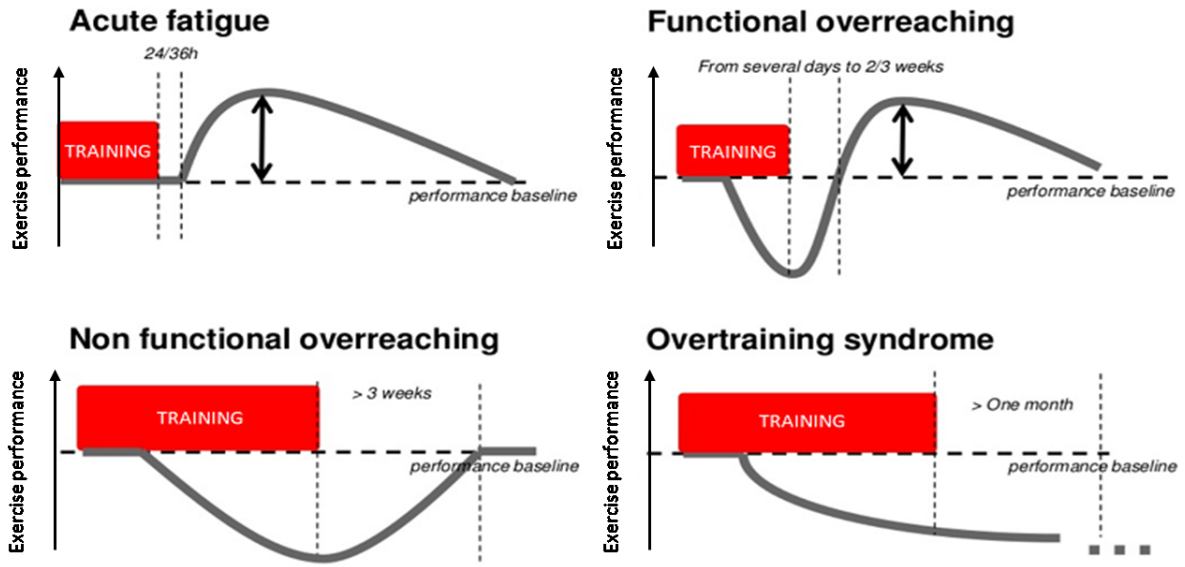


Figure 12. Schematic representation of the different stages of training and overtraining.
Gray line indicates alterations in performance in response to exercise training (above baseline indicates performance gain, below baseline indicates performance deterioration). Adapted from Yann Le Meur https://www.slideshare.net/YannLEMEUR/highintensity-training-ylmsportscience-2015

4.2 Symptoms

The primary indication of the development of overreaching is a stagnation or decrease in training-specific performance (169, 371), but this is often preceded by numerous other symptoms including elevated rate of perceived exertion for a given workload, disturbed mood-state and reduced reproductive drive (13, 132, 215, 217). Other symptoms are believed to depend on the effect of overload training on the autonomic nervous system (113, 235). From this perspective, two distinct overtraining types were defined: *i.e.* sympathetic *versus* parasympathetic overtraining. Sympathetic overtraining is characterized by elevated basal sympathetic tone and induces a clinical pattern similar to adrenal insufficiency (Addison's disease) including increased resting heart rate and blood pressure, insomnia and restlessness. Conversely, in the parasympathetic form the sympathetic drive is abnormally decreased resulting in a similar clinical pattern as thyroid hyperfunction (Basedow's disease) characterized by bradycardia, hypotension and motivation loss (179, 216, 217).

The development of one or the other type of overtraining was believed to depend on the type of training with the sympathetic form associated with high-intensity anaerobic exercise, *versus* the latter with endurance sports (219). More recent research, however, suggests that both forms rather exist as a continuum (111, 112). The sympathetic overtraining form develops as an initial stress response attempting to maintain physiological function, and is followed by a gradual predomination of parasympathetic symptoms due to fatigue of the sympathetic neuroendocrine system. Despite this suggestion, previous studies failed to identify consistent physiological dysregulations (35, 371) and as such there are currently no conclusive diagnostic criteria available for the early detection of overreaching.

4.3 Pathophysiology

In order to understand the physiological and psychological symptoms induced by overload training, numerous studies aimed to identify the underlying mechanisms of overtraining resulting in various hypothetical models.

4.3.1 Glycogen hypothesis

Exercise training on consecutive days results in low muscle glycogen content which is associated with increased fatigue and performance decrements (58, 59, 196). In addition, low muscle glycogen concentration mitigates immunological disturbances (57, 139), as well as increases the oxidation of branched chain amino acids for gluconeogenesis, which eventually may lead to elevated central fatigue (280). Although this association seems plausible, other studies indicated that subjects with normal muscle glycogen levels may still become overreached. This indicates that glycogen depletion *per se* is not a prerequisite for the development of overtraining (139, 341).

4.3.2 Central fatigue hypothesis

The development of overtraining involves an apparent loss of neural drive characterized by disrupted mood, sleep, and behavior. As the neurotransmitter serotonin (5-HT) is involved in the regulation of these functions (263) and as exercise has been shown to elevate 5-HT concentrations in the brain (126, 307, 392), it has been suggested that 5-HT is responsible for the development of central fatigue during overload training (3, 8, 27, 279). In favor of this hypothesis, increasing serotonergic activity in the brain by administering the serotonin re-uptake inhibitor paroxetine, has been shown to decrease performance (351). In addition, another

study reported that the sensitivity of 5-HT receptors was remarkably increased in overreached athletes compared to well-trained matched controls (27). However, other studies failed to reproduce this effect (261, 264, 291), suggesting that increased 5-HT levels in the brain can not explain the development of central fatigue in response to overtraining.

4.3.3 Hypothalamic hypothesis

The hypothalamic-pituitary-adrenal (HPA) axis is, together with the autonomic nervous system, the most important stress management system of the body. Upon stress, the hypothalamus secretes corticotropin-releasing hormone, which stimulates the pituitary gland to release adrenocorticotropic hormone (ACTH). In turn, ACTH stimulates the adrenal cortex to produce glucocorticoid hormones (e.g. cortisol) in order to restore physiological homeostasis. Overload training has been shown to dysregulate the HPA axis at the hypothalamic and pituitary level resulting in a blunted ACTH and cortisol response to exercise (13, 34, 36, 108). The decreased cortisol production subsequently alters many processes that may be implicated in the development of overtraining, including 5-HT production, protein breakdown and gluconeogenesis (217). However, alterations in the HPA axis demonstrate a high variability between individuals, and also depends on other hormones indicating that it is insufficient to explain all the symptoms of overtraining (36, 138, 371).

4.3.4 Integrated hypothesis

As no single hypothesis is able to explain all aspects of overtraining, it is the prevailing opinion today that overtraining results from an imbalance between (non)training-related stress and recovery causing a maladaptation at various levels **(Figure 13)** (37, 216, 235, 301). The mediating factors include, but are not limited to, changes in substrate metabolism, autonomic nervous system activity, oxidative stress, inflammation and neural drive. However, the precise pathophysiology of overtraining still remains poorly understood.

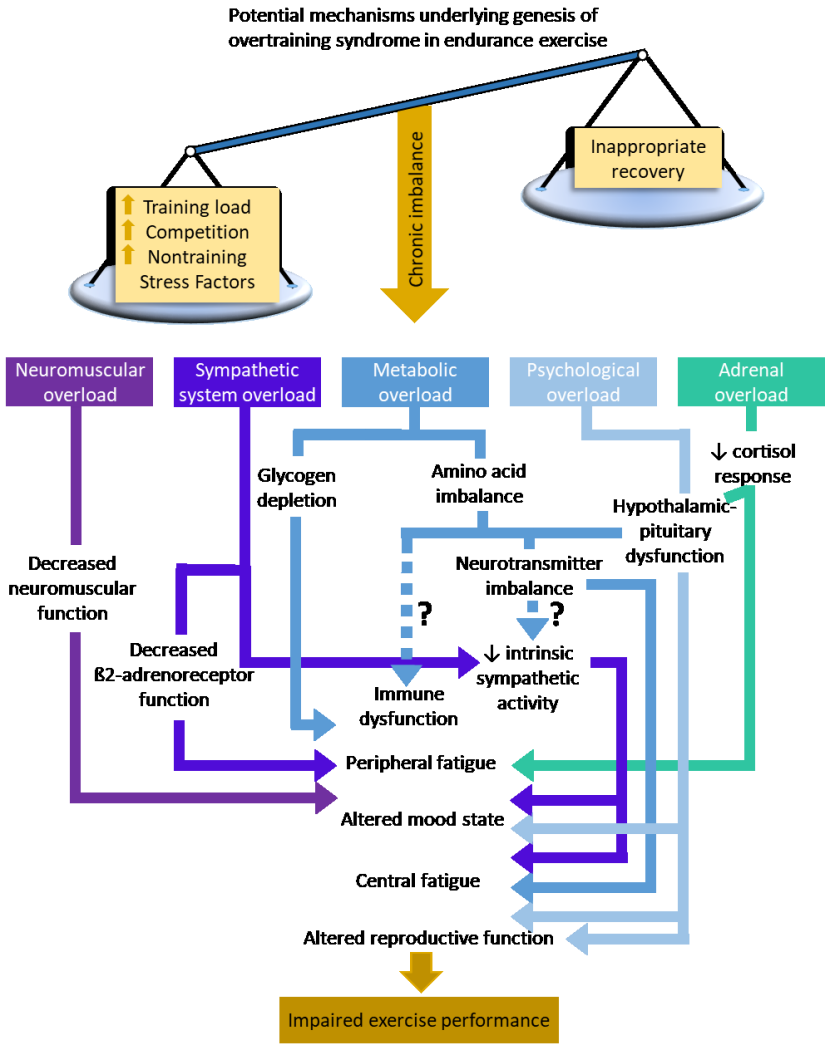


Figure 13. Integrated hypothesis of the development of overtraining.
Adapted from Lehmann et al., 1998 (235), with permission.

4.4 Strategies to counteract overtraining

4.4.1 Sleep and rest

The most effective strategy to counteract the development of overtraining is adequate rest together with a reduction in training and/or non-training related stress. In addition, it is clear that sleep quantity and quality are impaired during overload training (143, 202, 258). Regardless if impaired sleep predisposes athletes to the development of overreaching or occurs as a consequence of overtraining (16), improving sleep quality has been proposed to be one of the primary recovery strategies available to counteract the development of overtraining (135, 338).

4.4.2 Nutrition

Because reduced muscle glycogen content may also be implicated in the development of overtraining, it is tempting to suggest that increasing carbohydrate intake, especially immediately post-exercise, may contribute to prevent overtraining (340). Various studies investigating this potential have indicated that increased carbohydrate intake alleviates some overreaching symptoms and attenuates performance decrements (2, 57, 139), indeed. However, other studies observed no effect at all (341, 357).

Besides the importance of carbohydrate intake to restore muscle glycogen levels immediately post-exercise, additional protein intake may even further increase recovery by stimulating muscle repair (21). Witard et al. (395, 396) observed that ingestion of a high protein diet (3 $g \cdot kg^{-1} \cdot day^{-1}$), compared to an energy and carbohydrate matched control diet (1.5 g protein$\cdot kg^{-1} \cdot day^{-1}$) during intensified training, attenuated the decrease in performance, while it completely inhibited overload-induced disturbances in leukocyte kinetics and upper respiratory tract symptoms. These data indicate that high-carbohydrate, high-protein intake has the clear potential to reduce the development of overtraining symptoms, but is still insufficient to completely prevent overtraining.

In addition to the significance of sufficient protein and carbohydrate intake, two recent studies highlighted the importance of increasing total energy intake during intensified training (398, 399). These studies observed that rowers who were unable to match their energy intake with the increased energetic expenditure during overload training (398), as well as cyclists who did not maintain fat-free mass during overload training (399), demonstrated a clear reduction in resting metabolic rate. Therefore, increasing energy intake primarily by alterations in protein and carbohydrate intake may at least partly attenuate the development of overtraining.

4.4.3 Other interventions

Other interventions such as cold water immersion (CWI) or antioxidant intake have also been suggested to counteract the symptoms of overreaching (124, 136, 262). CWI is considered to attenuate, and speed the recovery of, excessive exercise-induced decrements in performance (24). Although the precise mechanisms by which CWI affects post-exercise recovery are not entirely clear, they are most probably related to accelerated removal of body heat, reduced tissue temperature and hydrostatic pressure effects (369, 391). This in turn may reduce oedema and inflammation, as well as the development of delayed onset muscle soreness (81, 233). To

investigate the potential of CWI to counteract the development of overreaching/overtraining, Halson et al. (136) randomized cyclists to either CWI (4 times per week for 15 min) or passive recovery during 3 weeks of overload training. The obtained results indicated that CWI was successful to counteract the performance decrements observed in the passive recovery group and as such may impede the development of overtraining. However, it should be noted that these effects may be caused by the placebo effect of CWI (23) and that CWI may even inhibit beneficial training adaptations (4, 24, 161, 404). Antioxidant intake on the other hand, has also been suggested to counteract the development of overtraining as increased oxidative stress has been observed in overtrained athletes (362). In a study by Peters et al. (299), daily supplementation of 600 mg vitamin C reduced the incidence of upper-respiratory-tract infections following an ultramarathon race compared to a placebo supplement. However, there are no other data available to indicate that antioxidant intake may attenuate symptoms of overtraining, and similarly to the use of CWI, long-term antioxidant intake may hamper beneficial training adaptations (266).

4.5 Ketone ester intake to counteract overtraining

Some recent publications stimulated interest in the use of KE to stimulate post-exercise recovery (165, 374), and as such KE may counteract the pathophysiological effects leading up to the development of overtraining. Although there are no data available on KE during (intensified) training, KE may counteract the development of overtraining via various mechanisms.

By decreasing muscle glycogen breakdown during exercise (63), KE may counteract low muscle glycogen content and its purported negative impact on central fatigue (280) and immunological disturbances (57, 139). Moreover, a recent study also indicated that KE ingestion before a 10 mM glucose clamp stimulated post-exercise glycogen resynthesis by 50% (165). However, in a study by Vandoorne *et al.*, KE did not affect post-exercise glycogen resynthesis following ingestion of a high-protein, high-carbohydrate recovery drink (374).

Optimal post-exercise recovery does not only involve increased glycogen resynthesis, but also requires a stimulation of muscle protein synthesis (19, 49). KE may stimulate net muscle anabolism via different mechanisms. First, it has been shown by Vandoorne *et al.,* (374) that KE post-exercise stimulates the protein synthesis markers S6K1 and 4E-BP. In addition, it has also been shown in mice that AcAc stimulates muscle regeneration by satellite cell activation and proliferation (409). Moreover, KB do not only stimulate muscle protein synthesis, but are

also able to inhibit muscle protein breakdown as infusion of ketone salts (sodium-ßHB) decreased phenylalanine release in the forearm in response to lipopolysaccharide-induced inflammation (368).

KE intake may not only counteract the development of overtraining by stimulating post-exercise recovery, but has also been shown to directly regulate a range of putative factors implicated in the development of overtraining. It is the prevailing opinion that the initial increase in sympathetic activity during overload training may cause exhaustion of the sympathetic neuroendocrine system and an ensuing predomination of parasympathetic output. However, ßHB has been shown to directly suppress the activity of GPR41, located in sympathetic ganglions, resulting in a depressed sympathetic tone (203). As such, KE might delay exhaustion of the sympathetic system.

Numerous studies suggested that overload training also results in excessive oxidative stress and an exacerbated inflammatory response (216, 245, 362) causing increased fatigue. KE may counteract this maladaptive response by decreasing oxidative stress and inflammation through the ability of ßHB to inhibit HDACs (333), and the activity of the NLRP3 inflammasome (405), respectively.

Besides the pivotal role of physiological alterations in the development of overtraining, a recent study indicated that a 3-week overload training period increases muscular oxidative capacity in subjects not developing overreaching, but not in the overreached subjects (17). This suggests that the timely development of adequate muscular responses to overload training may be crucial to avoid the occurrence of overtraining. Increased muscular oxidative capacity in response to training is likely initiated by enhanced skeletal muscle vascularization (i.e. angiogenesis) (155, 173). Interestingly, previous studies indicated that ßHB enhances lymphangiogenesis in mice as well as stimulates the generation of vascular endothelial growth factor (VEGF) in the aorta of diabetic rats and in mouse brain microvascular endothelial cells (117, 178, 401). Therefore, it is tempting to suggest that KE intake may alleviate the development of overtraining by stimulating muscular oxidative capacity.

Given the numerous direct and indirect effects of KB on post-exercise recovery and symptoms implicated in the development of overtraining, KE looks a promising strategy to counteract overload training-induced pathophysiological effects, eventually leading up to the

development of overtraining. However, the potential effects of KE supplementation during overload training have not been investigated yet.

CHAPTER II

OBJECTIVES

The general aim of the project is to investigate the potential of KB to alter the physiological and muscular adaptations during exercise and overload training and the consequent effect on endurance exercise performance and the development of non-functional overreaching. We will use KE as a tool to elevate blood KB concentration in the presence of optimal nutritional conditions for exercise performance and recovery.

KE may stimulate endurance performance by counteracting muscle glycogen breakdown during exercise (63). However, this glycogen sparing effect may also negatively affect performance by impairing high-intensity glycolytic flux at the end of the race (96, 97). Therefore, in a **first study**, we investigated whether KE provision during the initial phase of a ~3 h 15 min simulated cycling race i) may increase glycogen availability in the final phase of the event, and ii) result in improved high-intensity performance (15 min time-trial and sprint) at the end of the race.

Ingestion of KE causes a decrease in blood pH which may negatively impact exercise performance (76, 353). In a **second study**, we will therefore investigate whether ingestion of 0.3 $g \cdot kg^{-1}$ BW $NaHCO_3$ may counteract KE-induced acidosis resulting in a further improvement of high-intensity performance (15 min time-trial and sprint) at the end of a ~3 h 15 min simulated cycling race.

In a **third study**, we examined whether KE may counteract the development of non-functional overreaching. Subjects performed a three-week supra-maximal cycling overload training period, while receiving either KE or an isocaloric placebo post-exercise. We hypothesized that KE inhibits the development of pathophysiological effects leading up to non-functional overreaching resulting in an increased training performance. Specifically, we assessed hormonal markers (catecholamines, ACTH, cortisol) implicated in the development of overtraining, alterations in physiological parameters (heart rate, blood pressure) and exercise performance (sustained training load, 30 min time-trial and sprint performance).

In a **fourth study**, we aimed to identify the effect of KE on muscular adaptations to overload training. Therefore, biopsies from the *m. vastus lateralis* were obtained in the context of 'study 3', before and after completion of the three-week overload training period. We specifically focused on the effect of KE on angiogenesis as this is considered to be one of the primary muscular adaptations to exercise training (173). We hypothesized that KE stimulates muscle angiogenesis by increasing the expression of the pro-angiogenic factor VEGF.

Study 1 — Effect of KE on endurance exercise performance

- **Hypothesis 1:** KE intake during the initial phase of a simulated cycling race causes muscle glycogen sparing
- **Hypothesis 2:** Via this mechanism (hypothesis 1) KE intake increases high-intensity exercise performance at the end of a simulated cycling race

Study 2 — Effect of KE and bicarbonate on endurance exercise performance

- **Hypothesis 1:** Intake of sodium bicarbonate counteracts ketoacidosis during exercise
- **Hypothesis 2:** Co-ingestion of KE and bicarbonate increases high-intensity exercise performance at the end of a simulated cycling race

Study 3 — Effect of KE on the development of non-functional overreaching

- **Hypothesis 1:** KE intake attenuates pathophysiological effects leading up to non-functional overreaching during a three-week supramaximal overload training period
- **Hypothesis 2:** KE intake during a three-week overload training period increases exercise performance

Study 4 — Effect of KE on the muscular response to overload training

- **Hypothesis:** KE intake stimulates muscle angiogenesis during a three-week overload training period via stimulation of VEGF

CHAPTER III

RESEARCH ARTICLES

Article 1

Exogenous ketosis impacts neither performance nor muscle glycogen breakdown in prolonged endurance exercise

Chiel Poffé[1], Monique Ramaekers[1], Stijn Bogaerts[2, 3], Peter Hespel[1,4] #

[1] Exercise Physiology Research Group, Department of Movement Sciences, KU Leuven, Leuven, Belgium

[2] Department of Physical and Rehabilitation Medicine; University Hospitals Leuven, Leuven, Belgium

[3] Locomotor and neurological disorders, Department of Development and Regeneration, Faculty of Medicine, KU Leuven, Leuven, Belgium

[4] Bakala Academy-Athletic Performance Center, KU Leuven, Leuven, Belgium

Accepted, J. Appl. Physiol.

ABSTRACT

Available evidence indicates that ketone bodies inhibit glycolysis in contracting muscles. Therefore, we investigated whether acute exogenous ketosis by oral ketone ester (KE) intake early in a simulated cycling race, can induce transient glycogen sparing by glycolytic inhibition thereby increasing glycogen availability in the final phase of the event. In a randomized cross-over design, 12 highly-trained male cyclists completed a simulated cycling race (RACE), which consisted of 3 h intermittent cycling ($IMT_{180'}$), a 15-min time-trial ($TT_{15'}$) and a maximal sprint (SPRINT). During RACE subjects received 60 g carbohydrates per h combined with three boluses (25-20-20 g) (R)-3-hydroxybutyl (R)-3-hydroxybutyrate (KE) or a control drink (CON) at 60 and 20 min before, and at 30 min during RACE. KE intake transiently increased blood D-ß-hydroxybutyrate to ~3 mM (range: 2.6-5.2 mM) during the first half of RACE ($p < 0.001$ *vs.* CON). Blood pH concomitantly decreased from ~7.42 to 7.36 (range: 7.29-7.40), whilst bicarbonate dropped from 26.0 to 21.6 mM (range: 20.1-23.7) (both $p < 0.001$ *vs.* CON). Net muscle glycogen breakdown during $IMT_{180'}$ (KE: -78 ± 9; CON: -60 ± 6 mmol · kg^{-1} ww, $p = 0.08$) and $TT_{15'}$ (KE: -9 ± 5; CON: -18 ± 5 mmol · kg^{-1} ww, $p = 0.35$) was similar between KE and CON. Accordingly, mean power output during $TT_{15'}$ (KE: 273 ± 11; CON: 272 ±11 W, $p = 0.83$) and time-to-exhaustion in the SPRINT (KE: 59 ± 5; CON: 58 ± 5 s, $p = 0.66$) were similar between conditions. In conclusion, KE intake during a simulated cycling race does not cause glycogen sparing, neither does it affect all-out performance in the final stage of a simulated race.

Keywords: ketone ester, cycling performance, muscle glycogen, ketoacidosis

NEW & NOTEWORTHY

Exogenous ketosis produced by oral ketone ester ingestion during the early phase of prolonged endurance exercise and against the background of adequate carbohydrate intake, neither causes muscle glycogen sparing, nor improves performance in the final stage of the event. However, such exogenous ketosis may decrease buffering capacity in the approach of the final episode of the event. Furthermore, ketone ester intake during exercise may reduce appetite immediately post-exercise.

INTRODUCTION

Endurance exercise performance is largely determined by the ability of the body to generate sustained muscular work, which largely depends on muscular oxygen supply via cardiac output, as well as ATP production via oxidative energy turnover (10, 13, 14). During continuous exercise, the energy to synthetize ATP is derived from the degradation of both intra- and extra-muscular carbohydrate and lipid substrates, with only a trivial contribution of amino acids (41, 55). The balance between utilization of carbohydrates *versus* lipids is regulated by exercise intensity, with lower intensities resulting in higher fraction of fat utilization (41, 46). Furthermore, higher relative substrate availability also results in greater fractional utilization (29). From this perspective, numerous studies have explored the effect of changing substrate availability by dietary manipulation on endurance exercise performance (4, 27). These studies showed general improvements in performance with high-carbohydrate intake compared to consumption of large amounts of fat in events lasting ≥1 h (6, 48) Therefore, high carbohydrate intake before and during exercise is still considered as the optimal choice to attain maximal endurance exercise performance (28).

Recent findings also have stimulated interest in the role of ketone bodies in exercise metabolism (12, 37). Ketone bodies (KB), i.e. D-β-hydroxybutyrate (βHB) and acetoacetate (AcAc), are produced by liver mitochondria in response to metabolic stressors that cause reduced carbohydrate availability such as starvation or prolonged exercise (22, 35). Under these conditions, KB provide an alternative oxidizable carbon source for energy provision in muscle, which in turn also yields a thermodynamic advantage as it increases the energy release from ATP hydrolysis compared to glucose or lipid oxidation (23, 24, 39, 43). Given the ability of KB to provide an alternative energy source, it may potentially cause sparing of finite glucose resources such as muscle glycogen during exercise. However, hyperketonemia by ketogenic diet fails to enhance endurance performance due to drop of carbohydrate availability (6, 25).

Nonetheless, exogenous ketosis produced by the ingestion of oral KB supplements, either in the form of ketone salts, ketone precursors, or ketone esters, has emerged as a potential novel approach to impact endurance exercise performance via metabolic regulation. However, ingestion of ketone salts (34, 40, 59), ketone precursors (44, 45) or ketone diester (31) only marginally increased blood ketone concentrations ([ßHB] < 1 mM), yet caused substantial gastrointestinal distress (31, 51). Conversely, oral intake of the ketone monoester (R)-3-

hydroxybutyl (R)-3-hydroxybutyrate (KE) can significantly raise circulating ßHB concentration during exercise (> 3 mM), indeed, at low incidence of gastrointestinal complaints (11, 51). In a seminal study by Cox. et al. (12) co-ingestion of ~40 g of KE and ~70 g carbohydrates, compared to carbohydrate (~120 g) alone, slightly (~2%) improved 30 min time-trial performance following 1 h constant load cycling. This ergogenic effect was attributed to muscle glycogen sparing by inhibition of glycolytic flux in conjunction with stimulation of intramyocellular triglyceride oxidation. However, this trial was run in the fasted stated, which shifts energy metabolism from carbohydrate to fat metabolism (15, 56) and in fact is not an adequate nutritional condition to study endurance exercise performance. Hence, a follow-up study with adequate carbohydrate intake prior to exercise observed no effect of KE on a 10 km run preceded by 1 h submaximal exercise (20). This study speculated that reduced glycolytic flux may rather reflect glycogenolytic impairment than sparing, and may explain the lack of ergogenic effect in their exercise settings involving high-intensity efforts largely relying on carbohydrate utilization for energy provision (26).

Given the need for high-rate glycolytic ATP production in search of success in the final stage of most endurance cycling competitions, it is reasonable to postulate that KE supplementation should rather be used during the initial phase of an endurance event to spare muscle glycogen, and thereby maintain the capacity for high glycolytic flux in the final episode of the race. However, to date, no studies have investigated whether such approach can enhance endurance exercise performance. Therefore, we investigated whether increasing blood ketone concentration by KE ingestion early in a cycling race inhibits glycogen degradation, indeed, and via this mechanism stimulates final high-intensity power outputs.

METHODS

Ethical approval and subjects. Twelve highly-trained, male cyclists and triathletes [age: 25 ± 6 (mean ± SD) y; height: 1.77 ± 0.06 m; body mass: 72 ± 8 kg; $\dot{V}O_2$ max: 62.4 ± 6.6 $ml \cdot kg^{-1} \cdot min^{-1}$, range: 55 – 73 $ml \cdot kg^{-1} \cdot min^{-1}$; cycling activity: 11.4 ± 4.2 $h \cdot wk^{-1}$, range: 7 – 20 $h \cdot wk^{-1}$] participated in this study. Potential subjects were screened using a medical questionnaire and a physical examination, including a resting electrocardiogram, prior to involvement in the study. All participants were non-smokers and did not take any medication or ergogenic supplement for at least three months before the start of the study. They were instructed to maintain their habitual physical activity level and diet during the full study period, but avoiding strenuous

exercise 48 h before each experimental session. The study was approved by the KU Leuven Biomedical Ethics Committee (B322201939080), and conforms to the Declaration of Helsinki. All subjects were informed of the content and potential risks involved with the experimental procedures before providing their written consent.

General study design. The subjects performed two experimental sessions with a 1-week washout period in between, in a randomized, double-blind, placebo-controlled, crossover design. Both experimental sessions were scheduled on the same time of the day and involved a simulated cycling race (RACE) consisting of 3 h submaximal intermittent cycling ($IMT_{180'}$) followed by a 15 min time-trial ($TT_{15'}$) and an all-out SPRINT. Subjects received either 65 g (918 ± 29 $mg \cdot kg^{-1}$, range: 722 – 1072 $mg \cdot kg^{-1}$) of a ketone ester drink [>96% (R)-3-hydroxybutyl (R)-3-hydroxybutyrate] or a viscosity and taste-matched placebo (CON) drink. Drinks were provided in three boluses (25-20-20 g) to be ingested (i) 60 and (ii) 20 min before $IMT_{180'}$ and (iii) at min 30 of $IMT_{180'}$, aiming to establish physiological ketosis (2-5 mM βHB) during the initial 1.5-2 hours of $IMT_{180'}$. The ketone ester drinks were purchased from TdeltaS Ltd (Thame Oxfordshire, UK), while the CON drink was prepared by dissolving collagen peptan® (12.5% w/v; 6d Sports Nutrition, Oudenaarde, Belgium) and 1 mM bitter sucrose octaacetate (Sigma-Aldrich, Bornem, Belgium) in water. Total energy content for the three doses administered was 306 kcal for KE, versus 29 kcal for CON. Both drinks were provided in non-transparent 50 ml tubes to avoid visual identification of the treatments. All exercise tests were conducted in an air-conditioned laboratory (18°C; 60% relative humidity) using the subjects' own bicycle, which was mounted on a calibrated cycle ergometer (Avantronic Cyclus II, Leipzig, Germany).

Preliminary Testing. Two weeks before the first experimental session, the subjects participated in two familiarization trials with 4 days in between. On the first visit, they performed a maximal incremental-load cycling test to determine their lactate threshold (LT) as well as maximal oxygen uptake rate ($\dot{V}O_2$ max). Initial workload was set at 100 W, followed by 40 W increments per 8 min, until volitional exhaustion. Respiratory gas exchange was measured during the final phase of the test (Cortex Metalyzer II, Leipzig, Germany) with $\dot{V}O_2$ max defined as the highest oxygen uptake measured over a 30 s period. Capillary blood samples were obtained from a hyperaemic earlobe at min 4 and 8 of each intensity block for lactate determination (Lactate Pro2, Arkray, Japan). LT was defined as the workload corresponding to a 1 mM blood lactate increment from min 4 to 8 within the same stage. Following a 15 min

active recovery by cycling at 100 W, the subjects performed the final ~2 h of the simulated cycling race (see below) so as to reach an identical duration compared to the subsequent sessions. During the second visit, subjects completed the simulated cycling race as to be performed during the experimental trials.

Experimental sessions. The evening before both experimental sessions, the subjects reported to the laboratory for a standardized carbohydrate-rich dinner (~5600 kJ; 69% carbohydrate, 16% fat, 15% protein). Next morning and following an overnight fast, they received a standardized breakfast (~2600 kJ; 72% carbohydrate, 15% fat, 13% protein) followed by 500 ml of a 6% carbohydrate drink (6d Sports Nutrition, Oudenaarde, Belgium) 90 minutes later. Two hours following breakfast, subjects started the RACE (Fig. 1). The exercise protocol started with $IMT_{180'}$ which consisted of six identical 30 minute blocks during which the intensity was varied per 5-min intervals between 60 and 90% of the lactate threshold (0-5': 60%; 5-10': 70%; 10-15': 90%; 15-20': 70%; 20-25': 80%; 25-30': 60%). Throughout $IMT_{180'}$, subjects ingested 500 ml of a 6% carbohydrate drink as well as an energy bar (6d Sports Nutrition, Oudenaarde, Belgium) each hour, to obtain a carbohydrate intake of 60 g · h^{-1}. Following $IMT_{180'}$, subjects rested for 5 min before starting $TT_{15'}$ in which they aimed for the highest possible mean power output. Workload during the initial 3 min (t0-t3) was set equal to the mean power output effected during the last familiarization session for $TT_{15'}$. From t3 to t12, the subjects could adjust the workload at 3-min intervals according to their subjective perception of fatigue. During the last three minutes (t12-t15), 1-min adjustments were allowed to facilitate full exhaustion by the end of $TT_{15'}$. Upon completion of $TT_{15'}$, subjects rested for 5 minutes followed by 2 min cycling at 50 W whereafter they started an all-out cycling bout at 175% of LT. Time to exhaustion (TTE) was defined as drop of cadence to < 70 rev · min^{-1}. Five minutes after completion of the SPRINT, subjects received a 500 ml recovery drink delivering 60 g carbohydrates and 30 g proteins (6d Sports Nutrition, Oudenaarde, Belgium), after which they rested for 1 h. Water was provided ad libitum during the first experimental session, and intake was recorded to allow replication during the second session. During $IMT_{180'}$ and $TT_{15'}$, but not during SPRINT, a countdown timer was shown but power output and heart rate were blinded to the subjects.

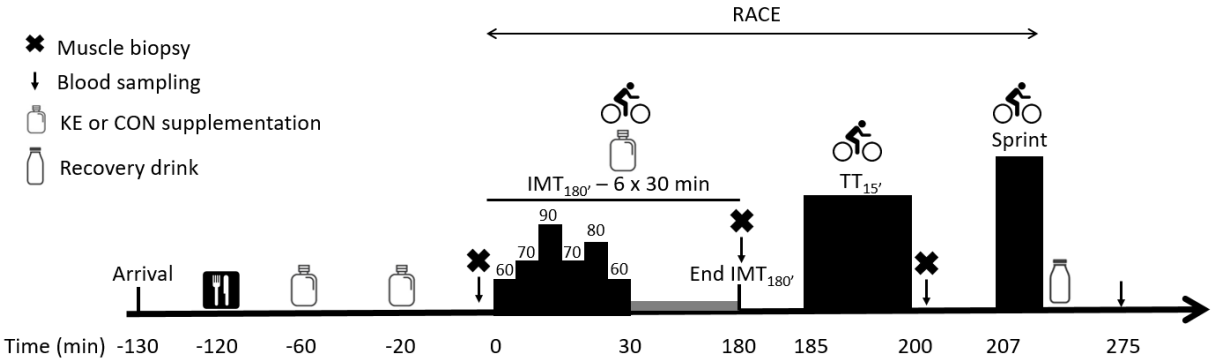


Figure 1. Schematic representation of the experimental sessions.
In a double-blind, randomized, cross-over design, subjects (n = 12) performed a simulated cycling race (RACE) consisting of 3 h intermittent cycling ($IMT_{180'}$) followed by a 15 min time-trial ($TT_{15'}$) and a SPRINT. Before and during exercise, subjects received ketone ester (KE) or control (CON) in addition to optimal carbohydrate intake (60 g · h^{-1}). Exercise intensity during $IMT_{180'}$ was set relative to the lactate threshold of each individual.

Assessment of perceived exertion, gastrointestinal distress and appetite perception. Ratings of perceived exertion were evaluated halfway $IMT_{180'}$ and immediately after $IMT_{180'}$, $TT_{15'}$ and the SPRINT (RPE, 6-20 Borg Scale) (3). Gastrointestinal discomfort was assessed 6 minutes after completion of the SPRINT using a validated 0-8 Likert scale questionnaire, evaluating distress at the systemic (dizziness, headache, muscle cramp, urge to urinate), upper- (reflux, bloating, nausea, vomiting), and lower abdominal (cramps, flatulence, abdominal pain, diarrhoea) level. Subsequently, appetite sensations were determined using a validated 10-points visual analogue scale (VAS) (60) assessing four appetite or satiety questions ("How hungry do you feel?", "How full do you feel?", "How satisfied do you feel?", "How much do you think you could eat now?").

Capillary blood sampling and analyses. Blood D-ß-hydroxybutyrate (ßHB) and glucose concentrations were determined (Glucomen Lx plus-meter with Lx ß-ketone and Lx glucose strips, Menarini Diagnostics, Firenze, Italy) from capillary blood samples obtained immediately before and 30 min following ingestion of the first KE bolus, as well as at 30-min intervals during $IMT_{180'}$ and at the end of $TT_{15'}$. In order to ensure double-blindness of the study these measurements were performed by an investigator who otherwise was not involved in the experiments. Blood lactate concentration was assessed (Lactate Pro 2, Arkray, Japan) in capillary blood obtained at regular intervals during $IMT_{180'}$ and $TT_{15'}$ and 5 minutes following the SPRINT. Furthermore, a 70 µl capillary blood sample from a hyperaemic earlobe was

obtained in a safeCLINITUBE (Radiometer Medical APS, Copenhagen, Denmark) (i) immediately before ingestion of the first KE bolus, (ii) at the start, (iii) mid and (iv) end of $IMT_{180'}$, (v) as well as 3 min after completion of the SPRINT. Following hypoxic mixing during 10 seconds, acid-base balance, blood gasses and plasma electrolytes were directly determined by an automated acid-base laboratory (ABL90 Flex analyzer, Radiometer Medical Aps, Copenhagen, Denmark).

Venous blood sampling and analyses. Venous blood samples were obtained from an antecubital vein (Venoject, Tokyo, Japan) (i) before, (ii) halfway, (iii) and at the end of $IMT_{180'}$, and (iv) at the end of $TT_{15'}$ and (v) 1 h post-exercise. Samples were collected into vacuum tubes containing either EDTA or Silica Clot Activator (BD Vacutainer). Tubes were centrifuged (1500 rpm for 10 min at 4°C) and the supernatant was stored at -80°C (EDTA plasma) or -20°C (serum) until later analysis. Commercially available ELISAs were performed to assess serum GDF15, leptin and total ghrelin (GDF15: DGD150, R&D, Minnesota; Leptin: BMS2039INST, Thermo Fisher Scientific, Massachusetts; Total Ghrelin: EZGRT-89K, Merck, Germany), while adrenaline and noradrenaline were determined in EDTA plasma (BA E-5400, LDN, Nordhorn, Germany). Plasma nonesterified free fatty acids (FFA) were determined using a colorimetric reagent kit (WAKO Chemicals, Neuss, Germany). All samples per parameter were assayed in duplicate and in a single batch according to the respective manufacturer's instructions. Coefficients of variation (CV) between the duplicates were 1.3 ± 0.3% for GDF15; 2.9 ± 0.8% for leptin; 1.7 ± 0.5% for ghrelin; 2.3 ± 0.9% for adrenaline; 2.6 ± 0.9% for noradrenaline; and 3.1 ± 1.1% for FFA.

Urine sampling and analyses. Urine was collected starting from the first KE or CON bolus up to 1 h post-exercise. Total urinary output volume was determined, and urinary ketone excretion was assessed using ketone reagent strips (Ketostix®, Ascensia Diabetes Care, Basel, Switzerland).

Muscle biopsy procedure. A percutaneous needle biopsy (100-200 mg) of the *m. vastus lateralis* was taken under local anaesthesia (2% xylocaine without adrenaline, 1 ml subcutaneously) immediately before exercise and after completion of $IMT_{180'}$ and $TT_{15'}$ using a 5-mm Bergström-type needle. To avoid interference between multiple biopsies (57), during the first experimental session, the pre-exercise biopsy was obtained from the left leg, while the biopsies at the end of $IMT_{180'}$ and $TT_{15'}$ were taken from the right leg with the needle pointing

distal *vs.* proximal, respectively. During the second experimental session, a similar procedure was followed using the other leg. Part of the muscle sample was immediately frozen in liquid nitrogen and stored at -80°C for biochemical assays at a later date. The remaining part was mounted in embedding medium (Tissue-Tek OCT), frozen in precooled isopentane, and kept at -80°C until histochemical analyses were done.

Muscle glycogen and intramyocellular triglyceride content. Muscle glycogen content was determined in triplicate as glucose residues following acid hydrolysis using a standard enzymatic fluorometric assay (32). For determination of fiber type-specific intramyocellular triglyceride (IMTG) content, serial sections (7 µm) from *m. vastus lateralis* were laid together on uncoated glass slides. The staining procedure to quantify IMCL was performed using Oil red O (ORO), as previously described by our laboratory (15). Cryosections were fixed for 10 min using 0.1% Triton X-100 added to 4% paraformaldehyde in phosphate-buffered saline (PBS). After two 5 min washes (0.5% BSA in PBS), sections were treated with 10 mM NH_4Cl to minimize autofluorescence and washed again (2 x 5 min). Following subsequent prehybridization with 1% BSA in PBS during 30 min, sections were incubated during 1 h at 37°C with primary antibodies directed against myosin heavy chain I (MHCI), MHCIIa (BA-F8 and SC-71, respectively, both 1:200 in PBS, Developmental Studies Hybridoma Bank, Iowa City, IA), and laminin (1:500 in PBS, Sigma-Aldrich, Bornem, Belgium). Following three 5 min washes, cryosections were incubated with the appropriate conjugated antibodies during 1 h at 37°C (type I: goat anti-mouse IgG2b Alexa Fluor 488, type IIa: goat anti-mouse IgG1 Cy5, both 1:500 in PBS, Jackson ImmunoResearch Laboratories Inc., West Grove, PA; laminin: donkey anti-rabbit Alexa Fluor 488, 1:500 in PBS, Invitrogen, Carlsbad, CA). After three additional washes, sections were treated for 10 min with formalin and rinsed with deionized water (3 x 30 s). Thereafter, samples were treated with the ORO working solution during 15 min. The working solution was prepared by adding 20 ml deionized water to 30 ml of the ORO stock solution [0.5 g Oil red O (Sigma-Aldrich, St Louis, MO, USA) in 100 ml 60% triethyl phosphate] and was filtered three times (prefilter: Millipore AP25; filter: Millipore-HV 0.45 µm; Millipore, Ireland) before application. Upon completion of the ORO procedure, sections were rinsed with deionized water (3 x 30 s) before being immersed under tap water during 7 min. Slides were mounted using Fluorescent Mounting Medium (DakoCytomation, Glostrup, Denmark). Samples were visualized by fluorescence microscopy (Nikon E1000, Nikon, Boerhavedorp, Germany) at 40x magnification. Images were analyzed in a blinded manner

resulting in a total of 115 ± 4 quantified muscle fibers per biopsy. Results are expressed as arbitrary units (AU).

Statistical analyses. Statistical analyses were performed using GraphPad Prism version 8.3.0 (GraphPad Software, La Jolla, CA, USA). A two-way repeated-measures analysis of variance (condition x time) was used to evaluate differences over time and between conditions. Whenever a significant interaction effect was detected, a post hoc analysis using Bonferroni correction was incorporated into the repeated-measures analysis of variance. In case of a significant interaction effect, P-values refer to the output of the post-hoc analyses, whilst otherwise P-values for main effects were reported. Alterations between conditions at one timepoint were assessed using a paired t-test. Correlations were calculated using a two-tailed Pearson's test. Statistical significance was set at $p < 0.05$. All data are expressed as mean ± SEM. 95% confidence intervals (CI) were included in the text for the primary outcome variables.

RESULTS

Blood ßHB concentration and urinary ketone excretion. Blood ßHB concentrations at baseline were low (~ 0.1–0.4 mM) in both KE and CON ($p = 0.92$, Fig. 2A), and remained low in CON throughout the trial. Following KE ingestion, blood ßHB increased to peak level within 30 min, causing a stable physiological ketosis of ~2-3 mM during the initial 2 hours of $IMT_{180'}$ ($p < 0.001$ *vs.* baseline). Thereafter, blood ßHB gradually dropped to <1 mM by the start of $TT_{15'}$ and SPRINT ($TT_{15'}$: 0.8 ± 0.1 mM; SPRINT: 0.6 ± 0.1 mM). Ketone bodies were undetectable (< 0.05 g/l) in urine in CON, whereas in KE a small fraction was excreted (0.2 ± 0.1 g, $p < 0.001$). Urine volume on average was lower in KE (300 ± 76 ml) than in CON (522 ± 116 ml, $p = 0.04$).

Blood glucose, lactate and plasma free fatty acid concentrations. Baseline blood glucose concentration was similar between KE (7.5 ± 0.3 mM) and CON (7.0 ± 0.4, $p = 0.23$, Fig. 2B). KE ingestion decreased blood glucose, resulting in ~0.5 mM lower concentration throughout the initial 150 min of $IMT_{180'}$ in KE *vs.* CON ($p = 0.03$). Thereafter, blood glucose concentrations were similar between conditions. Blood lactate concentrations during $IMT_{180'}$ and $TT_{15'}$, as well peak lactate concentrations following the SPRINT (KE: 8.5 ± 1.3 *vs.* CON: 9.5 ± 1.5 mM, $p = 0.37$, Fig. 2C) were similar between KE and CON. Baseline plasma FFA

was similar between the experimental conditions at ~0.15 mM (p = 0.99, Fig. 2D). Plasma FFA gradually increased during RACE in both conditions (p < 0.001), yet the rise was 50% lower in KE compared to CON. Thus, plasma FFA concentrations were lower in KE compared to CON by the end of $IMT_{180'}$ (KE: 0.38 ± 0.07 *vs.* CON: 0.75 ± 0.09 mM, 95% CI of difference: -0.52 to -0.22 mM, p < 0.001) and $TT_{15'}$ (KE: 0.39 ± 0.10 *vs.* CON: 0.59 ± 0.11 mM, 95% CI of difference: -0.36 to -0.05 mM, p < 0.01). One hour post-exercise, plasma FFA concentrations had returned to baseline values in both conditions.

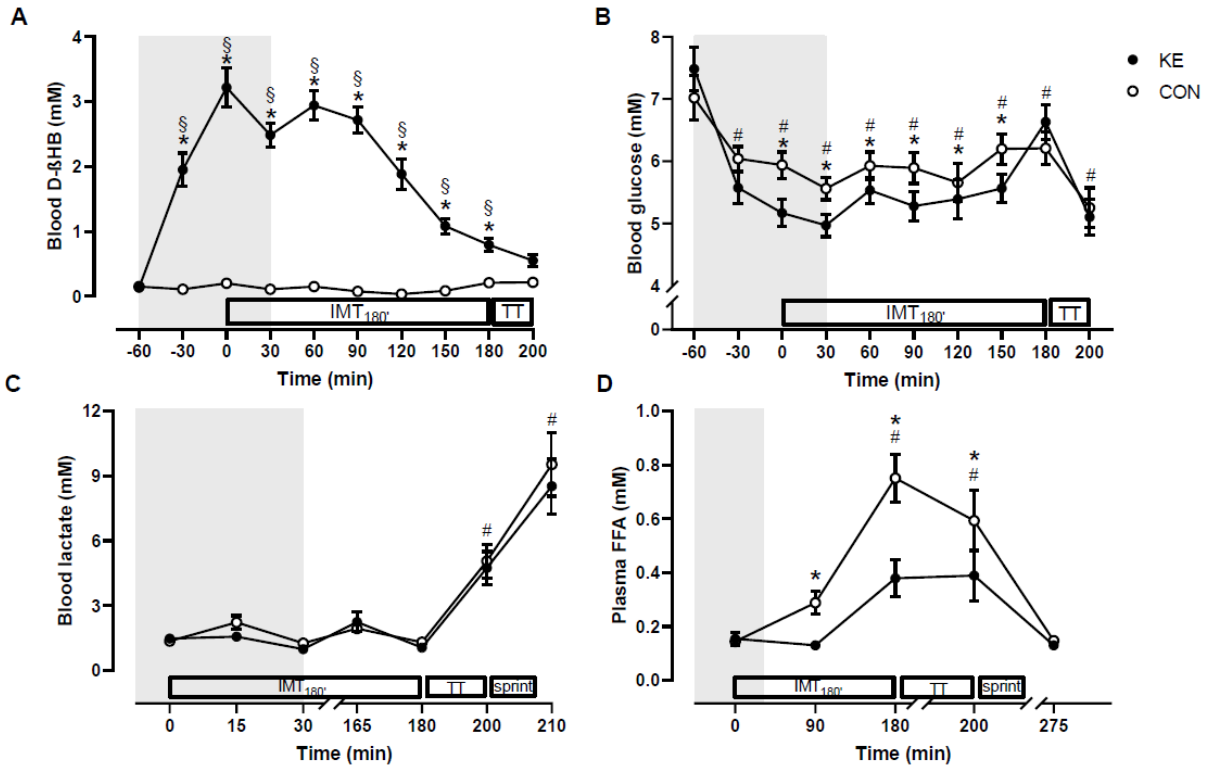


Figure 2. Effect of ketone ester supplementation on blood D-βHB, glucose, lactate and plasma free fatty acid concentrations.
Data are mean ± SEM for blood D-βHB (A), glucose (B), lactate (C) and plasma free fatty acid (FFA) concentration during each trial. Before and at the beginning of the exercise protocol subjects received control (◦, n = 12) or ketone ester supplements (•, n = 12). *P < 0.05 KE *vs.* CON at time points indicated; $^{\#}$P < 0.05 *vs.* baseline for both KE and CON; §P < 0.05 *vs.* baseline for the indicated condition. *$IMT_{180'}$* 3 h submaximal intermittent cycling, *TT* 15 min time-trial. Grey area indicates time zone during which subjects received KE or CON.

Exercise performance and RPE. Mean power output during $TT_{15'}$ (KE: 273 ± 11 *vs.* CON: 272 ± 11 W, 95% CI of difference: -5 to +4 W, p = 0.83, Fig. 3A-B) and time to exhaustion in the SPRINT (KE: 59 ± 5 *vs.* 58 ± 5 s, 95% CI of difference: -5 to +4 s, p = 0.66, Fig. 3C-D) were identical between conditions. Performance outcomes were also similar between the first and second experimental session for $TT_{15'}$ (95% CI of difference: -5 to +4 W, p = 0.74) and SPRINT (95% CI of difference: -4 to +4 s, p = 0.88), thus excluding a potential order effect.

Rate of perceived exertion tended to be higher in KE than in CON halfway $IMT_{180'}$ (KE: 13.3 ± 0.6 *vs.* CON: 12.3 ± 0.3, 95% CI of difference: -0.01 to +2.02, p = 0.06), but not in the final stage of the trial.

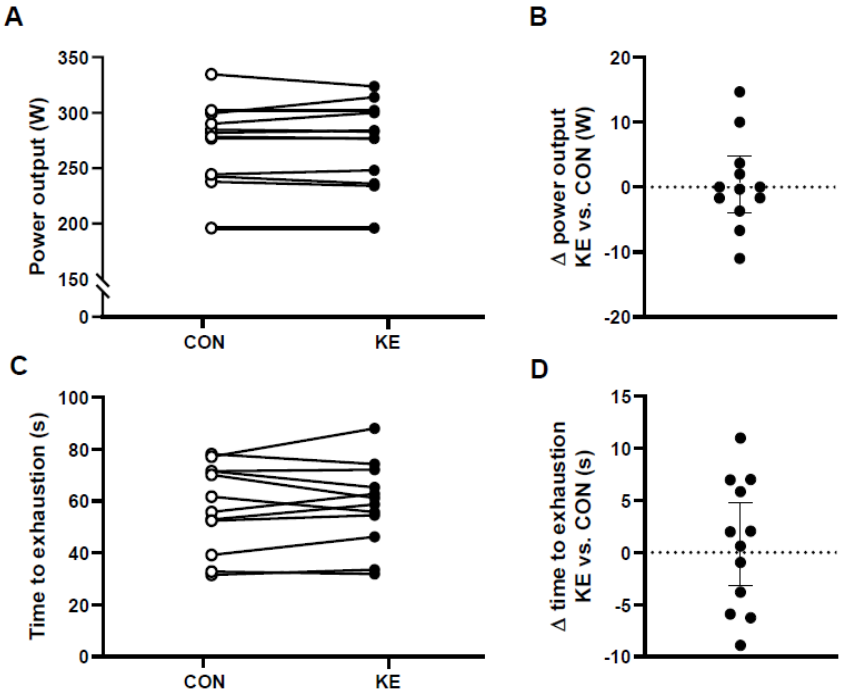


Figure 3. Effect of ketone ester supplementation on exercise performance.
Individual data points for mean power output during the 15 min simulated time-trial ($TT_{15'}$) (A), and difference in mean power output during $TT_{15'}$ for each individual between the KE and CON condition together with 95% CI (B). Individual data points for time to exhaustion in the SPRINT test (C), and difference in time to exhausting in the SPRINT test for each individual between the KE and CON condition together with 95% CI (D). Subjects received either control (◦, n = 12) or ketone ester supplements (•, n = 12). Panel B and D, points above dotted line favor KE.

Muscle glycogen and intramyocellular triglyceride content. Initial muscle glycogen content was 139 ± 7 mmol · kg^{-1} wet weight (ww) in KE *vs.* 130 ± 7 mmol · kg^{-1} in CON (p = 0.47, Fig. 4A). $IMT_{180'}$ tended to decrease muscle glycogen concentration more in KE (minus 78 ± 9 mmol · kg^{-1} ww) than in CON (minus 60 ± 6 mmol · kg^{-1} ww, 95% CI of difference: -38 to +2 mmol · kg^{-1} ww, p = 0.08). Nonetheless, muscle glycogen store at the start of $TT_{15'}$ was similar between conditions (KE: 61 ± 7 *vs.* CON: 69 ± 5 mmol · kg^{-1} ww, 95% CI of difference: -26 to +9 mmol · kg^{-1} ww, p = 0.70). $TT_{15'}$ further decreased muscle glycogen content (KE: minus 9 ± 5 *vs.* CON: minus 18 ± 5 mmo · kg^{-1} ww, p = 0.35) resulting in identical glycogen contents at the start of the SPRINT (KE: 52 ± 7 *vs.* CON: 52 ± 6 mmol · kg^{-1} ww, 95% CI of difference: -17 to +18 mmol · kg^{-1} ww, p = 0.99). IMTG content was similar between the conditions at baseline (KE: 4.4 ± 0.8 *vs.* CON: 4.1 ± 1.3 AU, 95% CI of difference: -2 to +3 AU., p = 0.99, Fig. 4B). IMTG breakdown during $IMT_{180'}$ (–3.8 ± 0.6 *vs.* – 2.9 ± 1.0 AU,

95% CI of difference: -2.9 to +1.2 AU, p = 0.61) and SPRINT (–0.3 ± 0.3 *vs.* – 0.2 ± 0.3 AU, 95% CI of difference: -2.1 to +2.0 AU, p = 0.99) were similar between KE *vs.* CON, respectively. Mean blood ßHB concentrations during $IMT_{180'}$ in KE were positively correlated with the degree of muscle glycogen breakdown (r = 0.58, p < 0.05, Fig. 4C), indicating higher rate of exercise-induced net glycogenolysis at higher circulating ßHB. Conversely, plasma FFA concentrations at the end of $IMT_{180'}$ were negatively correlated with the extent of muscle glycogen breakdown during $IMT_{180'}$ (r = -0.45, p = 0.03).

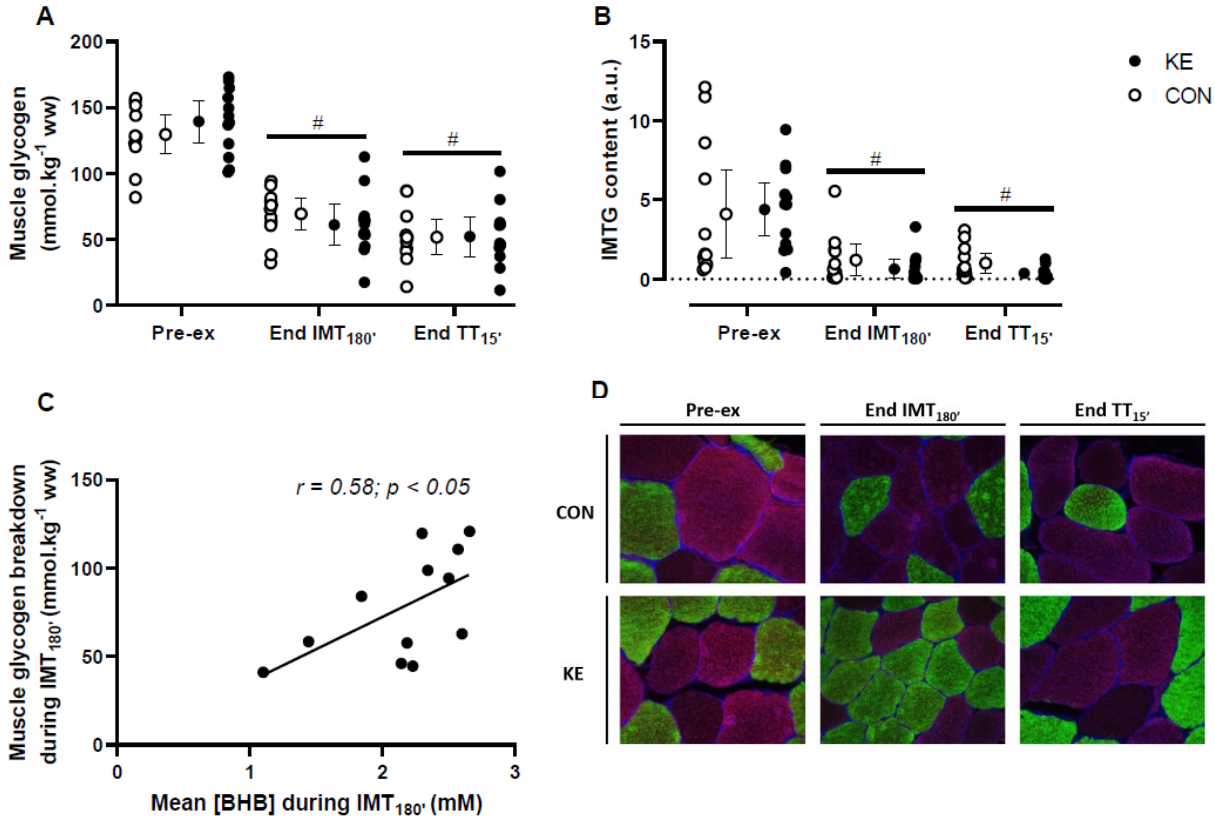


Figure 4. Effect of ketone ester on muscle glycogen and intramyocellular triglyceride content and relationship between blood ßHB and glycogen breakdown.
Data are mean ± 95% CI together with individual data points for muscle glycogen (A) and intramyocellular triglyceride (IMTG) content (B) before (Pre-ex), and immediately after 3 h submaximal intermittent cycling ($IMT_{180'}$), and a 15 min time-trial ($TT_{15'}$). Panel C, correlation analyses showing a positive correlation between mean blood ßHB concentrations and muscle glycogen breakdown during $IMT_{180'}$. Panel D, representative immunofluorescence images of the Oil-Red-O staining for determination of IMTG content of m. vastus lateralis. Intramyocellular lipid droplets (red), type I (green), type IIa (blue), type IIx (black) fibers. Subjects received either control (◦, n = 12) or ketone ester supplements (•, n = 12). * P < 0.05 KE vs. CON at time points indicated; $^{\#}$ P < 0.05 vs. baseline for both KE and CON.

Acid-base balance and blood gases. Blood pH and [HCO_3^-] at baseline were similar between conditions (pH: 7.42 ± 0.01 *vs.* 7.41 ± 0.01; [HCO_3^-]: 26.0 ± 0.2 *vs.* 25.7 ± 0.2 mM for KE *vs.* CON, both $p > 0.05$, Fig. 5A+B). Following KE intake, blood pH (7.37 ± 0.01) and [HCO_3^-] (22.5 ± 0.3 mM) slightly dropped, resulting in lower values compared to CON throughout $IMT_{180'}$ ($p < 0.05$). At the end of the SPRINT, blood pH and [HCO_3^-] were decreased to a similar extent in both conditions ($p > 0.05$). The KE-induced pH drop was associated with a lower pCO_2 halfway (-7 ± 2%) $IMT_{180'}$ for KE *vs.* CON ($p < 0.001$). There were no differences between the experimental conditions for the partial pressure of O_2 (pO_2).

Plasma electrolytes. Plasma calcium, sodium, chloride and potassium concentrations were similar between KE and CON at baseline. Plasma calcium concentrations were ~0.3 mM higher in KE compared to CON at the start and halfway $IMT_{180'}$ (both $p < 0.001$, Fig. 5C). Plasma sodium concentrations were 2.7 ± 2.5 mM higher in KE compared to CON halfway $IMT_{180'}$ ($p < 0.001$, Fig. 5D). Neither plasma chloride nor potassium concentrations were different between KE and CON at any timepoint.

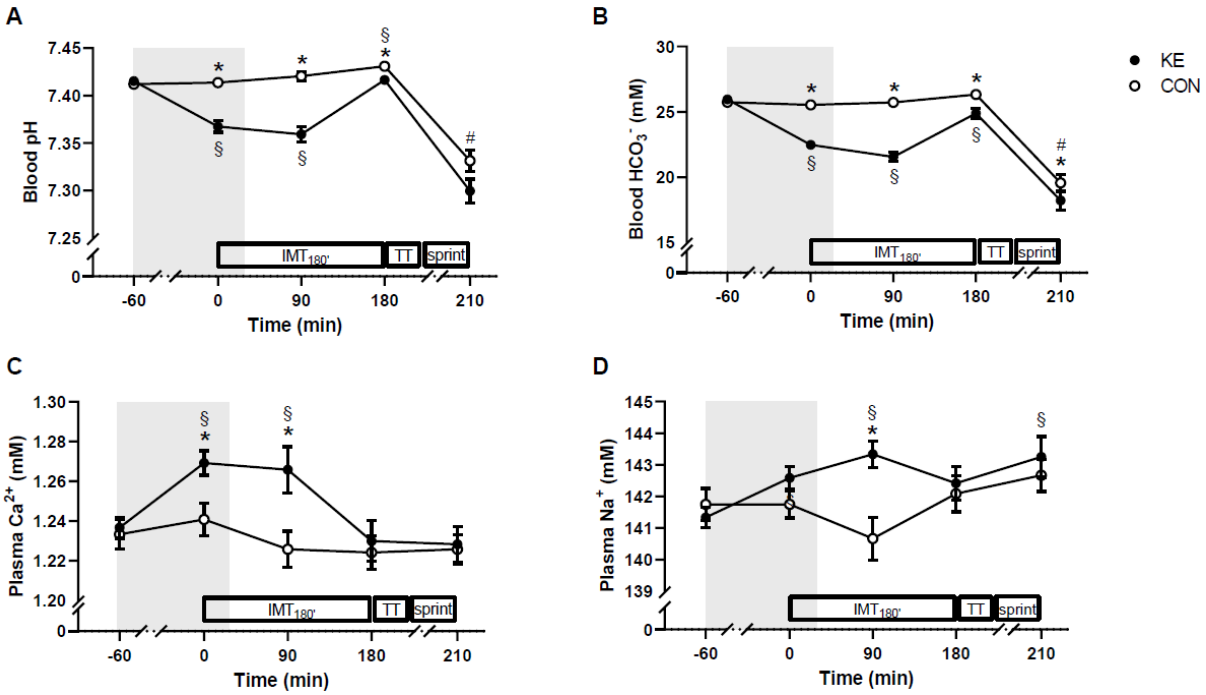


Figure 5. Effect of ketone ester supplementation on blood acid-base balance and plasma electrolytes.
Data are mean ± SEM for blood pH (A) and bicarbonate (HCO_3^-) (B), and plasma calcium (Ca^{2+}) (C) and sodium (Na^+) concentration at baseline (-60), immediately before (0), during (90) and after (180) 3 h submaximal intermittent cycling ($IMT_{180'}$), and immediately after the SPRINT (210). During the experimental trials subjects received either control (◦, n = 12) or ketone ester supplements (•, n = 12). * $P < 0.05$ KE *vs.* CON at time points indicated; # $P < 0.05$ *vs.* baseline (-60 min) for both KE and CON; § $P < 0.05$ *vs.* baseline (-60 min) for the indicated condition.

Hormonal parameters. We assessed serum hormones implicated in regulation of energy homeostasis and appetite (i.e. GDF15, ghrelin, leptin), as well as catecholamines (i.e. adrenaline and noradrenaline). All hormonal parameters measured were similar between experimental conditions at baseline (Fig. 6). Compared with baseline, RACE gradually increased GDF15 till 1 h post-exercise ($p < 0.001$ *vs.* baseline). However, compared with CON the exercise-induced increment of serum GDF15 was 10% smaller in KE. Thus at 1 h post RACE GDF15 was ~9% lower in KE (321 ± 27 pg·ml^{-1}) than in CON (353 ± 31 pg·ml^{-1} $p < 0.01$). Serum ghrelin was unaffected by the exercise in CON. However, in KE ghrelin rapidly dropped, yielding circulating concentrations at ~275 pg · ml^{-1} throughout the trial and ~17% lower than CON ($p < 0.05$ at all time points). Serum leptin and plasma adrenaline concentrations were affected neither by the exercise protocol nor by KE. But compared with CON noradrenaline concentrations tended to be slightly higher in KE (main effect of condition: $p = 0.07$).

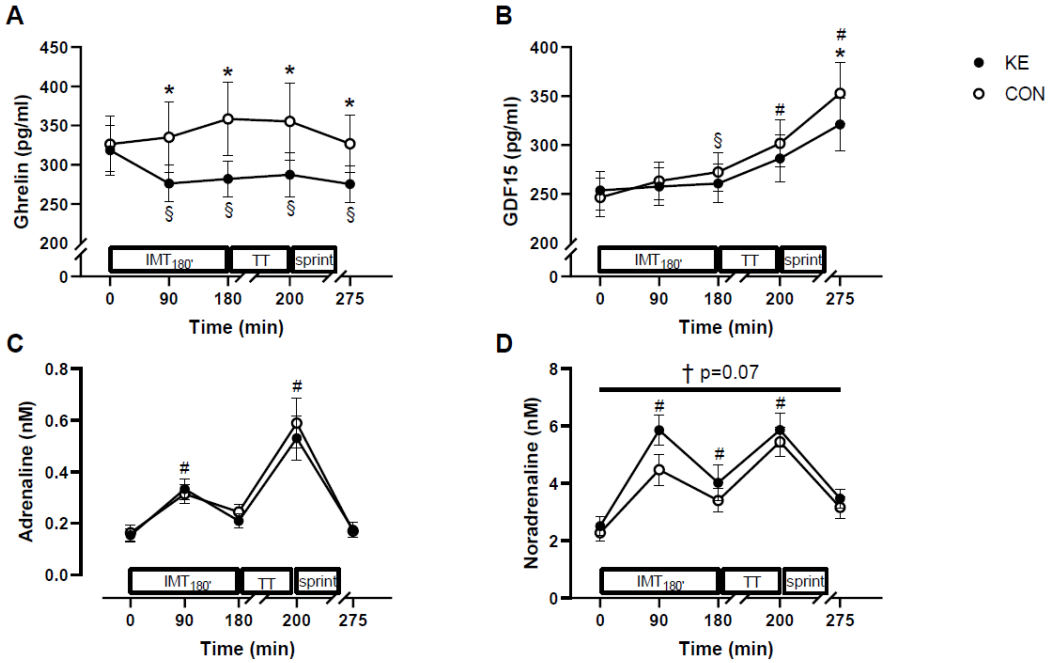


Figure 6. Effect of ketone ester supplementation on blood hormones.
Data are mean ± SEM for serum ghrelin (A) and GDF15 (B), and plasma adrenaline (C) and noradrenaline (D) before (0), during (90) and after (180) 3 h submaximal intermittent cycling ($IMT_{180'}$), immediately after the 15 min time-trial (TT; 200), and following a 1 h recovery period (275). Subjects received control (◦, n = 12) or ketone ester supplements (•, n = 12). * $P < 0.05$ KE *vs.* CON at time points indicated; # $P < 0.05$ *vs.* pre-ex (0) for both KE and CON; § $P < 0.05$ *vs.* pre-ex (0) for the indicated condition; † $P = 0.07$ KE *vs.* CON at time points indicated.

Appetite sensations and gastrointestinal tolerance. KE ingestion decreased the perception of hunger (KE: 3.6 ± 0.3 *vs.* CON: 4.9 ± 0.6, p = 0.03) as well as the desire to eat (KE: 5.1 ± 0.5 *vs.* CON: 6.5 ± 0.5, p < 0.01) after exercise, while no differences were observed for the perceived fullness and nutritional satisfaction. Total gastrointestinal discomfort scores were small in both experimental conditions (KE: 13 ± 3 *vs.* CON: 12 ± 3 out of 96, p = 0.52). Lower abdominal (~3 out of 32) and systemic (~6 out of 32) distress were similar between the conditions, while the incidence of upper abdominal discomfort was slightly higher in KE (KE: 4 ± 1 *vs.* CON: 2 ± 1 out of 32, p = 0.03).

Identification of condition and best performance trial. Upon completion of the second experimental session, the subjects were asked to identify the order of their experimental conditions (KE *vs.* CON) and their best $TT_{15'}$ performance. Seven of the twelve participants (58%) correctly identified their experimental conditions. However, all subjects were doubtful about their choice (certainty of response: 4 ± 2 out of 10; 0: no idea at all; 10: absolutely certain). Six subjects (50%) correctly identified their trial with the best performance on the $TT_{15'}$. These data indicate that the blinding of the conditions was successful.

DISCUSSION

Ketosis-induced glycogen sparing conceivably may be ergogenic in prolonged endurance exercise events wherein a low to moderate intensity initial phase is followed by a high-intensity final part involving intermittent short maximal exercise bouts and sprinting. Therefore, in the present study we investigated whether repeated oral ketone ester ingestion (KE) during the first half of a simulated cycling race (RACE) could 1) inhibit net muscle glycogen breakdown and 2) facilitate high power production in the final part of the event. It is well established indeed that residual muscle glycogen availability at the end of an endurance exercise event is an important determinant of the capacity to perform additional short maximal exercise (33). However, contrary to our hypothesis, in the conditions of the current study, KE neither reduced muscle glycogen breakdown nor improved performance in a 15-min time trial or a constant-load sprint at the end of the event. In fact, we rather demonstrate that KE, by causing transient metabolic acidosis, may result in reduced extracellular buffering capacity in the approach of the final stage of the race.

It has been suggested that acute exogenous ketosis during exercise causes upregulation of IMTG oxidation *versus* downregulation of muscle glycogenolysis in energy metabolism (16, 19). However, this opinion is based on observations in a single study where in a 2 h constant-load exercise bout @70% $\dot{V}O_2$ max was performed after an overnight fast, i.e. in the absence of a usual carbohydrate-rich pre-exercise meal. Furthermore, the rate of carbohydrate intake during exercise was ~40% higher in the control experiments (1.2 $g \cdot min^{-1}$) than during ketosis (0.7 $g \cdot min^{-1}$). But the rate of carbohydrate intake during exercise intake *per se* is a pivotal factor in regulating the fractional contribution of carbohydrates, including muscle glycogen, *versus* fats in energy metabolism (26, 30, 54). In addition, muscle glycogen concentration was measured by a semi-quantitative method (PAS-staining) which lacks specificity for glycogen (47), and only yields relative muscle glycogen values. In addition, inter-assay variation and sample freezing artefacts may impair the validity of repeated-measures comparisons. Therefore, in the current study we measured mixed muscle glycogen concentrations with a quantitative enzymatic assay. In addition, we reproduced the recommended nutritional background for optimal endurance exercise performance (5), i.e. a carbohydrate-rich meal delivering ~1-4 g CHO per kg BW ~1-4 h prior to the event, as well as an identical high rate carbohydrate intake during the exercise (60 gram per hour) in both KE and CON.

In this nutritional context KE impacted neither the magnitude of net muscle glycogen breakdown nor IMTG utilization during $IMT_{180'}$, nor during $TT_{15'}$. However, KE markedly inhibited the rise in plasma FFA during RACE, which probably resulted from ßHB-induced inhibition of adipolysis via activation of the nicotinic acid receptor PUMA-G (53). In addition, KE improved performance neither in $TT_{15'}$ nor in SPRINT. Surprisingly, in KE higher circulating blood ßHB was associated with more net glycogen breakdown during $IMT_{180'}$ ($r = 0.58$). This was likely caused by ßHB-induced suppression of plasma FFA content, resulting in less inhibition of muscle glycogenolysis by FFA (18, 42). In support of this hypothesis, lower plasma FFA concentrations correlated with increased glycogen breakdown during $IMT_{180'}$ ($r = -0.45$). Collectively our findings thus clearly indicate that in the current experimental conditions involving adequate carbohydrate supply to stimulate cycling performance, KE affected neither intramuscular energy substrate utilization nor performance. This suggests that the glycogen sparing effect and concomitant performance benefit due to KE seen by Cox et al. (2016) conceivably were due to the specific nutritional conditions used in their protocol because these effects were entirely negated by following recommended rates of carbohydrate intake before and during exercise in both the absence and presence of KE intake in the current study.

Besides the purported effect of KE on glycolytic rate (12), there is clear evidence to indicate that KE might also impact exercise performance by altering blood acid-base balance. Ketone ester ingestion at the dosages commonly recommended (20-30 g bolus) causes transient metabolic acidosis (17, 50), by increasing circulating βHB and AcAc concentrations for ~2-3 hours post ingestion (9, 50). Such effect is likely to impair high-intensity exercise performance (7). Accordingly, the repeated ingestion of a ketone ester supplement before and early in RACE (65 g KE over a 90 min period) gradually decreased blood pH from 7.42 to 7.37. Blood HCO_3^- content concomitantly dropped by ~15%. But after cessation of the ketone ester intake blood pH and HCO_3^- content returned to normal during the 2nd half of RACE. Still, compared with CON by the start of $TT_{15'}$, pH and [HCO_3^-] were slightly lower in KE. However, this significantly affected neither blood lactate, nor pH and [HCO_3^-] drop, nor performance in either $TT_{15'}$ or SPRINT. This is in agreement with a previous study showing that compensation mechanisms (e.g. hyperventilation and decrease in pCO_2), are sufficient to counteract potential ergolytic effects induced by the acid load of KE (17). Nonetheless, KE tended to cause higher RPE values during the initial phase of RACE, which is in apparent contrast with previous studies showing no effect of KE on RPE (20–21). This discrepancy probably results from differences in the exercise context. Faull et al. (21) assessed RPE during a maximal incremental-load cycling test, suggesting that KE does not affect RPE during short high-intensity exercise. Furthermore, Evans et al. (20) found exogenous ketosis not to alter RPE during a 1h constant-load running bout (65% of $\dot{V}O_2$ max) followed by a 10-km all-out running test. But in the conditions of this study blood ßHB levels during exercise were low (< 1.5 mM). The above literature data taken together with the current observations may suggest that KE may increase RPE, indeed, during prolonged low-intensity exercise associated with high (> 2 mM) blood ßHB concentrations. Support for such interpretation comes from an earlier study showing that leg discomfort during cycling was increased by KE at submaximal workloads, but not during maximal exercise, and that the extent of this effect was positively associated with the degree of ketoacidosis (21).

A recent study indicated that KE, compared to isocaloric glucose ingestion, acutely suppresses hunger and the desire to eat in healthy subjects at rest (49). Given the importance of adequate food intake immediately following exercise to stimulate muscle repair and recovery (1), we also evaluated the effect of KE on appetite regulation in the conditions of the current study. In line with earlier observations in healthy subjects at rest (49), here we add the observation that KE intake during exercise suppressed both ‘hunger’ perception and the ‘desire

to eat' immediately post-exercise. Attempting to explain this KE-induced appetite shift, we also measured the so-called 'appetite hormones'. By analogy with the earlier findings by Stubbs et al. (49), KE rapidly and consistently suppressed serum ghrelin concentrations, whilst serum leptin was unchanged. This probably indicates that acute appetite regulation during intermittent exogenous ketosis effected by ketone ester intake is at least partly effected by ghrelin, and is independent of leptin. Interestingly, we recently also demonstrated that KE during a period of training overload eventually increased spontaneous food intake to compensate for elevated energy expenditure in training, probably by short-term downregulating of GDF15 as neither ghrelin nor leptin were affected (38). Available literature indeed indicates that GDF15 suppresses food intake during episodes of metabolic stress (e.g. high-fat feeding) (36). Accordingly, here, we report the novel finding that KE blunted the acute exercise-induced rise in GDF15. Such effect eventually might also explain the lower baseline GDF15 levels seen during rapid succession of training sessions with short rest intervals in between, eventually explaining higher rate of spontaneous energy intake during training overload as we have recently reported (38). Taken together, our current and earlier data (38) provide evidence to indicate that GDF15 is an important long-term modulator of appetite during training. Conversely, ghrelin is probably more important in acute appetite regulation by KE, both in the absence (49) and presence of acute exercise.

Concerns have been raised about the gastrointestinal side-effects induced by oral ketone supplementation in conjunction with exercise (31, 58). However, previous studies have indicated that gastrointestinal complaints are less frequent with the ketone monoester (R)-3-hydroxybutyl (R)-3-hydroxybutyrate than with available ketone salts or the diester 1,3-butanediol acetoacetate (51, 52), and are similar to the discomfort due to ingestion of carbohydrate drinks during exercise (51). In the conditions of the current study, gastrointestinal discomfort scores were very low and were identical between KE and CON, indicating that KE usage in conjunction with high-rate carbohydrate intake during exercise in general is well tolerated.

Another novel observation in the current study is that KE ingestion markedly suppressed urine production. Against the background of standardized fluid intake before and during exercise (616 ± 23 $ml \cdot h^{-1}$, range: 508 – 732 $ml \cdot h^{-1}$), compared with CON urinary output from the start of KE intake till 1 h post exercise was reduced by ~40%. The precise physiological mechanism underlying this anti-diuretic effect remains to be elucidated. Interestingly, however,

we observed KE to slightly raise plasma sodium concentrations halfway $IMT_{180'}$. Such effect most probably is due to facilitated renal tubular sodium absorption. Support for such assumption comes from a study in diabetic rats showing elevated ßHB during ketoacidosis to stimulate vasopressin secretion (8). Vasopressin and the renin-angiotensin-aldosterone system are the primary regulators of renal tubular sodium and water co-absorption (2). Future studies must address the impact of exogenous ketosis on regulation of the anti-diuretic hormonal axis during exercise.

In conclusion, this study clearly demonstrates that exogenous ketosis during a simulated cycling race affects neither intramyocellular triglycerides or muscle glycogen breakdown, nor high-intensity exercise performance in the final stage of the event. However, the low degree of transient ketoacidosis induced by ketone ester intake may increase fatigue perception during low intensity episodes of a race. Finally, high-rate ketone ester intake during exercise may blunt post exercise appetite via ghrelin suppression. Therefore, the current findings and available literature data taken together do not support an ergogenic action of oral KE intake in endurance exercise whenever good practice with regard to carbohydrate intake before and during exercise is in place. To allow further identification of the potential benefits or drawbacks of ketone supplements for athletes, future studies need to incorporate research designs that are relevant for real life exercise events, as well as should include adequate nutritional conditions being recommended for optimal performance.

ADDITIONAL INFORMATION

Competing interests

The authors declare that they have no competing interests.

Author contributions

Conception and design of the study: CP and PH. Biopsy sampling: SB. Data collection and biochemical analyses: CP and MR. Analysis and interpretation of the data and manuscript drafting: CP and PH. All authors critically evaluated the manuscript and approved for submission.

Grants

This study was funded by Research Fund Flanders (Fonds voor Wetenschappelijk Onderzoek – Vlaanderen; research grant no. G080117N).

Acknowledgements

The authors would like to thank all subjects for their dedicated participation in this study.

REFERENCES

1. **American Dietetic Association**, **Dietitians of Canada**, **American College of Sports Medicine**, **Rodriguez N**, **Di Marco N**, **Langley S**. American College of Sports Medicine position stand. Nutrition and athletic performance. *Med Sci Sports Exerc* 41: 709-731, 2009.

2. **Bae EH, Ma SK.** Water and sodium regulation in heart failure. *Electrolyte Blood Press* 7: 38-41, 2009.

3. **Borg G**. Psychophysical scaling with applications in physical work and the perception of exertion. *Scand J Work Environ Heal* 16: 55-58, 1990.

4. **Burke LM**, **Hawley JA**. Fat and carbohydrate for exercise. *Curr Opin Clin Nutr Metab Care* 9: 476-81, 2006.

5. **Burke LM**, **Hawley JA**, **Wong SHS**, **Jeukendrup AE**. Carbohydrates for training and competition. *J Sports Sci* 29: 17-27, 2011.

6. **Burke LM**, **Ross ML**, **Garvican-Lewis LA**, **Welvaert M**, **Heikura IA**, **Forbes SG**, **Mirtschin JG**, **Cato LE**, **Strobel N**, **Sharma AP**, **Hawley JA**. Low carbohydrate, high fat diet impairs exercise economy and negates the performance benefit from intensified training in elite race walkers. *J Physiol* 595: 2785-2807, 2017.

7. **Carr AJ**, **Hopkins WG**, **Gore CJ**. Effects of acute alkalosis and acidosis on performance. A Meta-Analysis. *Sport Med* 41: 801-814, 2011.

8. **Charlton J**, **Thompson C**, **Thornton S**, **Palmer J**, **Baylis P**. Does beta-hydroxybutyrate stimulate plasma vasopressin in the streptozotocin-diabetic rat? In: *Clinical Science*. Portland Press Ltd., 1989, p. 28.

9. **Clarke K**, **Tchabanenko K**, **Pawlosky R**, **Carter E**, **Todd King M**, **Musa-Veloso K**, **Ho M**, **Roberts A**, **Robertson J**, **VanItallie TB**, **Veech RL**. Kinetics, safety and tolerability of (R)-3-hydroxybutyl (R)-3-hydroxybutyrate in healthy adult subjects. *Regul Toxicol Pharmacol* 63: 401-408, 2012.

10. **Conley KE**, **Kemper WF**, **Crowther GJ**. Limits to sustainable muscle performance: interaction between glycolysis and oxidative phosphorylation. *J Exp Biol* 204: 3189-3194, 2001.

11. **Cox PJ**, **Clarke K**. Acute nutritional ketosis: implications for exercise performance and metabolism. *Extrem Physiol Med* 3: 1-9, 2014.

12. **Cox PJ**, **Kirk T**, **Ashmore T**, **Willerton K**, **Evans R**, **Smith A**, **Murray AJ**, **Stubbs B**, **West J**, **McLure SW**, **King MT**, **Dodd MS**, **Holloway C**, **Neubauer S**, **Drawer S**, **Veech RL**, **Griffin JL**, **Clarke K**. Nutritional ketosis alters fuel preference and thereby endurance performance in athletes. *Cell Metab* 24: 256-268, 2016.

13. **Coyle EF**. Physiological determinants of endurance exercise performance. *J Sci Med Sport* 2: 181-189, 1999.

14. **Davis JA**. Anaerobic threshold: review of the concept and directions for future research. *Med. Sci. Sports Exerc.* 17: 6-21, 1985.

15. **De Bock K**, **Richter EA**, **Russell AP**, **Eijnde BO**, **Derave W**, **Ramaekers M**, **Koninckx E**, **Léger B**, **Verhaeghe J**, **Hespel P**. Exercise in the fasted state facilitates fibre type-specific intramyocellular lipid breakdown and stimulates glycogen resynthesis in humans. *J Physiol* 564: 649-660, 2005.

16. **Dearlove DJ**, **Faull OK**, **Clarke K**. Context is key: exogenous ketosis and athletic performance. *Curr Opin Physiol* 10: 81-89, 2019.

17. **Dearlove DJ**, **Faull OK**, **Rolls E**, **Clarke K**, **Cox PJ**. Nutritional ketoacidosis during incremental exercise in healthy athletes. *Front Physiol* 10: 1-6, 2019.

18. **Dyck DJ**, **Putman CT**, **Heigenhauser GJF**, **Hultman E**, **Spriet LL**. Regulation of fat-carbohydrate interaction in skeletal muscle during intense aerobic cycling. *Am J Physiol - Endocrinol Metab* 265: E852, 1993.

19. **Evans M**, **Cogan KE**, **Egan B**. Metabolism of ketone bodies during exercise and training: physiological basis for exogenous supplementation. *J Physiol* 595: 2857-2871, 2017.

20. **Evans M**, **McSwiney FT**, **Brady AJ**, **Egan B**. No benefit of ingestion of a ketone monoester supplement on 10-km running performance. *Med Sci Sports Exerc* 51: 2506-2515, 2019.

21. **Faull OK**, **Dearlove DJ**, **Clarke K**, **Cox PJ**. Beyond RPE: the perception of exercise under normal and ketotic conditions. *Front Physiol* 10: 1-10, 2019.

22. **Fery F**, **Balasse EO**. Ketone body turnover during and after exercise in overnight-fasted and starved humans. *Am J Physiol - Endocrinol Metab* 8: E318-E325, 1983.

23. **Fery F**, **Balasse EO**. Response of ketone body metabolism to exercise during transition from postabsorptive to fasted state. *Am J Physiol - Endocrinol Metab* 250: E495-E501, 1986.

24. **Fery F**, **Balasse EO**. Effect of exercise on the disposal of infused ketone bodies in humans. *J Clin Endocrinol Metab* 67: 245-250, 1988.

25. **Harvey KL**, **Holcomb LE**, **Kolwicz SC**. Ketogenic diets and performance. *Nutrients* 11: 2296-2311, 2019.

26. **Hawley JA**, **Leckey JJ**. Carbohydrate dependence during prolonged, intense endurance exercise. *Sport Med* 45: 5-12, 2015.

27. **Hawley JA**, **Maughan RJ**, **Hargreaves M**. Exercise metabolism: historical perspective. *Cell Metab* 22: 12-17, 2015.

28. **Helge JW**. A high carbohydrate diet remains the evidence based choice for elite athletes to optimise performance. *J Physiol* 595: 2775, 2017.

29. **Jeukendrup AE**. High-carbohydrate versus high-fat diets in endurance sports. *Sport Med Sport Traumatol* 51: 17-23, 2003.

30. **Jeukendrup AE**. Carbohydrate intake during exercise and performance. *Nutrition* 20: 669-677, 2004.

31. **Leckey JJ**, **Ross ML**, **Quod M**, **Hawley JA**, **Burke LM**. Ketone diester ingestion impairs time-trial performance in professional cyclists. *Front Physiol* 8: 1-10, 2017.

32. **Lowry OH**, **Passoneau JV**. A flexible system of enzymatic analysis. In: *Academic Press, New York, NY*. 1972.

33. **Mata F**, **Valenzuela PL**, **Gimenez J**, **Tur C**, **Ferreria D**, **Domínguez R**, **Sanchez-Oliver AJ**, **Sanz JMM**. Carbohydrate availability and physical performance: physiological overview and practical recommendations. *Nutrients* 11: 1084-1093, 2019.

34. **O'Malley T**, **Myette-Cote E**, **Durrer C**, **Little JP**. Nutritional ketone salts increase fat oxidation but impair high-intensity exercise performance in healthy adult males. *Appl Physiol Nutr Metab* 42: 1031-1035, 2017.

35. **Owen OE**, **Morgan AP**, **Kemp HG**, **Sullivan JM**, **Herrera MG**, **Cahill GF**. Brain metabolism during fasting. *J Clin Invest* 46: 1589-1595, 1967.

36. **Patel S**, **Alvarez-guaita A**, **Melvin A**, **Coll AP**, **Savage DB**, **Rahilly SO**. GDF15 provides an endocrine signal of nutritional stress in mice and humans. *Cell Metab* 29: 707-718, 2019.

37. **Pinckaers PJM**, **Churchward-Venne TA**, **Bailey D**, **van Loon LJC**. Ketone bodies and exercise performance: the next magic bullet or merely hype? *Sport Med* 47: 383-391, 2017.

38. **Poffé C**, **Ramaekers M**, **Van Thienen R**, **Hespel P**. Ketone ester supplementation blunts overreaching symptoms during endurance training overload. *J Physiol* 597: 3009-3027, 2019.

39. **Robinson AM**, **Williamson DH**. Physiological roles of ketone bodies as substrates and signals in mammalian tissues. *Physiol Rev* 60: 143-187, 1980.

40. **Rodger S**, **Plews D**, **Laursen P**, **Driller M**. Oral β-hydroxybutyrate salt fails to improve 4- minute cycling performance following submaximal exercise. *J Sci Cycl* 6: 26-31, 2017.

41. **Romijn JA**, **Coyle EF**, **Sidossis LS**, **Gastaldelli A**, **Horowitz JF**, **Endert E**, **Wolfe RR**. Regulation of endogenous fat and carbohydrate metabolism in relation to exercise intensity and duration. *Am J Physiol - Endocrinol Metab* 265: 380-391, 1993.

42. **Romijn JA**, **Coyle EF**, **Sidossis LS**, **Zhang XJ**, **Wolfe RR**. Relationship between fatty acid delivery and fatty acid oxidation during strenuous exercise. *J Appl Physiol* 79: 1939-1945, 1995.

43. **Sato K**, **Kashiwaya Y**, **Keon CA**, **Tsuchiva N**, **King MT**, **Radda GK**, **Chance B**, **Clarke K**, **Veech RL**. Insulin, ketone bodies, and mitochondrial energy transduction. *FASEB J* 9: 651-658, 1995.

44. **Scott BE**, **Laursen PB**, **James LJ**, **Boxer B**, **Chandler Z**, **Lam E**, **Gascoyne T**, **Messenger J**, **Mears SA**. The effect of 1,3-butanediol and carbohydrate supplementation on running performance. *J Sci Med Sport* 22: 702-706, 2019.

45. **Shaw DM**, **Merien F**, **Braakhuis A**, **Plews D**, **Laursen P**, **Dulson DK**. The effect of 1,3-butanediol on cycling time-trial performance. *Int J Sport Nutr Exerc Metab* 29: 466-473, 2019.

46. **Sherman WM**, **Kingman S**. Metabolism of sugars and physical performance. *Am J Clin Nutr* 62: 228-241, 1995.

47. **Skurat A V.**, **Segvich DM**, **Depaoli-Roach AA**, **Roach PJ**. Novel method for detection of glycogen in cells. *Glycobiology* 27: 416-424, 2017.

48. **Stellingwerff T**, **Cox GR**. Systematic review: carbohydrate supplementation on exercise performance or capacity of varying durations. *Appl Physiol Nutr Metab* 39: 998-1011, 2014.

49. **Stubbs BJ**, **Cox PJ**, **Evans RD**, **Cyranka M**, **Clarke K**, **de Wet H**. A ketone ester drink lowers human ghrelin and appetite. *Obesity* 26: 269-273, 2018.

50. **Stubbs BJ**, **Cox PJ**, **Evans RD**, **Santer P**, **Miller JJ**, **Faull OK**, **Magor-Elliott S**, **Hiyama S**, **Stirling M**, **Clarke K**. On the metabolism of exogenous ketones in humans. *Front Physiol* 8: 848, 2017.

51. **Stubbs BJ**, **Cox PJ**, **Kirk T**, **Evans RD**, **Clarke K**. Gastrointestinal effects of exogenous ketone drinks are infrequent, mild, and vary according to ketone compound and dose. *Int J Sport Nutr Exerc Metab* 29: 596-603, 2019.

52. **Stubbs BJ**, **Koutnik AP**, **Poff AM**, **Ford KM**, **D'Agostino DP**. Commentary: ketone diester ingestion impairs time trial performance in professional cyclists. *Front Physiol* 9: 279, 2018.

53. **Taggart AKP**, **Kero J**, **Gan X**, **Cai TQ**, **Cheng K**, **Ippolito M**, **Ren N**, **Kaplan R**, **Wu K**, **Wu TJ**, **Jin L**, **Liaw C**, **Chen R**, **Richman J**, **Connolly D**, **Offermanns S**, **Wright SD**, **Waters MG**. (D)-β-hydroxybutyrate inhibits adipocyte lipolysis via the nicotinic acid receptor PUMA-G. *J Biol Chem* 280: 26649-26652, 2005.

54. **Tsintzas K**, **Williams C**. Human muscle glycogen metabolism during exercise. *Sport Med* 25: 7-23, 1998.

55. **van Loon LJC**, **Greenhaff PL**, **Constantin-Teodosiu D**, **Saris WHM**, **Wagenmakers AJM**. The effects of increasing exercise intensity on muscle fuel utilisation in humans. *J Physiol* 536: 295-304, 2001.

56. **Van Proeyen K**, **Szlufcik K**, **Nielens H**, **Ramaekers M**, **Hespel P**. Beneficial metabolic adaptations due to endurance exercise training in the fasted state. *J Appl Physiol* 110: 236-245, 2011.

57. **Van Thienen R**, **D'Hulst G**, **Deldicque L**, **Hespel P**. Biochemical artifacts in experiments involving repeated biopsies in the same muscle. *Physiol Rep* 2: 1-14, 2014.

58. **Vandoorne T**, **De Smet S**, **Ramaekers M**, **Van Thienen R**, **De Bock K**, **Clarke K**, **Hespel P**. Intake of a ketone ester drink during recovery from exercise promotes mTORC1 signaling but not glycogen resynthesis in human muscle. *Front Physiol* 8: 1-12, 2017.

59. **Waldman HS**, **Basham SA**, **Price FG**, **Smith JW**, **Chander H**, **Knight AC**, **Krings BM**, **McAllister MJ**. Exogenous ketone salts do not improve cognitive responses after a high-intensity exercise protocol in healthy college-aged males. *Appl Physiol Nutr Metab* 43: 711-717, 2018.

60. **Woods AL**, **Rice AJ, Garvican-Lewis LA, Wallet AM, Lundy B, Rogers MA, Welvaert M, Halson S, McKune A, Thompson KG.** The effects of intensified training on resting metabolic rate (RMR), body composition and performance in trained cyclists. *PLoS One* 13: 1-24, 2018.

Article 2

Oral bicarbonate unlocks the ergogenic action of exogenous ketosis in endurance exercise

Chiel Poffé[1], Monique Ramaekers[1], Stijn Bogaerts[2, 3], Peter Hespel[1,4] #

[1] Exercise Physiology Research Group, Department of Movement Sciences, KU Leuven, Leuven, Belgium

[2] Department of Physical and Rehabilitation Medicine; University Hospitals Leuven, Leuven, Belgium

[3] Locomotor and neurological disorders, Department of Development and Regeneration, Faculty of Medicine, KU Leuven, Leuven, Belgium

[4] Bakala Academy-Athletic Performance Center, KU Leuven, Leuven, Belgium

Revised manuscript in preparation for MSSE

ABSTRACT

Purpose: We recently reported that exogenous ketosis during endurance exercise did not affect endurance exercise performance, but causes a transient drop in pH together with a depletion of extracellular alkaline reserves. Therefore, we hypothesized that the ergogenic potential of exogenous ketones may be overruled by its concomitant acid-base disturbances. **Methods:** Nine well-trained male cyclists performed a simulated cycling race (RACE), which consisted of 3 h intermittent cycling ($IMT_{180'}$) followed by a 15-min time-trial ($TT_{15'}$) and an all-out sprint (SPRINT). In a randomized cross-over design, subjects received either i) 65 g ketone ester (KE), ii) 300 mg · kg^{-1} body weight $NaHCO_3$ (BIC), iii) KE+BIC or iv) a control drink (CON) during RACE, together with 60 g carbohydrates per h. **Results:** KE ingestion transiently elevated blood D-ß-hydrxybutyrate to ~2-4 mM during the initial 2 hours of RACE in KE and KE+BIC ($p < 0.05$ *vs.* CON). In KE, blood pH and bicarbonate concentration concomitantly dropped from 7.43 to 7.35 and from 25.5 to 20.5 mM, respectively during the first half of the RACE (both $p < 0.05$ *vs.* CON). Additional intake of BIC completely counteracted KE-induced acidosis and resulted in higher blood pH (7.51 ± 0.01) and bicarbonate concentration (31.1 ± 0.6 mM) by the end of $IMT_{180'}$, together with 50% higher HCO_3^- utilization during $TT_{15'}$ and SPRINT (all $p < 0.05$ *vs.* CON). Mean power output during the $TT_{15'}$ was similar between KE, BIC and CON at ~255 W, but was 5% higher in KE+BIC ($p < 0.05$). Time-to-exhaustion in the sprint was similar between all conditions at ~60 s ($p = 0.88$). **Discussion:** Co-ingestion of oral bicarbonate and KE enhances high-intensity performance at the end of an endurance exercise event in the absence of gastrointestinal distress.

Keywords: ketone, bicarbonate, exercise performance, ketoacidosis

INTRODUCTION

The popularity of oral ketone supplementation in athletic populations has exponentially grown over recent years. From the scientific perspective, this popularity was originally triggered by a seminal publication by Cox and his co-workers (1) showing that exogenous ketosis generated by high-dose ingestion of the ketone ester (R)-3-hydroxybutyl (R)-3-hydroxybutyrate (KE) improved performance in a 30-min time trial. More recently we also demonstrated that consistent ingestion of KE after training sessions during a 3-week training overload period not only markedly suppressed the development of overreaching symptoms, but also significantly enhanced exercise tolerance as well as improved endurance exercise performance (2-5).

Nonetheless, the effects of acute exogenous ketosis in endurance exercise performance remain a matter of considerable debate. In contrast to the original findings by Cox et al. (1), follow-up studies failed to demonstrate an ergogenic effect of exogenous ketosis on shuttle run time to exhaustion (6), repeated sprint performance (6, 7), 5 and 10 km running performance (8, 9), 4-60 min cycling time-trial performance (10-13), or during an incremental cycle test (14). We recently also found that KE intake during the initial phase of a 3 h 15 min simulated cycling race did not boost all-out performance in the final part of the event (Poffé *et al.,* accepted, *J. Appl. Physiol.*). These conflicting results are at least partly explained by different sources of ketone supplements used. For instance, poor gastrointestinal tolerance of high-dose ketone salts, i.e. sodium-potassium or magnesium-calcium ß-hydroxybutyrate (7, 12, 13), ketone precursors (9, 10), and ketone diester (11), limits actual elevation of circulating ketone body levels above 2 $mmol \cdot L^{-1}$ which is considered to be required to possibly impact exercise performance (15). Conversely, KE is in general well tolerated (16, 17) and allows to establish blood ketone concentrations at ~2-5 $mmol \cdot L^{-1}$ throughout exercise (1, 14). But with the exception of the aforementioned study by Cox *et al.* (1), all other studies available to date failed to demonstrate an ergogenic effect of acute KE administration in prolonged exercise performance (8, 14).

Also the nutritional background conceivably plays an important role in the physiological effects of acute KE during exercise. It is well-established that ketone bodies provide a valuable alternative for glucose as an energy substrate during episodes of glucose deficiency, such as fasting (18), or low-carb high-fat 'ketogenic' diet (19). Thus one may logically expect

exogenous ketosis to more readily improve performance in endurance exercise in the fasted state (1, 14), rather than during similar exercise supported by recommended rates of carbohydrate supply before and during exercise (31). However, in the only study that reported a positive effect of KE in endurance performance to date (20), no pre-exercise meal was provided. Moreover, compared with control (~120 $g \cdot h^{-1}$) the rate of carbohydrate intake during exercise was substantially lower during KE (~70 $g \cdot h^{-1}$). Such nutritional background is likely to shift substrate oxidation towards increased fat metabolism in KE (21).

Different physiological mechanisms are probably implicated in modulation of endurance exercise performance during exogenous ketosis. First, early studies in perfused rat hearts have demonstrated that both β-hydroxybutyrate and acetoacetate can directly suppress phosphofructokinase activity in striated muscle (22). Such mechanisms may explain the effect of oral KE to reduce blood lactate accumulation and impair performance during high-intensity exercise requiring high rates of glycol(geno)lytic ATP production for energy provision (6, 8). However, whenever glycogen availability per se limits performance, KE-induced glycolytic inhibition may exert an ergogenic action by glycogen sparing. In addition, exogenous ketosis may also cause glycogen sparing by substituting for glucose as a readily oxidizable carbon source for muscle ATP production (23), as well as by reducing the need for hepatic glucose output on behalf of blood glucose homeostasis. Along such rationale, in the aforementioned study by Cox *et al.* high-dose KE intake during 120-min cycling resulted in glycogen sparing (1), at lower rate of oral carbohydrate intake though than control. We recently investigated whether exogenous ketosis against the background of high-rate carbohydrate intake (60 $g \cdot h^{-1}$), during the initial low-intensity phase of a 3 h 15 min simulated cycling race could increase performance by increasing glycogen availability in the final high-intensity stage of the race (Poffé *et al.*, accepted, *J. Appl. Physiol.*). However, KE induced neither glycogen sparing nor enhanced sustained maximal power outputs in the final part of the race.

Exogenous ketosis may also impact performance by shifting acid-base balance. Early studies have shown that the acid load caused by the ingestion of 20-40 grams KE decreases blood pH by about 0.05-0.10 units at rest (24) as well as during submaximal exercise (14). By analogy, in the conditions of our aforementioned study involving a 3 h 15 min simulated cycling race, blood pH transiently decreased by ~0.05 units following KE ingestion. Importantly, the intermittent ingestion of KE also decreased blood alkaline reserve in the approach of the final

stage of the event. Moreover, compared with the control condition the subjects reported higher degree of fatigue perception during the early phase of the event when blood pH was slightly reduced due to KE. Although the precise role of [H^+] in the development of muscular fatigue is still a matter of debate (25) a recent meta-analysis has clearly indicated that extracellular acidosis negatively impacts exercise performance (26), even at moderately decreased pH values (27) as observed following KE ingestion.

Literature data (14, 24) and our own recent observations (Poffé *et al.*, accepted, *J. Appl. Physiol.*) taken together, it is reasonable to assume that the potential of exogenous ketosis to enhance endurance exercise performance via beneficial metabolic regulation, may be overruled by ketosis-induced acid-base dysregulation, i.e. acidosis due to high circulating concentrations of β-hydroxybutyrate and acetoacetate. Hence we postulated that prevention of KE-induced acidosis by concomitant oral bicarbonate ($NaHCO_3$) ingestion may unlock the ergogenic potential of KE. It is well established that oral $NaHCO_3$ ingestion before exercise can counteract exercise-induced pH drop and thereby stimulate performance in short all-out exercise (26, 28, 29). In addition, we have recently also demonstrated that consistent low-dose $NaHCO_3$ ingestion during prolonged submaximal exercise can elevate alkaline reserve without causing gastrointestinal distress (Dalle *et al.,* in revision). Against this background, the aim of the present study was to investigate whether $NaHCO_3$ intake and exogenous ketosis produced by oral KE administration, may be synergistic in improving endurance exercise performance.

METHODS

Ethical approval and subjects. Nine well-trained, male cyclists [age: 29 ± 5 (mean ± SD) y; height: 1.81 ± 0.07 m; body mass: 71 ± 7 kg; $\dot{V}O_2$ max: 61.0 ± 2.9 $ml \cdot kg^{-1} \cdot min^{-1}$, range: 55 – 64 $ml \cdot kg^{-1} \cdot min^{-1}$; cycling activity: 11.2 ± 4.6 $h \cdot wk^{-1}$, range: 7 – 20 $h \cdot wk^{-1}$] volunteered in this study, which was approved by the KU Leuven Biomedical Ethics Committee (B322201939080), and conforms to the Declaration of Helsinki. Subjects gave their written informed consents after they were informed of the content and potential risks involved with the experimental procedures. Exclusion criteria for participation were smoking and intake of any medication or ergogenic supplement during the last three months before the start of the study. Subjects were instructed to maintain their habitual physical activity level and diet during the full study period.

General study design. The research design (Figure 1) was adopted from our recent study in which we investigated the effect of KE in endurance exercise performance (Poffé et al., accepted, *J. Appl. Physiol.*). The study was of a double-blind, placebo-controlled, crossover design and involved four experimental sessions, each separated by a 1-week washout period. All experimental sessions were conducted at the same time of the day and involved a simulated cycling race (RACE). The race started with 3 h submaximal intermittent cycling ($IMT_{180'}$) followed by a 15 min time-trial ($TT_{15'}$) and an all-out exercise bout at 175% of the anaerobic threshold (SPRINT).

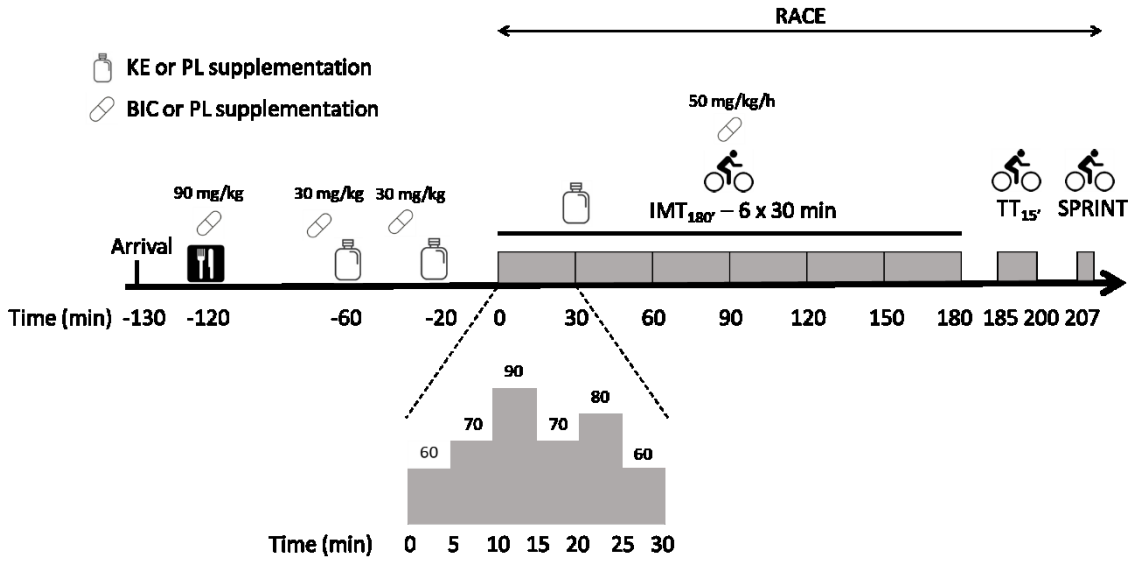


Figure 1. Schematic overview of the experimental sessions.
The study involved four experimental sessions in a double-blind, randomized, crossover design. At each occasion subjects (n = 9) participated in a 6x30 min simulated intermittent cycling race ($IMT_{180'}$) while ingesting 60 g carbohydrates per hour, followed by a 15 min simulated time-trial ($TT_{15'}$) and a ~1min all-out exercise bout at 175% of lactate threshold (SPRINT). Before and during exercise the subjects received either placebo (CON), or ketone ester (KE), or bicarbonate (BIC) or ketone ester plus bicarbonate supplements (KE+BIC). Intensities during $IMT_{180'}$ were set relative to each individual's lactate threshold. *PL* placebo.

On each of the experimental days, the subjects were allocated to one of the following four supplemental conditions in a stratified, randomized order: (i) ketone ester (KE), (ii) $NaHCO_3$ (BIC), (iii) KE+BIC, or (iv) placebo (CON). In the KE conditions (KE, KE+BIC), subjects received a 65 g ketone ester dose in the form of a drink [96% (R)-3-hydroxybutyl (R)-3-hydroxybutyrate, TdeltaS Ltd, Thame Oxfordshire, UK). Drinks were provided in three aliquots (25-20-20 g) to be ingested (i) 60 and (ii) 20 min before $IMT_{180'}$, and (iii) at min 30 of $IMT_{180'}$. By analogy with our earlier study (Poffé *et al.,* accepted, *J. Appl. Physiol.*) we aimed to induce physiological ketosis (2-5 mM βHB) during the initial 1.5-2 h of $IMT_{180'}$. The other conditions

(CON, BIC) received a taste and viscosity-matched placebo, which was prepared by dissolving collagen (12.5% w/v; 6d Sports Nutrition, Oudenaarde, Belgium) and 1 mM bitter sucrose octaacetate (Sigma-Aldrich, Bornem, Belgium) in water. Both drinks were provided in non-transparent 50 ml tubes to avoid visual identification of the treatments. In the BIC conditions (BIC, KE+BIC), subjects received a total of 300 mg · kg^{-1} body weight (BW) $NaHCO_3$ in the form of 700 mg gelatin capsules. They ingested 90 mg · kg^{-1} with breakfast, followed by two boluses of 30 mg · kg^{-1} at 75 and 30 min before RACE. Throughout $IMT_{180'}$, subjects received $NaHCO_3$ at a rate of 50 mg · kg^{-1} · h^{-1} dissolved in 500 ml of a 6% maltodextrin solution. Conversely, in the other conditions (CON, KE) subjects ingested an appearance-matched placebo (NaCl), containing an equimolar amount of sodium. Furthermore, during $IMT_{180'}$ subjects received one energy bar delivering 30 g carbohydrates per hour (6d Sports Nutrition, Oudenaarde, Belgium) in order to obtain a carbohydrate intake of 60 g · h^{-1} in each experimental arm. All exercise tests were performed in an air-conditioned laboratory (18°C, 60% relative humidity) and on the subject's own bicycle which was mounted on a calibrated cycle ergometer (Avantronic Cyclus II, Leipzig, Germany).

Preliminary Testing. Two weeks before the first experimental session, the subjects participated in two familiarization trials with 4 days in between. During the first visit, subjects performed a maximal incremental cycling test to determine their lactate threshold (LT) and maximal oxygen uptake rate ($\dot{V}O_2$ max). Initial workload was set at 100 W, and was increased by 40 W per 8 min until volitional exhaustion. Respiratory gas exchange was measured during the final phase of the test (Cortex Metalyzer II, Leipzig, Germany) and the highest oxygen uptake measured over a 30 s period was defined as the $\dot{V}O_2$ max. At min 4 and 8 of each intensity block, capillary blood samples were obtained from a hyperaemic earlobe for determination of blood lactate (Lactate Pro2, Arkray, Japan). LT was determined as the workload corresponding to a 1 mM blood lactate increment from min 4 to 8 within the same stage. After 15 min of active recovery by cycling at 100 W, the subjects performed the final 2 hours of the simulated cycling race (see below) to simulate an identical exercise duration (~3 h 15 min) compared to the experimental sessions. On the second visit the subjects then completed the full simulated cycling race as to be performed during the later experimental trials.

Experimental sessions. On the evening before each experimental session, the subjects received a standardized carbohydrate-rich dinner (~5600 kJ; 69% carbohydrate, 16% fat, 15% protein). Following an overnight fast the subjects reported to the laboratory 2 h before the start

of the RACE (between 6:00 AM and 10:00 AM) and received a standardized breakfast (~2600 kJ; 72% carbohydrate, 15% fat, 13% protein) followed by 500 ml of a 6% carbohydrate drink (6d Sports Nutrition, Oudenaarde, Belgium) 90 minutes later. The RACE (Fig. 1) started with $IMT_{180'}$ during which the intensity was varied between 60 and 90% (5-min stages) of the previously determined LT. Upon completion of $IMT_{180'}$ and an ensuing 5 min rest period, subjects started the $TT_{15'}$ in which they aimed for the highest possible mean power output. During the initial 3 min (t0-t3), workload was fixed at the mean power output effected during the $TT_{15'}$ of the last familiarization session. From t3 to t12, subjects could voluntarily increase or decrease the workload at 3-min intervals according to their perception of fatigue. From t12 to t15, 1-min adjustments were allowed to establish full exhaustion by the end of $TT_{15'}$. After completing $TT_{15'}$, subjects rested for 5 min followed by 2 min cycling at 50 W. Thereafter, subjects performed a constant-power (175% of LT) all-out exercise bout (SPRINT) to exhaustion which was defined as drop of cadence to < 70 rev · min^{-1}. During the first experimental session, water was provided ad libitum to the subjects, and intake was recorded to allow replication during the ensuing sessions. Throughout each exercise session, a countdown timer was shown but power output and heart rate were blinded to the subjects. During SPRINT, subjects received no information about the elapsed time.

Respiratory gas measurements. Minute ventilation (V_E), oxygen consumption ($\dot{V}O_2$) and carbon dioxide production ($\dot{V}CO_2$) were measured using breath-by-breath indirect calorimetry (Cortex Metalyzer II, Leipzig, Germany) at the beginning (t40-t55) and end (t160-t175) of $IMT_{180'}$. The reported values for both 15 min periods represent the overall average of the last minute of each 5 min block.

Assessment of perceived exertion, gastrointestinal distress and appetite perception. Subjective scores of the rate of perceived exertion (RPE) were assessed halfway $IMT_{180'}$ and immediately after $IMT_{180'}$, $TT_{15'}$ and the SPRINT using a 6-20 Borg Scale (30). Gastrointestinal discomfort was rated 6 minutes after completion of the SPRINT by means of a validated 0-8 Likert scale questionnaire (31). The questionnaire evaluated distress at the systemic (dizziness, headache, muscle cramp, urge to urinate), upper- (reflux, bloating, nausea, vomiting), and lower abdominal (cramps, flatulence, abdominal pain, diarrhoea) level. Subsequently, “appetite sensations” were determined using a validated 10-points visual analogue scale (VAS) (32) for the following four questions: “How hungry do you feel?”; “How full do you feel?”; “How satisfied do you feel?”; “How much do you think you could eat now?”.

Capillary blood sampling and analyses. Capillary blood samples were obtained from a hyperaemic earlobe for evaluation of blood D-ß-hydroxybutyrate (ßHB) and glucose concentrations (Glucomen Lx plus-meter with Lx ß-ketone and Lx glucose strips, Menarini Diagnostics, Firenze, Italy) immediately before and 30 min following ingestion of the first KE bolus, as well as at 30-min intervals during $IMT_{180'}$ and at the end of $TT_{15'}$. An investigator who was otherwise not involved in the experiments performed both measurements to ensure double-blindness during the experiments. Blood lactate levels were determined (Lactate Pro 2, Arkray, Japan) in capillary blood obtained at regular intervals during $IMT_{180'}$ and $TT_{15'}$, and 5 minutes after completion of the SPRINT. In addition, a 70µl capillary blood sample from a preheated earlobe was collected into a safeCLINITUBE (Radiometer Medical APS, Copenhagen, Denmark) (i) before breakfast, (ii) immediately before ingestion of the first KE bolus, (iii) at the start, (iv) mid and (v) end of $IMT_{180'}$, (vi) as well as 3 min following SPRINT. Thereafter, samples were mixed during 10 seconds followed by determination of acid-base balance and blood gasses using an automated acid-base laboratory (ABL90 Flex analyser, Radiometer Medical Aps, Copenhagen, Denmark).

Urine sampling and analyses. Urine was collected in flasks from breakfast up to 1 h post-exercise. Total urinary output volume was noted, and urinary ketone excretion was evaluated using ketone reagent strips (Ketostix®, Ascensia Diabetes Care, Basel, Switzerland).

Statistical analyses. Differences between conditions and over time were evaluated using a three-way repeated-measures analysis of variance, while alterations between conditions at one timepoint were assessed using a two-way repeated-measures analysis of variance (GraphPad Prism version 8.3.0, La Jolla, CA, USA). When the sphericity assumption was violated (Mauchly's test), a Geisser-Greenhouse correction was used. If significant, post hoc analyses were performed using Bonferroni correction. A p-value < 0.05 was defined as statistically significant. All data are presented as mean ± SEM.

RESULTS

Blood ßHB concentration and urinary ketone excretion. Initial blood ßHB levels were below 0.4 mM in each condition ($p > 0.05$, Fig. 2A), and remained stable in CON and BIC. Conversely, ingestion of KE similarly elevated blood ßHB to ~2-4 mM during the initial 2 hours of $IMT_{180'}$ ($p < 0.05$ *vs.* baseline) in KE and KE+BIC, but by the end of $IMT_{180'}$ ßHB had reverted to 0.5 ± 0.1 mM ($p < 0.05$ *vs.* CON and BIC). Only a minor fraction (0.2 ± 0.1 g) of the ketone bodies ingested in the form of the ketone ester (65 g) in both KE and KE+ BIC was excreted via urine. Ketone bodies were undetectable (< 0.05 $g \cdot L^{-1}$) in urine during CON and BIC.

Blood glucose and lactate concentrations. Baseline blood glucose and lactate concentrations were similar between conditions (Fig. 2B and 3A). Compared with CON, KE as well as KE+BIC slightly reduced blood glucose levels (main effect of KE: $p < 0.05$), yet *post-hoc* analyses did not reveal a significant difference between conditions at any time point. KE did not affect blood lactate concentrations during RACE ($p > 0.05$ *vs.* CON, Fig 3A). However, in BIC and KE+BIC blood lactate concentrations by the end of $IMT_{180'}$, $TT_{15'}$ and the SPRINT were ~1-2 mM higher than in CON ($p < 0.05$).

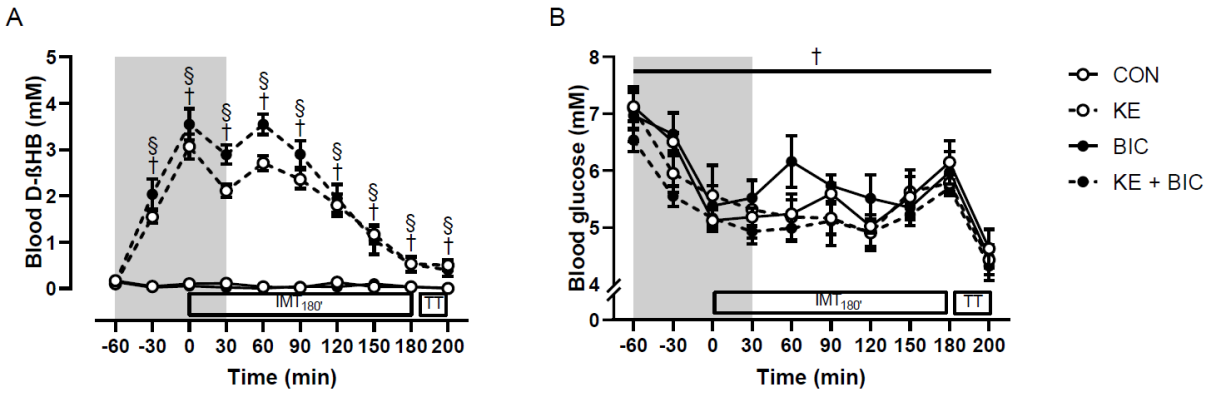


Figure 2. Effect of ketone ester intake, alone or combined with bicarbonate supplementation, on blood D-βHB and glucose during the simulated cycling race.
Data are mean ± SEM (n = 9) and represent blood D-βHB (A) and glucose (B) concentrations. Subjects participated in a simulated cycling race consisting of 3 h intermittent submaximal cycling ($IMT_{180'}$) followed by a 15 min simulated time trial (TT). Before and during the initial phase of $IMT_{180'}$ (grey zone) the subjects received either control (-○-, CON), ketone ester (-○·, KE), bicarbonate (-●-, BIC) or ketone ester plus bicarbonate supplements (-●·, KE + BIC). † $P < 0.05$ for main effect of KE (KE, KE + BIC *vs.* CON, BIC); # $P < 0.05$ for main effect of BIC (BIC, KE + BIC *vs.* CON, KE); § $P < 0.05$ *vs.* baseline (-60 min) for the indicated conditions.

Acid-base balance and arterial pCO_2. Baseline blood pH (~7.42, range: 7.37–7.45) and [HCO_3^-] (25.4 mM, range: 23.7–28.3 mM) were similar between conditions ($p > 0.05$, Fig. 3B-C). Compared with CON, KE caused blood pH (7.35 ± 0.01) and [HCO_3^-] (20.5 ± 0.5 mM) to drop by halfway $IMT_{180'}$ ($p < 0.05$). But intake of BIC fully negated this mild KE-induced acidosis. Moreover, KE alone or in conjunction with BIC resulted in higher blood pH (7.51 ± 0.01) and [HCO_3^-] (31.1 ± 0.6 mM) by the end of $IMT_{180'}$ compared to CON ($p < 0.05$). $TT_{15'}$ and SPRINT caused similar pH-drop between conditions, yet due to the higher initial value, at the end of the exercise protocol blood pH still was ~0.1 unit higher in KE and KE+BIC than in CON ($p < 0.05$). Conversely, from start $TT_{15'}$ to end of SPRINT blood [HCO_3^-] dropped more in BIC (- 9.1 ± 1.2 mM) and KE+BIC (-8.8 ± 1.0 mM) than in CON and KE (-6.2 ± 0.6 mM, $p < 0.05$). The transient pH-drop caused by KE ingestion during $IMT_{180'}$ was associated with ~10 mmHg lower pCO_2 compared to CON ($p < 0.05$). Conversely, pCO_2 was similar between CON, BIC and KE+BIC, at all times ($p > 0.05$).

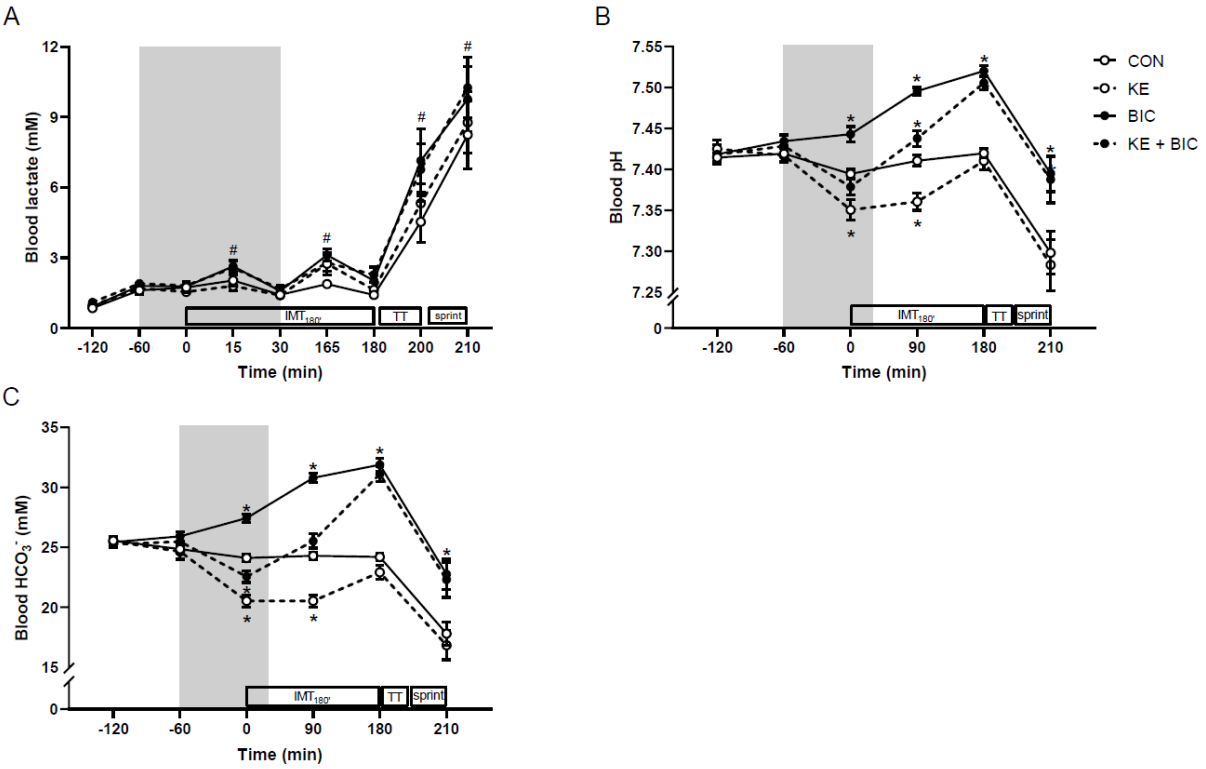


Figure 3. Effect of ketone ester and/or bicarbonate supplementation on blood lactate, pH and bicarbonate concentration.

Data are mean ± SEM for blood lactate (A), pH (B) and bicarbonate (HCO_3^-) concentration (C). In a crossover design, subjects (n = 9) received control (-O-, CON), ketone ester (-O·, KE), bicarbonate (-●-, BIC) or ketone ester and bicarbonate (-●·, KE + BIC) before and during exercise (grey zone). * $P < 0.05$ *vs.* CON for indicated condition; $^{\#}P < 0.05$ for main effect of BIC (BIC, KE + BIC *vs.* CON, KE). *$IMT_{180'}$* 3 h submaximal intermittent cycling, *TT* 15 min time-trial, *sprint* ~1min all-out exercise bout at 175% of lactate threshold.

Blood electrolytes (Figure 4). Blood calcium levels tended to be higher in KE compared to CON ($p = 0.06$). Concomitant ingestion of BIC significantly decreased blood calcium concentrations throughout RACE resulting in 0.08 mM lower values at the end of the SPRINT in KE+BIC compared to KE ($p < 0.05$). Blood sodium (+ 1.1 mM) and chloride (+ 1.2 mM) levels tended to be higher in KE compared to CON during RACE ($p = 0.08$ and $p = 0.05$, respectively). Additional BIC ingestion did not affect sodium concentrations ($p > 0.05$ *vs.* KE), but resulted in 9.2 mM lower blood chloride levels by the end of the RACE in KE+BIC compared to KE ($p < 0.05$). Blood potassium concentrations were not affected by any of the experimental conditions.

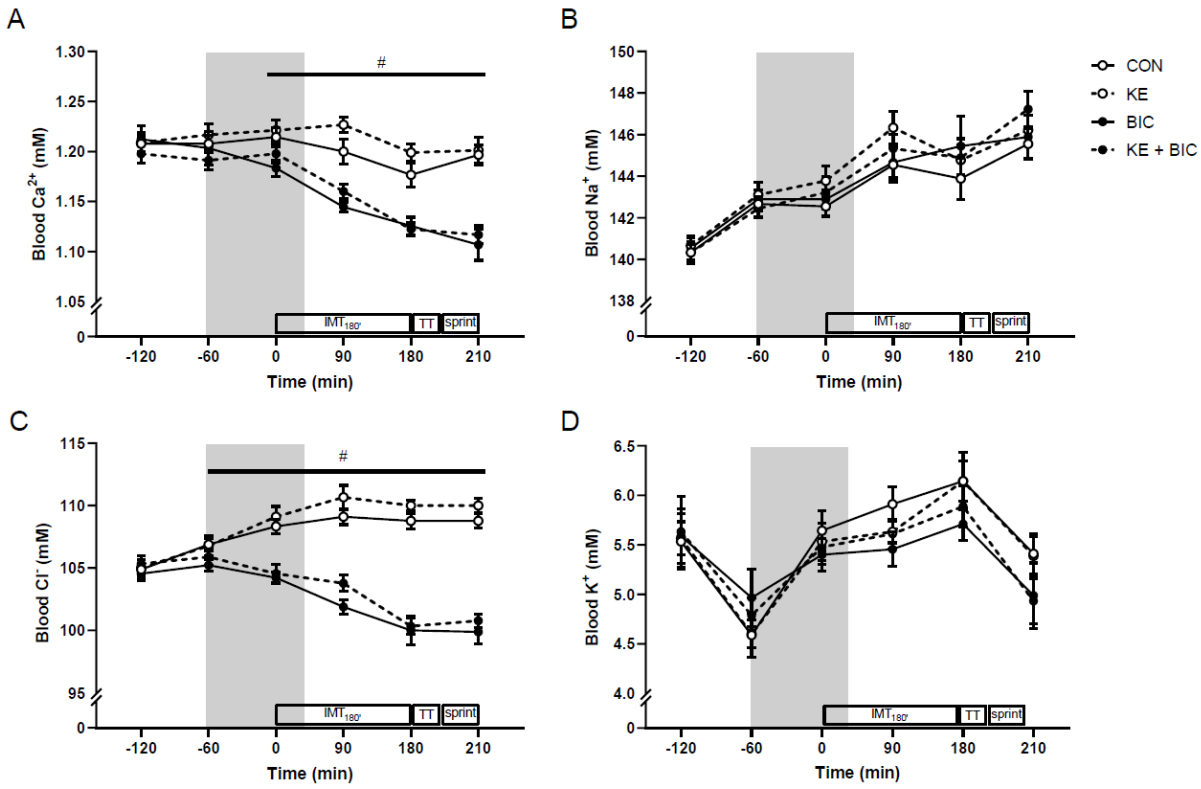


Figure 4. Effect of ketone ester and/or bicarbonate supplementation on blood electrolytes.
Data are mean ± SEM and represent blood calcium (Ca^{2+}) (A), sodium (Na^{+}) (B), chloride (Cl^{-}) (C) and potassium (K^{+}) (D) concentration. In a randomized, crossover design subjects (n = 9) received control (-○-, CON), ketone ester (-○·, KE), bicarbonate (-●-, BIC) or ketone ester and bicarbonate (-●·, KE + BIC) before and during exercise (grey zone). # $P < 0.05$ main effect of BIC (BIC, KE + BIC *vs.* CON, KE). *$IMT_{180'}$* 3 h submaximal intermittent cycling, *TT* 15 min time-trial, *sprint* ~1min all-out exercise bout at 175% of lactate threshold.

Exercise performance and RPE. Mean power output during $TT_{15'}$ in CON, BIC, and KE was ~255 W and was similar between these conditions ($p > 0.05$, Fig. 5A). Conversely, compared with CON, BIC, and KE, mean power output in KE+BIC from min 3 to 12 of $TT_{15'}$ was ~15 W higher, resulting in a 5% higher mean power output throughout $TT_{15'}$ (266 ± 4 W,

$p < 0.05$). In the final 3 min of $TT_{15'}$, KE conditions tended to increase their power output compared to CON and BIC ($p = 0.07$). Time to exhaustion in the SPRINT following $TT_{15'}$ was similar between conditions (CON: 55 ± 7; KE: 55 ± 6; BIC: 58 ± 6; KE+BIC: 58 ± 7 s, $p = 0.88$, Fig. 5B). No order effect was observed for both the performance outcomes ($p = 0.30$ and $p = 0.66$ for $TT_{15'}$ and SPRINT, respectively). Rate of perceived exertion was similar between experimental conditions at all times.

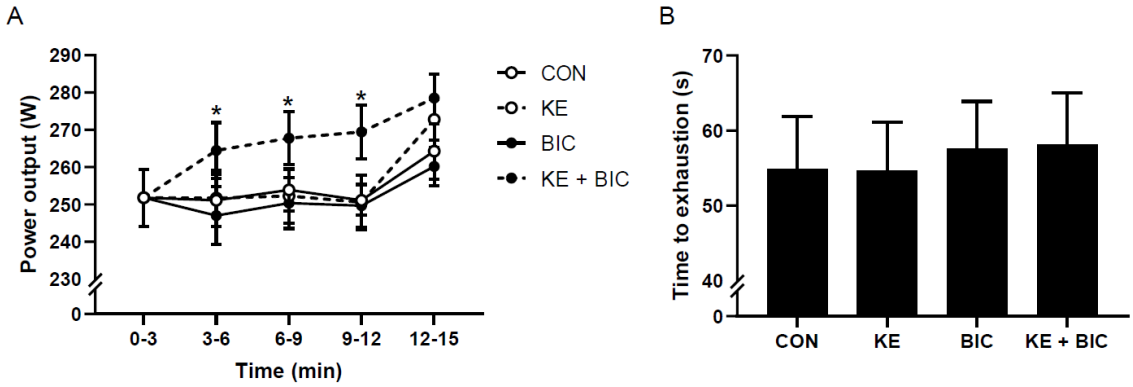


Figure 5. Effect of ketone ester and/or bicarbonate supplementation on exercise performance. Data are mean ± SEM and represent power output during each 3 min block of the 15 min simulated time-trial ($TT_{15'}$) (A) and time to exhaustion in the SPRINT (B). Subjects (n = 9) received in a crossover design control (-○-, CON), ketone ester (-○·, KE), bicarbonate (-●-, BIC) or ketone ester and bicarbonate (-●·, KE + BIC) before and during exercise. * $P < 0.05$ for indicated condition *vs.* CON.

Respiratory measurements and heart rate. Pulmonary gas exchange and heart rate were measured from min 40 to 55 and from min 165 to 175 during $IMT_{180'}$. The first time window (TW1) represents the episode of peak blood βHB concentration upon KE intake, whilst blood βHB had nearly returned to baseline by the start of the second time window immediately before $TT_{15'}$ (TW2). Compared to CON, V_E during TW1 was ~15% higher in KE and KE+BIC ($p < 0.05$, Table 1). In contrast, during TW2 V_E was higher in KE ($p < 0.05$ *vs.* CON) but not in KE+BIC ($p > 0.05$ *vs.* CON). The higher V_E during TW1 in KE and KE+BIC also resulted in a ~10% higher $\dot{V}O_2$ and $\dot{V}CO_2$ ($p < 0.05$ *vs.* CON). The respiratory exchange ratio (RER) was similar between conditions at all times ($p > 0.05$). Also heart rate during $IMT_{180'}$ and $TT_{15'}$ was similar between all conditions. But peak heart rate elicited by SPRINT on average was 6 ± 2 bpm higher in BIC and KE+BIC (~179 bpm) than in CON or KE (~173 bpm, main effect of bicarbonate: $p < 0.05$).

Table 1. Effect of ketone ester and/or bicarbonate supplementation on respiration during exercise.

	CON	KE	BIC	KE + BIC
V_E ($L \cdot min^{-1}$)				
40 – 55′	76 ± 4	88 ± 4 *	78 ± 5	88 ± 5 *
160 – 175′	84 ± 5	90 ± 3 *	80 ± 5	84 ± 5
$\dot{V}O_2$ ($L \cdot min^{-1}$)				
40 – 55′	2.9 ± 0.1	3.1 ± 0.1 *	3.2 ± 0.2 *	3.1 ± 0.1 *
160 – 175′	3.0 ± 0.1	3.0 ± 0.1	3.1 ± 0.1	3.0 ± 0.1
$\dot{V}CO_2$ ($L \cdot min^{-1}$)				
40 – 55′	2.7 ± 0.2	3.0 ± 0.1 *	3.0 ± 0.2 *	3.0 ± 0.2 *
160 – 175′	2.8 ± 0.1	2.8 ± 0.1	2.8 ± 0.1	2.8 ± 0.1

Values are mean ± SEM for minute ventilation (V_E), oxygen consumption ($\dot{V}O_2$) and carbon dioxide production ($\dot{V}CO_2$) from min 40 to 55 (TW1) and from min 160 to 175 (TW2) of the 3 h intermittent cycling bout ($IMT_{180'}$). In a crossover design, subjects (n = 9) received control (CON), ketone ester (KE), bicarbonate (BIC) or ketone ester and bicarbonate (KE+BIC) before and during exercise. * $P < 0.05$ for indicated condition *vs.* CON.

Gastrointestinal comfort and appetite perception. The incidence and severity of gastrointestinal symptoms was low to moderate (mean score of 17 on a 96-points scale, range: 2-43) and there was no difference between conditions ($p>0.05$). KE, but not KE+BIC suppressed the perception of hunger (KE: 3.8 ± 0.4 *vs.* CON: 5.6 ± 0.6 and KE+BIC: 5.9 ± 0.5, $p < 0.05$) as well as the desire to eat (KE: 4.0 ± 0.3 *vs.* CON: 5.8 ± 0.6 and KE+BIC: 5.8 ± 0.7, $p < 0.05$) after exercise, but perceived fullness and nutritional satisfaction was similar between conditions.

Identification of experimental conditions. After the final experimental session, the subjects were asked to identify the order of their experimental conditions. None of the subjects was able to correctly identify the four experimental conditions, indicating successful blinding.

DISCUSSION

The acute ergogenic effect of exogenous ketosis in endurance exercise is currently being debated. But in fact, apart from a single study yielding positive results in a 30-min time trial (1), most studies have failed to demonstrate acute ketogenic supplement intake before or during exercise to enhance performance (6-14). From a physiological perspective one could even logically speculate that high-dose oral ketone ester intake may negatively impact high-intensity endurance exercise performance by glycolytic inhibition on the one hand (22), but also by causing metabolic acidosis. Indeed, it is well documented that oral ketone ester intake in sufficient dosage to establish circulating βHB concentrations at ~3-5 mM during exercise, results in a significant pH drop (14, 24). Therefore, in this study we investigated whether counteracting KE-induced acidosis by $NaHCO_3$ co-ingestion may improve endurance exercise performance. We recently found that KE administration alone and in conjunction with adequate high rates of carbohydrate intake before and during exercise, did not enhance the capacity to sustain high power outputs in the final phase of a 3 h 15 min simulated cycling race (Poffé *et al.,* accepted, *J. Appl. Physiol.*). Here we repeated this experiment but administered KE either alone, or in conjunction with $NaHCO_3$. In line with our earlier findings, KE alone did not beneficially impact performance. However, concomitant $NaHCO_3$ administration, which was effective to fully counteract the anticipated ketoacidosis, resulted in 5% higher power outputs during the final 15 min of the race simulation. This effect was specifically due to a KE~$NaHCO_3$ interaction because neither KE alone nor $NaHCO_3$ alone improved performance. Our findings thus for the first time show that counteracting pH drop due to intake of oral ketogenic supplements can unmask the ergogenic effect of acute exogenous ketosis in endurance exercise.

Previous studies showed that blood pH and blood HCO_3^- content decrease for up to 1 h post ingestion of KE both at rest (24) and during submaximal exercise (14). In line with these observations, we observed that ingestion of 65 g KE over a 90 min period before and during the initial half an hour of a 3 h 15 min simulated cycling race resulted in a transient mild ketoacidosis (pH: ~7.35) with concomitant drop of alkaline reserve (see figure 3B-C). However, following the final KE bolus at min 30, values gradually restored to normal within the next 150 min. KE induced hyperventilation to compensate for the metabolic acidosis. This resulted in transient pCO_2 reduction from 40 to 32 mm Hg as well as slightly elevated $\dot{V}O_2$ and $\dot{V}CO_2$, which was probably largely due to increased repiratory work. Yet RER remained unaffected. It is not possible to exactly calculate substrate oxidation rates due to the inability to correct for

ketone oxidation. Nonetheless, the unaltered RER values further support our previous observation that during submaximal exercise under exogenous ketosis, but against the background of ample exogenous carbohydrate supply before and during exercise, ketone body oxidation does not substitute for carbohydrate oxidation (Poffé *et al.,* accepted, *J. Appl. Physiol.*).

Aiming to elucidate the physiological mechanisms underlying the ergogenic effect of KE+BIC in $TT_{15'}$, we evaluated both the respiratory and metabolic impact of the interventions by the end of $IMT_{180'}$. Interestingly, compared with KE alone, KE+BIC counteracted the increase in V_E at the end of $IMT_{180'}$. But neither $\dot{V}O_2$, $\dot{V}CO_2$ nor RER were different between KE and KE+BIC. This indicates that alterations in either pulmonary ventilation or substrate hierarchy did not account for the increased power output seen in $TT_{15'}$. However, the addition of $NaHCO_3$ ingestion to KE produced moderate alkalosis (pH @ ~7.51) in conjunction with elevated alkaline reserve (blood $[HCO_3^-]$ @ ~31.1 mM) by the start of $TT_{15'}$. Expectedly, the elevated pre-exercise alkaline reserve resulted in ~ 50% more HCO_3^- utilization during $TT_{15'}$ and SPRINT in KE+BIC than in KE. Unfortunately we did not assess blood acid-base balance at the end of $TT_{15'}$, i.e. prior to the start of SPRINT. However, blood lactate was measured at repeated time before and during $TT_{15'}$ and SPRINT. Clearly, $TT_{15'}$ increased circulating blood lactate concentration at a much faster rate during KE+BIC than during KE alone, resulting in 50% higher blood lactate by the end of $TT_{15'}$ in the former condition. These data are in line with previous findings indicating elevated extracellular alkaline reserve due to bicarbonate ingestion to facilitate the co-transport of H^+ and La^- out of skeletal muscle cells during exercise (33). Still, it cannot be excluded that the elevated La^- values also resulted from higher glyco(geno)lytic rate (34).

There is also some evidence to indicate that modulation of intra- and extracellular ion distributions, most prominently potassium, may be implicated in the ergolytic effect of exercise-induced acidosis (35). To date, one study has reported that KE intake decreases extracellular potassium concentrations at rest (24), which may depress muscular contractility during submaximal exercise (35). In contrast, we found KE and KE+BIC to impact serum $[K^+]$ level neither at rest nor during exercise. Furthermore, we found blood calcium and chloride concentrations at the start of $TT_{15'}$ to be significantly lower in KE+BIC than in KE. However, this effect was independent of KE because $NaHCO_3$ alone caused similar drop in extracellular calcium and chloride without affecting performance. In apparent contrast with the present

findings, we recently demonstrated that oral $NaHCO_3$ supplementation before and during a 3 h simulated cycling race improved power output by ~3% in a 90 s all-out exercise bout at the end of the event (Dalle *et al.*, in revision). However, the final SPRINT in the current study was preceded by $TT_{15'}$, which already raised blood lactate from ~2 to ~7 mM, and thus conceivably also reduced blood pH and blood alkaline reserve prior to the start of SPRINT. Hence the potential benefit of elevated pre-SPRINT pH and alkaline reserve probably was largely absorbed during $TT_{15'}$ (36), leaving hardly any benefit in terms of buffering capacity available for SPRINT.

We and others recently also reported that acute exogenous ketosis by KE ingestion suppresses hunger and the desire to eat both at rest (37) and immediately post-exercise (Poffé *et al.*, accepted, *J. Appl. Physiol.*), Hence KE may suppress nutritional intake after exercise, which in turn might impair muscle repair and training adaptation (38). The data of the current study confirm these previous observations. Although the precise mechanism by which KE decreases appetite remains to be confirmed, it is the prevailing opinion that the appetite-suppressing effect of ketosis is directly caused by the ketone body ßHB. ßHB has been shown to decrease appetite both via central actions in the brain (39) as well as by decreasing ghrelin concentrations in the blood (37). However, the reduced appetite following KE may also result from ketoacidosis (40). In support of the latter hypothesis, here we show that co-ingestion of bicarbonate completely negated the effect of KE to suppress appetite.

Gastrointestinal distress is a potential source of concern whenever considering KE or bicarbonate supplementation before and during exercise. However, in line with recent studies from our group (2) and others (16), KE resulted in similar gastrointestinal discomfort as ingestion of carbohydrate drinks. Moreover, the addition of $NaHCO_3$ intake did not increase symptoms. Thus, in the conditions of the current study, co-ingestion of KE and $NaHCO_3$, against the background of high rate carbohydrate intake during exercise, was well-tolerated.

In conclusion, in this study we for the first time demonstrate that co-ingestion of KE and $NaHCO_3$ to induce transient exogenous ketosis while avoiding ketoacidosis, is a safe and well-tolerated nutritional intervention to stimulate high-intensity exercise performance in the final stage of a prolonged endurance exercise event.

ADDITIONAL INFORMATION

Competing interests

The authors declare that they have no competing interests.

Author contributions

Conception and design of the study: CP and PH. Data collection: CP, MR and SB. Analysis and interpretation of the data and manuscript drafting: CP and PH. All authors critically evaluated the manuscript and approved for submission. All authors agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

Funding

This study was funded by Research Fund Flanders (Fonds voor Wetenschappelijk Onderzoek – Vlaanderen; research grant no. G080117N).

Acknowledgements

The authors wish to thank all subjects for their dedicated cooperation in this demanding trial.

REFERENCES

1. Cox PJ, Kirk T, Ashmore T, et al. Nutritional ketosis alters fuel preference and thereby endurance performance in athletes. *Cell Metab.* 2016;24(2):256-68.

2. Poffé C, Ramaekers M, Van Thienen R, Hespel P. Ketone ester supplementation blunts overreaching symptoms during endurance training overload. *J Physiol.* 2019;597(12):3009-27.

3. Poffé C, Ramaekers M, Van Thienen R, Hespel P. Reply from Chiel Poffé, Monique Ramaekers, Ruud Van Thienen and Peter Hespel. *J Physiol.* 2019;597(16):4409-10.

4. Poffé C, Ramaekers M, Van Thienen R, Hespel P. Reply from Chiel Poffé, Monique Ramaekers, Ruud Van Thienen and Peter Hespel. *J Physiol.* 2019;597(17):4679-80.

5. Poffé C, Ramaekers M, Hespel P. Reply from Chiel Poffé, Monique Ramaekers and Peter Hespel. *J Physiol.* 2019;597(21):5309-10.

6. Evans M, Egan B. Intermittent running and cognitive performance after ketone ester ingestion. *Med Sci Sport Exerc.* 2018;50(11):2330-8.

7. Waldman HS, Basham SA, Price FG, et al. Exogenous ketone salts do not improve cognitive responses after a high-intensity exercise protocol in healthy college-aged males. *Appl Physiol Nutr Metab.* 2018;43(7):711-7.

8. Evans M, McSwiney FT, Brady AJ, Egan B. No benefit of ingestion of a ketone monoester supplement on 10-km running performance. *Med Sci Sports Exerc.* 2019;51(12):2506-15.

9. Scott BE, Laursen PB, James LJ, et al. The effect of 1,3-butanediol and carbohydrate supplementation on running performance. *J Sci Med Sport.* 2019;22(6):702-6.

10. Shaw DM, Merien F, Braakhuis A, Plews D, Laursen P, Dulson DK. The effect of 1,3-butanediol on cycling time-trial performance. *Int J Sport Nutr Exerc Metab.* 2019;29(5):466-73.

11. Leckey JJ, Ross ML, Quod M, Hawley JA, Burke LM. Ketone diester ingestion impairs time-trial performance in professional cyclists. *Front Physiol.* 2017;8(OCT):1-10.

12. O'Malley T, Myette-Cote E, Durrer C, Little JP. Nutritional ketone salts increase fat oxidation but impair high-intensity exercise performance in healthy adult males. *Appl Physiol Nutr Metab.* 2017;42(10):1031-5.

13. Rodger S, Plews D, Laursen P, Driller M. Oral β-hydroxybutyrate salt fails to improve 4-minute cycling performance following submaximal exercise. *J Sci Cycl.* 2017;6(1):26-31.

14. Dearlove DJ, Faull OK, Rolls E, Clarke K, Cox PJ. Nutritional ketoacidosis during incremental exercise in healthy athletes. *Front Physiol.* 2019;10(290):1-6.

15. Margolis LM, O'fallon KS. Utility of ketone supplementation to enhance physical performance: a systematic review. *Adv Nutr.* 2019;1-8.

16. Stubbs BJ, Cox PJ, Kirk T, Evans RD, Clarke K. Gastrointestinal effects of exogenous ketone drinks are infrequent, mild, and vary according to ketone compound and dose. *Int J Sport Nutr Exerc Metab.* 2019;29(6):596-603.

17. Cox PJ, Clarke K. Acute nutritional ketosis: implications for exercise performance and metabolism. *Extrem Physiol Med.* 2014;3(17):1-9.

18. Owen OE, Morgan AP, Kemp HG, Sullivan JM, Herrera MG, Cahill GF. Brain metabolism during fasting. *J Clin Invest.* 1967;46(10):1589-95.

19. Burke LM, Ross ML, Garvican-Lewis LA, et al. Low carbohydrate, high fat diet impairs exercise economy and negates the performance benefit from intensified training in elite race walkers. *J Physiol.* 2017;595(9):2785-807.

20. Burke LM, Hawley JA, Wong SHS, Jeukendrup AE. Carbohydrates for training and competition. *J Sports Sci.* 2011;29(1):17-27.

21. De Bock K, Richter EA, Russell AP, et al. Exercise in the fasted state facilitates fibre type-specific intramyocellular lipid breakdown and stimulates glycogen resynthesis in humans. *J Physiol.* 2005;564(November):649-60.

22. Newsholme EA, Randle PJ, Manchester KL. Inhibition of the phosphofructokinase reactin in perfused rat heart by respiration of ketone bodies, fatty acids, and pyruvate. *Nature.* 1962;193(4812):270-1.

23. Robinson AM, Williamson DH. Physiological roles of ketone bodies as substrates and signals in mammalian tissues. *Physiol Rev.* 1980;60(1):143-87.

24. Stubbs BJ, Cox PJ, Evans RD, et al. On the metabolism of exogenous ketones in humans. *Front Physiol.* 2017;8:848.

25. Westerblad H. Acidosis is not a significant cause of skeletal muscle fatigue. *Med Sci Sports Exerc.* 2016;48(11):2339-42.

26. Carr AJ, Hopkins WG, Gore CJ. Effects of acute alkalosis and acidosis on performance. A meta-analysis. *Sport Med.* 2011;41(10):801-14.

27. Robergs R, Hutchinson K, Hendee S, Madden S, Siegler J. Influence of pre-exercise acidosis and alkalosis on the kinetics of acid-base recovery following intense exercise. *Int J Sport Nutr Exerc Metab.* 2005;15(1):59-74.

28. McNaughton L, Curtin R, Goodman G, Perry D, Turner B, Showell C. Anaerobic work and power output during cycle ergometer exercise: Effects of bicarbonate loading. *J Sports Sci.* 1991;9(2):151-60.

29. Dalle S, De Smet S, Geuns W, Van Rompaye B, Hespel P, Koppo K. Effect of stacked sodium bicarbonate loading on repeated all-out exercise. *Int J Sport Med.* 2019;40(11):711-6.

30. Borg G. Psychophysical scaling with applications in physical work and the perception of exertion. *Scand J Work Environ Heal.* 1990;16(SUPPL. 1):55-8.

31. Pfeiffer B, Cotterill A, Grathwohl D, Stellingwerff T, Jeukendrup AE. The effect of carbohydrate gels on gastrointestinal tolerance during a 16-km run. *Int J Sport Nutr Exerc Metab.* 2009;19(5):485-503.

32. Woods AL, Rice AJ, Garvican-Lewis LA, et al. The effects of intensified training on resting metabolic rate (RMR), body composition and performance in trained cyclists. *PLoS One.* 2018;13(2):1-24.

33. Spriet LL, Lindinger MI, Heigenhauser GJF, Jones NL. Effects of alkalosis on skeletal muscle metabolism and performance during exercise. *Am J Physiol - Regul Integr Comp Physiol.* 1986;251(5):833-9.

34. Siegler JC, Marshall PWM, Bishop D, Shaw G, Green S. Mechanistic insights into the efficacy of sodium bicarbonate supplementation to improve athletic performance. *Sport Med.* 2016;2(1):1-13.

35. Cairns SP, Lindinger MI. Do multiple ionic interactions contribute to skeletal muscle fatigue? *J Physiol.* 2008;586(17):4039-54.

36. Gough LA, Deb SK, Sparks SA, McNaughton LR. Sodium bicarbonate improves 4 km time trial cycling performance when individualised to time to peak blood bicarbonate in trained male cyclists. *J Sports Sci.* 2018;36(15):1705-12.

37. Stubbs BJ, Cox PJ, Evans RD, Cyranka M, Clarke K, de Wet H. A ketone ester drink lowers human ghrelin and appetite. *Obesity.* 2018;26(2):269-73.

38. American Dietetic Association, Dietitians of Canada, American College of Sports Medicine, Rodriguez N, Di Marco N, Langley S. American College of Sports Medicine position stand. Nutrition and athletic performance. *Med Sci Sports Exerc.* 2009;41(3):709-31.

39. Laeger T, Pöhland R, Metges CC, Kuhla B. The ketone body β-hydroxybutyric acid influences agouti-related peptide expression via AMP-activated protein kinase in hypothalamic GT1-7 cells. *J Endocrinol.* 2012;213(2):193-203.

40. Zheng ZH, Anderstam B, Yu X, Qureshi AR, Heimbürger O, Lindholm B. Bicarbonate-based peritoneal dialysis solution has less effect on ingestive behavior than lactate-based peritoneal dialysis solution. *Perit Dial Int.* 2009;29(6):656-63.

Article 3

Ketone ester supplementation blunts overreaching symptoms during endurance training overload

Chiel Poffé[1], Monique Ramaekers[1], Ruud Van Thienen[1], Peter Hespel[1,2] #

[1] Exercise Physiology Research Group, Department of Movement Sciences, KU Leuven, Leuven, Belgium

[2] Bakala Academy-Athletic Performance Center, KU Leuven, Leuven, Belgium

#, Correspondence: Peter Hespel, Exercise Physiology Research Group, Department of Movement Sciences, KU Leuven, Tervuursevest 101, Heverlee, 3001, Belgium

peter.hespel@kuleuven.be

Published in J Physiol (2019) 597: 12, 3009-3027

ABSTRACT

It is well known that elevated blood ketones attenuate net muscle protein breakdown, as well as negate catabolic events, during energy deficit. Therefore, we hypothesized that oral ketones can blunt endurance training-induced overreaching. Fit male subjects participated in two daily training sessions (3 weeks, 6 days/week) while receiving either a ketone ester (KE, n = 9) or a control drink (CON, n = 9) following each session. Sustainable training load in week 3, as well as power output in the final 30 min of a 2 h standardized endurance session were 15% higher in KE than in CON (both $p < 0.05$). KE inhibited the training-induced increase in nocturnal adrenaline ($p < 0.01$) and noradrenaline ($p < 0.01$) excretion, as well as blunted the decrease in resting (CON: -6 ± 2 bpm; KE: +2 ± 3 bpm, $p < 0.05$), submaximal (CON: -15 ± 3 bpm; KE: -7 ± 2 bpm, $p < 0.05$) and maximal (CON: -17 ± 2 bpm; KE: -10 ± 2 bpm, $p < 0.01$) heart rate. Energy balance during the training period spontaneously turned negative in CON (-2135 kJ/d), but not in KE (+198 kJ/d). The training consistently increased growth differentiation factor 15 (GDF15), but ~2-fold more in CON than in KE ($p < 0.05$). In addition, delta GDF15 correlated with the training-induced drop in maximal heart rate ($r = 0.60$, $p < 0.001$) and decrease in osteocalcin ($r = 0.61$, $p < 0.01$). Other measurements such as blood ACTH, cortisol, IL-6, leptin, ghrelin, and lymphocyte count, and muscle glycogen content, did not differentiate KE from CON. In conclusion, KE during strenuous endurance training attenuates the development of overreaching. We also identify GDF15 as a possible marker of overtraining.

Keywords: ketone, overreaching and overtraining, exercise recovery, GDF15

KEY POINTS SUMMARY

- Overload training is required for sustained performance gain in athletes (functional overreaching). However, excess overload may result in a catabolic state which causes performance decrements for weeks (non-functional overreaching) up to months (overtraining).
- Blood ketone bodies can attenuate training- or fasting-induced catabolic events. Therefore, we investigated whether increasing blood ketone levels by oral ketone ester intake (KE), can protect against endurance training-induced overreaching.
- We show for the first time that KE intake post-exercise markedly blunts the development of physiological symptoms indicating overreaching, and at the same time significantly enhances endurance exercise performance.
- We provide preliminary data to indicate that GDF15 may be a relevant hormonal marker to diagnose the development of overtraining.
- Collectively, our data indicate that ketone ester intake is a potent nutritional strategy to prevent the development of non-functional overreaching and to stimulate endurance exercise performance.

INTRODUCTION

Endurance athletes intermittently participate in overload training (*e.g.,* training camps) or competition (*e.g.,* multi-stage cycling races) with the express purpose of eliciting physiological responses that are crucial for sustained performance gain. Training overload, however, must be consistently well-balanced with recovery, allowing physiological repair mechanisms to produce beneficial adaptations that eventually yield performance gains (*e.g.,* functional overreaching). Inadequate recovery, with or without other stress factors, such as sleep deprivation, negative energy balance, disease, or mental fatigue, eventually can result in a maladaptive catabolic state requiring days to weeks (non-functional overreaching), or even months (overtraining) to fully recover (Meeusen *et al.* 2013). Since sufficient recovery will only result in performance improvements during functional overreaching, prevention of non-functional overreaching is pivotal in training management.

The primary indication of overreaching is a stagnation or decrease in training-specific performance (Hooper *et al.* 1995; Urhausen & Kindermann, 2002). But this is often preceded by numerous other symptoms, such as mood disturbances (Morgan *et al.* 1987; Killer *et al.* 2017) and dysregulation in various physiological systems, including the autonomic nervous system (Lehmann *et al.* 1998), immunity (Fry *et al.* 1994b) and energy metabolism (Lombardi *et al.* 2012). Nevertheless, previous studies have failed to identify consistent physiological outcomes (Urhausen & Kindermann, 2002; Cadegiani & Kater, 2017) that can be used to predict overtraining. In fact, the pathophysiology of overtraining remains poorly understood, which impairs the design of optimal preventive interventions (Armstrong & Vanheest, 2002; Kreher & Schwartz, 2012).

Numerous studies have focused on the use of post-exercise nutrition to facilitate recovery between training sessions and thereby counteract overtraining (Kreider *et al.* 2010; Hawley *et al.* 2011). Amongst various nutritional interventions, protein-carbohydrate co-ingestion is recognized to be the best strategy to enhance recovery by stimulating both muscle glycogen resynthesis (van Loon *et al.* 2000) and muscle repair (Breen *et al.* 2011). However, protein plus carbohydrate ingestion is insufficient to prevent overtraining (Achten *et al.* 2004; Halson *et al.* 2004; Witard *et al.* 2011; D'Lugos *et al.* 2016; Svendsen *et al.* 2016). Other nutritional interventions, such as anti-oxidant intake to protect against exercise-induced oxidative stress, fail to negate overtraining (Gleeson & Bishop, 2000; Meeusen & Watson, 2007). In contrast,

consistent anti-oxidant intake may even inhibit beneficial training adaptations (Merry & Ristow, 2016).

Some recent publications have stimulated interest in the use of exogenous ketone supplements as a novel fuelling strategy to modulate metabolic responses both during (Cox *et al.* 2016; Leckey *et al.* 2017) and after exercise (Holdsworth *et al.* 2017; Vandoorne *et al.* 2017). Ketone bodies, namely D-ß-hydroxybutyrate (ßHB), acetoacetate (AcAc) and acetone, are fatty-acid derived compounds that can serve as an alternative energy substrate for active metabolic tissues including brain (Owen *et al.* 1967), heart (Aubert *et al.* 2016), or skeletal muscles (Balasse & Féry, 1989) under conditions of metabolic stress (Johnson *et al.* 1969; Cahill, 1976). Aside from their role in energy supply, ketone bodies also can play a role in metabolic regulation by inhibiting muscle proteolysis (Thomsen *et al.* 2018) and glucose depletion (Robinson & Williamson, 1980), and stimulation of muscle regeneration or remodelling by enhancing satellite cell activation and differentiation (Zou *et al.* 2016). Moreover, ketone bodies directly regulate a range of putative factors involved in the development of overtraining, such as autonomic neural output, inflammation and oxidative stress (Kimura *et al.* 2011; Puchalska & Crawford, 2017). These actions clearly indicate a potential role for ketone bodies in overtraining prevention, but the dietary conditions required to elevate blood ketone levels, by sustained low carbohydrate high fat diet, are detrimental to endurance exercise performance (Cox & Clarke, 2014).

However, exogenous ketone supplements recently emerged as a novel approach to induce ketosis. Exogenous supplements are available either in the form of ketone salts or ketone esters (Cox *et al.* 2016; Brownlow *et al.* 2017; Leckey *et al.* 2017), but ketone esters allow blood ketones to reach ~3-fold higher levels than salts, with less incidence of gastrointestinal problems (Stubbs *et al.* 2017; Sansone *et al.* 2018). Recent studies in our and other laboratories showed that post-exercise ingestion of the ketone monoester (R)-3-hydroxybutyl (R)-3-hydroxybutyrate (KE) stimulates markers of protein synthesis and potentially also muscle glycogen repletion following exercise (Holdworth *et al.* 2017; Vandoorne *et al.* 2017). But these experiments have only looked at the acute effects following a single, high-intensity exercise bout. The effects of long-term ketone ester intake in training and recovery today are entirely unknown. Given the evidence that βHB infusion antagonizes starvation-induced catabolic processes (Sherwin *et al.* 1975; Pawan & Semple, 1983), it is reasonable to postulate that ketosis

induced by consistent exogenous ketone intake during strenuous training may alleviate detrimental catabolic events.

Therefore, we performed a double-blind, placebo-controlled study to assess the effect of KE during strenuous endurance training with the express purpose of producing a state of non-functional overreaching. We hypothesized that KE can attenuate training-induced pathophysiological effects leading up to non-functional overreaching, and eventually enhance endurance exercise performance.

METHODS

Ethical approval and subjects. Twenty healthy, physically active males were recruited to participate in this study, which was approved by the KU Leuven Biomedical Ethics Committee (B322201733747), and conforms to the Declaration of Helsinki. Potential subjects were screened using a medical questionnaire and a physical examination, including a resting electrocardiogram, prior to involvement in the study. From the initial twenty recruits, one subject did not complete the study due to adverse reactions to the protein-carbohydrate drinks prescribed by the study protocol, and another withdrew for reasons unrelated to the study protocol. Eighteen subjects eventually completed the full study protocol and were included in the final data analyses (for subjects' characteristics see **Table 1)**. All subjects were regularly involved in sports and physical activity at a rate of 4.8 ± 0.4 $h \cdot wk^{-1}$ (mean ± SEM), but none were consistently engaged in cycling. Throughout the entire study period, subjects were instructed to refrain from strenuous exercise other than prescribed by the study protocol. All subjects were informed of the content and potential risks involved with the experimental procedures before providing their written consent.

TABLE 1. Subject characteristics.

	Control	Ketone Ester
Age (y)	21.2 ± 2.9	21.4 ± 2.4
Height (m)	1.81 ± 0.04	1.80 ± 0.04
Body mass (kg)	74.6 ± 10.5	72.8 ± 6.5
$\dot{V}O_2$ max ($ml \cdot kg^{-1} \cdot min^{-1}$)	55.3 ± 6.1	55.9 ± 5.5

Values are mean ± SD and represent baseline characteristics of the subjects receiving either control (n = 9) or ketone ester (n = 9).

Preliminary Testing and Subject Randomization. Two weeks before baseline measurements, the subjects completed 3 preliminary sessions with a 48-h rest interval in between. During the first visit, subjects performed a maximal incremental exercise test on a bicycle ergometer (Avantronic Cyclus II, Leipzig, Germany) to determine their $\dot{V}O_2$ max. Initial workload was set at 70 W, followed by 25 W increments per min, until volitional exhaustion. Respiratory gas exchange was measured continuously during the test (Cortex Metalyzer II, Leipzig, Germany) and the highest oxygen uptake measured over a 30 s period was defined as the maximal oxygen uptake rate ($\dot{V}O_2$ max). During the second and third session, subjects were familiarized with the exercise testing procedures to undergo in the experimental sessions. Subjects started with a 10 min warming up (5 min at 100 W, 5 min at 150 W) followed by a 30 min simulated time-trial (TT_{30min}) on a cycling ergometer (Avantronic Cyclus II, Leipzig, Germany). They were instructed to maintain their cadence between 80 and 100 $rev \cdot min^{-1}$, and to adjust the resistance at 5 min intervals from t5 to t25, and every min from t25 to t30 to develop the highest possible mean power output (W) over 30 min. Following 15 min of active recovery by cycling at 50 W, the subjects performed a 90 s all-out sprint (90S) on a self-constructed isokinetic cycling ergometer (210, 211) with cadence fixed at 90 $rev \cdot min^{-1}$. Following familiarization, the subjects were pair-matched to obtain two groups with similar distributions for $\dot{V}O_2$ max, mean power outputs in TT_{30min} and 90S, training history (hours/week), and body mass and height. The matched pairs were then randomly split into two experimental groups by an investigator who was otherwise not involved in the trial.

General Experimental Design. After randomization the subjects were enrolled in a fully-controlled three-week intensive cycling training program which was designed with the express purpose of inducing a state of non-functional overreaching. During the training period the subjects received either a ketone ester drink (KE, n = 9) or a corresponding control drink (CON, n = 9). Following the training period, the subjects were followed-up during a tapering week without training.

Training Intervention. A detailed overview of the training program is shown in **Figure 1**. The three-week overload training program (28 training sessions) consisted of a combination of high-intensity interval training (HIIT), intermittent endurance training (IMT), and constant-load endurance training (ET) sessions. All training sessions were performed in the laboratory on an electromagnetically braked ergometer and under the careful supervision of the investigators. The HIIT and IMT sessions were done on calibrated cycling ergometers (Avantronic Cyclus II,

Leipzig, Germany) in order to be able to monitor the power output~heart rate relationship (Watt). The ET sessions were alternatingly performed on the same calibrated ergometers or on routine cycling ergotrainers (Tacx Neo Smart, Wassenaar, The Netherlands). The workload in ET sessions on the routine ergotrainers was based on the heart rate~power relationship obtained from the sessions on the calibrated ergometers (2-3x/wk). During the ET sessions, subjects cycled at a continuous load (70-95% of mean power output TT_{30min}) for 60 to 150 minutes. HIIT sessions consisted of 30 s maximal sprints at a cadence fixed at 100 rev·min^{-1}, interspersed by 4 min 30 s active recovery intervals at 50 W. The number of sprints was increased from four in week 1, to five in week 2, and six in week 3. Subjects were given verbal encouragement to perform maximally during each sprint. IMT sessions consisted of five 6-min bouts at 100-110% of mean power output taken from TT_{30min}, separated by 8 min lower intensity at 55-85%. During the final week, the duration of the high-intensity bouts was increased to 8 min, whilst decreased to 6 min for the lower intensity bouts. Exercise intensities of IMT and ET were calculated relative to the mean power output effected during TT_{30min} in the pre-test. The intensity of each training session can be found in **Figure 1**. If a subject failed to maintain a cadence ≥ 70 rev·min^{-1} at the prescribed workload, the workload was decreased to the level that allowed the subjects to return and maintain a cadence ≥70 rev·min^{-1}.

		Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
Week 1	AM	70 min **IMT** 100/55%	Rest	30 min **HIIT**	70 min **IMT** 100/55%	30 min **HIIT**	70 min **IMT** 100/55%	***Test Wk1***
	PM	Rest	Rest	60 min **ET** 70%	60 min **ET** 70%	60 min **ET** 70%	60 min **ET** 70%	60 min **ET** 77.5%
Week 2	AM	70 min **IMT** 100/65%	Rest	30 min **HIIT**	70 min **IMT** 105/65%	30 min **HIIT**	70 min **IMT** 110/80%	***Test Wk2***
	PM	Rest	Rest	90 min **ET** 77.5%	60 min **ET** 85%	90 min **ET** 80%	60 min **ET** 90%	90 min **ET** 85%
Week 3	AM	120 min **HIIT & ET** 85%	Rest	70 min **IMT** 110/80%	120 min **EPT_{120min}** 85% - 30' all-out	70 min **IMT** 110/80%	70 min **IMT** 110/85%	***Posttest Test Wk3***
	PM	Rest	Rest	90 min **ET** 90%	Rest	120 min **ET** 95%	150 min **HIIT & ET** 92.5%	Rest

FIGURE 1. Overview of the training program.
HIIT, High-Intensity Interval Training. 30 s all-out sprints interspersed by 4.5 min active recovery intervals (50 W). The number of sprints was increased from 4 in week 1, to 5 in week 2, and 6 in week 3; IMT, Intermittent endurance sessions. Week 1 and 2: 5 x 6 min with 8 min recovery. Week 3: 5 x 8 min with 6 min recovery; ET, Constant-load endurance training sessions. EPT_{120min}, 120 min endurance performance test. Training intensities for IMT and ET are expressed as a percentage of the mean power output effected during the 30 min time-trial (TT_{30min}) in the pre-test.

Post-exercise Nutritional Intervention. Subjects from both experimental groups received a 500 ml high-dose protein-carbohydrate drink (**Table 2**) thirty minutes after each exercise session. In addition, immediately following each session and 30 min pre-sleep, KE subjects received 25 g of ketone-ester [96% (R)-3-hydroxybutyl (R)-3-hydroxybutyrate] to elevate post-exercise circulating plasma ketone concentrations, as previously shown by our lab (Vandoorne *et al.* 2017). The ketone ester supplements were purchased from TdeltaS Ltd (Thame Oxfordshire, UK). Subjects in CON received an isocaloric drink (CON) containing 16.4 g pure medium-chain triglycerides (Now Foods, Bloomingdale, Illinois). To equalize the taste and appearance of CON and KE drinks, 1 mM bitter sucrose octaacetate (Sigma-Aldrich, Bornem, Belgium) was added to the CON, whilst a red colorant (AVEVE Bloem, Merksem, Belgium) was added to both the drinks to obtain similar appearance. Subjects received 50 ml of diet coke for mouth rinsing immediately following the KE or CON drink to improve palatability (Leckey *et al.* 2017). Capillary blood samples from the earlobe were obtained pre- and post-exercise and 30 min following ingestion of the supplements to assess circulating blood β-hydroxybutyrate concentrations (Glucomen Lx plus-meter with Lx β-ketone strips, Menarini Diagnostics, Firenze, Italy) during IMT sessions on day 6, 13 and 20 of the training period.

TABLE 2.Composition of protein-carbohydrate recovery drinks.

	Protein-carbohydrate recovery mixture
Total carbohydrates	60.6 g
Maltodextrin	30.6 g
Sucrose	20.0 g
Fructose	10.0 g
Total protein	31.0 g
Non-essential amino acids	15.9 g
Essential amino acids	15.1 g
Of which leucine	*3.13 g*

Composition of protein-carbohydrate drinks given 30 min following each training session. Data represent grams of ingredients per 500 ml drink.

Experimental Trials. Before (pre-test), during (at day 7 and 14), and after (post-test) the training period, as well as during the recovery phase (3d and 7d post), the subjects participated in an experimental session involving a TT_{30min} and 90 s isokinetic sprint. On the evening before each session, they received a standardized carbohydrate-rich dinner (~5400 kJ; 69%

carbohydrate, 16% fat, 15% protein). Next morning and after an overnight fast, a blood sample was collected from both an earlobe (capillary blood) and from an antecubital vein (Venoject, Tokyo, Japan). The subjects then received a standardized breakfast (~2700 kJ; 71% carbohydrate, 15% fat, 14% protein). Following a 1.5 h rest in a comfortable chair they warmed up for 10 min at incremental workloads corresponding to 70% (5 min) and 85% (5 min) of their average power output recorded during TT_{30min} in the last familiarization session. Thereafter, they performed a TT_{30min} in which they aimed for the highest possible mean power output. During the first five minutes (t0-t5), the workload was set equal to the average power obtained during the last familiarization session. From t5 to t25, subjects were allowed to adjust the workload at 5-min intervals according to their subjective perception of fatigue. From t25 to t30, 1-min adjustments were allowed to facilitate full exhaustion by the end of TT_{30min}. Subjects were allowed to drink water *ad libitum* and received on-line feedback about the time to completion. On completion of the TT_{30min}, they recovered for 15 min by cycling at 50 W, followed by the 90S. On day 18 of the training period, a 120 min endurance exercise performance test (EPT_{120min}) was included to assess endurance performance (186). This session consisted of a 90 min preload (cycling at 85% of the mean power output effected during TT_{30min} in the pre-test) to induce fatigue, followed by an all-out 30 min time trial. During these tests, heart rate was monitored continuously (Polar RS800CX, Kempele, Finland), while blood lactate concentration was measured (Lactate Pro2, Arkray, Japan) in a capillary blood sample from the earlobe at 5-min intervals during TT_{30min}, and 3 min after completion of 90S. Ratings of perceived exertion were recorded immediately after completion of TT_{30min}, 90S and EPT_{120min} (RPE, 6-20 Borg Scale) (Borg, 1990). Power outputs were blinded to the subjects during all tests. Standardized verbal encouragement was provided only during 90S. During the pre- and post-test, an additional blood sample was obtained from an antecubital vein (Venoject, Tokyo, Japan) immediately before and after the TT_{30min}.

Body Composition and Anthropometry. Whole-body dual-energy X-ray absorptiometry (DXA) scans (Discovery W, Hologic Inc, Bedford, MA) were made during both the pre-test and the post-test. Scans were performed in the fasted-state and at the same time of the day during both sessions to minimise measurement errors (Bone & Burke, 2016). Output parameters considered were whole-body bone mineral content (BMC) and density (BMD), and percent body fat and lean soft tissue mass. All scans were performed by a single certified technician, and subject positions over the different sessions were standardized according to the manufacturer's recommendations. The densitometer was calibrated daily against a spinal

phantom to account for potential day-to-day variability. Following the DXA-scan, skinfold thickness was measured at twelve sites (biceps, triceps, subscapular, supra-iliac, midaxillary, iliac-crest, abdomen, chin, anterior thigh, posterior thigh, lateral calf, and medial calf) according to standard procedures.

Subjective Feelings of Appetite, Gastro-intestinal Discomfort and Recovery-stress State. During each experimental session, subjects completed 3 questionnaires to assess their (i) appetite sensations, (ii) gastro-intestinal discomfort, and (iii) recovery-stress state. Appetite was assessed immediately before breakfast using a validated 0-10 Likert visual analogue scale (VAS), adapted from Woods *et al.* (2018). Subjects were provided with four appetite or satiety questions ("How hungry do you feel?", "How full do you feel?", "How satisfied do you feel?", "How much do you think you could eat now?"). Gastro-intestinal discomfort was rated after completion of breakfast by means of a 0-8 Likert scale questionnaire adapted from *Pfeiffer et al.* (2009). The questionnaire comprised three sections, *i.e.* upper abdominal problems (reflux, bloating, nausea, vomiting); lower abdominal problems (cramps, flatulence, abdominal pain, diarrhea); and systemic problems (dizziness, headache, muscle cramp, urge to urinate). Subsequently, the Recovery-Stress Questionnaire for Athletes (RESTQ-76 Sport questionnaire) was administered to assess subjects' recovery stress state and to classify whether they became overreached or overtrained (Kellmann & Kallus, 2001). In accordance to a previous study, the recovery-stress balance was calculated by subtracting the total recovery score (∑9 recovery subscales) from the total stress score (∑10 stress subscales) (Coutts *et al.* 2007). High scores in the recovery-associated scales represent adequate recovery, while high scores for the stress-oriented subscales represent intense subjective strain.

Nutritional control. Nutritional intake was monitored via an on-line dietary platform (Mijn Eetmeter, Stichting Voedingscentrum Nederland; https://mijn.voedingscentrum.nl). The user modalities and dietary recording procedure were explained in detail to the subjects following the last familiarization session. Diaries were obtained during two subsequent days at the beginning (day 3-4), mid (day 12-13), and at the end (day 19-20) of the training period. Diaries were analysed for both macronutrient and energy intake.

Muscle biopsy procedure and muscle glycogen content. During the pre- and post-test, a percutaneous needle biopsy (100-200 mg) was obtained under local anaesthesia (2% xylocaine without adrenaline, 1 ml subcutaneously) before and immediately after the TT_{30min} from the *m.*

vastus lateralis using a 5-mm Bergström-type needle. Biopsies during the pre- and post-test were taken from the left and right leg, respectively, while pre- and post-exercise biopsies were obtained through the same incision, but with the needle pointing distal *vs.* proximal, respectively (Van Thienen *et al.* 2014). Part of the muscle sample was immediately frozen in liquid nitrogen and stored at -80°C for assay of muscle glycogen content at a later date. Muscle glycogen content was determined as glucose residues after acid hydrolysis using a standard enzymatic fluorometric assay (Lowry & Passoneau, 1972).

Urine sampling and analysis. Nocturnal urine was collected over 12 hours (8-10 pm till 8-10 am) the night before each experimental session in flasks prepared with 10 ml hydrochloric acid. Total urinary output volume was noted, and urinary ketone concentration was measured using ketone reagent strips (Ketostix®, Ascensia Diabetes Care, Basel, Switzerland). An aliquot of the urine sample was stored at -80°C until assayed in a single run for adrenaline and noradrenaline concentration using a commercially available enzyme linked immunosorbent assay (ELISA) (BA E-5400, LDN, Nordhorn, Germany).

Analysis of blood samples. Capillary blood samples from the earlobe were immediately analysed for blood D-β-hydroxybutyrate (Glucomen Lx plus-meter with Lx β-ketone strips, Menarini Diagnostics, Firenze, Italy). D-β-hydroxybutyrate measurements were performed by an investigator who was otherwise not involved in the experimental testing to ensure double-blindness. Venous blood samples were collected into vacuum tubes containing either EDTA or lithium heparin or Silica Clot Activator (BD Vacutainer). Tubes were centrifuged (1500 rpm for 10 min at 4°C) and the supernatant was stored at -20°C until later analysis. Commercially available ELISAs were performed to determine leptin, total ghrelin, GDF15, and total osteocalcin in serum, while IL-6 levels were determined in EDTA plasma (Leptin: BMS2039INST, Thermo Fisher Scientific, Massachusetts; Total Ghrelin: EZGRT-89K, Merck, Germany; GDF15: DGD150, R&D, Minnesota; Total osteocalcin: KAQ1381, Thermo Fisher Scientific, Massachusetts; IL-6: HS-600B, R&D, Minnesota). Cortisol and ACTH levels were assayed using electrochemiluminescence immunoassays (ECLIA) in serum and EDTA plasma samples, respectively. TRAP5b activity was measured in serum by a direct capture enzyme-immunoassay (#8036, TECOmedical, Switzerland). Fasted whole blood samples obtained during the pre- and post-test were analysed for the proportion of lymphocyte subset types, *i.e.* T-cell (CD3+), T-helper/inducer cell (CD4+), T-Suppressor/cytotoxic cell (CD8+), and CD4+/CD8+ cell count by flow cytometer. Plasma glucose levels were determined using a

standard enzymatic fluorometric assay (Lowry & Passoneau, 1972). All assays were run in a single batch, which included all samples, according to the respective protocols supplied by the manufacturer.

Statistical Analysis. Statistical analyses were performed in R version 3.3.3 using the nlme package for mixed-effects models and the stats package for unpaired t-tests (R Development Core Team, Vienna, Austria). Differences between mean values over time and between conditions were analysed using a two-way repeated measures analysis of variance (group x time). D-ßHB levels during the training sessions, as well as muscle glycogen content and blood glucose levels before and after TT_{30min} were assessed by a three-way repeated measures analysis of variance (group x time x number of training/experimental session). Pearson correlation coefficients were calculated using percent change scores unless otherwise stated. Holm-Sidak multiple comparison test was used for post-hoc analysis, when appropriate. Where relevant, Cohen's d was calculated as index of effect size (ES). Outliers were identified using the ROUT method in GraphPad Prism version 8.00 (GraphPad Software, La Jolla, California, USA). If outliers were detected, statistics were performed both with and without the outlier and both results are included in the text. Statistical significance was defined as $p < 0.05$. Data are presented as mean ± SEM.

RESULTS

Blood D-ßHB and urinary ketone excretion. In the pre-test fasted blood D-ßHB levels were similar between the groups at ~0.1 mM. During the training period, fasted D-ßHB levels gradually increased, reaching peak levels at ~0.35 mM in the post-test ($p < 0.01$; **Figure 2A**). During the recovery week, blood D-ßHB levels returned towards baseline within 3 days. There were no differences between the groups at any time. Ketone bodies were undetectable (< 0.05 g/l) in urine during the pre-test (**Figure 2B**), yet transiently increased at wk1 and wk2 ($p < 0.01$ and $p < 0.001$, respectively), irrespective of the experimental condition. During the training sessions, blood D-ßHB levels were low (~0.1-0.3 mM) in both the groups before and immediately post exercise (**Figure 2C**). But ketone ester intake immediately after exercise increased blood D-ßHB levels to 2.6 ± 0.2 mM within 30 min (30' post-ex), whilst values were unchanged in CON ($p < 0.001$ *vs.* KE).

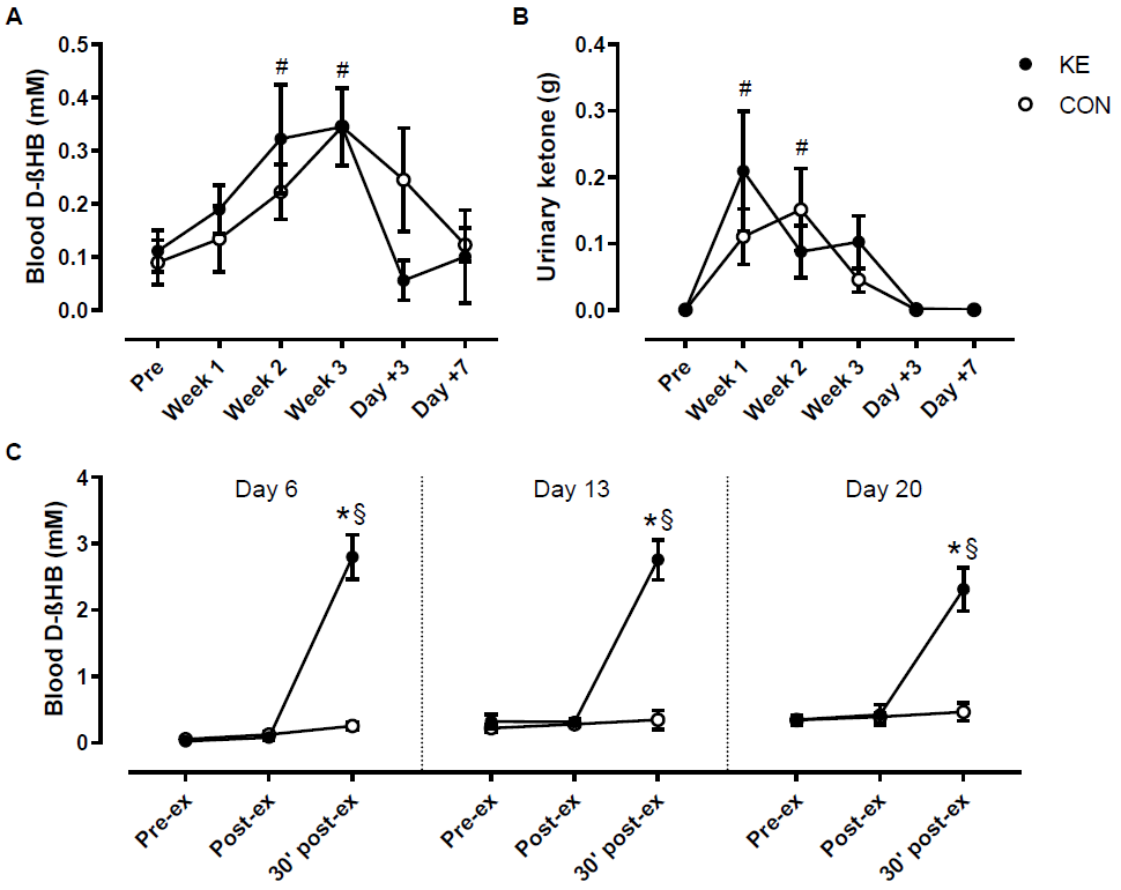


FIGURE 2. Effect of ketone ester supplementation on blood D-ßHB concentrations and urinary ketone excretion.

Data are means ± SEM for fasted morning blood D-ßHB concentration (panel A) and nocturnal urinary ketone excretion (panel B) before (Pre) and at the end of week 1 (Week 1), 2 (Week 2), 3 (Week 3) of the training period, and after 3 (Day +3) and 7 (Day +7) recovery days after training. During the training period the subjects received either control (○, n = 9) or ketone ester supplements (●, n = 9) immediately after each training session. Panel C shows blood D-ßHB concentrations before (Pre-ex), and immediately (Post-ex) and 30 min after (30' post-ex) the IMT sessions on day 6, 13 and 20. * $p < 0.05$ KE *vs.* CON at time points indicated; # $p < 0.05$ *vs.* PRE for both KE and CON; § $p < 0.05$ *vs.* pre-ex for indicated group.

Training load. Total weekly training load progressively increased from ~4600 kJ in week 1, to ~6400 kJ in week 2, and ~9600 kJ in week 3 (**Figure 3A**). Compared to their normal training volume (4.8 ± 0.4 $h \cdot wk^{-1}$), this corresponds to a ~120% increment in week 1, 165% in week 2, and a ~3-fold increment in week 3. Training load (kJ) was similar between the groups in week 1 and 2, but was ~15% higher in KE than in CON at week 3 (KE: 10266 ± 321 kJ *vs.* CON: 8962 ± 646 kJ, 95% CI: +237 to +2372 kJ, $p < 0.05$, $d = 0.80$). Differences in work output between the groups were most explicit for the prolonged endurance training sessions (ET) at the end of the training period, with no significant differences for the HIIT and IMT sessions (**Figure 3B**).

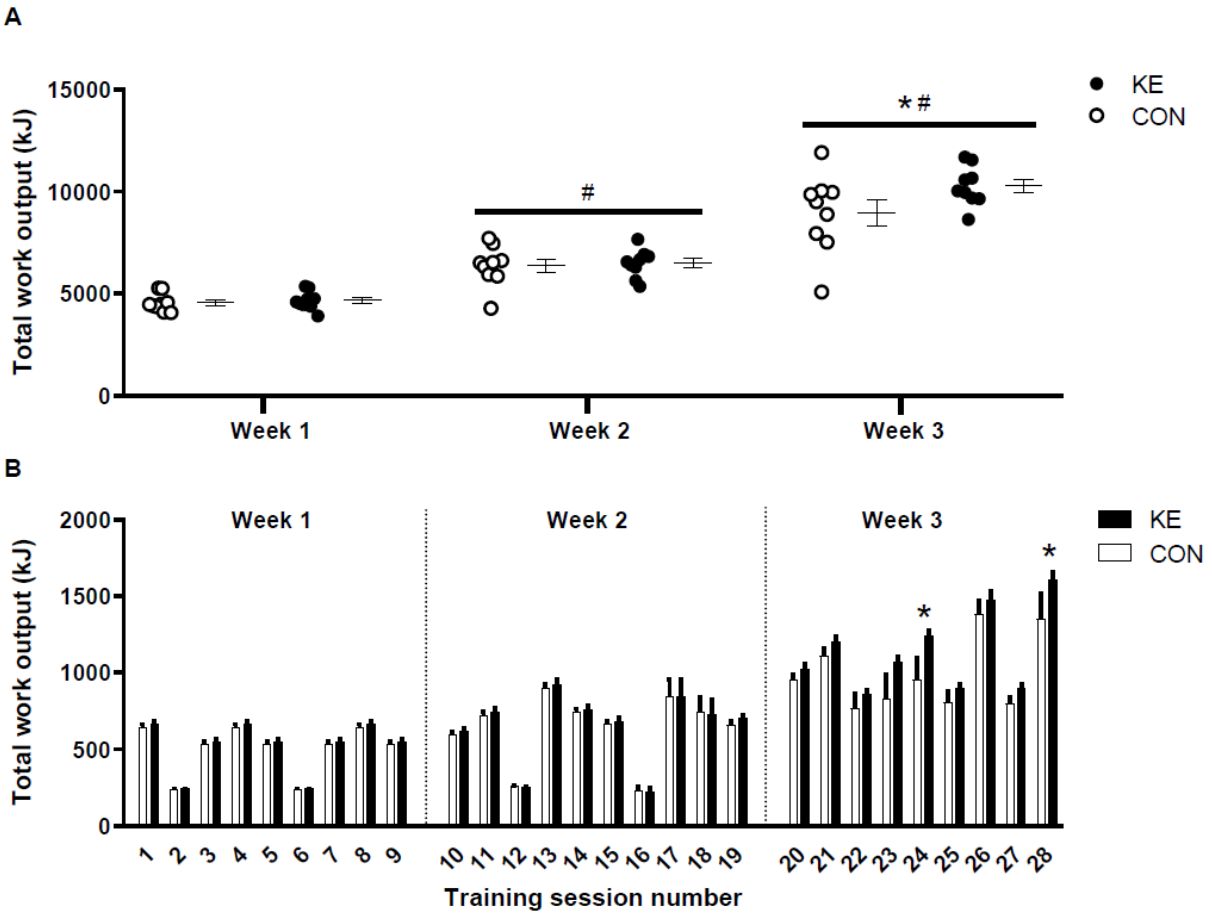


FIGURE 3. Effect of ketone ester supplementation on training workload.
Individual data points together with means ± SEM representing total work output per week (panel A). Means ± SEM for work output per training session (panel B) in subjects receiving either control (open circles/bars, n = 9) or ketone ester (filled circles/bars, n = 9) supplements. The subjects performed 28 training sessions over a 3-week training period. * $p < 0.05$ KE *vs.* CON at time points indicated; # $p < 0.05$ *vs.* PRE for both KE and CON.

Exercise performance and peak lactate concentration. Mean power outputs in TT_{30min} and 90S in the pre-test were similar between the groups (TT_{30min}: 216 ± 7 W *vs.* 215 ± 8 W; 90S: 492 ± 20 W *vs.* 499 ± 17 W for KE and CON, respectively). In KE, compared to the pre-test mean power output in TT_{30min} was 4.9 ± 1.5% higher (95% CI: +3 to +18 W, $p < 0.05$, $d = 0.47$) in the post-test, and 7.5 ± 1.7% (95% CI: +8 to +24 W, $p < 0.001$, $d = 0.71$) and 8.3 ± 2.1% (95% CI: +8 to +28 W, $p < 0.001$, $d = 0.83$) higher at day +3 and day +7, respectively (**Figure 4A**). Conversely, training did not improve TT_{30min} performance in CON (95% CI pre *vs.* post-test: -2 to +14 W, $p > 0.05$), except at day +7 (+7.0 ± 2.0%, 95% CI: +5 to +25 W, $p < 0.001$, $d = 0.52$). However, TT_{30min} mean power outputs were not significantly different between CON and KE at any time (95% CI for KE *vs.* CON during post-test: -33 to +14 W, $p > 0.05$). Furthermore, mean power output during EPT_{120min} on day 18, was ~15% higher in KE than in CON (KE: 216 ± 8 W *vs.* CON: 188 ± 14 W, 95% CI for KE *vs.* CON: +5 to +52 W, $p < 0.05$, $d = 0.77$; **Figure 4C**). Compared to the pre-test, mean power output effected in 90S was stable in wk1 and wk2, but decreased by 5.5 ± 1.4% in the post-test ($p < 0.05$, $d = 0.30$; **Figure 4B**). Yet during the recovery period 90S power outputs returned to baseline by day +3. Performance in 90S was not significantly different between the groups at any time. Blood lactate levels following 90S peaked at ~13-15 mM in the pre-test as well as in wk1 and wk2. However, in the post-test peak blood lactate levels decreased by ~5 mM ($p < 0.001$) to 9.8 ± 1.0 mM in CON and 8.2 ± 0.8 mM in KE. During the recovery period, blood lactate concentrations rapidly returned to baseline in CON (day +3: 13.2 ± 1.9 mM, $p = 0.29$ *vs.* PRE; day +7: 12.6 ± 1.5 mM, $p = 0.10$ *vs.* PRE), but not in KE group (day +3: 10.9 ± 0.8 mM, $p < 0.05$ *vs.* PRE; day +7: 9.6 ± 1.1 mM, $p < 0.01$ *vs.* PRE).

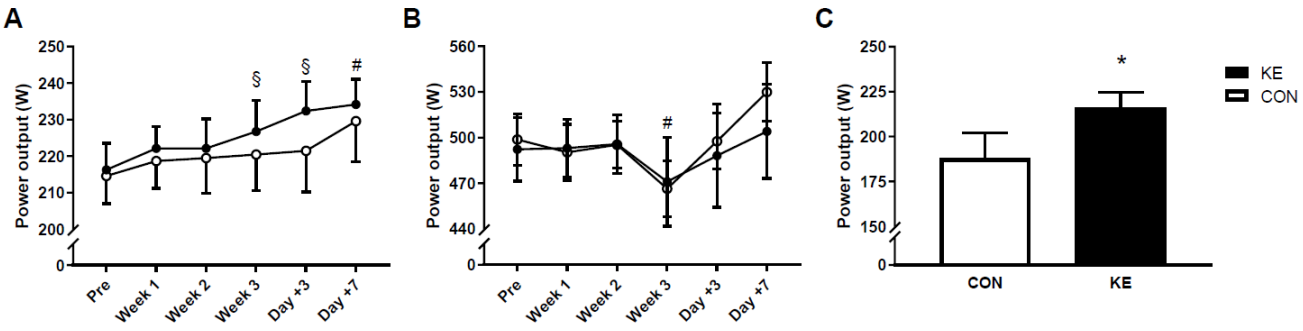


FIGURE 4: Effect of ketone ester supplementation on exercise performance.
Data are means ± SEM. Mean power output during the 30min simulated time-trial (TT_{30min}) (panel A) and in a 90s all-out cycling bout (90S) (panel B) before (Pre) and at the end of week 1 (Week 1), 2 (Week 2), 3 (Week 3) of the training period, and after 3 (Day +3) and 7 (Day +7) days of recovery. Mean power output in the final half an hour of a 120 min endurance performance test (EPT_{120min}) on day 18 of the training period (panel C). Subjects received either control (○/open bars, n = 9) or ketone ester (●/filled bars, n = 9) during each training session. * $p < 0.05$ KE *vs.* CON; # $p < 0.05$ *vs.* PRE for both KE and CON; § $p < 0.05$ *vs.* PRE for indicated group.

Heart rate and blood pressure. Resting heart rate (HR) was 62 ± 4 bpm in KE *vs.* 66 ± 2 bpm in CON ($p = 0.73$). In CON the training intervention gradually decreased resting HR to 60 ± 1 bpm in the post-test ($p < 0.05$), and even further to 55 ± 2 bpm at day +3 ($p < 0.001$; **Figure 5A**). At day +7, resting HR still was ~6 bpm lower than in the pre-test ($p < 0.05$). In contrast, in KE resting HR was stable throughout the full training period, and consistently was ~2-5 bpm higher than in CON ($p < 0.05$). Only at day +3, resting HR in KE was slightly lower than at baseline ($p < 0.05$). The training also substantially decreased HR during submaximal and maximal exercise. In CON submaximal and maximal HR on average decreased by ~16 bpm (range: -5 to –28 bpm) from the pre-test to the post-test ($p < 0.001$; **Figure 5B-C**). Thus, submaximal HR during TT_{30min} was 167 ± 4 in the pre-test, decreasing to 152 ± 4 bpm in the post-test. By analogy, maximal HR taken from 90S dropped from 189 ± 2 to 172 ± 3 bpm ($p < 0.001$). In KE submaximal and maximal HRs also dropped during the training period, yet the drop was markedly smaller than in CON ($p < 0.05$ and $p < 0.01$ *vs.* CON, respectively). Submaximal HR dropped from 163 ± 3 in the pre-test to 157 ± 3 bpm in the post-test ($p < 0.05$). Corresponding maximal HRs were 189 ± 3 and 179 ± 2 bpm ($p < 0.001$). In both the groups the training-induced suppression of submaximal and maximal HR was rapidly inverted during the recovery week. Within a week, submaximal HR returned to baseline in KE, but not in CON ($p < 0.001$). Clearly, ketone ester intake consistently blunted (over)training-induced bradycardia both at rest and during submaximal and maximal exercise. Furthermore, the decrement of resting HR effected by the training intervention was associated with a drop of diastolic blood pressure from 68 ± 1 to 57 ± 3 mm Hg in CON ($p < 0.001$), but not in KE (pre-test 67 ± 2, post-test 62 ± 2 mm Hg, $p = 0.20$). Systolic blood pressures on average were ~126 mm Hg and were not altered by either training or ketone ester intake.

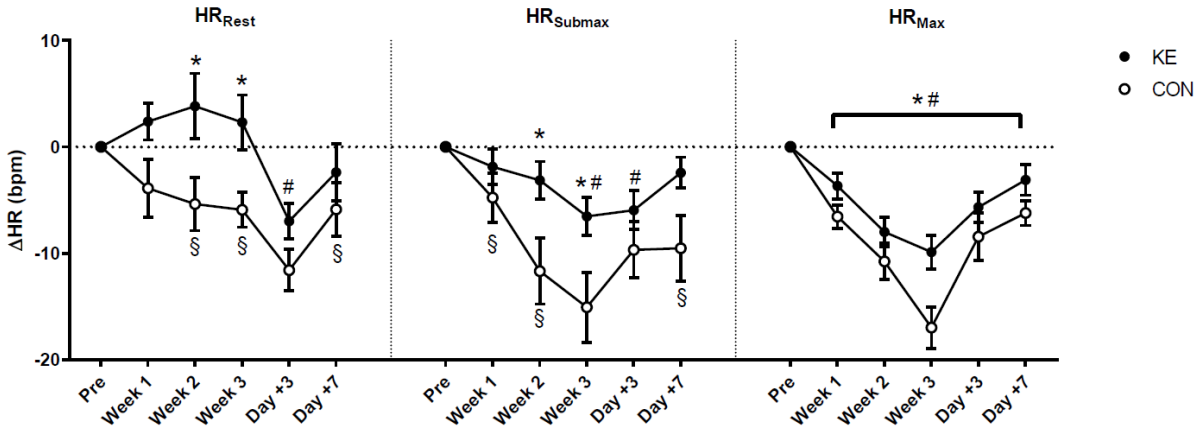


FIGURE 5. Effect of ketone ester supplementation on heart rate and blood pressure.
Data are means ± SEM and represent changes in resting (HR_{Rest}), submaximal (HR_{Submax}) and maximal (HR_{Max}) heart rate before (Pre), and at the end of week 1 (Week 1), 2 (Week 2), 3 (Week 3) of the training period, and after 3 (Day +3) and 7 (Day +7) recovery days. Subjects received either control (○, n = 9) or ketone ester supplements (●, n = 9) following each training session. * $p < 0.05$ KE *vs.* CON; # $p < 0.05$ *vs.* PRE for both KE and CON; § $p < 0.05$ *vs.* PRE for indicated group.

Body composition and bone mineralization. Baseline values for body composition and bone mineralization were similar between both experimental groups (**Table 3**). The training period decreased body fat percentage by 1.4 ± 0.2% ($p < 0.001$, $d = 0.36$) and sum of skinfolds by 7.3 ± 1.4 mm ($p < 0.001$, $d = 0.28$), while both lean mass and body weight remained unchanged irrespective of the experimental conditions. In KE, BMC from the pre-test to the post-test increased by 24 ± 10 g ($p < 0.01$, $d = 0.07$), whilst it was stable in CON (-6 ± 8 g, $p = 0.53$). BMD was unaffected by the experimental conditions.

TABLE 3. Effect of ketone ester supplementation on body composition and bone mineralization.

	Control		Ketone Ester	
	PRE	POST	PRE	POST
Body weight (kg)	72.9 ± 3.6	73.1 ± 3.2	72.1 ± 2.1	71.9 ± 2.2
Lean mass (kg)	60.4 ± 2.6	61.6 ± 2.4	61.1 ± 1.9	61.8 ± 1.9
Percent body fat (%)	13.1 ± 1.3	11.6 ± 1.1 #	11.4 ± 0.9	10.1 ± 0.8 #
Sum skinfolds (mm)	103.6 ± 10.6	95.3 ± 8.9 #	87.1 ± 7.7	80.8 ± 7.2 #
BMC (g)	2749 ± 133	2744 ± 130	2758 ± 106	2782 ± 112 #§
BMD ($g \cdot cm^{-2}$)	1.19 ± 0.04	1.19 ± 0.04	1.23 ± 0.03	1.24 ± 0.03

Data are mean ± SEM for body weight, body composition and bone mineralization parameters measured by DXA scan, in subjects receiving either ketone ester supplements (n = 9) or control (n = 9). BMC, bone mineral content; BMD, bone mineral density. # $p < 0.05$ PRE *vs.* POST; § $p < 0.05$ group x time interaction.

Total energy and macronutrient intake and appetite sensations. With the exception of the ketone ester/control drink and the post exercise recovery shakes prescribed by the study protocol, food intake during the full study period was ad libitum. Concurrent with the increase in training workload from training week 1 to 3, KE subjects spontaneously increased their total energy intake by ~20% from ~14700 to ~17600 kJ·day^{-1}. Total energy intake increased proportionately (+1966 ± 826 kJ·day^{-1} at week 2 and +2880 ± 489 kJ·day^{-1} at week 3, $p < 0.01$ and $p < 0.001$ *vs.* wk1, respectively) with the training load and the concomitant increase in energy expenditure (+1089 ± 135 kJ·day^{-1} at week 2 and +3362 ± 104 kJ·day^{-1} at week 3; assuming a mechanical efficiency of 23.8% (94)) in KE, while it remained stable in CON (energy intake: -384 ± 794 kJ·day^{-1} at week 2 and -157 ± 751 kJ·day^{-1} at week 3, $p = 0.86$) (**Table 4**). The increasing energy intake in KE was largely effected by greater amounts of carbohydrate intake (+25.5 ± 6.3% at week 2 and +29.9 ± 5.9% at week 3, both $p < 0.001$) at fairly constant fat and protein intake. Subjective ratings of appetite were similar between the groups and over time (data not shown).

TABLE 4. Effect of ketone ester supplementation on total energy and macronutrient intake.

	Control			Ketone Ester		
	Week 1	Week 2	Week 3	Week 1	Week 2	Week 3
Energy intake	15439 ± 741	15055 ± 493	15282 ± 808	14688 ± 1042	16655 ± 854 #	17568 ± 1121 #
Carbohydrate	7211 ± 311	7412 ± 329	7417 ± 397 §	6934 ± 354	8647 ± 464 #	9014 ± 614 #§
Protein	2655 ± 228	2673 ± 65	2613 ± 163	2807 ± 230	3019 ± 217	3273 ± 235
Fat	3362 ± 221	3293 ± 205	3394 ± 380	2878 ± 502	3240 ± 328	3413 ± 578

Data are mean ± SEM (kJ/day) for total energy and macronutrient intake, in subjects receiving either ketone ester supplements (n = 9) or control (n = 9). Macronutrient data are exclusive the KE and CON drinks. # $p < 0.05$ *vs.* week 1; § $p < 0.05$ KE *vs.* CON at indicated time point.

Hormonal parameters. We measured 'energy homeostasis and appetite hormones' (GDF15, leptin, ghrelin), urinary catecholamines, and the ACTH~cortisol hypothalamic pituitary axis. All hormonal levels were similar between the groups at baseline (**Figures 6 and 7**). Serum GDF15 gradually increased during the training period in all subjects ($p < 0.001$), yet the rise was greater in CON than in KE ($p < 0.05$). Thus in the post-test serum GDF15 levels were markedly lower in KE (361 ± 19 pg/ml) than in CON (435 ± 29 pg/ml, 95% CI of KE *vs.* CON: -17 to -133 pg/ml, $p < 0.05$, $d = 0.91$). Yet values rapidly returned to baseline during the recovery week. Compared to baseline, the 3-week training intervention decreased serum leptin

3-fold in CON ($p < 0.001$), but not in KE ($p = 0.26$), returning to baseline within one week after training. Nonetheless, serum leptin levels were not significantly different between the groups at any time. Serum ghrelin was affected neither by the training program nor by ketone ester intake (KE_{Pre}: 486 ± 38; KE_{Post}: 471 ± 31; CON_{Pre}: 466 ± 31; CON_{Post}: 461 ± 30 pg/ml; group effect: $p = 0.74$; time-effect: $p = 0.32$). Nocturnal urinary catecholamine excretions were stable throughout the full intervention period in KE. In contrast, in CON both the adrenaline and noradrenaline excretions increased about 2-fold from the pre-test to the post-test (main group effect for adrenaline, $p < 0.01$; interaction effect for noradrenaline, $p < 0.01$). Thus in the post-test urinary noradrenaline output was 108 ± 20 nmol in CON ($p < 0.01$ *vs.* pre-test) and 56 ± 17 nmol in KE ($p = 0.82$ *vs.* pre-test). Corresponding values for adrenaline excretion were 41 ± 17 nmol in CON *versus* 17 ± 7 nmol in KE (group effect: $p < 0.01$). Plasma ACTH levels tended to decrease in both groups from the pre-test to the post-test (pre-test 54.4 ± 6.4 *vs.* post-test 44.1 ± 5.5 ng/l, $p = 0.08$), while serum cortisol levels were unchanged (pre-test 184.5 ± 8.0 *vs.* post-test 182.2 ± 7.8 µg/l, $p = 0.83$). ACTH/cortisol ratio tended to decrease in CON (pre-test 340 ± $91 \cdot 10^{-6}$ *vs.* post-test 196 ± $22 \cdot 10^{-6}$, $p = 0.07$), but not in KE (pre-test 306 ± $46 \cdot 10^{-6}$ *vs.* post-test 278 ± $55 \cdot 10^{-6}$, $p = 0.72$).

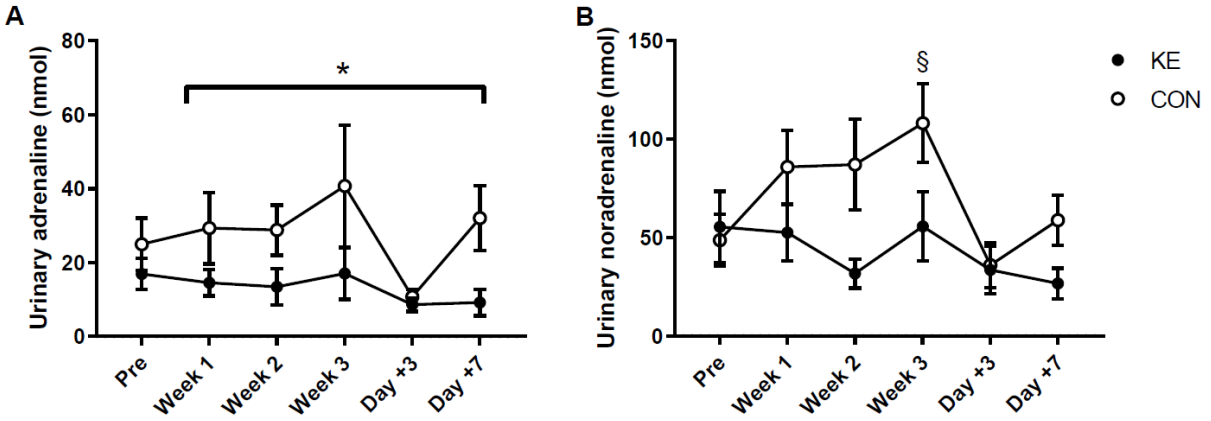


FIGURE 6. Effect of ketone ester supplementation on urinary catecholamine excretion.
Data are means ± SEM for urinary **(A)** adrenaline and **(B)** noradrenaline excretion before (Pre) and at the end of week 1 (Week 1), 2 (Week 2), 3 (Week 3) of the training period and after 3 (Day +3) and 7 (Day +7) days of recovery after training. Subjects received either control (○, n = 9) or ketone ester supplements (●, n = 9) following each training session. * $p < 0.05$ KE *vs.* CON; # $p < 0.05$ *vs.* PRE for both KE and CON; § $p < 0.05$ *vs.* PRE for indicated group.

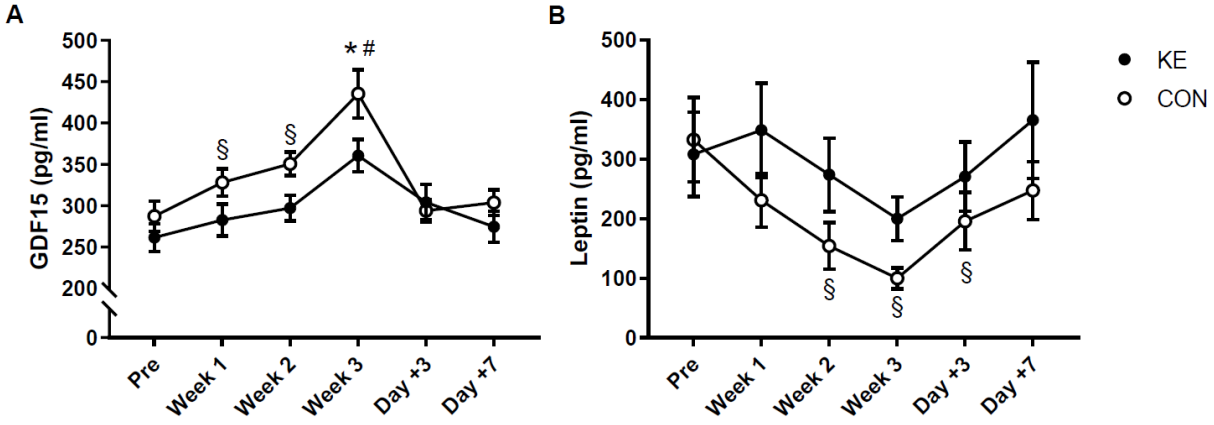


FIGURE 7. Effect of ketone ester supplementation on 'appetite' hormones involved in energy balance regulation.
Data are means ± SEM and represent changes in **(A)** GDF15, and **(B)** leptin concentration before (Pre), after 1 (Week 1), 2 (Week 2), 3 (Week 3) weeks of training and following 3 (Day +3) and 7 (Day +7) days of recovery. Subjects received either control (○, n = 9) or ketone ester supplements (●, n = 9) during the training period. * $p < 0.05$ KE *vs.* CON; # $p < 0.05$ *vs.* PRE for both KE and CON; § $p < 0.05$ *vs.* PRE for indicated group.

Moderate to strong negative correlations were found between both absolute and delta GDF15 levels and changes in maximal heart rate during the training period ($r = -0.64$ and $r = -0.59$, respectively, both $p < 0.001$). One outlier was identified in both data sets, but its removal only marginally affected the correlation coefficients ($r = -0.64$ and $r = -0.60$, respectively, both $p < 0.001$, **Figure 8**). Changes in leptin levels were positively correlated with alterations in BMC ($r = 0.56$, $p < 0.05$).

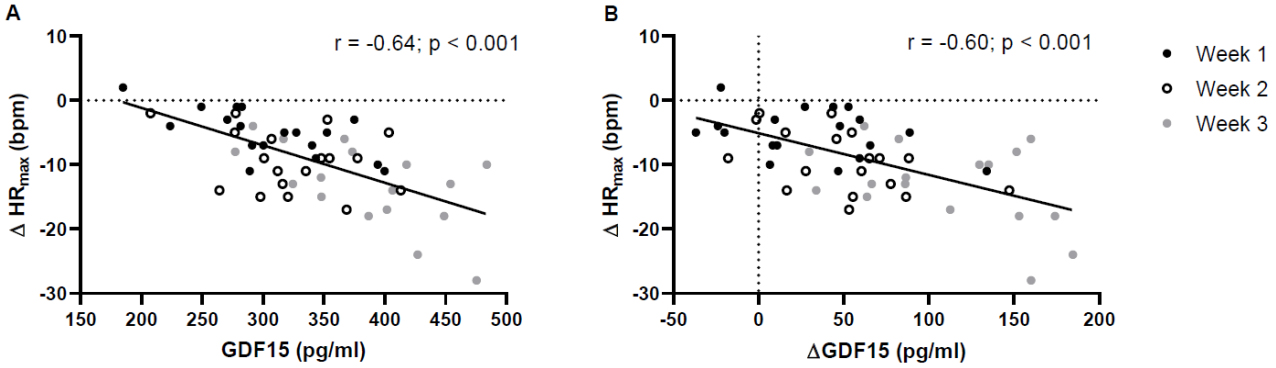


FIGURE 8. Relation between GDF15 and maximal heart rate.
Correlation analyses showing a strong negative correlation between alterations in maximal heart rate (ΔHR_{max}) and both **(A)** absolute and **(B)** delta GDF15 during the training period. Individual data points represent changes after 1 (Week 1), 2 (Week 2), and 3 (Week 3) weeks of training, compared to pre-test values.

Cytokine and immune response. Plasma IL-6 levels were similar between both groups in the pre-test (KE: 0.68 ± 0.06 *vs.* CON: 0.85 ± 0.12 pg/ml; p = 0.71) and were stable throughout the study in both the groups (data not shown). Baseline values for lymphocyte subtype counts, *i.e.* $CD3^+$, $CD4^+$ and $CD8^+$ were similar between the groups (**Table 5**). Compared to the pre-test, in the post-test $CD3^+$ and $CD8^+$ cell counts were decreased by ~5 and 14%, respectively ($p < 0.001$), whereas $CD4^+$ increased by ~6% ($p < 0.001$). As a result $CD4^+/CD8^+$ ratio increased by ~25% ($p < 0.001$). Lymphocyte changes were similar between the groups.

TABLE 5. Effect of ketone ester supplementation on lymphocyte cell counts.

	Control		Ketone Ester	
	PRE	POST	PRE	POST
Mature T ($CD3^+$)	1.13 ± 0.02	1.07 ± 0.01 #	1.11 ± 0.02	1.06 ± 0.01 #
T-helper ($CD4^+$)	0.57 ± 0.02	0.59 ± 0.02 #	0.56 ± 0.04	0.60 ± 0.03 #
T-cytotoxic ($CD8^+$)	0.48 ± 0.03	0.41 ± 0.02 #	0.43 ± 0.03	0.37 ± 0.02 #
$CD4^+/CD8^+$	1.23 ± 0.09	1.49 ± 0.12 #	1.35 ± 0.14	1.67 ± 0.15 #

Data are means ± SEM (n * 10^9 cells/L) for lymphocyte subtype counts in subjects receiving either ketone ester supplements (n = 9) or control (n = 9). # $p < 0.05$ PRE *vs.* POST.

Bone metabolism markers. The training period decreased total serum osteocalcin concentration by ~30% (pre-test: 19.5 ± 0.6 *vs.* post-test: 13.1 ± 0.6 ng/ml, $p < 0.05$), irrespective of the experimental condition. Levels returned to baseline within the 1-week recovery period. TRAP5b activity was modified neither by the training nor by ketone ester intake (KE_{Pre}: 4.25 ± 0.25; KE_{Post}: 4.23 ± 0.23; CON_{Pre}: 4.06 ± 0.36; CON_{Post}: 3.92 ± 0.26 U/L; group effect: p = 0.30; time-effect: p = 0.18). Alterations in osteocalcin showed a strong inverse correlation with changes in GDF15 ($r = -0.61$, $p < 0.01$).

Blood glucose levels and muscle glycogen content. Initial muscle glycogen content was 140 ± 8 mmol·kg^{-1} wet weight in CON *vs.* 122 ± 9 mmol·kg^{-1} in KE (p = 0.41). The training period slightly decreased resting muscle glycogen content in CON (-28 ± 9 mmol·kg^{-1}, $p < 0.05$) but not in KE (+2.4 ± 13.2 mmol·kg^{-1}, p = 0.99), yielding identical muscle glycogen contents between CON (111 ± 10 mmol·kg^{-1}) and KE (124 ± 9 mmol·kg^{-1}) in the post-test. The TT_{30min} decreased muscle glycogen content in both groups by ~44% during the pre-test (KE: 59 ± 10 mmol·kg^{-1}; CON: 69 ± 12 mmol·kg^{-1}; both $p < 0.001$ compared to pre-exercise), while muscle glycogen was unaltered by the TT_{30min} in the post-test (KE: 92 ± 9 mmol·kg^{-1}; CON: 86 ± 8 mmol·kg^{-1}; p = 0.10 and p = 0.19 *vs.* pre-exercise, respectively) for both groups. Plasma

glucose levels immediately before the TT_{30min} were similar between the groups during both the pre- and post-test (KE_{Pre}: 4.1 ± 0.3; KE_{Post}: 4.9 ± 0.2; CON_{Pre}: 3.9 ± 0.2; CON_{Post}: 4.2 ± 0.4 mM; group effect: $p = 0.45$; time-effect: $p = 0.37$). At the end of the TT_{30min}, glucose levels were increased by ~1.5 mM ($p < 0.001$ *vs.* pre-exercise) during both the pre- and post-test independent of KE supplementation (KE_{Pre}: 5.6 ± 0.3; KE_{Post}: 5.9 ± 0.2; CON_{Pre}: 6.0 ± 0.4; CON_{Post}: 5.3 ± 0.6 mM).

Stress-recovery state. The training period substantially decreased the stress-recovery state in both groups (pre-test: 80 ± 5 *vs.* post-test: -6 ± 7, $p < 0.001$). This was due to an increase in 'total stress' scores (pre-test: 49 ± 3 *vs.* post-test: 110 ± 6, $p < 0.001$) together with a decrement in 'total recovery' scores (pre-test: 129 ± 4 *vs.* post-test: 104 ± 4, $p < 0.001$). Stress-recovery state rapidly improved during the recovery week, yet values remained below baseline even at day 7 (69 ± 6, $p < 0.05$ *vs.* pre-test). There were no differences between the groups at any time.

Gastro-intestinal tolerance. Gastro-intestinal discomfort scores were slightly higher in the post-test than in the pre-test (pre-test: 7 ± 1 *vs.* post-test: 11 ± 2 out of a maximum of 96, $p < 0.05$). This small increase was due to higher incidence of lower abdominal symptoms (pre-test: 2 ± 1 *vs.* post-test: 4 ± 1 out of a maximum of 32, $p < 0.05$) and more systemic discomfort (pre-test: 3 ± 1 *vs.* post-test: 4 ± 1 out of a maximum of 32, $p < 0.05$). Upper abdominal distress was stable between the pre-test and post-test ($p = 0.73$). Gastro-intestinal discomfort scores returned to baseline within the 7-day recovery period. No differences were observed between the experimental conditions at any time point.

Identification of experimental condition. Upon completion of the study the subjects were asked to identify their presumed group allocation. Nobody was certain of his experimental condition, but 10 out of 18 (5 in KE and 5 in CON) subjects correctly guessed the condition based on subjective perceptions, indicating successful blinding of the treatments.

DISCUSSION

Early detection and prevention of overtraining is pivotal in athlete training management. From this perspective we investigated whether post-exercise ketone ester intake (KE) can prevent non-functional overreaching and performance impairment during an episode of excessive training load. The endurance training program used induced explicit cardiovascular, hormonal, as well as perceptual symptoms of overreaching in all subjects. Interestingly, KE markedly inhibited the appearance of these symptoms, whilst enhancing tolerable training load, increasing energy intake, and stimulating endurance exercise performance. Our data indicate that KE is a potent strategy to prevent overtraining and stimulate endurance training adaptation. In addition, we provide preliminary evidence that GDF15 may be a valid hormonal marker of overtraining.

Although the complex pathophysiology is still poorly understood, it is the prevailing opinion that autonomic neural imbalance plays an important role in the development of overtraining (Fry *et al.* 1991; Lehmann *et al.* 1998). From this perspective two distinct overtraining types were defined. 'Sympathetic' overtraining is characterized by elevated basal sympathetic tone, whilst sympathetic drive is abnormally decreased in the 'parasympathetic' overtraining form (Israel, 1976). The former has primarily been associated with high-intensity anaerobic exercise, *versus* the latter with endurance training activities (Kuipers & Keizer, 1988), but both forms probably rather exist as a continuum (Fry *et al.* 1994a, 2006). Initially sympathetic overtraining develops as a stress-response attempting to maintain functional status. However, in a later stage, parasympathetic symptoms gradually predominate due to fatigue at the site of the sympathetic neuroendocrine system. In the CON conditions of the current study, the subjects exhibited the initial basal sympathetic stress-response as evidenced by elevated nocturnal catecholamine excretion, predominantly noradrenaline, which reflects spill-over from the sympathetic nervous system (Esler *et al.* 1988; Fry *et al.* 1994a). Nonetheless, against the face of this elevated sympathetic activity, resting heart rate significantly decreased, which indicates that at the cardiac site the elevated central sympathetic drive was probably overruled by excess vagal output (Hedelin *et al.* 2000; Pichot *et al.* 2002) either or not in conjunction with downregulation of cardiac β_2-adrenoceptor sensitivity (Gleeson, 2002; Fry *et al.* 2006). But these autonomic responses were substantially altered by KE during training. KE fully counteracted the initial training-induced sympathetic overdrive, as evidenced by stable nocturnal catecholamine excretion throughout the full training period. Nonetheless resting heart

rate slightly increased in weeks 1-2, probably indicating transient sympathetic dominance at the site of the heart. KE intake also markedly impacted on exercise tachycardia, which also depends on sympathetic-parasympathetic interactions. In CON the training markedly suppressed the exercise-induced rise in heart rate, including a substantial drop in maximal heart rate by 10-28 bpm. KE administration clearly counteracted this effect, which is most likely due to sympathetic regulation (Lehmann *et al.* 1991; Stanojevic *et al.* 2013). However, we did not measure plasma catecholamine levels during exercise. But central autonomic command of exercise heart rate is also controlled by input from mechano- and metaboreceptors in active muscles (Fisher, 2014), which also might be changed by KE intake. Furthermore, regulation might also occur at the site of cardiac adrenergic sensitivity or GPR41 activity. GPR41 is present in sympathetic ganglions and is thereby directly involved in sympathetic control (Kimura *et al.* 2011). βHB suppresses GPR41 activity in mice, resulting in a depressed sympathetic tone and heart rate (Kimura *et al.* 2011).

Apart from the KE or CON supplements, the subjects received a standardized protein-carbohydrate solution to stimulate recovery immediately after each training session. Intake of other foods and drinks was ad libitum because we wanted to evaluate the effect of KE on appetite regulation and spontaneous food intake. It is well known that overtraining in endurance athletes often results in appetite suppression (Fry *et al.* 1992). Furthermore, decreased appetite during the ketogenic diet has been linked to elevated plasma ketone levels (Paoli *et al.* 2015). However, in the current study, neither the training overload nor KE decreased appetite/hunger perception. Stubbs et al. (2018) demonstrated that acute KE intake which elevated plasma βHB level to ~3-4 mM compared to isocaloric glucose ingestion suppressed appetite in healthy subjects. However, appetite scoring in the current study was done in early morning in the fasted state when blood βHB levels were basal in both the experimental groups (< 0.4 mM). Nonetheless, actual food intake pattern was significantly different between CON and KE. Spontaneous energy intake in CON was constant throughout the study (~15000 kJ per day) despite the gradual increase in training load from week 1 to week 3 (see **Figure 3**), resulting in an energetic deficiency of ~1470 and ~2800 kJ per day during weeks 2 and 3, respectively. Conversely, KE gradually increased energy intake to ~17600 kJ per day, predominantly via extra carbohydrate intake, resulting in an energetic balance during both week 2 and 3 (average energy surplus of 198 kJ per day). It is important to note that the effect of KE to blunt the appearance of overreaching symptoms such as decreased heart rates, elevated urinary noradrenaline excretion, as well as elevated serum GDF15 level was obvious before any

significant difference in daily energy intake occurred. This suggests that modulation of food intake per se was not the primary mechanism of action of KE. Aiming to elucidate the underlying mechanism, we measured serum levels of the 'appetite hormones' leptin, ghrelin, and GDF15. GDF15 is a peptide which has only recently been discovered to act as a stress-induced hormone that is involved in appetite regulation by decreasing food intake (Johnen *et al.* 2007; Macia *et al.* 2012; Wang-Wei Tsai *et al.* 2013; Patel *et al.* 2019). Here, for the first time, we demonstrate that training overload increased systemic GDF15 level. Interestingly, this effect was negated by KE intake, which might at least partly explain the higher energy intake during training in the latter group. This observation together with literature data (Patel *et al.* 2019) indicates that the GDF15 increment in CON reflects training-induced physiological stress, rather than a moderate energy deficit. Additional support for such contention comes from a recent study in our laboratory showing that four weeks of hypocaloric diet (30% energy deficit), aimed to induce body weight loss (minus 2-4 kg) in fit lean females, did not alter systemic GDF15 levels either in the presence or absence of KE supplementation (Hiroux *et al.,* unpublished observations). It is important to note that this suppression of GDF15 occurred in the absence of elevated plasma βHB concentration, indicating adaptation of GDF15 secretion by short-term KE intake. Conversely, neither leptin nor ghrelin were directly involved in food intake regulation during the intervention period. Serum leptin, an appetite suppressor, was even higher during training in KE than in CON, while ghrelin was unaffected. But it is well established that leptin during episodes of energy deficit operates as a 'starvation signal' by decreasing basal energy expenditure through suppression of heart rate, blood pressure, thyroid hormone levels, and sympathetic nervous system activity (Flier, 1998; Pandit *et al.* 2017). Thus lower basal metabolic rate effected by leptin decrement (Woods *et al.* 2017, 2018) might explain the absence of body weight drop in CON despite energy expenditure in training exceeding energy intake during the later stage of the training period.

It is also well-known that the dysregulation of hormonal and energy balance in overtraining, especially in non-weight-bearing sports such as cycling and swimming, can stimulate bone demineralization and thereby impair long-term bone health (Nagle & Brooks, 2011; Olmedillas *et al.* 2012). In both the experimental groups, we observed a decrease of the bone formation marker osteocalcin against stable activity of the osteoclast marker TRAP5b. This may indicate net bone resorption (Crockett *et al.* 2011; Ferreira *et al.* 2015). These results are in accordance with a study showing similar symptoms of net bone resorption to occur in professional cyclists during a 3-week cycling race, *i.e.* Giro d'Italia (Lombardi *et al.* 2012).

Nonetheless, the training intervention significantly altered neither bone mineral density (BMD) nor bone mineral content (BMC) measured by whole body DXA scan. This may be due to the short duration of the intervention, because months rather than weeks of strenuous cycling training are needed to induce a measurable degree of bone demineralization (Barry & Kohrt, 2008). In addition, consistent ingestion of a protein-carbohydrate mixture may be sufficient to maintain bone mineral status (Townsend *et al.* 2017). Other studies also have suggested a link between bone metabolism and energy balance regulation (Confavreux *et al.* 2009; Lombardi *et al.* 2012). Along such interaction, Δleptin during training was positively correlated with ΔBMC ($r = 0.56$). In addition, training-induced rise of GDF15 was associated with drop of osteocalcin ($r = -0.61$), which for the first time indicates that GDF15 may be implicated in regulation of bone metabolic activity. Taken together the above observations support a tight link between maintenance of energy balance and bone metabolism during intensified training. In fact, the beneficial effect of KE on bone mineral content during the overtraining period might be largely explained by better matching of energy intake to energy expenditure in training driven by the concerted actions of appetite hormones, most prominently GDF15.

It is well established that low muscle glycogen levels in endurance athletes impairs the capacity to sustain strenuous training. Based on longitudinal observations in endurance (over)trained individuals it has been postulated that glycogen depletion may be involved in the development of overtraining (Costill *et al.* 1971, 1988). Against such opinion, our current observations show that despite a multiplicity of other symptoms of overreaching, resting glycogen levels remained within the normal range in both groups, confirming previous studies showing that glycogen depletion is not a prerequisite for overtraining to develop (Snyder *et al.* 1995; Halson *et al.* 2004). In addition, pre and post exercise muscle glycogen contents were similar between CON and KE. Furthermore, in line with earlier findings (Achten *et al.* 2004), the training overload markedly blunted exercise-induced net muscle glycogen breakdown, which has been attributed to decreased adrenergic sensitivity (Lehmann *et al.* 1993; Jeukendrup & Hesselink, 1994). Again, KE did not impact on exercise-induced net glycogen breakdown during a 30 min maximal exercise bout. However, the training intervention also substantially reduced peak blood lactate levels produced by a 90 s all-out exercise bout. This effect faded within 3 recovery days in CON, whilst persisting in KE till day +7.

The overload training program elicited clear physiological dysregulations as well as negated positive training adaptations, which clearly indicates a state of functional overreaching

in some, *versus* non-functional overreaching in others. Sprint performance decreased in all subjects (range: -1 to -12%), whilst endurance performance in TT_{30min} on average was stable (range: -4 to +12%), which corroborates earlier findings (Woods *et al.* 2018). Sprint performance restored to baseline within 3 recovery days, while performance improvements in TT_{30min} only occurred by day +7. Nonetheless, in some subjects performance impairments persisted until the end of the recovery period (90S: -5 to +13%; TT_{30min}: -5 to +14%). KE administration did not alter sprint power output, but clearly stimulated endurance exercise performance during the final week of the overload period, as evidenced by a 15% increase in both training load as well as in EPT_{120min} compared to CON. It should be noted that the observed increase in endurance performance during the final stage of the training period coincided with higher rate of daily carbohydrate intake in KE than in CON. But this caused neither different blood glucose levels nor different muscle glycogen contents between the groups. Nonetheless, it cannot be excluded that beneficial regulation of blood glucose turnover during exercise contributed to explaining the ergogenic effect of KE in EPT_{120min}.

The training overload program induced many responses indicating the development of a physiological overtraining status. Concomitantly, mental well-being consistently deteriorated. Results from the RESTQ-76 Sport questionnaire indicate impaired general well-being, attention issues, and both physical and emotionally exhaustion. The decrement in RESTQ-76 scores in fact was similar to the effect of a four week overload training period in well-trained triathletes (Coutts *et al.* 2007), but was independent of the experimental group. This shows that in the conditions of the current study RESTQ-76 Sport scoring was less sensitive than the physiological measures to identify the development of overtraining.

The primary aim of the study was to assess the effect of KE in overtraining. At the same time, this is the first longitudinal study to explore such a wide spectrum of physiological responses induced by a period of deliberate and well-controlled endurance training overload. Our observations corroborate the prevailing opinion that overtraining is a complex integrative response involving dysregulations in multiple systems, including the autonomous nervous system, energy balance, and hormonal status (Lehmann *et al.* 1998; Kreher & Schwartz, 2012), which eventually also result in immunological impairment. Therefore, identification of a harmful degree of overreaching by a single biomarker is conceivably irrelevant (Urhausen & Kindermann, 2002; Hecksteden *et al.* 2016; Greenham *et al.* 2018)(129, 150, 371). Attempts to use hormonal markers of overtraining, such as ACTH, cortisol, growth hormone, thyroid

hormones and prolactin, have generally failed. Nonetheless, here we show GDF15 to consistently increase with training overload. Furthermore, GDF15 changes clearly discriminated the less (KE) *versus* the more overtrained (CON) experimental group, and both delta and absolute GDF15 was highly correlated with the training-induced drop of peak exercise heart rate ($r = 0.60$ and $r = 0.64$, respectively). Moreover, our data indicate that GDF15 is not only implicated in regulation of energy balance during training, but is probably also implicated in regulation of bone metabolism. Clearly GDF15 deserves further attention as a potential sensitive hormonal marker of overtraining development.

Previous studies have raised concerns about the implementation of ketone supplementation because of high incidence of gastro-intestinal symptoms due to acute ketone ingestion (Leckey *et al.* 2017; Vandoorne *et al.* 2017). However, compared to ketone ester intake, gastro-intestinal symptoms are more explicit with ingestion of ketone salts (Veech, 2004). In the conditions of the current study, involving KE three times daily, the incidence of gastro-intestinal complaints was low and similar between KE and CON. This suggests that gastro-intestinal distress was due to the overtraining per se (De Oliveira & Burini, 2009), rather than to the supplements.

In conclusion, here we demonstrate that an oral ketone ester is a potent nutritional strategy that prevents the development of physiological overtraining symptoms and non-functional overreaching. In addition we provide preliminary observations to indicate that GDF15 may be an adequate hormonal marker for the development of overreaching/overtraining.

ADDITIONAL INFORMATION

Competing interests

The authors declare that they have no competing interests.

Author contributions

Conception and design of the study: CP and PH. Biopsy sampling: RVT. Data collection and biochemical analyses: CP and MR. Analysis and interpretation of the data and manuscript drafting: CP and PH. All authors critically evaluated the manuscript and approved for submission. All authors agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

Funding

This study was funded by Research Fund Flanders (Fonds voor Wetenschappelijk Onderzoek – Vlaanderen; research grant no. G080117N).

Acknowledgements

The authors wish to thank all subjects for their dedicated cooperation in this demanding trial.

REFERENCES

Achten J, Halson SL, Moseley L, Rayson MP, Casey A & Jeukendrup AE (2004). Higher dietary carbohydrate content during intensified running training results in better maintenance of performance and mood state. J Appl Physiol 96, 1331-1340.

Armstrong LE & Vanheest JL (2002). The unknown mechanism of the overtraining syndrome Clues from depression and psychoneuroimmunology. Sport Med 32, 185-209.

Aubert G, Martin OJ, Horton JL, Lai L, Vega RB, Leone TC, Koves T, Gardell SJ, Krüger M, Hoppel CL, Lewandowski ED, Crawford PA, Muoio DM & Kelly DP (2016). The failing heart relies on ketone bodies as a fuel. Circulation 133, 698-705.

Balasse EO & Féry F (1989). Ketone body production and disposal: effects of fasting, diabetes, and exercise. Diabetes Metab Rev 5, 247-270.

Barry DW & Kohrt WM (2008). BMD decreases over the course of a year in competitive male cyclists. J Bone Miner Res 23, 484-491.

Bone J & Burke LM (2016). DXA estimates of body composition and carbohydrate loading. Ann Nutr Metab 68, 228-229.

Borg G (1990). Psychophysical scaling with applications in physical work and the perception of exertion. Scand J Work Environ Heal 16, 55-58.

Breen L, Philp A, Witard OC, Jackman SR, Selby A, Smith K, Baar K & Tipton KD (2011). The influence of carbohydrate-protein co-ingestion following endurance exercise on myofibrillar and mitochondrial protein synthesis. J Physiol 589, 4011-4025.

Brownlow ML, Jung SH, Moore RJ, Bechmann N & Jankord R (2017). Nutritional ketosis affects metabolism and behavior in Sprague-Dawley rats in both control and chronic stress environments. Front Mol Neurosci 10, 1-17.

Cadegiani FA & Kater CE (2017). Hormonal aspects of overtraining syndrome: a systematic review. BMC Sports Sci Med Rehabil 9, 1-15.

Cahill GF (1976). Starvation in man. Clin Endocrinol Metab 5, 397-415.

Confavreux CB, Levine R & Karsenty G (2009). A paradigm of integrative physiology, the crosstalk between bone and energy metabolisms. Mol Cell Endocrinol 310, 21-29.

Costill DL, Bowers R, Branam G & Sparks K (1971). Muscle glycogen utilization exercise on successive days. J Appl Physiol 31, 834-838.

Costill DL, Flynn MG, Kirwan JP, Houmard JA, Mitchell JB, Thomas R & Park SH (1988). Effects of repeated days of intensified training on muscle glycogen and swimming performance. Med Sci Sport Exerc 20, 249-254.

Coutts AJ ·, Wallace LK & Slattery KM (2007). Monitoring changes in performance, physiology, biochemistry, and psychology during overreaching and recovery in triathletes. Int J Sports Med 28, 125-134.

Cox PJ & Clarke K (2014). Acute nutritional ketosis: implications for exercise performance and metabolism. Extrem Physiol Med 3, 17.

Cox PJ, Kirk T, Ashmore T, Willerton K, Evans R, Smith A, Murray AJ, Stubbs B, West J, McLure SW, King MT, Dodd MS, Holloway C, Neubauer S, Drawer S, Veech RL, Griffin JL & Clarke K (2016). Nutritional ketosis alters fuel preference and thereby endurance performance in athletes. Cell Metab 24, 256-268.

Crockett JC, Rogers MJ, Coxon FP, Hocking LJ & Helfrich MH (2011). Bone remodelling at a glance. J Cell Sci 124, 991-998.

D'lugos AC, Luden ND, Faller JM, Akers JD, Mckenzie AI & Saunders MJ (2016). Supplemental protein during heavy cycling training and recovery impacts skeletal muscle and heart rate responses but not performance. Nutrients 8, 1-19.

De Oliveira EP & Burini RC (2009). The impact of physical exercise on the gastrointestinal tract. Curr Opin Clin Nutr Metab Care 12, 533-538.

Esler M, Jennings G, Korner P, Willett IAN, Dudley F, Hasking G & Anderson W (1988). Assessment of human sympathetic nervous system activity from measurements of norepinephrine spillover. Hypertension 11, 3-20.

Ettema G & Loras HW (2009). Efficiency in cycling: a review. Eur J Appl Physiol 106, 1-14.

Ferreira A, Alho I, Casimiro S & Costa L (2015). Bone remodeling markers and bone metastases: from cancer research to clinical implications. Bonekey Rep 4, 1-9.

Fisher JP (2014). Autonomic control of the heart during exercise in humans: role of skeletal muscle afferents. Exp Physiol 99, 300-305.

Flier JS (1998). What's in a name? In search of leptin's physiological role. J Clin Endocrinol Metab 83, 1407-1413.

Fry AC, Kraemer WJ, Van Borselen F, Lynch JM, Triplett NT, Koziris LP, Fleck SJ & Fleck Cate SJ (1994a). Catecholamine responses to short-term high-intensity resistance exercise overtraining. J Appl Physiol 77, 941-946.

Fry AC, Schilling BK, Weiss LW & Chiu LZF (2006). B2 -Adrenergic receptor downregulation and performance decrements during high-intensity resistance exercise overtraining. J Appl Physiol 101, 1664-1672.

Fry RW, Grove JR, Morton AR, Zeronit PM, Gaudieri S & Keast D (1994b). Psychological and immunological correlates of acute overtraining. Br J Sp Med 28, 241-246.

Fry RW, Morton AR, Garcia-Webb P, Crawford GPM & Keast D (1992). Biological responses to overload training in endurance sports. Eur J Appl Physiol Occup Physiol 64, 335-344.

Fry RW, Morton AR & Keast D (1991). Overtraining in athletes. Sport Med 12, 32-65.

Gleeson M (2002). Biochemical and immunological responses to overtraining. J Sport Sci Med 1, 31-41.

Gleeson M & Bishop NC (2000). Modification of immune responses to exercise by carbohydrate, glutamine and anti-oxidant supplements. Immunol Cell Biol 78, 554-561.

Greenham G, Buckley JD, Garrett J, Eston R & Norton K (2018). Biomarkers of physiological responses to periods of intensified, non-resistance-based exercise training in well-trained male athletes: a systematic review and meta-analysis. Sport Med 48, 2517-2548.

Halson SL, Lancaster GI, Achten J, Gleeson M & Jeukendrup AE (2004). Effects of carbohydrate supplementation on performance and carbohydrate oxidation after intensified cycling training. J Appl Physiol 97, 1245-1253.

Hawley JA, Burke LM, Phillips SM, Spriet LL & Hawley JA (2011). Nutritional modulation of training-induced skeletal muscle adaptations. J Appl Physiol 110, 834-845.

Hecksteden A, Skorski S, Schwindling S, Hammes D, Pfeiffer M, Kellmann M, Ferrauti A, Meyer T & Lucia A (2016). Blood-borne markers of fatigue in competitive athletes – Results from simulated training camps. PLoS One 11, 1-13.

Hedelin R, Wiklund U, Bjerle P & Henriksson-Larsen K (2000). Cardiac autonomic imbalance in an overtrained athlete. Med Sci Sports Exerc 32, 1531-1533.

Holdsworth DA, Cox PJ, Kirk T, Stradling H, Impey SG & Clarke K (2017). A ketone ester drink increases postexercise muscle glycogen synthesis in humans. Med Sci Sport Exerc 49, 1789-1795.

Hooper SL, Mackinnon LT, Howard A, Gordon RD & Bachmann AW (1995). Markers for monitoring overtraining and recovery. Med Sci Sport Exerc 27, 106-112.

Israel S (1976). The problems of overtraining with reference to performance physiology and internal medicine [in German]. Med Sport 16, 1-12.

Jeukendrup A, Saris WHM, Brouns F & Kester ADM (1996). A new validated endurance performance test. Med Sci Sports Exerc 2, 266-270.

Jeukendrup AE & Hesselink MKC (1994). Overtraining-what do lactate curves tell us? Br J Sp Med 28, 239-240.

Johnen H et al. (2007). Tumor-induced anorexia and weight loss are mediated by the TGF-b superfamily cytokine MIC-1. Nat Med 13, 1333-1340.

Johnson RH, Walton JL, Krebs HA & Williamson DH (1969). Metabolic fuels during and after severe exercise in athletes and non-athletes. Lancet 2, 452-455.

Kellmann M & Kallus KW (2001). Recovery-stress questionnaire for athletes: user manual. In Champaign, IL: Human Kinetics.

Killer SC, Svendsen IS, Jeukendrup AE & Gleeson M (2017). Evidence of disturbed sleep and mood state in well-trained athletes during short-term intensified training with and without a high carbohydrate nutritional intervention. J Sports Sci 35, 1402-1410.

Kimura I, Inoue D, Maeda T, Hara T, Ichimura A, Miyauchi S, Kobayashi M, Hirasawa A, Tsujimoto G & Lefkowitz RJ (2011). Short-chain fatty acids and ketones directly regulate sympathetic nervous system via G protein-coupled receptor 41 (GPR41). PNAS 108, 8030-8035.

Koninckx E, Van Leemputte M & Hespel P (2008). Effect of a novel pedal design on maximal power output and mechanical efficiency in well-trained cyclists. J Sport Sci 26, 1015-1023.

Koninckx E, Van Leemputte M & Hespel P (2010). Effect of isokinetic cycling versus weight training on maximal power output and endurance performance in cycling. Eur J Appl Physiol 109, 699-708.

Kreher & Schwartz (2012). Overtraining syndrome: a practical guide. Sports Health 4, 128-138.

Kreider RB et al. (2010). ISSN exercise & sport nutrition review: research & recommendations. J Int Soc Sports Nutr 7, 1-43.

Kuipers H & Keizer HA (1988). Overtraining in elite athletes, review and directions for the future. Sport Med 6, 79-92.

Leckey JJ, Ross ML, Quod M, Hawley JA & Burke LM (2017). Ketone diester ingestion impairs time-trial performance in professional cyclists. Front Physiol 8, 1-10.

Lehmann M, Dickhuth H, Gendrisch G, Lazar W, Thum M, Kaminski R, Aramendi J, Peterke E, Wieland W & Keul J (1991). Training - overtraining. A prospective, experimental study with experienced middle- and long-distance runners. Int J Sports Med 12, 444-452.

Lehmann M, Foster C, Dickhuth H-H & Gastmann U (1998). Autonomic imbalance hypothesis and overtraining syndrome. Med Sci Sport Exerc 30, 1140-1145.

Lehmann M, Foster C & Keul J (1993). Overtraining in endurance athletes: a brief review. Med Sci Sport Exerc854-862.

Lombardi G, Lanteri P, Graziani R, Colombini A, Banfi G, Corsetti R & Lucia A (2012). Bone and energy metabolism parameters in professional cyclists during the Giro d'Italia 3-weeks stage race. PLoS One 7, 1-9.

Lowry OH & Passoneau JV (1972). A flexible system of enzymatic analysis.

Macia L, Wang V, Tsai W, Nguyen AD, Johnen H, Kuffner T, Shi Y-C, Lin S, Herzog H, Brown DA, Breit SN, Sainsbury A & Aguila MB (2012). Macrophage Inhibitory Cytokine 1 (MIC-1/GDF15) decreases food intake, body weight and improves glucose tolerance in mice on normal & obesogenic diets. PLoS One 7, 1-8.

Meeusen R, Duclos M, Foster C, Fry A, Gleeson M, Nieman D, Raglin J, Rietjens G, Steinacker J & Urhausen A (2013). Prevention, diagnosis, and treatment of the overtraining syndrome: Joint consensus statement of the european college of sport science and the American College of Sports Medicine. Med Sci Sports Exerc 45, 186-205.

Meeusen R & Watson P (2007). Amino acids and the brain: Do they play a role in "central fatigue"? Int J Sport Nutr Exerc Metab 17, 37-46.

Merry TL & Ristow M (2016). Do antioxidant supplements interfere with skeletal muscle adaptation to exercise training? J Physiol 594, 5135-5147.

Morgan WP, Brown DR, Raglin JS, O'Connor PJ & Ellickson KA (1987). Psychological monitoring of overtraining and staleness. Br J Sports Med 21, 107-114.

Nagle KB & Brooks MA (2011). A systematic review of bone health in cyclists. Sports Health 3, 235-243.

Olmedillas H, González-Agüero A, Moreno LA, Casajus JA & Vicente-Rodríguez G (2012). Cycling and bone health: a systematic review. BMC Med 10, 1-10.

Owen OE, Morgan AP, Kemp HG, Sullivan JM, Herrera MG & Cahill GF (1967). Brain metabolism during fasting. J Clin Invest 46, 1589-1595.

Pandit R, Beerens S & Adan RAH (2017). Role of leptin in energy expenditure: the hypothalamic perspective. Am J Physiol Regul Integr Comp Physiol 312, 938-947.

Paoli A, Bosco G, Camporesi EM & Mangar D (2015). Ketosis, ketogenic diet and food intake control: A complex relationship. Front Psychol; DOI: 10.3389/fpsyg.2015.00027.

Patel S, Alvarez-guaita A, Melvin A, Coll AP, Savage DB & Rahilly SO (2019). GDF15 provides an endocrine signal of nutritional stress in mice and humans. Cell Metab 29, 707-718.

Pawan GLS & Semple SJG (1983). Effect of 3-hydroxybutyrate in obese subjects on very-low-energy diets and during therapeutic starvation. Lancet 1, 15-17.

Pfeiffer B, Cotterill A, Grathwohl D, Stellingwerff T & Jeukendrup AE (2009). The effect of carbohydrate gels on gastrointestinal tolerance during a 16-km run. Int J Sport Nutr Exerc Metab 19, 485-503.

Pichot V, Busso T, Roche F, Garet M, Costes F, Duverney D, Lacour JR & Barthélémy JC (2002). Autonomic adaptations to intensive and overload training periods: a laboratory study. Med Sci Sports Exerc 34, 1660-1666.

Puchalska P & Crawford PA (2017). Multi-dimensional roles of ketone bodies in fuel metabolism, signaling, and therapeutics. Cell Metab 25, 262-284.

Robinson AM & Williamson DH (1980). Physiological roles of ketone bodies as substrates and signals in mammalian tissues. Physiol Rev 60, 143-187.

Sansone M, Sansone A, Borrione P, Romanelli F, Luigi L Di & Sgro P (2018). Effects of ketone bodies on endurance exercise. Curr Sports Med Rep 17, 444-453.

Sherwin RS, Hendler RG & Felig P (1975). Effect of ketone infusions on amino acid and nitrogen metabolism in man. J Clin Invest 55, 1382-1390.

Snyder AC, Kuipers H, Cheng B, Servais R & Fransen E (1995). Overtraining following intensified training with normal muscle glycogen. Med Sci Sport Exerc 22, 1063-1070.

Stanojevic D, Stojanovic Tosic J & Djorjevic D (2013). Heart rate modulations in overtraining syndrome. Serbian J Exp Clin Res 14, 23-28.

Stubbs BJ, Cox PJ, Evans RD, Cyranka M, Clarke K & de Wet H (2018). A ketone ester drink lowers human ghrelin and appetite. Obesity 26, 269-273.

Stubbs BJ, Cox PJ, Evans RD, Santer P, Miller JJ, Faull OK, Magor-Elliott S, Hiyama S, Stirling M & Clarke K (2017). On the metabolism of exogenous ketones in humans. Front Physiol 8, 848.

Svendsen IS, Killer SC, Carter JM, Randell RK, Asker ·, Jeukendrup E & Gleeson · Michael (2016). Impact of intensified training and carbohydrate supplementation on immunity and markers of overreaching in highly trained cyclists. Eur J Appl Physiol 116, 867-877.

Thomsen HH, Rittig N, Johannsen M, Møller AB, Jørgensen JO, Jessen N & Møller N (2018). Effects of 3-hydroxybutyrate and free fatty acids on muscle protein kinetics and signaling during LPS-induced inflammation in humans: anticatabolic impact of ketone bodies. Am J Clin Nutr 108, 1-11.

Townsend R, Elliott-Sale KJ, Currell K, Tang J, Fraser WD & Sale C (2017). The effect of postexercise carbohydrate and protein ingestion on bone metabolism. Med Sci Sports Exerc 49, 1209-1218.

Urhausen A & Kindermann W (2002). Diagnosis of overtraining. What tools do we have? Sport Med 32, 95-102.

van Loon LJ, Saris WH, Kruijshoop M & Wagenmakers AJ (2000). Maximizing postexercise muscle glycogen synthesis: carbohydrate supplementation and the application of amino acid or protein hydrolysate mixtures. Am J Clin Nutr 72, 106-111.

Van Thienen R, D'Hulst G, Deldicque L & Hespel P (2014). Biochemical artifacts in experiments involving repeated biopsies in the same muscle. Physiol Rep 2, 286-299.

Vandoorne T, De Smet S, Ramaekers M, Van Thienen R, De Bock K, Clarke K & Hespel P (2017). Intake of a ketone ester drink during recovery from exercise promotes mTORC1 signaling but not glycogen resynthesis in human muscle. Front Physiol 8, 1-12.

Veech RL (2004). The therapeutic implications of ketone bodies: the effects of ketone bodies in pathological conditions: ketosis, ketogenic diet, redox states, insulin resistance, and mitochondrial metabolism. Prostaglandins, Leukot Essent Fat Acids 70, 309-319.

Wang-Wei Tsai V, Macia L, Johnen H, Kuffner T, Manadhar R, Beck Jørgensen S, Ki Michelle Lee-Ng K, Ping Zhang H, Wu L, Peter Marquis C, Jiang L, Husaini Y, Lin S, Herzog H, Brown DA, Sainsbury A, Breit SN & Morrison C (2013). TGF-b superfamily cytokine MIC-1/GDF15 is a physiological appetite and body weight regulator. PLoS One 8, 1-10.

Witard OC, Jackman SR, Kies AK, Jeukendrup AE & Tipton KD (2011). Effect of increased dietary protein on tolerance to intensified training. Med Sci Sports Exerc 43, 598-607.

Woods AL, Garvican-Lewis LA, Lundy B, Rice AJ & Thompson KG (2017). New approaches to determine fatigue in elite athletes during intensified training: Resting metabolic rate and pacing profile. PLoS One 12, 1-17.

Woods AL, Rice AJ, Garvican-Lewis LA, Wallett AM, Lundy B, Rogers MA, Welvaert M, Halson S, McKune A & Thompson KG (2018). The effects of intensified training on resting metabolic rate (RMR), body composition and performance in trained cyclists. PLoS One; DOI: 10.1371/journal.pone.0191644.

Zou X, Meng J, Li L, Han W, Li C, Zhong R, Miao X, Cai J, Zhang Y & Zhu D (2016). Acetoacetate accelerates muscle regeneration and ameliorates muscular dystrophy in mice. J Biol Chem 291, 2181-2195.

Article 4

Response to the letter to the editor: Ketone ester supplementation in endurance athletes: a miracle drink or 'spin'?

Chiel Poffé[1], Monique Ramaekers[1], Ruud Van Thienen[1], Peter Hespel[1,2] #

[1] Exercise Physiology Research Group, Department of Movement Sciences, KU Leuven, Leuven, Belgium

[2] Bakala Academy-Athletic Performance Center, KU Leuven, Leuven, Belgium

#, Correspondence: Peter Hespel, Exercise Physiology Research Group, Department of Movement Sciences, KU Leuven, Tervuursevest 101, Heverlee, 3001, Belgium

peter.hespel@kuleuven.be

Published in J Physiol (2019) 597: 16, 4409-4410

REPLY FROM CHIEL POFFE, MONIQUE RAMAEKERS, RUUD VAN THIENEN AND PETER HESPEL

In our recent article (Poffé *et al.* 2019) published in this journal, we demonstrated that ketone ester (KE) intake during a 3-week endurance training overload-period blunts the development of overreaching symptoms as well as enhances endurance exercise performance. The criticisms by Korevaar *et al.* (2019) mainly relate to (i) the media coverage of the data, (ii) the presumed absence of predefined primary endpoints, and (iii) excess focus on positive outcomes, termed 'cherry picking'. We fully agree with Korevaar et al. that the lay public would benefit from a more balanced presentation of the results in the media. However, the arguments with regard to the lack of predefined primary outcomes and 'cherry picking' are fully speculative.

The major contribution of public funding to scientific research (Van Dalen *et al.* 2014) implies that media coverage is important in translating scientific output to the lay public. Some media channels provided a very well-balanced and nuanced discussion of our findings (Hutchinson, 2019; Van Teeffelen, 2019), but others did not. This resulted in invalid statements like 'miracle drink' or 'superfuel', as indicated by Korevaar et al. However, we are not responsible for these wordings and obviously do not support such inappropriate terminology. Therefore, in this reply we confine our response to scientific facts and figures.

Korevaar et al. correctly state that an a priori definition of primary output variables, with corresponding power calculation, is essential to avoid selective reporting in clinical trials. Good point, indeed. However, their criticism that both aspects were absent in our trial is incorrect. The primary outcomes and a power calculation were included in the study file which was approved by the Ethics Committee Research UZ/KU Leuven (registration number: B322201733747). Moreover, the Ethics Committee Research UZ/KU Leuven procedures comply with national and international clinical trials regulations, including the Declaration of Helsinki (for detailed information: https://www.uzleuven.be/nl/ethische-commissie/onderzoek/wet-en-regelgeving-voor-studies). Therefore, the suggestion by Korevaar et al. that we falsely report to comply with the Declaration of Helsinki is irrelevant. But we agree that registration of the trial in an open access database would facilitate such verification, indeed. However, registration in a public data base actually is only routinely done

for clinical trials involving medicinal products (https://www.clinicaltrialsregister.eu/;https://databankklinischeproeven.be/en).

Furthermore, the definition of primary endpoints in 'classical' clinical trials is often easy and straightforward. For instance, blood pressure is the logic primary output variable in studies addressing antihypertensive agents. However, in trials exploring novel fields, wherein limited a priori consensus exists about clear-cut endpoints, such as in overreaching/overtraining (Urhausen & Kindermann, 2002), predefinition of primary endpoints is more complex. Nevertheless, we defined training load to be the primary endpoint of our study. We aimed to assess whether stimulation of anabolic signaling and muscle glycogen repletion by KE ingestion post-exercise (Holdsworth *et al.* 2017; Vandoorne *et al.* 2017) could attenuate training overload-induced fatigue, and thereby facilitate higher training volume to be tolerated. Therefore, we imposed an expectedly 'supramaximal' training load to induce failure in adhering to the prescribed training program. This resulted in higher training loads in the subjects on ketone ester supplements during the days before the performance tests (e.g. TT_{30min} and 90S). Nonetheless, compared with baseline, subjects on KE, but not subjects on placebo, performed better in TT_{30min} and 90S.

Our data showed that training load in the final week of the training program was ~15% higher in KE than in placebo. This effect has been excessively highlighted in the media. But clearly, this effect in healthy volunteers cannot be extrapolated to well-trained athletes. Because this would imply that in some endurance events, female elite athletes on KE could probably outperform male athletes without KE, which makes no sense. But one should also recognize that in elite athletes even an ergogenic effect of < 1% may be crucial (Paton & Hopkins, 2006). Furthermore, training performance was the pre-defined primary endpoint of our study. But post hoc, the effects of KE on the physiological parameters reflecting overreaching, such as maximal heart rate, urinary catecholamine excretion, hormonal food intake regulation, and training-induced increase in GDF15, are far more interesting, and conceivably will trigger further research in this promising field.

Korevaar et al. also claim that we created a 'spin' by adopting a 'cherry picking' strategy in the data presentation. They substantiate such conclusion by indicating that confidence intervals around the 15% difference in training load and EPT_{120min} 'would show the uncertainty of this finding'. This statement is bizarre, not to say inappropriate, given that 95% confidence

intervals (CI) as well as effect sizes (ES) are clearly reported in the results section (training load: CI = +237 to +2372 kJ, ES = 0.80; EPT_{120min}: CI = +5 to +52 W, ES = 0.77) and in fact further underscore the relevance of the findings. Similarly, Korevaar et al. criticize the absence of adjustment for multiple statistical comparisons. But the statistics section in the manuscript clearly mentions that adjustment for multiple comparisons was done, indeed. Finally, the accusation of 'cherry picking' is entirely false. Positive outcomes were presented most transparently by showing individual data points as well as effect sizes and confidence intervals. In addition, also all negative observations were reported. Even in the abstract the authors report seven parameters that were unaffected by KE.

From our perspective, Korevaar et al. highlight some valuable hallmarks in the context of good clinical practice. However, their criticisms regarding the output of our clinical trial are merely based on speculation and can be entirely rejected by facts and figures. The data shown in the manuscript justify the valid conclusions of the study, and will hopefully stimulate further research to elucidate the role of ketone bodies in physiological regulation at rest and during exercise.

ADDITIONAL INFORMATION

Competing interests

The authors declare that they have no competing interests.

Author contributions

All authors have read and approved the final version of this manuscript and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

Funding

This study was funded by Research Fund Flanders (Fonds voor Wetenschappelijk Onderzoek – Vlaanderen; research grant no. G080117N).

REFERENCES

Holdsworth DA, Cox PJ, Kirk T, Stradling H, Impey SG & Clarke K (2017). A ketone ester drink increases postexercise muscle glycogen synthesis in humans. Med Sci Sport Exerc 49, 1789-1795.

Hutchinson A (2019). There might finally be a cure for overtraining. Available at: https://www.outsideonline.com/2396013/ketones-overtraining-research.

Korevaar DA, Cohen J & McInnes MD (2019). Ketone ester supplementation in endurance athletes: a miracle drink or 'spin'? J Physiol 597, 4407-4408.

Paton CD & Hopkins WG (2006). Variation in performance of elite cyclists from race to race. Eur J Sport Sci 6, 25-31.

Poffé C, Ramaekers M, Van Thienen R & Hespel P (2019). Ketone ester supplementation blunts overreaching symptoms during endurance training overload. J Physiol 597, 3009-3027.

Urhausen A & Kindermann W (2002). Diagnosis of overtraining. What tools do we have? Sport Med 32, 95-102.

Van Dalen R, Mehmood S, Verstraten P & Van Der Wiel K (2014). Public funding of science: an international comparison.

Van Teeffelen J (2019). Ketonen: wonderdrank voor wielrenners? Available at: https://jurgenvanteeffelen.nl/ketonen-wonderdrank-voor-wielrenners/.

Vandoorne T, De Smet S, Ramaekers M, Thienen R Van, De Bock K, Clarke K & Hespel P (2017). Intake of a ketone ester drink during recovery from exercise promotes mTORC1 signalling but not glycogen resynthesis in human muscle. Front Physiol 8, 1-12.

Article 5

Response to the letter to the editor: Type I error

Chiel Poffé[1], Monique Ramaekers[1], Ruud Van Thienen[1], Peter Hespel[1,2] #

[1] Exercise Physiology Research Group, Department of Movement Sciences, KU Leuven, Leuven, Belgium

[2] Bakala Academy-Athletic Performance Center, KU Leuven, Leuven, Belgium

#, Correspondence: Peter Hespel, Exercise Physiology Research Group, Department of Movement Sciences, KU Leuven, Tervuursevest 101, Heverlee, 3001, Belgium

peter.hespel@kuleuven.be

Published in J Physiol (2019) 597: 17, 4679-4680

REPLY FROM CHIEL POFFE, MONIQUE RAMAEKERS, RUUD VAN THIENEN AND PETER HESPEL

We thank P. Mullie and P. Autier for providing their interest in our recent article published in this journal (Poffé *et al.* 2019). The article reported the effects of ketone ester (KE) intake on physiological markers of overreaching, as well as on exercise performance during a three-week endurance training overload period. The authors state that the observed 15% increase in training load during the final week in KE is likely a statistical type I error caused by (i) a presumed one-sided testing of the null hypothesis and (ii) the inclusion of all subjects including an "intuitive", but not statistically confirmed outlier (Mullie & Autier, 2019). They make their point by performing unpaired statistical testing on a reconstructed data set derived from our original data. But, given the pair-matched design of our study, unpaired t-testing is inappropriate. Therefore, statistical results reported in the manuscript refer to the output of two-way repeated measures analyses of variance, or paired t-tests in places. However, in the statistics section we erroneously stated that unpaired t-tests were used. The authors apologize for this mistake.

In the methods section of our manuscript, we clearly indicated that we adopted a study design involving matched-pairs randomization. The pair-matching was primarily based on mean power outputs in TT_{30min} in the pretest. Appropriate statistical analysis thus requires to account for this matched-pairs randomization used. Therefore, and as clearly reported in the manuscript, we consistently used two-way repeated-measures analyses of variance to obtain two-sided p-values and relevant confidence limits. However, in the statistical section of the manuscript it was accidentally written that also unpaired-test were used, whilst in fact paired t-tests were performed. Mullie and Autier were probably mislead by this statement, and reactively performed unpaired t-tests to challenge our findings, yet yielding incorrect p-values and confidence intervals. To further document the validity of our data, we here present the training load per experimental pair in week 3 (figure 1, left panel). Importantly, given the pair-matching procedure, training loads obviously were very similar within pairs in week 1 (intra-pair difference: 2 ± 4% (mean ± SE)). The intra-pair differences depicted in figure 1 clearly show that in week 3 seven out of nine subjects on KE outperformed their paired control (95% confidence interval (CI) KE *vs.* CON: +237 to +2372 kJ, $P < 0.05$) , which clearly supports the conclusions of the study.

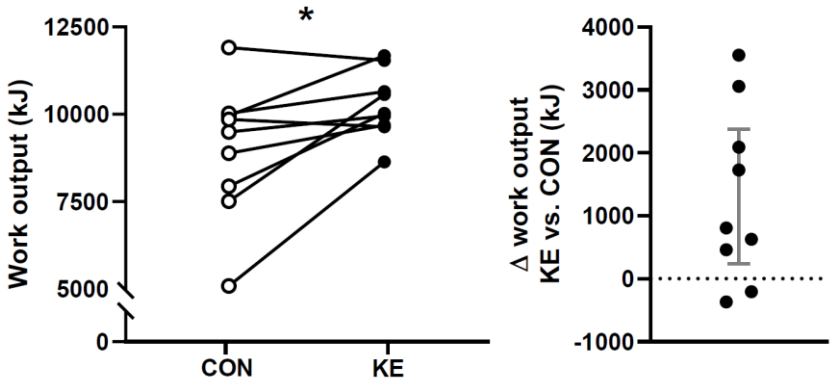


Figure 1. Effect of ketone ester supplementation on training workload during week 3.
Individual data points of pair-matched subjects (open circles, control; filled circles, KE; $n = 9$) for total work output (left panel), and difference in total work output between KE subjects and their corresponding control together with 95% confidence intervals (right panel), in week 3 of the training period. * $P < 0.05$ KE *vs.* CON.

In a second point Mullie and Autier argue that the effect of KE intake on training load in week 3 has reached statistical significance because of one subject exhibiting a remarkably low work output (5084 kJ). They even propose to exclude this subject, as this would result in a more similar data distribution of the experimental groups. However, this statement is rather bizarre, given that this subject is not qualified as an outlier by appropriate statistical analysis. In addition, the difference in training load in week 3 between this subject and his pair-matched control perfectly falls within the range of all intra-pair differences (Figure 1, right panel). This indicates that the supposedly 'abnormal' value simply reflects the normal physiological variability in responding to overload training. In fact, using the invalid approach by Mullie and Autier, one could also intuitively exclude the subject with the highest training load (11912 kJ) amongst the controls. Notwithstanding the above debate, even after exclusion of this intuitively identified outlier and his corresponding control, the training load in week 3 is significantly higher in KE (95% CI: +37 to +2009 kJ; $P < 0.05$).

Additional support for our conclusion that KE intake enhanced endurance performance, indeed, comes from other performance tests, i.e. EPT_{120min} and TT_{30min}. Because exercise performance is often subject to high day-to-day variation, we calculated a composite score for endurance performance by averaging the relative performance of each subject for EPT_{120min}, TT_{30min} and training load in week 3. Such global score probably well reflects the true endurance exercise performance capacity in the final stage of the overload training protocol. Compared to their paired controls, endurance performance so calculated was higher in 8 out of 9 KE subjects.

Mean improvement in endurance performance was 11% (95% CI: +3 to +18%, P < 0.01, Figure 2).

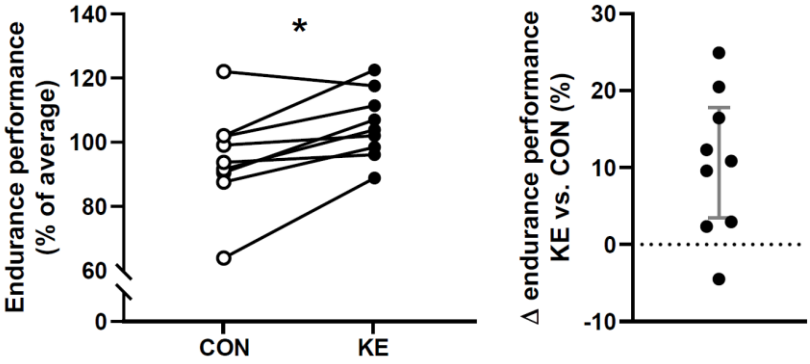


Figure 2. Effect of ketone ester supplementation on overall endurance performance.
Individual data points of pair-matched subjects (open circles, control; filled circles, KE; n = 9) representing their composite endurance performance score (weighted score for EPT120min, TT30min and training load during week 3; left panel), and difference in endurance performance between KE subjects and their corresponding control together with 95% confidence intervals (right panel). * $P < 0.05$ KE *vs.* CON.

The data outlined above clearly support our conclusions with regard to the effect of KE on endurance exercise performance in the conditions of the current study. But extrapolating these data obtained in healthy volunteers to elite endurance athletes, would be very naïve, indeed (Close *et al.* 2019). Therefore, the statement by Mullie and Autier regarding the linear translation of our data to the Tour de France of 2018 (incorrectly noted as 1998 in their letter to the editor) is irrelevant. A similar irrelevant extrapolation could suggest that the use of KE could push the world record in marathon running to below 1 h 45min, which is just pleasant phantasy.

Last but not least we want to emphasize that unfortunately the Letters to the Editor by both Mullie and Autier and Korevaar *et al.* (2019) tend to narrow the scope of our study to exercise performance. But the physiological observations with regard to the effect of KE on heart rate regulation, basal adrenergic activity, food intake, and GDF15 regulation during training overload are far more intriguing. And these novel data are highly statistically significant, whatever statistical procedure is used.

In summary, we show here that the criticism raised by Mullie and Autier on our exercise performance data may result from a misleading statement in the statistical section of the manuscript. However, the correct statistical output and the individual data included in the

original manuscript, together with the additional analyses provided in this response, prove that the increase in training load due to KE definitely is not a type I error but a valid observation that is likely to stimulate further research on this topic.

ADDITIONAL INFORMATION

Competing interests

The authors declare that they have no competing interests.

Author contributions

All authors have read and approved the final version of this manuscript and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

Funding

This work is supported by Research Fund Flanders (Fonds voor Wetenschappelijk Onderzoek – Vlaanderen; research grant no. G080117N).

REFERENCES

Close GL, Kasper AM & Morton JP (2019). From paper to podium: quantifying the translational potential of performance nutrition research. Sports Med 49 (Suppl. 1), 25-37.

Korevaar DA, Cohen J & McInnes MD (2019). Ketone ester supplementation in endurance athletes: a miracle drink or 'spin'? J Physiol 597, 4407-4408.

Mullie P & Autier P (2019). Type 1 error. J Physiol 597, 4667-4678.

Poffé C, Ramaekers M, Van Thienen R & Hespel P (2019). Ketone ester supplementation blunts overreaching symptoms during endurance training overload. J Physiol 597, 3009-3027.

Article 6

Response to the letter to the editor: Does ketone ester supplementation really blunt overreaching symptoms during endurance training overload?

Chiel Poffé[1], Monique Ramaekers[1], Peter Hespel[1,2] #

[1] Exercise Physiology Research Group, Department of Movement Sciences, KU Leuven, Leuven, Belgium

[2] Bakala Academy-Athletic Performance Center, KU Leuven, Leuven, Belgium

Published in J Physiol (2019) 597: 21, 5309-5310

REPLY FROM CHIEL POFFE, MONIQUE RAMAEKERS AND PETER HESPEL

We appreciate the interest of P. Bellinger in our recent article in which we reported that ketone ester (KE) intake blunts the development of overreaching symptoms and stimulates endurance exercise performance (Poffé *et al.* 2019a). In his letter to the editor, Bellinger states that our conclusion that KE blunts the development of overreaching symptoms is unfounded, as the subjects were not in a state of non-functional overreaching (NFOR) but only functionally overreached (FOR) (Bellinger, 2019). The author substantiates his commentary by (intentionally) omitting a crucial part of the definition of NFOR as proposed in the consensus statement of the European College of Sport Science and the American College of Sports Medicine (Meeusen *et al.* 2013). We respectfully disagree with Bellinger's statements, as our conclusions are independent of whether or not NFOR occurred, but are based on the observation that physiological symptoms indicative of the development of NFOR were clearly blunted by KE. Moreover, we argue that any discussion about whether subjects were either FOR or NFOR is simply nonsensical as there are no objective criteria to discriminate between both at the individual level. Finally, our study did not at all intend to identify symptoms that are able to diagnose NFOR. We wanted to isolate symptoms, and explore the effect of KE intake here on, which in a longitudinal follow-up of an athlete allow to identify the transition from FOR to NFOR, in order to timely reduce training load.

Bellinger correctly states that FOR results in a short-term decrease in performance, in which restoration of performance may take days to weeks. However, the author states that NFOR causes a decrease in performance that will not resume for several weeks or months, thereby neglecting that a stagnation of performance may also indicate NFOR (Meeusen *et al.* 2013). Therefore, the observed absence of improvements in the 30min time-trial as well as in 90s sprint performance in some subjects following 7 rest days, does not justify the claim by Bellinger that all subjects were merely FOR. But again, in fact this doesn't even matter in the context of the current study.

In a second point, the author states that subjects should be classified on an individual basis as FOR or NFOR, and that such a classification is typically employed in other studies while completely lacking in our manuscript. We completely agree that such a classification would be highly desirable. However, although cross-sectional studies have suggested criteria to discriminate between NFOR *vs.* OTS (Meeusen *et al.* 2004, 2010; Buyse *et al.* 2019), clear-cut

criteria to adequately discriminate FOR from NFOR are entirely missing. In fact, not a single published paper, including the ones cited by Bellinger, has been able to make such a distinction at the individual level. Therefore, we believe that it would be inappropriate to classify each subject as either FOR or NFOR based on unvalidated criteria. However, in agreement with the aforementioned consensus statement (Meeusen *et al.* 2013), we indicated in our article that in the total group of subjects both FOR and NFOR were present, indeed. Following 7 days of recovery, performance was still decreased in some, *versus* increased in others (90s sprint: -5 to + 13%; 30min time-trial: -5 to +14%). Adequate prevention of NFOR requires pertinent longitudinal follow-up of an athlete. Therefore, and also from a purely scientific perspective, it is bizarre that Belllinger challenges our longitudinal findings, based on earlier cross-sectional observations. For sure, identification of adequate markers indicating transition from FOR to NFOR will require more well-controlled longitudinal studies to be done.

Given the inability to discriminate objectively between FOR and NFOR, the argument by Bellinger that growth differentiation factor 15 (GDF15) levels should be studied in response to an individual's classification to serve as a marker for the development of overreaching/overtraining, does not make sense. Only longitudinal observations can really serve such purpose. Therefore, we believe that (i) the consistent increase in GDF15 with training overload in all subjects, (ii) the divergent response in GDF15 between the less (KE) *versus* the more overreached (CON) group; and (iii) the strong correlation between GDF15 and the training-induced drop in peak exercise heart rate, clearly document the potential of GDF15 to serve as a biological marker of the degree of overreaching in a given individual. But, more longitudinal studies are needed to support such statement, of course.

Notwithstanding the above debate, the aim of our study was not to diagnose whether NFOR occurred, but to investigate in a hands-on approach whether KE intake could blunt the development of overreaching symptoms, thereby allowing higher training volumes to be tolerated. Therefore, we employed an ecologically valid study design by imposing a 'supramaximal' training load to induce failure in adherence to the prescribed training programme. This resulted in higher training loads for subjects receiving ketone supplements. Nonetheless, subjects from the KE group exhibited remarkably fewer symptoms indicating overreaching development. These data clearly indicate that KE intake is a potent nutritional strategy to reduce the incidence of overreaching symptoms, independent of whether or not a subject is classified as FOR or NFOR.

In a final point, Bellinger argues that the 15% increase in 30min time-trial performance following a 90 min preload (EPT_{120min}) at day 18 of the training period observed in KE, might be the result of initial between-group differences. We fully agree that this cannot be excluded. However, the ability of KE subjects to manage a 15% higher training load at the end of the training period, together with the pair-matched study design, provide strong arguments to believe that these between-group differences are merely caused by the experimental conditions, indeed. Nevertheless, as indicated in our reply to Korevaar et al. (Poffé *et al.* 2019b), we predefined only training load as the primary endpoint. Therefore, the conclusion that KE intake stimulates endurance performance is primarily based on the differences in training load, but is further supported by alterations in other performance tests (90s sprint, 30min time-trial, EPT_{120min}), as already shown in our recent response to Mullie and Autier (Poffé *et al.* 2019c).

In summary, the criticism by Bellinger that our conclusions are invalid because we did not individually classify subjects as FOR *versus* NFOR, is in fact irrelevant given the absence of well-validated criteria to perform such a classification. Moreover, the original data presented in our article clearly support the conclusion that KE intake blunts the development of overreaching symptoms, while improving endurance exercise performance, indeed.

ADDITIONAL INFORMATION

Competing interests

The authors declare that they have no competing interests.

Author contributions

All authors have read and approved the final version of this manuscript and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

REFERENCES

Bellinger P (2019). Does ketone ester supplementation really blunt overreaching symptoms during endurance training overload? *J Physiol* 597, 5307-5308.

Buyse L, Decroix L, Timmermans N, Barbé K, Verrelst R & Meeusen R (2019). Improving the diagnosis of nonfunctional overreaching and overtraining syndrome. *Med Sci Sports Exerc* (in press; https://doi.org/10.1249/ MSS.0000000000002084).

Meeusen R, Duclos M, Foster C, Fry A, Gleeson M, Niemand D, Raglin J, Rietjens G, Steinacker J & Urhausen A (2013). Prevention, diagnosis and treatment of the overtraining syndrome: Joint consensus statement of the European College of Sport Science and the American College of Sports Medicine. *Med Sci Sports Exerc* 45, 186-205.

Meeusen R, Nederhof E, Buyse L, Roelands B, De Schutter G & Piacentini MF (2010). Diagnosing overtraining in athletes using the two-bout exercise protocol. *Br J Sports Med* 44, 642-648.

Meeusen R, Piacentini MF, Busschaert B, Buyse L, De Schutter G & Stray-Gundersen J (2004). Hormonal responses in athletes: the use of a two bout exercise protocol to detect subtle differences in (over)training status. *Eur J Appl Physiol* 91, 140-146.

Poffé C, Ramaekers M, Van Thienen R & Hespel P (2019a). Ketone ester supplementation blunts overreaching symptoms during endurance training overload. *J Physiol* 597, 3009-3027.

Poffé C, Ramaekers M, Van Thienen R & Hespel P (2019b). Reply from Chiel Poffé, Monique Ramaekers, Ruud Van Thienen and Peter Hespel. *J Physiol* 597, 4409-4410.

Poffé C, Ramaekers M, Van Thienen R & Hespel P (2019c). Reply from Chiel Poffé, Monique Ramaekers, Ruud Van Thienen and Peter Hespel. *J Physiol* 597, 4679-4680.

Article 7

Exogenous ketosis enhances the angiogenic response to exercise

Chiel Poffé[1], Ruben Robberechts[1], Ruud Van Thienen[1], Peter Hespel[1,2] #

[1] Exercise Physiology Research Group, Department of Movement Sciences, KU Leuven, Leuven, Belgium

[2] Bakala Academy-Athletic Performance Center, KU Leuven, Leuven, Belgium

Submitted

ABSTRACT

De novo capillarization (angiogenesis) is a primary muscular adaptation to exercise training and is crucial to improve endurance exercise tolerance. Excess training load, however, impedes such beneficial adaptations, yet we recently reported that such downregulation may be counteracted by intermittent exogenous ketosis (IEK) post-exercise. Therefore, here we investigated whether IEK could stimulate muscular angiogenesis during a three-week endurance training-overload period involving 10 weekly training sessions in healthy volunteers. Subjects received either 25 g of a ketone ester (KE, n = 9) or a control drink (CON, n = 9) immediately after each training session and before sleep. Biopsies from *m. vastus lateralis* were obtained on the day before (Pre) and after (Post) the training period, in the absence of acute ketosis. Subjects receiving KE showed a 44% (pre: 4.6 ± 0.2 *vs.* post: 6.6 ± 0.6 capillary contacts) and 42% (pre: 8.9 ± 0.4 *vs.* post: 12.6 ± 0.8 capillaries per 1000 µm, both $p < 0.01$) increase in capillary contacts and capillary-to-fiber perimeter exchange index, respectively. In contrast, both parameters were unaffected in CON. Concomitant with this increased capillarization in KE, VEGF and eNOS expression substantially increased both at the protein (VEGF: +33 ± 17%; eNOS: +259 ± 149%, both $p < 0.05$) and mRNA (VEGF: +181 ± 71%; eNOS: +140 ± 40%, both $p < 0.01$) level. Conversely, in CON only the protein content of eNOS increased (+98 ± 23%, $p < 0.01$). KE impacted neither muscle morphology nor muscle fiber type distribution. In conclusion: intermittent exogenous ketosis during overload training markedly stimulates exercise-induced angiogenesis in skeletal muscle. This novel finding opens an interesting perspective to consider the potential of exogenous ketosis to stimulate muscular adaptations to exercise and to counteract inactivity or disease-related capillary rarefaction.

Keywords: ketones, angiogenesis, VEGF, overreaching, overtraining, exercise performance

INTRODUCTION

Enhanced skeletal muscle vascularization is an early adaptive response to endurance exercise training, and improves the potential for oxygen diffusion, nutrient uptake and metabolite clearance to and from skeletal muscles (16). The increase in muscle capillarization usually precedes or parallels exercise-induced adaptations at the myofiber level, such as shift from type IIb/IIx to type IIa fibers (34) and increase in mitochondrial density and oxidative enzyme activity (1, 32). Hence it has been postulated that angiogenesis is required for these latter adaptations to occur (32). The angiogenic response to training clearly is an important mechanism involved in the development of muscular endurance capacity and performance (24, 25). But capillarization also plays a pivotal role in maintenance of metabolic health. From a clinical perspective, stimulation of de novo angiogenesis eventually resulting in elevated capillary density, also significantly contributes to the beneficial effects of exercise in the prevention and treatment of various diseases such as peripheral arterial disease, diabetes and chronic obstructive pulmonary disease (20). Collectively, the above findings clearly indicate that the angiogenic response to exercise is highly important both from a clinical and exercise performance perspective.

Exercise training induces beneficial muscular adaptations, indeed, yet excessive exercise may also result in so-called overreaching, and eventually overtraining in a later stage (2, 9, 22). Recent studies indicated that exercise training overload impairs skeletal muscle adaptations. In this regard, Bellinger et al. (2), demonstrated that 3 weeks of high-volume overload training improved muscular oxidative capacity only in the subjects who did not develop symptoms of overreaching, but not in the overreached. This suggests that timely development of adequate muscular responses is important in the development of beneficial muscular metabolic and functional adaptation.

Recently, we demonstrated that intermittent exogenous ketosis (IEK) produced by the ingestion of the ketone ester (R)-3-hydroxybutyl (R)-3-hydroxybutyrate (KE) during a 3-week endurance training overload period markedly blunted the development of overreaching symptoms (28-31). This included suppression of overload training-induced bradycardia both at rest and during exercise, as well as prevention of energy deficiency by increasing energy intake. Moreover, IEK resulted in a 15% higher sustainable training load in the final week, while total training load over the three-week period was similar between both groups. This study for the

first time demonstrated the potential of IEK to facilitate exercise adaptations during training overload. Interestingly, previous studies in mice also showed that ketone bodies, in particular β-hydroxybutyrate (βHB), enhanced lymphangiogenesis (10) as well as stimulated the synthesis of vascular endothelial growth factor (VEGF) in mouse brain microvascular endothelial cells (17) and in the aorta of diabetic rats (36). Given these pro-angiogenic effects of ketone bodies in diverse tissues, it is reasonable to postulate that ketone bodies may also stimulate de novo capillarization in skeletal muscle. Therefore, here we investigated the effects of IEK on muscular angiogenesis. For this purpose we used the muscle biopsy samples obtained in our recent study showing a wide spectrum of beneficial physiological effects due to IEK during endurance training overload (29).

METHODS

Subject recruitment. Twenty healthy, recreationally active male subjects participated in this study. Two subjects withdrew within the first week of the study, one due to adverse reactions to the protein-carbohydrate drinks prescribed by the study protocol, and one for reasons unrelated to the experimental procedures. Therefore, the data analyses presented in this report included eighteen subjects (mean ± SD: age 21.3 ± 2.6 years, height 1.81 ± 0.04 m, body mass 73.7 ± 8.5 kg, maximal oxygen uptake ($\dot{V}O_2$ max) 55.7 ± 5.7 $ml \cdot kg^{-1} \cdot min^{-1}$) who completed the full study protocol. During the entire study period, subjects were asked to abstain from any strenuous exercise other than prescribed by the study protocol.

Study design. The muscle biopsy samples used for this study were obtained in the context of a larger project involving a randomized, double-blind experimental design of which the details have been reported elsewhere (29). Briefly, subjects were pair-matched for $\dot{V}O_2$max, exercise performance, training history, body mass and height. Thereafter, subjects were enrolled in a fully controlled 3-week cycling training program aimed to cause a state of overreaching. During the training period, subjects received either a ketone ester drink (KE, n = 9) or a corresponding control drink (CON, n = 9). Muscle biopsies were obtained on the day before (Pre) and after (Post) the overload training period.

Supervised training intervention. The training program consisted of two training sessions per day (6 days/week) and included a combination of high-intensity interval training (HIIT), intermittent endurance training (IMT) and constant-load endurance training (ET) sessions (for

detailed overview see Poffé *et al.*, 2019). The training load was gradually increased over the three-week period (week 1: ~4600 kJ, week 2: ~6400 kJ, week 3: ~9600 kJ). Total training load was not different between the experimental groups (KE: 21411 ± 585 kJ *vs.* CON: 19868 ± 965 kJ, p = 0.19). However, in the final training week training load was 15% higher in KE than in CON. Each training session was performed in the laboratory on calibrated cycling ergometers (Avantronic Cyclus II, Leipzig, Germany and Tacx Neo Smart, Wassenaar, The Netherlands) and under the careful supervision of at least one of the investigators.

Post-exercise nutritional supplementation. Thirty minutes following each exercise session, subjects received a 500 ml high-dose protein-carbohydrate drink delivering 61 g carbohydrates and 31 g protein (6d Sports Nutrition, Oudenaarde, Belgium). In addition, subjects from KE received 25 g of ketone ester [96% (R)-3-hydroxybutyl (R)-3-hydroxybutyrate; TdeltaS Ltd, Thame, UK] immediately following each training session and 30 min pre-sleep, whilst CON ingested an isocaloric control drink (CON) containing 16.4 g pure medium-chain triglycerides (Now Foods, Bloomingdale, IL, USA). To achieve a similar taste and appearance, bitter sucrose octaacetate (1 mM; Sigma-Aldrich, Bornem, Belgium) was added to CON, whilst a red colorant (AVEVE Bloem, Merksem, Belgium) was added to both the drinks. Immediately following ingestion of KE or CON subjects received 50 ml of diet coke for mouth rinsing.

Muscle biopsy sampling. On the day before (Pre) and after (Post) the training period, and 10-13 hours after the last training session a percutaneous needle biopsy (100-200 mg) from the midportion of *m. vastus lateralis* was obtained under local anesthesia (2% xylocaine without adrenaline, 1 ml subcutaneously). Biopsies during Pre and Post were taken in the rested state from the left and right leg, respectively. After removal of visible blood, muscle samples were separated into two parts. One part was immediately frozen in liquid nitrogen and stored at -80°C for further biochemical analyses. Remaining muscle was mounted in embedding medium (Tissue-Tek OCT), frozen in precooled isopentane, and kept at -80°C until later histochemical analyses. To minimize potential diet-induced variations in muscle metabolism, subjects received a standardized carbohydrate-rich dinner (~5400 kJ; 69% carbohydrate, 16% fat, 15% protein) and breakfast (~2700 kJ; 71% carbohydrate, 15% fat, 14% protein) on the evening before and 1.5 h before the biopsy samplings, respectively.

Muscle analyses.

Fiber type and fiber cross-sectional area. Serial sections (7 µm) from *m. vastus lateralis* were obtained using a cryostat at -20°C. Pre- and post-biopsies of each subject were laid together on uncoated glass slides for determination of muscle fiber typing and fiber type-specific CSA. Cryosections were fixed in ice-cold acetone for 10 min at -20°C. Thereafter, slides were incubated for 1 h at 37°C with primary antibodies directed against myosin heavy chain I (MHCI), MHCIIa (BA-F8 [AB_10572253] and SC-71 [AB_2147165], respectively, both 1:200 in PBS, Developmental Studies Hybridoma Bank, Iowa City, IA), and laminin (1:500 in PBS, Sigma-Aldrich, Bornem, Belgium, AB_477163). After three 5 min washes with PBS, cryosections were incubated with the appropriate conjugated secondary antibodies during 1 h at 37°C (type I: goat anti-mouse IgG2b Alexa Fluor 488 [AB_2338856], type IIa: goat anti-mouse IgG1 Cy5 [AB_2338715], both 1:500 in PBS, Jackson ImmunoResearch Laboratories Inc., West Grove, PA; laminin: donkey anti-rabbit Alexa Fluor 488, 1:500 in PBS, Invitrogen, Carlsbad, CA, AB_2535792). Following a last washing step, cover glasses were mounted with Fluorescent Mounting Medium (DakoCytomation, Glostrup, Denmark). Images were visualized by fluorescence microscopy (Nikon E1000, Nikon, Boerhavedorp, Germany) at 20x magnification. Images were manually analyzed with ImageJ (version 1.52, National Institutes of Health, USA) in a blinded manner. On average 497 ± 36 (mean ± SEM) fibers were analyzed for cross-sectional area and fiber type per biopsy.

Capillarization. Uncoated glass slides with serial cryosections (7 µm) from pre- and post-biopsies were air dried for 20 min at room temperature, followed by prehybridization in 1% BSA in PBS for 60 min. Thereafter, slides were incubated for 2 h at room temperature with a primary antibody against the endothelial cell maker CD31 (1:500 in 0.5% BSA in PBS; DakoCytomation, AB_2114471) for visualization of capillaries (34). Following three 5 min washes (0.5% BSA in PBS), a biotinylated rabbit anti-mouse IgG (H&L) antibody (1:500; DakoCytomation, AB_2687571) was added for 1 h. Slides were then washed for 3 x 5 min and incubated for 1 h at 37°C with primary antibodies against myosin heavy chain I and IIa (BA-F8 [AB_10572253] and SC-71 [AB_2147165], respectively, dilution 1:200, Developmental Studies Hybridoma Bank). After washing (2 x 5 min), slides were incubated with the appropriate secondary antibodies (type I: goat anti-mouse IgG2b Alexa Fluor 488 [AB_2338856], type IIa: goat anti-mouse IgG1 Cy5 [AB_2338715], both 1:500, Jackson ImmunoResearch Laboratories Inc., West Grove, PA) during 1 h at 37°C. After three additional 5 min washes, streptavidin was applied for 30 min (1:100 in PBS, PerkinElmer, Waltham, MA),

sections were washed again (3 x 5 min) and incubated with cyanine (1:100 in PBS, PerkinElmer) for 10 min. After a final washing step, sections were covered using Fluorescent Mounting Medium (DakoCytomation). Cryosections were captured at 20x by fluorescence microscopy and subsequent images were analyzed with ImageJ by an investigator blinded to subject coding. The number of capillaries was counted for each interior muscle fiber by a blinded investigator and expressed as capillary contacts. In addition, the capillary-to-fiber perimeter exchange index (CFPE: capillaries per 1000 μm perimeter) was calculated by correcting for the capillary sharing factor and muscle fiber perimeter (13). On average, 229 ± 25 (mean ± SEM) fibers were analyzed per cryosection.

RNA extraction, reverse transcription and real-time quantitative PCR analysis. Total RNA was extracted from 15-20 mg frozen muscle tissue using TRIzol (Invitrogen, Vilvoorde, Belgium). Following isolation, the quality and quantity of the RNA were determined using a SimpliNano spectrophotometer (GE Healthcare, Chicago, IL). cDNA was synthesized from 0.9 μg RNA using the cDNA Reverse Transcription kit (Qiagen, Hilden, Germany) according to the manufacturer's recommendations. Real-time quantitative polymerase chain reaction (PCR) was performed using the GoTaq qPCR Master Mix (Promega, Madison, WI) on a QuantStudio 3 thermocycler (Thermo Fisher Scientific, Waltham, MA). Real-time PCR primers were designed for human cyclophilin A (CycloA), vascular endothelial growth factor A (VEGFa) and endothelial nitric-oxide synthase (eNOS) (Table 1). Each sample was run in duplicate and gene expression was calculated using the delta delta threshold cycle method with CycloA as a housekeeping gene.

Table 1. Primer sequences used for real-time PCR.

	Forward	Reverse
VEGFa	TTTCTGCTGTCTTGGGTGCATTGG	ACCACTTCGTGATGATTCTGCCCT
eNOS	CAGTTACCAGCTAGCCAAAGT	CTCATTCTCCAGGTGCTTCAT
CycloA	CTTCATCCTAAAGCATACGGGTC	TGCCATCCAACCACTCAGTCT

Western blot. Frozen muscle tissue (15-20 mg) was homogenized 4 x 20 s in ice-cold lysis buffer (1:10 w/v; 50 mM Tris-HCl, pH 7.0; 270 mM sucrose; 5 mM EGTA; 1 mM EDTA; 1 mM sodium orthovanadate; 50 mM glycerophosphate; 5 mM sodium pyrophosphate; 50 mM sodium fluoride; 1 mM dithiothreitol; 0.1% Triton X-100; and a complete protease inhibitor tablet [Roche Applied Science, Vilvoorde, Belgium]) using a FastPrep instrument (MP Biomedicals, Santa Ana, CA). Homogenates were then centrifuged at 10 000 g for 10 min at

4°C. The supernatants were collected and stored at -80°C. Protein concentration was measured using the DC protein assay kit (Bio-Rad Laboratories, Nazareth, Belgium). Proteins (20-30 µg) were separated by SDS-PAGE (8-15% gels) and transferred to polyvinylidene difluoride membranes. Subsequently, membranes were blocked in TBS-T (tris-buffered saline with Tween-20) containing 5% non-fat milk for 1 h. Thereafter, membranes were incubated overnight at 4°C in TBS-T containing 5% non-fat milk or 5% BSA for 1 h with the following antibodies: VEGF (sc-7269, AB_628430), eNOS (sc-376751, AB_2832203), ERRα (sc-65718, AB_1122673), AMP-activated protein kinase α (AMPKα) (sc-74461, AB_1118940) and PGC1α (sc-517380, AB_2755043) from Santa Cruz Biotechnology (Dallas, TX); HIF-1α (#3716, AB_2116962), phospho-AMPKα Thr172 (#2535, AB_331250) and GAPDH (#2118, AB_561053) from Cell Signaling (Leiden, The Netherlands). Membranes were then incubated for 45 min at room temperature with the appropriate horseradish peroxidase-conjugated secondary antibodies (1:5000; Sigma-Aldrich, Bornem, Belgium). Membranes were scanned and quantified with Genesnap and Genetools Softwares (Syngene, Cambridge, UK). Results are presented as the ratio protein of interest/GAPDH or as the ratio phosphorylated/total form if the phosphorylation status of the protein was measured.

Statistics. Statistical analyses were performed in GraphPad Prism version 8.3.0 (GraphPad Software, La Jolla, CA). A two-way repeated measures analysis of variance (ANOVA) (group x time) was performed to assess differences in mean values between the experimental groups and over time, followed by a Bonferroni post-hoc test if a significant interaction was detected. Reported P-values refer to the output of the post-hoc analyses in case of a significant interaction effect, whilst otherwise P-values for main effects were reported. A probability level of $p < 0.05$ was set as statistically significant. Data are expressed as mean ± SEM.

Study approval. All subjects gave their written informed consent before inclusion in the study, which was approved by the KU Leuven Biomedical Ethics Committee (registration number: B322201733747) and according to the Declaration of Helsinki.

RESULTS AND DISCUSSION

Capillary contacts (CC) as well as capillary-to-fiber perimeter exchange index (CFPE) were not altered in response to the training period in CON (CC: 5.1 ± 0.4 *vs.* 5.5 ± 0.2 capillary contacts; CFPE: 9.2 ± 0.7 *vs.* 10.3 ± 0.6 capillaries per 1000 µm for pre *vs.* post, respectively,

p = 0.99 and p = 0.33, Figure 1). This is in line with earlier reports showing that overload training impairs the normal muscular adaptations to exercise (2, 9), which probably explains the lack of improvement in muscle oxidative capacity previously observed in young volunteers submitted to overreaching (2). In contrast, the training increased both capillarization indices in all subjects on KE, resulting in an increase of 44% (pre: 4.6 ± 0.2 *vs.* post: 6.6 ± 0.6 capillary contacts, $p < 0.01$) and 42% (pre: 8.9 ± 0.4 *vs.* post: 12.6 ± 0.8 capillaries per 1000 µm, $p < 0.001$) on average in CC and CFPE, respectively.

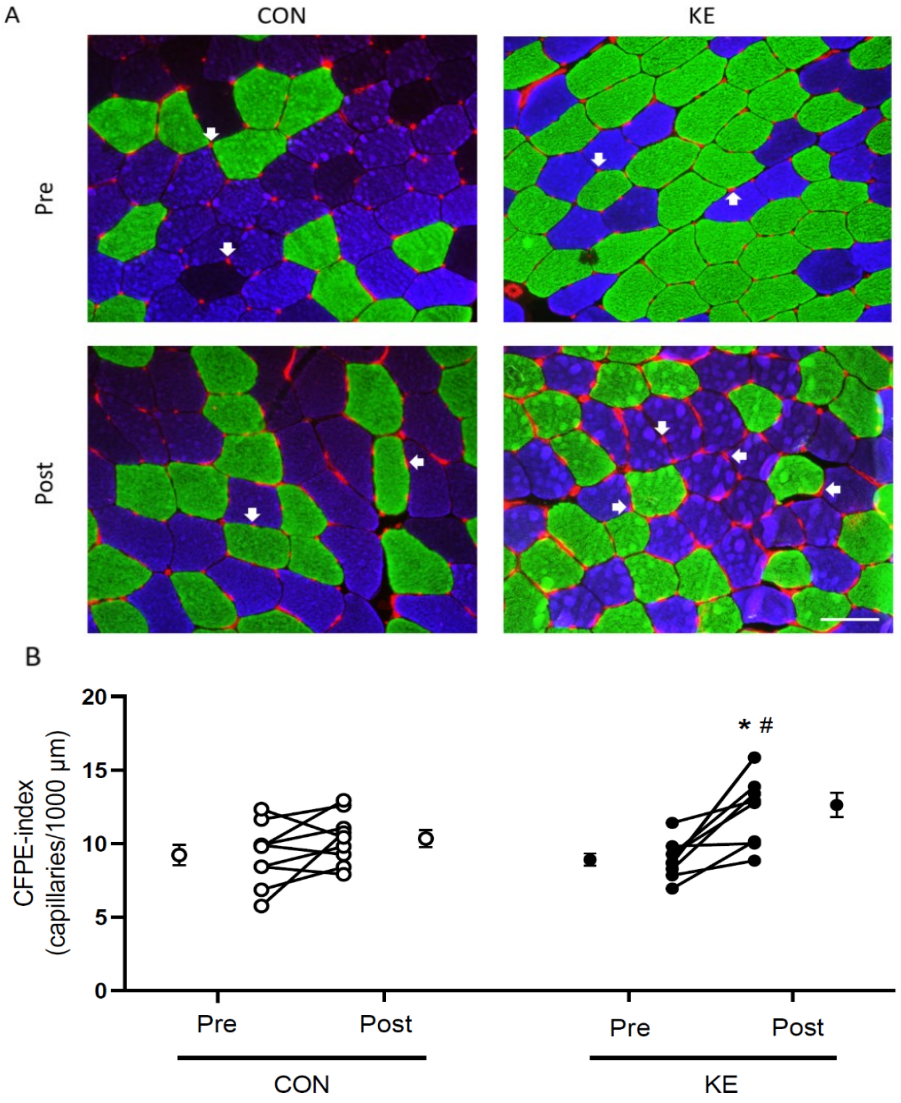


Figure 1. Ketone ester intake stimulates muscle capillarization in response to overload training. Panel A. Representative immunofluorescence images of *m. vastus lateralis* before (Pre) and after (Post) training in subjects receiving ketone ester (KE, n = 9) or control (CON, n = 9). The training increased capillarization in KE subjects but not in CON. CD31 (red), type I (green), type IIa (blue), type IIx (black). Scale bar = 100µm. Panel B. Dot plot together with mean (circle) ± SEM (whiskers) showing an increased capillary-to-fiber perimeter exchange index (CFPE-index) in KE (●, n = 9) compared to CON (○, n = 9) following training. * $p < 0.05$ KE *vs.* CON at indicated time point; # $p < 0.05$ *vs.* Pre for indicated group.

Previous data indicated that ketone body oxidation rather than ketone bodies *per se*, stimulates vessel sprouting (10). Therefore, we assessed whether the degree of de novo capillarization was more explicit for type I than for type II muscle fibers. Compared with type II, type I fibers have higher capacity for sarcolemmal ketone body transport (3) and oxidation (35), indeed. However, the effect of KE was similar between type I and type II fibers, indicating that in human muscle the effect does not depend on fiber-specific oxidative capacity. As the observed angiogenic response in KE might be mediated by alterations in muscle fiber morphology or fiber type distribution, we also assessed fiber-specific cross-sectional areas (CSA), perimeters, minimum Feret diameters, and relative distribution in *m. vastus lateralis*. However, none of these parameters was affected by either the training intervention or KE (Table 2). This is in keeping with previous observations showing that shifts in muscle fiber type composition or morphology require at least four weeks of training (6, 15, 18). Thus, the KE-induced increase in muscular capillarization occurred as a primary response to training and was independent of fiber type shifts or alterations in muscle fiber morphology.

Table 2. Neither ketone ester intake nor overload training modulated muscle fiber type composition or cross-sectional area.

	CON			KE		
	Pre	**Post**	**P-value**	**Pre**	**Post**	**P-value**
Fiber type distribution (% total number)						
Type I	43 ± 5	42 ± 5	0.99	50 ± 6	52 ± 4	0.99
Type IIa	47 ± 5	53 ± 4	0.42	43 ± 5	45 ± 3	0.99
Type IIx	11 ± 4	5 ± 2	0.28	7 ± 2	3 ± 2	0.62
Fiber CSA (µm²)						
Type I	4794 ± 439	4435 ± 490	0.99	4675 ± 383	4625 ± 323	0.99
Type IIa	5358 ± 533	4917 ± 476	0.99	4812 ± 406	4508 ± 403	0.99
Type IIx	4445 ± 353	4511 ± 536	0.99	4581 ± 382	4001 ± 351	0.76

Values are mean ± SEM and represent fiber type distribution and fiber cross-sectional area (CSA) of *m. vastus* lateralis before (Pre) and after (Post) overload training in subjects receiving ketone ester (KE, n = 9) or control (CON, n = 9).

Next, we explored the molecular mechanisms conceivably promoting the angiogenic effect of KE. Although angiogenesis is mediated by various factors (21, 23, 33), it is mainly controlled by the pro-angiogenic VEGF. Moreover, myocyte-expressed VEGF has been shown to be pivotal in training-induced angiogenesis (7, 27). Therefore, we first assessed whether KE stimulated VEGF at the mRNA and protein level. The training in KE increased muscle VEGF

protein content by 33% (pre: 0.16 ± 0.02 *vs.* post: 0.21 ± 0.03 a.u., p = 0.03), while mRNA expression increased 2-fold (pre: 2.3 ± 0.6 *vs.* post: 4.6 ± 0.9 a.u., p < 0.01, Figure 2A-B). In contrast, VEGF protein and mRNA content were unchanged in CON (VEGF protein: 0.15 ± 0.03 *vs.* 0.14 ± 0.03 a.u.; VEGF mRNA: 2.0 ± 0.5 *vs.* 1.9 ± 0.3 a.u. for pre *vs.* post, respectively, both p > 0.05). This supports the prevailing opinion that excess endurance training load blunts exercise-induced VEGF production (11, 14). Interestingly, the increase in VEGF mRNA expression in KE occurred more than 10 hours following the last training session, while previous studies indicated that the exercise-induced stimulation of VEGF mRNA fades within 6 hours (4, 19). These data indicate that the exercise-induced angiogenic response in KE was mediated by VEGF, which is in line with previous data showing a direct stimulatory effect of KE on VEGF in mouse brain endothelial cells (17) and in the aorta of diabetic rats (36). It is also important to note that the elevated VEGF mRNA and protein expressions in KE occurred 9-12 hours post the last ketone ester dose, i.e. at identical low blood ketone body concentrations between KE and CON (KE: 0.3 ± 0.1 *vs.* CON: 0.3 ± 0.1 mM, p = 0.99). The effect of intermittent KE to stimulate VEGF thus reflects a short-term adaptation which occurred independent of acute regulation of VEGF by KE.

In line with the training-induced increase in VEGF mRNA and protein content in KE, we observed a twofold increase in mRNA content of its downstream target eNOS (pre: 1.0 ± 0.2 *vs.* post: 2.2 ± 0.3 a.u., p < 0.001), while no alterations occurred in CON (pre: 1.2 ± 0.1 *vs*. post: 1.2 ± 0.3 a.u., p > 0.05, Figure 2C). Conversely, total eNOS protein content was similarly increased in both experimental groups at the end of the training period (KE: +259 ± 149 *vs.* CON: +98 ± 23%, main effect of time: p < 0.01, Figure 2D).

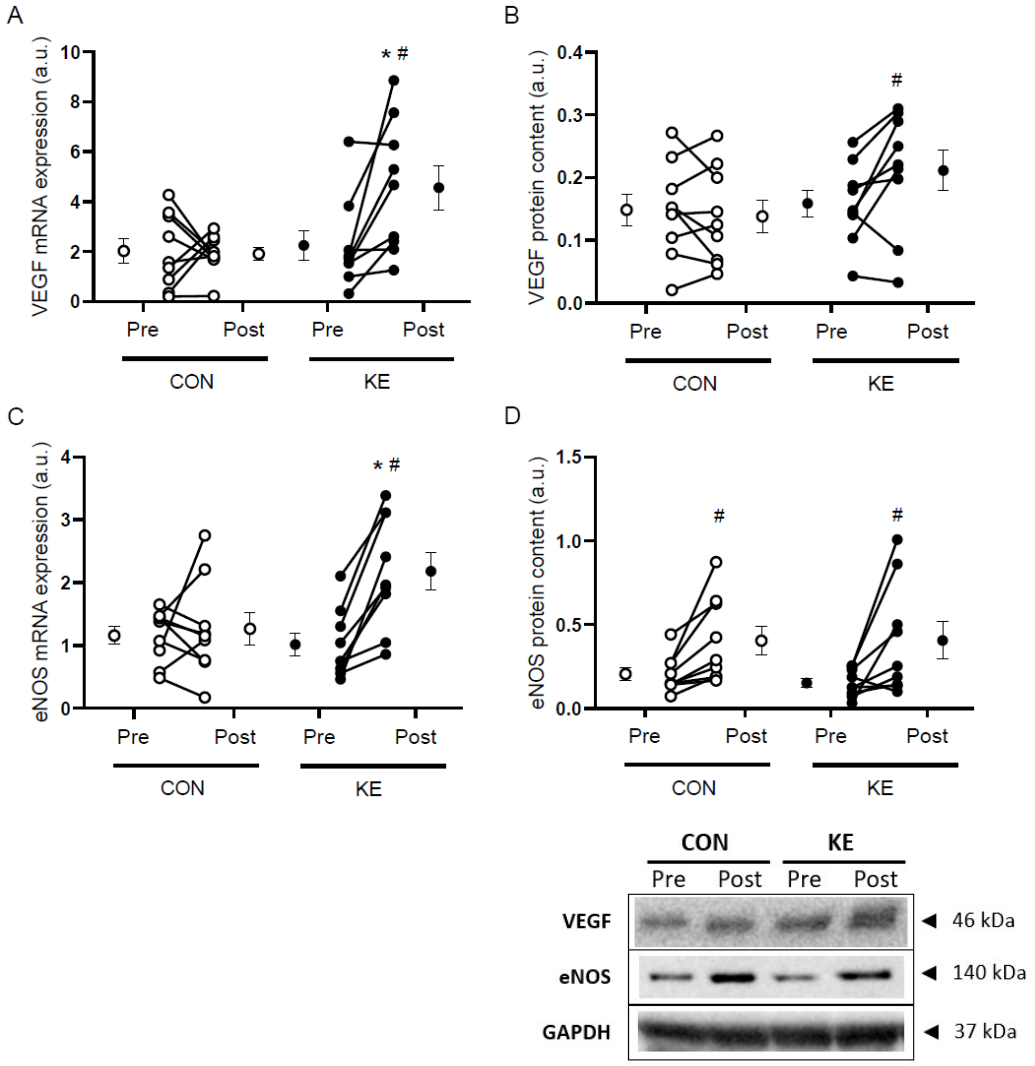


Figure 2. Ketone ester intake increases muscle angiogenesis through upregulation of VEGF. Dot plots together with mean (circle) ± SEM (whiskers) for VEGF mRNA (A) and protein (B) content and for eNOS mRNA (C) and protein (D) content before (Pre) and after (Post) training. During the training period the subjects received either control (○, n = 9) or ketone ester supplements (●, n = 9). KE but not CON increased both VEGF mRNA and protein levels. KE also elevated eNOS mRNA and protein expression, whilst CON increased protein expression only. Two-way repeated-measures ANOVA with a Bonferonni post-hoc test. * $p < 0.05$ KE *vs.* CON at indicated time point; # $p < 0.05$ *vs.* Pre for indicated group. *VEGF* vascular endothelial growth factor, *eNOS* endothelial nitric oxide synthase.

To further characterize the effect of KE on exercise-induced angiogenesis, we assessed upstream transcription factors of VEGF implicated in both baseline (AMPK) and exercise-induced (PGC1α, HIF1α and ERRα) angiogenesis (12). However, none of these factors differed between KE and CON (Figure 3). This suggests that these metabolic regulators do not mediate the KE-induced activation of VEGF. We did not attempt to further elucidate the molecular mechanisms by which KE stimulates training-induced VEGF expression. However, a recent study in mice showed that elevating NAD^+ improved the VEGF-dependent angiogenic response

to exercise through promotion of SIRT1 (5). Given the lower utilization of NAD^+ to produce acetyl-CoA during ketone oxidation compared to glucose breakdown (8, 26), and the resultant increase in NAD^+ during ketosis (8, 37), it is reasonable to postulate that intermittent KE might exert its angiogenic effect via transient elevation of intramyocellular NAD^+ during the 2-3 h time windows of elevated blood βHB following ketone ester ingestion three times daily. Nonetheless, other potential mechanisms might exist including a ketone body-induced increase in intramyocellular calcium concentration, which has been reported to be responsible for the stimulatory effect of the ketone body βHB on VEGF in brain endothelial cells (17). In support of such hypothesis, we recently observed acute KE intake to increase blood calcium concentrations during exercise (Poffé *et al.,* accepted, *J. Appl. Physiol.*).

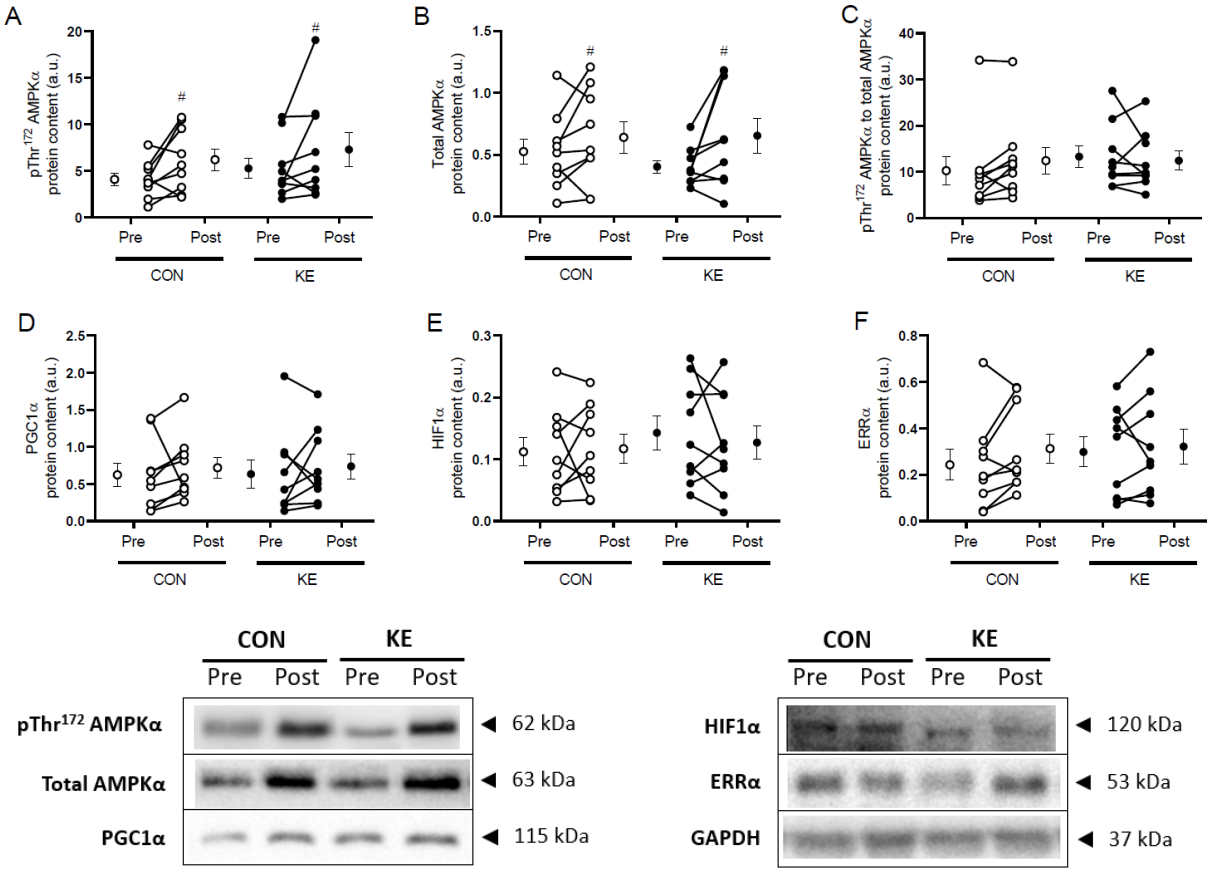


Figure 3. Ketone ester intake does not modulate transcriptional factors implicated in angiogenesis. Dot plots together with mean (circle) ± SEM (whiskers) for (A) phospho-AMPKα Thr172, (B) total AMPKα, (C) phospho over total AMPKα, (D) PGC1α, (E) HIF1α, and (F) ERRα protein content before (Pre) and after (Post) overload training. During the training period subjects ingested either a control drink (○, n = 9) or ketone ester supplements (●, n = 9). All transcriptional factors were similar between KE and CON. Two-way repeated-measures ANOVA with a Bonferonni post-hoc test. # $p < 0.05$ *vs.* Pre for indicated group. *AMPKα* AMP-activated protein kinase alpha, *PGC1α* peroxisome proliferator activated receptor gamma coactivator 1 alpha, *HIF1α* hypoxia-inducible factor 1-alpha, *ERRα* estrogen-related receptor alpha.

We previously reported that, compared with CON, KE in the conditions of the current study increased dietary energy intake by ~20%, as well as allowed for 15% higher training load to be tolerated during the final week of the training intervention (306). Hence it may be argued that the angiogenic effect of KE during the training overload period was at least partly, if not largely due to differential energy balance and training load between groups. However, evidence to indicate that slight short-term modifications in food intake or energy balance, as occurred in the current study, can impact the angiogenic response to exercise in young, healthy subjects is lacking. Similarly, the 15% higher training load effected by KE in the final week of the overload training period is also unlikely to explain the differences in angiogenesis as a further increase in training load during strenuous training would rather inhibit beneficial muscular adaptations (17, 102). In fact, training load and dietary energy intake were identical during the initial two weeks of training, and from a physiological perspective it is impossible to explain a ~40% increment in capillarization by the small differences in energy intake and training load during the 3rd week of training only. Nonetheless, it remains to be established whether the angiogenic response to KE seen here is specific for conditions of explicit training overload, or also emerges during optimal training which is balanced by adequate recovery, indeed.

In conclusion, in this study we for the first time demonstrate that intermittent exogenous ketosis due to oral ketone ester ingestion markedly stimulates the angiogenic response to endurance training via upregulation of VEGF. This effect is pertinent in the context of training-induced stimulation of endurance exercise performance. This novel finding also opens new perspectives for clinical research aiming to explore the potential of intermittent exogenous ketosis in muscular rehabilitation in clinical therapeutical conditions characterized by capillary rarefaction including peripheral arterial disease and COPD.

ADDITIONAL INFORMATION

Competing interests

The authors have declared that no conflict of interest exists.

Author contributions

Conception and design of the study: CP and PH. Data collection, biochemical and histological analyses: CP and RR. Analysis and interpretation of the data and manuscript drafting: CP and PH. Medical supervision and muscle biopsy procedures: RVT. All authors critically revised the manuscript and approved the final version of the manuscript.

Acknowledgements

This study was funded by Research Fund Flanders (Fonds voor Wetenschappelijk Onderzoek – Vlaanderen; research grant no. G080117N). The authors wish to thank Monique Ramaekers for skillful technical support.

REFERENCES

1. **Andersen P, Henriksson J**. Capillary supply of the quadriceps femoris muscle of man: adaptive response to exercise. J Physiol 270: 677-690, 1977.

2. **Bellinger PM, Sabapathy S, Craven J, Arnold B, Minahan C**. Overreaching attenuates training-induced improvements in muscle oxidative capacity. Med Sci Sport Exerc 52: 77-85, 2019.

3. **Bonen A**. The expression of lactate transporters (MCT1 and MCT4) in heart and muscle. Eur J Appl Physiol 86: 6-11, 2001.

4. **Breen EC, Johnson EC, Wagner H, Tseng HM, Sung LA, Wagner PD**. Angiogenic growth factor mRNA responses in muscle to a single bout of exercise. J Appl Physiol 81: 355-361, 1996.

5. **Das A, Huang GX, Bonkowski MS, Longchamp A, Li C, Schultz MB, Kim L, Osborne B, Joshi S, Lu Y, Treviño-villarreal JH, Kang M, Hung T, Lee B, Williams EO, Igarashi M, Mitchell JR, Wu LE, Turner N, Arany Z, Guarente L, Sinclair DA**. Impairment of an endothelial NAD+-H2S signaling network is a reversible cause of vascular aging. Cell 173: 74-89, 2018.

6. **De Smet S, Van Thienen R, Deldicque L, James R, Sale C, Bishop DJ, Hespel P**. Nitrate intake promotes shift in muscle fiber type composition during sprint interval training in hypoxia. Front Physiol 7: 1-11, 2016.

7. **Delaver H, Nogueira L, Wagner PD, Hogan MC, Metzger D, Breen EC**. Skeletal myofiber VEGF is essential for the exercise training response in adult mice. Am J Physiol - Regul Integr Comp Physiol 306: 586-595, 2014.

8. **Elamin M, Ruskin DN, Masino SA, Sacchetti P**. Ketone-based metabolic therapy: is increased nad + a primary mechanism? Front Mol Neurosci 10: 1-9, 2017.

9. **Ferraresso RLP, Buscariolli De Oliveira R, MacEdo DV, Alessandro Soares Nunes L, Brenzikofer R, Damas D, Hohl R**. Interaction between overtraining and the interindividual variability may (not) trigger muscle oxidative stress and cardiomyocyte apoptosis in rats. Oxid Med Cell Longev 2012: 1-11, 2012.

10. **García-Caballero M, Zecchin A, Souffreau J, Truong A-CK, Teuwen L-A, Vermaelen W, Martín-Pérez R, de Zeeuw P, Bouché A, Vinckier S, Cornelissen I, Eelen G, Ghesquière B, Mazzone M, Dewerchin M, Carmeliet P**. Role and therapeutic potential of dietary ketone bodies in lymph vessel growth. Nat Metab 1: 666-675, 2019.

11. **Gliemann L, Gunnarsson TP, Hellsten Y, Bangsbo J**. 10-20-30 training increases performance and lowers blood pressure and VEGF in runners. Scand J Med Sci Sport 25: e479-e489, 2015.

12. **Gorski T, De Bock K**. Metabolic regulation of exercise-induced angiogenesis. Vasc Biol 1: 1-8, 2019.

13. **Hepple RT**. A new measurement of tissue capillarity: the capillary-to-fibre perimeter exchange index. Can J Appl Physiol 22: 11-22, 1997.

14. **Hoier B, Passos M, Bangsbo J, Hellsten Y**. Intense intermittent exercise provides weak stimulus for vascular endothelial growth factor secretion and capillary growth in skeletal muscle. Exp Physiol 98: 585-597, 2013.

15. **Howald H, Hoppeler H, Claassen H, Mathieu O, Straub R**. Influences of endurance training on the ultrastructural composition of the different muscle fiber types in humans. Pflügers Arch - Eur J Physiol 403: 369-376, 1985.

16. **Hudlicka O, Brown M, Egginton S**. Angiogenesis in skeletal and cardiac muscle. Physiol Rev 72: 369-417, 1992.

17. **Isales CM, Min L, Hoffman WH**. Acetoacetate and β-hydroxybutyrate differentially regulate endothelin-1 and vascular endothelial growth factor in mouse brain microvascular endothelial cells. J Diabetes Complications 13: 91-97, 1999.

18. **Jansson E, Esbjörnsson M, Holm I, Jacobs I**. Increase in the proportion of fast-twitch muscle fibres by sprint training in males. Acta Physiol Scand 140: 359-363, 1990.

19. **Jensen L, Pilegaard H, Neufer PD, Hellsten Y**. Effect of acute exercise and exercise training on VEGF splice variants in human skeletal muscle. Am J Physiol - Regul Integr Comp Physiol 287: 397-402, 2004.

20. **Kissane RW, Egginton S**. Exercise-mediated angiogenesis. Curr Opin Physiol 10: 193-201, 2019.

21. **Liekens S, De Clercq E, Neyts J**. Angiogenesis: regulators and clinical applications. Biochem Pharmacol 61: 253-270, 2001.

22. **Meeusen R, Duclos M, Foster C, Fry A, Gleeson M, Nieman D, Raglin J, Rietjens G, Steinacker J, Urhausen A**. Prevention, diagnosis, and treatment of the overtraining syndrome: joint consensus statement of the European College of Sport Science and the American College of Sports Medicine. Med Sci Sports Exerc 45: 186-205, 2013.

23. **Milkiewicz M, Ispanovic E, Doyle JL, Haas TL**. Regulators of angiogenesis and strategies for their therapeutic manipulation. Int J Biochem Cell Biol 38: 333-357, 2006.

24. **Mitchell EA, Martin NRW, Bailey SJ, Ferguson RA**. Critical power is positively related to skeletal muscle capillarity and type i muscle fibers in endurance-trained individuals. J Appl Physiol 125: 737-745, 2018.

25. **Moro T, Brightwell CR, Phalen DE, McKenna CF, Lane SJ, Porter C, Volpi E, Rasmussen BB, Fry CS**. Low skeletal muscle capillarization limits muscle adaptation to resistance exercise training in older adults. Exp Gerontol 127: 1-10, 2019.

26. **Newman JC, Verdin E**. β-Hydroxybutyrate: a signaling metabolite. Annu Rev Nutr 37: 51-76, 2017.

27. **Olfert IM, Howlett RA, Wagner PD, Breen EC**. Myocyte vascular endothelial growth factor is required for exercise-induced skeletal muscle angiogenesis. Am J Physiol - Regul Integr Comp Physiol 299: 1059-1067, 2010.

28. **Poffé C, Ramaekers M, Hespel P**. Reply from Chiel Poffé, Monique Ramaekers and Peter Hespel. J Physiol 597: 5309-5310, 2019.

29. **Poffé C, Ramaekers M, Van Thienen R, Hespel P**. Ketone ester supplementation blunts overreaching symptoms during endurance training overload. J Physiol 597: 3009-3027, 2019.

30. **Poffé C, Ramaekers M, Van Thienen R, Hespel P**. Reply from Chiel Poffé, Monique Ramaekers, Ruud Van Thienen and Peter Hespel. J Physiol 597: 4679-4680, 2019.

31. **Poffé C, Ramaekers M, Van Thienen R, Hespel P**. Reply from Chiel Poffé, Monique Ramaekers, Ruud Van Thienen and Peter Hespel. J Physiol 597: 4409-4410, 2019.

32. **Škorjanc D, Jaschinski F, Heine G, Pette D**. Sequential increases in capillarization and mitochondrial enzymes in low-frequency-stimulated rabbit muscle. Am J Physiol - Cell Physiol 274: 810-818, 1998.

33. **Suárez Y, Sessa WC**. MicroRNAs as novel regulators of angiogenesis. Circ Res 104: 442-454, 2009.

34. **Waters RE, Rotevatn S, Li P, Annex BH, Yan Z**. Voluntary running induces fiber type-specific angiogenesis in mouse skeletal muscle. Am J Physiol - Cell Physiol 287: 1342-1348, 2004.

35. **Winder WW, Baldwin KM, Holloszy JO**. Enzymes involved in ketone utilization in different types of muscle: adaptation to exercise. Eur J Biochem 47: 461-467, 1974.

36. **Wu X, Miao D, Liu Z, Liu K, Zhang B, Li J, Li Y, Qi J**. β-hydroxybutyrate antagonizes aortic endothelial injury by promoting generation of VEGF in diabetic rats. Tissue Cell 64: 101345, 2020.

37. **Xin L, Ipek Ö, Beaumont M, Shevlyakova M, Christinat N, Masoodi M, Greenberg N, Gruetter R, Cuenoud B**. Nutritional ketosis increases NAD+/NADH ratio in healthy human brain: an in vivo study by 31P-MRS. Front Nutr 5: 1-8, 2018.

CHAPTER IV

GENERAL DISCUSSION

1. General overview

Study 1 — Effect of KE on endurance exercise performance

- KE intake during the initial phase of a simulated cycling race does not affect net muscle glycogen breakdown
- KE intake does not enhance high-intensity exercise performance at the end of a simulated cycling race

Study 2 — Effect of KE and bicarbonate on endurance exercise performance

- Intake of sodium bicarbonate counteracts KE-induced decrease of blood pH and [HCO_3^-] during prolonged exercise
- Co-ingestion of KE and sodium bicarbonate enhances high-intensity endurance exercise performance but not sprint performance at the end of a simulated cycling race

Study 3 — Effect of KE on the development of non-functional overreaching

- KE intake attenuates overload training-induced bradycardia and hyperactivity of the sympathetic nervous system, while counteracting energy deficiency during a three-week supramaximal overload training period
- KE intake during a three-week overload training period increases exercise tolerance and thereby elevates sustainable training load

Study 4 — Effect of KE on the muscular response to overload training

- KE intake increases the number of capillary contacts and capillary-to-fiber perimeter exchange index in m. vastus lateralis during a three-week overload training period via increased VEGF expression at both the mRNA and protein level

2. Acute effects of ketone ester supplementation during endurance exercise

Increasing circulating KB concentrations has for a long time been considered to evoke an ergogenic effect due to the ability of KB to spare glucose oxidation in skeletal muscle (319) and to increase muscle thermodynamics (328). Nevertheless, only recently with the development of the oral ingestible ketone ester (R)-3-hydroxybutyl (R)-3-hydroxybutyrate (KE) (50, 51), this potential could be investigated in the presence of high-carbohydrate intake, which is crucial to achieve optimal endurance performance (147, 152). Yet, studies investigating the effect of KE on exercise performance show equivocal results, and either assessed performance under conditions that are irrelevant for a real-world athletic setting (*e.g.* exercise testing in the fasted state) (63, 76, 180) and/or did not provide any insight into the impact of KE on substrate metabolism (76, 97, 180). Moreover, as KB would indeed inhibit glucose oxidation during exercise, KE may only be used during the initial part of an endurance exercise event, to eventually allow optimal high-rate glycolytic flux from muscle glycogen in the final high-intensity phase of the event. Therefore, the aim of our study was to identify the effect of KE ingestion on endurance exercise performance and muscle substrate use in an ecologically valid context.

2.1 Effect on exercise performance, muscle substrate use and plasma FFA content

As a potential ergogenic effect of KE on endurance exercise performance has primarily been attributed to a muscle glycogen-sparing action of KB (87), one might raise the question whether KE indeed lowers muscle glycogen utilization during exercise. In our studies, we observed neither alterations in net breakdown of muscle glycogen or intramyocellular triglycerides **(Article 1, Figure 4A+B)**, nor in the respiratory exchange ratio (RER) **(Article 2)** or blood lactate levels **(Article 1 and 2)** during 3 h intermittent exercise following ingestion of 65 g KE. In contrast, we even observed a trend towards increased net glycogen breakdown in KE, and a significant association between circulating blood ßHB concentrations and increased net glycogenolysis during $IMT_{180'}$. This likely resulted from ßHB-induced suppression of plasma FFA during exercise **(Article 1, Figure 2D)**, causing less inhibition of muscle glycogenolysis by FFA (84, 324). The lower plasma FFA content in KE is in line with previous research (63) and has been attributed to an inhibitory effect of ßHB on adipolysis via agonism of the nicotinic acid receptor PUMA-G (361). Nonetheless, the association between ßHB and

increased net glycogen breakdown may also result from KE-induced elevation of blood noradrenalin levels (100).

Our observation that KE did not counteract muscle glycogen breakdown is in apparent contrast with an earlier seminal study by Cox *et al.* (63), who observed a reduction in glycolytic intermediates and glycogen breakdown during respectively 1 and 2 h cycling following ingestion of 573 $mg \cdot kg^{-1}$ BW KE. Their opposite results are likely mediated by differences in the research design. In our studies, the KE and control conditions received an isocaloric amount of carbohydrates (60 $g \cdot h^{-1}$), while in Cox *et al.*, all trials were performed with at least 40% lower rate of carbohydrate intake in the KE *versus* the control condition. As such, their observed alterations in muscle substrate use following KE, were likely mediated by the decreased provision of carbohydrates, rather than by KE, as lower carbohydrate intake shifts substrate oxidation towards increased fat utilization (187).

In accordance to the absence of a glycogen-sparing action of KE in our study, we did not observe an increase in performance during a 15 min time-trial or sprint performed following 3 h of intermittent submaximal cycling. These data are in line with a recent study showing no effect of KE on 10km running performance (97), but contrast the 2% increase in 30 min time-trial performance following 1 h submaximal cycling as observed by Cox *et al.* (63). However, exercise testing in the latter study did not only include lower carbohydrate intake in the KE condition, but in addition was performed in the fasted state, thereby further increasing the reliance on fat oxidation (71, 373).

A recent systematic review suggested that the discrepant effects of KE on exercise performance could be attributed to differences in blood ßHB concentrations and that an ergogenic effect requires at least circulating [ßHB] of 2 mM (244). However, our studies induced ßHB concentrations above this 2 mM threshold but observed null effects on performance. Therefore, the contrasting effects on exercise performance compared to the reports by Cox *et al.* (63) were not due to differences in circulating ßHB concentrations, but were likely mediated by different research designs. Taken together, our results and available literature indicate that there is currently no evidence for either a glycogen sparing effect or an ergogenic effect of KE under nutritional conditions that are relevant for optimal athletic performance in endurance exercise.

2.2 Effect on acid-base balance and electrolytes

Since KB are weak organic acids, they may also impact exercise performance by altering blood acid-base balance. Previous studies indeed showed that blood pH and [HCO_3^-] decreased for up to 1 h following KE-ingestion at rest (353) and during submaximal exercise (76, 99). In accordance, we observed that ingestion of 65 g KE over a 90 min period before and during the initial half an hour of a ~3 h simulated cycling race resulted in a mild, transient ketoacidosis (pH: ~7.35) accompanied by a drop of alkaline reserve **(Article 2, Figure 3B+C)**. A previous study indicated that a decrease in blood pH, even at the small extent as observed in our studies, negatively affects exercise performance (318). Therefore, it is possible that ketoacidosis inhibited a potential ergogenic action of KE. Although the precise underlying mechanisms by which acidosis impairs performance remain unknown, it has been suggested that acidosis inhibits contractile cycling and/or reduces glyco(geno)lytic rate (77, 166, 348). The latter mechanism seems implausible in the context of our studies, as we did not detect differences in muscle glycogen content between KE and CON. Besides a potential negative effect of decreased blood pH, the ketoacidosis was also accompanied by an increase in blood sodium and chloride concentrations, which may also impair contractile function (44, 336). These alterations in electrolyte concentrations are in line with a previous study at rest (353), wherein these alterations were attributed to a stimulating effect of ßHB metabolism on myocellular sodium efflux. However, the impact on exercise performance is currently unknown.

2.3 Effect on cardiorespiratory parameters and blood gases

To compensate for the observed metabolic acidosis, subjects in our study increased their pulmonary ventilation by ~10% to remove excess CO_2 resulting in a lower pCO_2. These data are in line with a previous study showing decreased $P_{ET}CO_2$ levels during a maximal incremental exercise test following ingestion of 330 mg·kg^{-1} BW KE (76). In addition, the KE-induced increase in V_E in our study was accompanied by an elevated $\dot{V}O_2$. Conversely, three other studies (63, 76, 180) observed no alterations in $\dot{V}O_2$. These contrasting results most likely resulted from the higher KE intake in our studies (65 g *vs.* ~23, ~28 and ~40 g). We also observed elevated plasma noradrenalin concentrations in response to KE, which is known to increase $\dot{V}O_2$ (91, 92). However, whether this may explain the contrasting results between our study and previous reports can not be verified as others did not assess blood catecholamine levels. Similar to a recent study (96), KE affected heart rate neither during submaximal nor during maximal exercise.

2.4 Effect on the subjective perception of fatigue

Besides the potential negative effects of KE on blood acid-base balance and ventilation, we observed, in apparent contrast with other studies (96, 97, 99, 180), higher RPE values for KE *versus* CON during ketosis in **article 1**. This discrepancy probably results from the fact that we assessed RPE at lower intensities and/or at lower pH values. Support for such interpretation comes from an earlier study showing that KE increased leg discomfort during cycling only at submaximal workloads, and in particular at the lower pH levels (99). This study also indicated that low pH is an integral predictor for increased leg discomfort. Therefore, the increased RPE in KE compared to CON in **article 1** most probably resulted from the KE-induced acidosis. The fact that ketoacidosis only increased leg discomfort at submaximal but not at maximal exercise intensities has been attributed to a larger KE-induced mismatch between actual pH and expected pH at low than at higher intensities (76, 99, 296). An alternative explanation may be the observed hyperventilatory response observed in ours but not in other studies (63, 76, 180), as breathlessness is an important contributor to elevated RPE (99).

2.5 Effect on appetite and gastro-intestinal discomfort

Another source of concern is the potential negative effect of KE on appetite. A recent study indicated that acute KE ingestion, compared to isocaloric glucose intake, suppressed hunger and the desire to eat at rest (352). Although this effect may be beneficial for instance in the context of long-term weight loss in obese populations (80), it may impede adequate post-exercise nutritional intake causing suboptimal muscle repair and recovery (5). In line with these observations at rest (352), we for the first time observed that KE also decreases 'perceived hunger' and the 'desire to eat' immediately post-exercise. The KE-induced suppression of appetite is likely mediated by ghrelin, as by analogy with the earlier findings by Stubbs *et al*. at rest (352), KE decreased serum ghrelin levels without affecting serum leptin concentrations. On the other hand, other hormones like the appetite reducing hormone GDF15 may also be implicated in the regulation of appetite post-exercise (295). However, we observed that KE attenuated the exercise-induced rise in GDF15 suggesting that GDF15 is not involved in the acute effect of KE on appetite. Although these data and the observations by Stubbs *et al.* (352) suggest that the appetite-suppressing effect of KE is caused by inhibition of ghrelin, it can not be excluded that the associated acidosis (148, 407), or an inhibitory effect of ßHB on agouti-related peptide expression in the brain (7, 221), were also involved in this response. Previous studies also indicated that, although to a lesser extent compared to ketone salts or ketone diesters (232, 354, 355), KE may increase gastrointestinal distress (374). However, in line with a

previous study (354), KE in the conditions of our studies did not result in higher distress compared to ingestion of carbohydrate drinks alone.

2.6 Modulating effect of bicarbonate co-ingestion

The available data provide no evidence at all for an acute ergogenic effect of KE in endurance exercise, and even demonstrate that KE is associated with potential negative effects including metabolic acidosis, increased perception of fatigue and decreased appetite post-exercise. As all these responses may be related to the observed ketoacidosis, we assessed whether counteracting KE-induced acidosis, by co-ingestion of 300 $mg \cdot kg^{-1}$ BW $NaHCO_3$ (BIC), would improve endurance exercise performance. Therefore, we adopted a similar research design as described above, but with the addition of either BIC alone or KE+BIC.

2.6.1 Effect on performance, metabolism and respiration

Co-ingestion of KE and BIC increased $TT_{15'}$ performance at the end of the simulated cycling race on average by 5%, while consequent sprint performance remained similar **(Article 2, Figure 5)**. This effect was specifically due to the interaction between KE and BIC as neither KE alone, nor BIC alone improved performance. The ergogenic effect of KE + BIC probably did not result from alterations in pulmonary ventilation or substrate hierarchy as only small differences were observed in V_E, and no differences between KE + BIC *versus* KE alone were apparent for RER.

We observed that concomitant BIC ingestion induced a moderate alkalosis (pH: ~7.51) and elevated the alkaline reserve ($[HCO_3^-]$: 31.1 mM) by the start of $TT_{15'}$. This resulted in ~50% more HCO_3^- utilization during the $TT_{15'}$ and the ensuing sprint in KE + BIC compared to KE. Moreover, this was accompanied by ~1-2 mM higher blood lactate levels during the $TT_{15'}$ and after the sprint in KE + BIC than in KE. These data suggest that the elevated extracellular alkaline reserve by bicarbonate ingestion facilitated the co-transport of H^+ and La^- out of skeletal muscle cells during exercise (347) and/or increased glycol(geno)lytic rate (336), causing increased performance during the $TT_{15'}$. However, it can not be excluded that the decreased extracellular $[Ca^{2+}]$ and $[Cl^-]$ in KE + BIC *versus* KE, were also implicated in the observed ergogenic effect.

In contrast to a previous study showing that a similar dose of BIC resulted in a 3% higher power output during a 90 s isokinetic sprint at the end of an identical 3 h intermittent cycling

bout (Dalle *et al.,* in revision), we did not observe an increased sprint performance in BIC. These contrasting results are likely due to the addition of a $TT_{15'}$ before the final sprint in our study. This $TT_{15'}$ probably absorbed the bulk of the increased alkaline reserve in BIC (89, 127), leaving almost no increased buffering capacity available for the sprint.

2.6.2 Effect on appetite and gastro-intestinal discomfort

We observed that additional intake of BIC counteracted the KE-induced decrease in 'hunger' and 'desire to eat' post-exercise. This suggests that the negative effect of KE on appetite is not only caused by depressed serum ghrelin levels (352), but may also result from the ketoacidosis *per se* (148, 407). According to KE (354, 374), also BIC has previously been linked to increased gastrointestinal distress (32). However, KE + BIC did not result in higher gastrointestinal distress compared to exclusive high-carbohydrate intake in the conditions of our study.

2.7 Conclusion

Ingestion of KE during the initial phase of a ~3 h 15 min simulated cycling race in highly trained cyclists neither affected skeletal muscle substrate use nor high-intensity exercise performance at the end of a race. Moreover, KE intake decreased blood pH and the extracellular alkaline reserve causing hyperventilation and higher perceived fatigue during exercise. In addition, KE may impair post-exercise recovery and repair by decreasing hunger and the desire to eat immediately after exercise. Therefore, acute ingestion of KE during endurance exercise is definitely not recommended for athletes. In contrast, additional ingestion of BIC counteracts these negative effects of KE and significantly enhanced $TT_{15'}$ performance at the end of the race. Consequently, co-ingestion of KE and BIC is an appropriate, safe, and well-tolerated approach to increase performance at the end of a prolonged endurance event.

3. Physiological and muscular responses to endurance overload training

Despite the high prevalence (216) and long-term implications of overreaching/overtraining (258), there is still an incomplete understanding of the underlying pathophysiological mechanisms (8, 236, 258). This is largely due to the inconsistent physiological responses that have previously been observed in response to overload training and the limited availability of well-controlled studies investigating the chronological sequence of these pathophysiological effects (35, 371). Therefore, we assessed the effect of a 3-week endurance training overload program (two training sessions/day, 6 days/week) on a wide spectrum of physiological and muscular responses in healthy, recreationally active subjects. Thirty minutes after each training session, subjects received a recovery drink delivering ~60 g carbohydrates and ~30 g protein.

3.1 Effect on performance

The training program decreased sprint performance in all subjects (range: -1 to -12%), while performance in TT_{30min} on average remained stable (range: -4 to +12%). This is in line with a previous study (399) showing that predominantly anaerobic efforts are more sensitive to training distress than endurance efforts. Following 7 days of recovery, performance was still decreased in some subjects *versus* increased in others (90 s sprint: -5 to +13%; TT_{30min}: -5 to +14%). This indicates that, according to the ACSM consensus statement (258), some subjects were most likely FOR *versus* others NFOR. However, it is not possible to individually classify subjects as there are no clear-cut criteria available to adequately perform such classification (34, 260). This once more indicates that an increase in training load results in variable responses (16, 17, 229, 230, 398, 399), whereby some athletes improve without noticeable deleterious performance effects, while others demonstrate severely impaired performance. However, the underlying characteristics determining these individual responses remain largely unknown.

3.2 Effect on physiological parameters

Despite the incomplete understanding of the pathophysiology of overload training (258, 259, 371), it is the prevailing opinion that increased sympathetic activity occurs as an initial stress response to intensified training and may underlie subsequent physiological alterations (93, 111). Our results are in line with this belief, as we observed increased nocturnal catecholamine excretion, predominantly noradrenaline **(Article 3, Figure 6)**. This elevated intrinsic sympathetic activity coincided with an early decrease in resting, submaximal and

maximal heart rates **(Article 3, Figure 5)**. Although the decrease in submaximal and maximal heart rate is considered to be a typical response to overload training, previous studies observed both increased, decreased or stable resting heart rates (20, 52, 228, 229). The observed bradycardia at rest and during exercise in our study probably reflects excess vagal output (151, 304) either or not in conjunction with downregulation of $ß_2$-adrenoreceptor sensitivity (112, 123) or alterations in the cardiac input from mechanically and metabolically sensitive skeletal muscle afferents (105). Despite the consistent and rapid decrease in exercising heart rate in response to overload training, a recent study (363) indicated that these decreases are not related to the changes in performance and as such are insufficient to explain the development of overtraining.

Previous studies suggested that alterations in cytokine production and in the hypothalamic-pituitary-adrenal (HPA) axis may be involved the development of overtraining (258). However, in agreement with a recent systematic review (36), we observed no alterations in either resting IL-6, ACTH or cortisol levels. Despite the lack of evidence for a disturbed HPA axis at rest during overload training, Meeusen *et al.* (260) suggested that the response of the HPA axis to a two-bout maximal incremental exercise protocol may discriminate between adequately trained and overreached/overtrained subjects. Indeed, they observed that the exercise-induced rise in ACTH was impaired after the second exercise bout in overreached/overtrained subjects compared to normal trained subjects (260). Nonetheless, these results could not be reproduced in a larger cohort (34). Therefore, literature data together with our observations indicate that alterations in the HPA axis or in cytokine production do not (significantly) contribute to the development of overtraining, at least not in the early phase of overtraining which is crucial from the perspective of prevention.

Another proposed mechanism that may underlie overreaching/overtraining is a decrease in basal muscle glycogen concentrations (58, 59, 196). However, resting glycogen levels remained within the normal range in our study despite the development of multiple overreaching symptoms. This further supports the general belief that glycogen depletion is not required for overreaching to develop (139, 341). Nonetheless, we observed that overload training markedly reduced net glycogenolysis during exercise and lowered peak lactate levels after exercise, which is likely to impair optimal exercise performance (149). This is in line with other studies and has been attributed to a decreased adrenergic sensitivity of skeletal muscle (188, 234). However, the fact that decreased peak lactate levels were only evident at the end of the training

period, suggests that alterations in substrate utilization during exercise only occur as a secondary response to overload training.

Two recent studies from Woods *et al.* proposed that a reduced resting metabolic rate (RMR) may be an important contributor to the development of overtraining (398, 399). Although the mechanism responsible for a lower RMR remained elusive, they suggested that RMR decreases to protect against energy imbalance resulting from the inability to increase energy intake proportionally to the increased energetic demands of training. In line with these observations, energy intake remained stable around 15 000 kJ per day in our study despite a gradual increase in training load, resulting in an energetic deficiency of ~2800 kJ per day at the end of the overload period. As this energetic deficiency was not coupled to a decrease in body weight, RMR was also likely decreased in our study. This indicates that the inability to increase energy intake proportionally to the increased energy requirements may be an important factor underlying the development of overtraining. Interestingly, the energetic imbalance in our study did not coincide with alterations in subjective ratings of appetite.

The mechanism underlying the inability to increase energy intake during overload training was not related to the traditional appetite hormones ghrelin and leptin, as serum ghrelin levels remained stable throughout the overload period, while the circulating concentrations of the appetite suppressor leptin decreased. Nonetheless, depressed leptin levels may be responsible for the potential decrease in RMR. Indeed, previous research indicated that leptin deficiency operates as a starvation signal during episodes of energy deficit, serving to decrease basal energy expenditure by lowering heart rate, blood pressure and thyroid hormone activity (106, 290). In addition, we observed, for the first time, that the appetite suppressor GDF15 increases during training overload **(Article 3, Figure 7A)**. Previous literature data (295) together with the recent observation of our lab that GDF15 levels are not altered during hypocaloric diet-induced weight loss (Hiroux *et al.*, submitted), suggest that the increment in GDF15 did not occur as a secondary response to decreased energy intake. Therefore, the augmented GDF15 levels likely resulted from training-induced physiological stress **(cfr. Article 1, Figure 6B)** and may have triggered the inability to increase energy intake. Moreover, alterations in GDF15 were highly correlated with the training-induced drop of peak heart rate ($r = 0.60$).

To validate whether GDF15 also increases in response to overload training in elite athletes, we assessed serum GDF15 concentrations in blood samples of elite cyclists during the *Tour de France* 2019. Seven professional cyclists and four team-directors (control group) belonging to

the Deceuninck-Quick-Step team volunteered to provide fasted blood samples in the morning on (i) the day before the start (d0), (ii) at the first (d11) and (iii) second rest day (d17), and (iv) on the final day (d23). In line with our observations in healthy volunteers **(Article 3, Figure 7A)**, serum GDF15 levels were increased above baseline levels in all cyclists at d11 and remained ~50% above baseline values until the end of the Tour ($p < 0.05$ *vs.* baseline, **Figure 1)**. In contrast, GDF15 levels remained stable in the team-directors throughout the race. These data indicate that GDF15 may be considered as a potential hormonal marker to adequately monitor the development of overtraining.

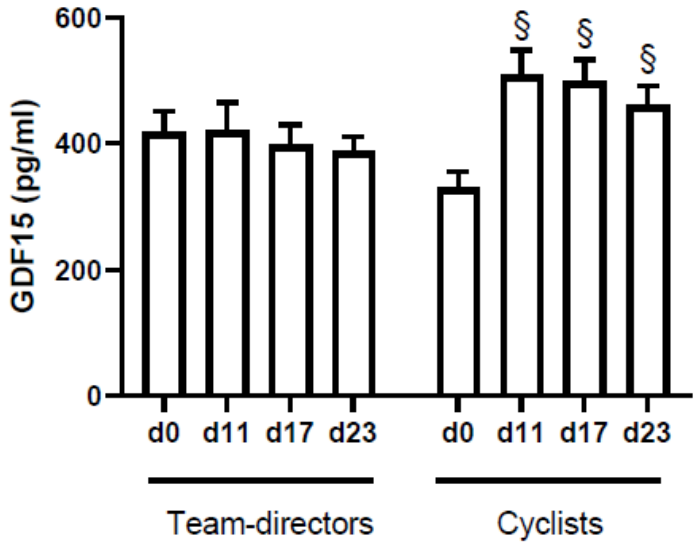


Figure 1. Serum GDF15 concentrations increase during the Tour de France in elite cyclists.
Data are means ± SEM for serum GDF15 levels in team directors (n = 4) and elite cyclists (n = 7) during the Tour de France 2019. § $p < 0.05$ *vs.* baseline.

3.3 Effect on muscular adaptations

In addition to the profound physiological dysregulations induced by overload training, recent work (17) indicated that subjects developing overreaching did not increase their muscle oxidative capacity in response to 3 week high-volume training, while subjects who were not overreached showed a 15% increase. Similarly, a previous study in rats observed that overreaching attenuated training-induced alterations in citrate synthase activity and mitochondrial complex IV activity (102). Along this line, we observed no alterations in capillary contacts nor in the capillary-to-fiber perimeter exchange index at the end of the 3-week overload training period. Although most studies agree that increased muscle capillarization requires between 2 to 4 weeks of training (163, 168, 182), a recent study

observed a higher amount of capillary contacts per myofiber already after 2 training weeks (164). Although these data are definitely not conclusive, they suggest that the development of adequate muscular adaptations to training may be required to avoid the occurrence of overtraining.

3.4 Conclusion

Three weeks of overload training induced a state of (non)functional overreaching which involved dysregulations in multiple systems, including increased sympathetic activity, energy deficiency, bradycardia at rest and during exercise, as well as impaired exercise-induced angiogenesis. Our data suggest that elevated sympathetic activity together with decreased heart rates at rest and during exercise occur as an early response to overload training, while energy deficiency, impaired exercise-induced glycogenolysis and reduced exercise performance seem to arise as a secondary response. Previously proposed hormonal markers to monitor the development of overtraining (e.g. ACTH, cortisol, IL-6) remained unaltered in our study, which once more indicates that these parameters are inadequate markers for timely detection of NFOR in the early phase of a malignant overtraining process. However, we provide preliminary evidence that GDF15 may evolve as an adequate and early hormonal marker for the development of overtraining.

4. Effects of ketone ester supplementation during endurance training overload

An imbalance between (non)training related stress and recovery results in the development of overreaching/overtraining, which is characterized by numerous physiological and muscular maladaptations (cfr. section 3). Elevating ketone levels in the blood has been shown to increase post-exercise recovery as well as to modulate a range of physiological symptoms implicated in the development of overreaching, Therefore, we hypothesized that KE supplementation can blunt the development of NFOR. Recreationally active male subjects performed a 3-week supramaximal cycling overload period as described in section 3 and were randomly assigned to receive either 25 g KE or an isocaloric placebo (medium-chain triglycerides) immediately after each training session and 30 min pre sleep.

4.1 Effect on performance

Sustained training load and performance in the exercise tests (e.g. 30 min time-trial and 90 s sprint) did not differ between KE and CON during the first two weeks. However, in the final week subjects in KE were able to tolerate a 15% higher training load compared to CON, mostly due to enhanced performance in the longer training sessions **(Article 3, Figure 3)**. Similarly, in the final week, KE intake increased performance on a 30 min time-trial, and on a 30 min time-trial following a 90 min preload (EPT_{120min}), while 90 s sprint performance decreased to a similar extent in both groups. This suggests that KE supplementation specifically enhanced endurance performance, while not altering predominantly anaerobic performances.

4.2 Effect on physiological parameters

KE completely counteracted the overload training induced increase in nocturnal noradrenaline excretion and substantially attenuated the decrease in resting, submaximal and maximal heart rate observed in CON. Although we did not measure circulating catecholamine levels after exercise, this suggests that KE intake may have decreased the rise in catecholamine concentrations post-exercise (218) resulting in a delayed exhaustion of the sympathetic nervous system during the next training sessions (111, 112). This is in line with a previous study in mice showing that ßHB directly suppresses sympathetic activity by downregulation of GPR41 in the sympathetic ganglions (203).

Besides the clear effect of KE on sympathetic activity and heart rate, subjects in KE were also able to maintain an energetic balance while subjects in CON were unable to increase their energy intake proportionally to the increased energetic demands of the training **(Article 3, Table 4)**. Interestingly, the increased energy intake in KE during training overload was exclusively due to higher carbohydrate intake as protein and fat intake remained unaltered. This indicates that although acute KE supplementation reduces hunger and the desire to eat immediately post-exercise **(article 1 and 2)**, long term KE intake during training overload induces an opposite effect. However, this was not accompanied by alterations in appetite sensations. As body mass remained stable in KE as well as in CON, this suggests that KE intake counteracted the overload training-induced decline in RMR that likely occurred in CON. These data are in line with a recent study by our lab (Hiroux *et al.,* submitted) showing that KE intake attenuated hypocaloric diet-induced decline in RMR by negating the drop in free T3. In addition, previous research also observed that a ketogenic diet reduced the drop in RMR following a 4-week hypocaloric diet (85).

The inability to increase energy intake in CON likely resulted from training-induced increases in circulating GDF15 levels. Conversely, KE markedly reduced the rise in GDF15 **(Article 3, Figure 7A)**, suggesting that lower GDF15 values may underlie the ability of KE to counteract training overload-induced energy imbalance and drop in RMR. GDF15 levels were measured in the fasted state in the absence of elevated blood KB. This suggests that acute suppression of post-exercise increments in GDF15 by KE intake **(Article 1, Figure 6B)**, results in less accumulation of GDF15 in the blood causing lower values even in the absence of acute ketosis. Nonetheless, KE may also have attenuated the potential drop in RMR by negating overload training-induced drop in blood leptin concentrations. In contrast to the lower ghrelin concentrations after acute KE intake **(Article 1, Figure 6A)**, no alterations in fasted ghrelin levels were observed during the training overload study **(Article 3)**. In line with these observations, serum ghrelin concentrations only slightly increased above baseline levels at day 17 ($p < 0.05$ *vs.* baseline) in the elite cyclists during the *Tour de France* 2019 **(Figure 2)**. This indicates that while ghrelin primarily determines the acute appetite response to KE ingestion **(article 1)** (352)**,** GDF15 conceivably is the predominant long-term regulator of appetite during training.

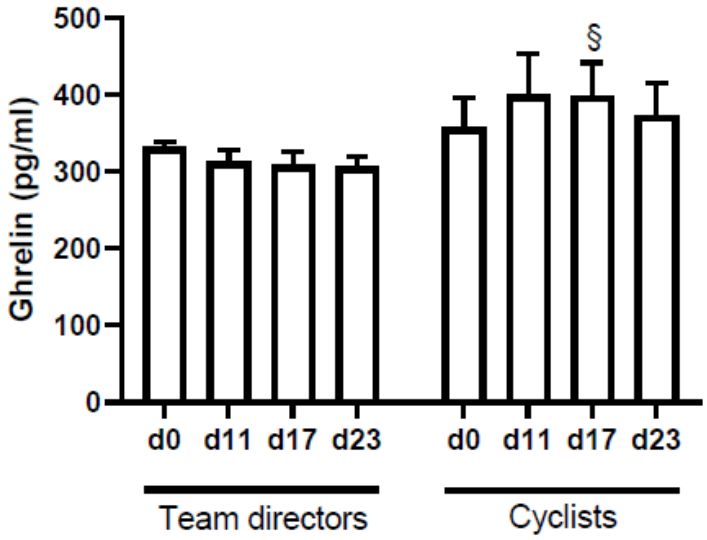


Figure 2. Serum ghrelin concentrations during the Tour de France in elite cyclists and team directors.
Data are means ± SEM for serum ghrelin concentrations in team directors (n = 4) and elite cyclists (n = 7) during the Tour de France 2019. § $p < 0.05$ *vs.* baseline for indicated group.

KE intake did not counteract overload-induced decreases in exercise-induced net glycogenolysis or peak lactate levels, nor did it protect against the small alterations in blood leukocyte count. Physiological markers that were unaffected by the overload period (*i.e.* IL-6, ACTH and cortisol) neither were altered by KE. In addition, KE was unable to prevent the decrement in mental well-being, as evidenced by a similar increase in total stress scores together with similarly depressed total recovery scores compared to CON. This suggests that the subjective measurement of training stress was less sensitive compared to the physiological measures to discriminate between the less (KE) *versus* the more (CON) overreached subjects. This is in line with the majority of the literature showing consistent alterations in subjective perceptions of training stress/recovery independent of the extent of physiological or performance disturbances (14, 15, 60, 82, 115, 137, 335). In contrast, a recent study by Ten Haaf *et al.* (364) indicated that the combination of questionnaire-based alterations in fatigue and readiness to train after only 3 days of overload training correctly predicted 78% of subjects as either acutely fatigued or functionally overreached. However, there is no evidence that a similar approach would be able to distinguish between functionally and non-functionally overreached subjects.

4.3 Effect on muscular adaptations

Subjects in KE showed a substantial increase in capillary contacts and CFPE-index in response to the training-overload period **(Article 7, Figure 1)**. This indicates that KE

counteracts overload training induced impairments of muscular adaptations to exercise (17, 102). Increased capillarization enhances the potential for oxygen diffusion and metabolite clearance from skeletal muscle, and has been shown to improve aerobic capacity (173). In addition, previous animal studies reported a close correlation between capillarization indices in skeletal muscle and mitochondrial density as well as maximal aerobic performance (170, 173). This suggests that improved capillarization may have contributed to the higher exercise tolerance and improved endurance performance in KE. The enhanced capillary response in KE was not coupled with alterations in muscle fiber morphology or fiber type distribution, indicating a purely angiogenic response. The absence of alterations in muscle fiber type composition or morphology are in keeping with literature indicating that these adaptations require at least four weeks of training (1, 74, 172, 181).

The increased capillarization in KE was accompanied by enhanced mRNA expression and protein content of VEGF **(Article 7, Figure 2A+B)**, the main regulator of exercise-induced angiogenesis (238, 268, 356). In contrast, mRNA and protein content of VEGF remained unaltered in CON. These data clearly indicate that KE probably increased muscle capillarity via upregulation of VEGF. These data are in line with previous observations in rodents showing that ßHB directly enhances generation of VEGF in the aorta and in the brain (178, 401). The increase in VEGF by KE is likely not mediated by any of the upstream transcription factors of VEGF, as AMPK, PGC1α, HIF1α and ERRα were all similar between KE and CON. A recent study in mice reported that higher NAD^+ enhanced the VEGF-dependent angiogenic response to exercise by stimulating SIRT1 (69). Given the lower utilization of NAD^+ to produce acetyl-CoA from ßHB compared to glucose (90, 278), and the concomitant increase in NAD^+ during ketosis (403), KE might have exerted its angiogenic effect via elevation of intramyocellular NAD^+. Nonetheless, the pro-angiogenic effect of KE may also be triggered by increases in intramyocellular calcium concentrations, which has been shown to be responsible for the increase in VEGF by ßHB in brain endothelial cells (178). In line with this hypothesis, we observed a small increase in blood calcium levels during ketosis in **article 1**.

4.4 Conclusion

KE intake counteracted the development of training overload-induced alterations in heart rate, sympathetic activity and energy imbalance thereby enhancing endurance performance and sustainable training load. Besides counteracting multiple physiological dysregulations implicated in the development of non-functional overreaching, KE intake also increased muscle

angiogenesis, indicating that KE stimulates beneficial muscular adaptations to exercise training. These data clearly demonstrate that KE supplementation is a potent strategy to counteract the development of non-functional overreaching.

5. Methodological considerations and limitations

In order to allow adequate interpretation of the obtained research findings, some methodological considerations should be taken into account.

Study participants and sample size. All participants were non-smoking, healthy men between 18 and 35 years old, not taking any supplements or medication. In **article 1 and 2**, subjects were highly-trained (>6h/week) cyclists or triathletes, while subjects in **article 3 and 7** were only recreationally active in various sports. These populations were chosen both to minimize as many confounding variables (*e.g.* age, sex, training history) as well as to allow optimal translation to the target population (*i.e.* highly-trained up to elite endurance athletes) taking practical (availability and willingness of subjects, feasibility of measurements) and financial limitations into consideration. Therefore, caution is required for extrapolation of the obtained research findings to other populations, such as females, elderly taking medication, or elite athletes. The practical and financial limitations also resulted in limited sample sizes, which may have reduced the statistical power or inflated the false discovery rate and the estimated effect size. Nonetheless, the implemented sample sizes (n = 9-12 per group/condition) are in line with the majority of studies in the field of exercise and muscle physiology. In addition, the obtained results concerning the effect of acute KE on exercise performance **(article 1)**, were successfully reproduced in **article 2**, indicating that this limited sample size indeed may produce reliable data. Furthermore, the effect of KE on overtraining symptoms in **article 3** were of such magnitude and consistent between individuals, that they can hardly be due statistical errors, i.e. represent true biological effects.

Specificity of exercise. For practical reasons (availability of equipment and complexity of measurements), we evaluated the effect of KE and BIC during cycling, instead of during other endurance sports (running, walking, cross-country skiing, triathlon). Given the differences in external load on the musculoskeletal system as well as in physiological responses (269) between these sports, the results may not be linearly generalized to other endurance-based sports. For instance, as KE and BIC have previously been linked to increased gastrointestinal distress (32, 374), its applicability may be smaller in triathlon compared to cycling given the already higher gastrointestinal complaints in triathlon (~30%) *versus* cycling (< 5%) (73, 302). In contrast, the ability of KE to delay central fatigue during exercise (96, 381), may induce more beneficial effects in running compared to cycling given the seemingly higher contribution of central fatigue to performance impairments during running (237, 269–272, 310).

Duration of training program. The overload training program in **article 3 and 7** was limited to three weeks in duration. As many muscular adaptations to exercise training, such as alterations in fiber type distribution (385) or increased activity of oxidative enzymes (6, 339), require at least four weeks of training, it has to be determined whether KE may also impact these muscle fiber characteristics during longer periods of overload training.

Individual response to training and KE/BIC supplementation. Despite the general improvement in 15 min time-trial performance following KE + BIC ingestion in **article 2**, still 3 out of 9 subjects performed better in one of the other experimental conditions. Similarly, in the training overload study, two out of nine subjects in the control condition were able to tolerate a higher training load compared to their matched pair receiving KE. The underlying characteristics determining this individual response remain unknown. Therefore, from the perspective of coaching in athletic performance, the applicability of KE or KE + BIC to counteract overload training or increase endurance performance must be verified on an individual basis.

Compatibility with other ergogenic supplements. We aimed to investigate the effect of KE (and BIC) in a design that was as ecologically valid as possible (*e.g.* appropriate carbohydrate and fluid intake before and during exercise). However, athletes commonly use multiple dietary supplements (e.g. caffeine, nitrate, beta-alanine) before and during exercise in order to increase endurance performance (247). Therefore, the compatibility of KE (and BIC) with these dietary supplements remains to be determined.

Determination of muscle glycogen content. Muscle glycogen concentration was determined using a quantitative enzymatic fluorometric assay (240) on wet muscle tissue. Although this is a valid and commonly used method, alterations in skeletal muscle water content may have affected the results obtained. However, to date there are no data available to suggest that KE may alter the amount of water stored in skeletal muscle.

Timing of muscle biopsy sampling. Skeletal muscle biopsies at the end of the overload training period in **article 3 and 7** were obtained 10-13 hours after the last training session. Therefore, it can not be excluded that acute effects of the last training session were still present. From another perspective, we did not observe between-group differences in the upstream transcription factors of VEGF in **article 7** and suggested that these are likely not implicated in the pro-angiogenic effect of KE. However, it can not be excluded that KE may have increased

these transcription factors during the 2-3 h time window of elevated blood KB following KE ingestion.

6. Implications and future perspectives

Our results indicate that acute ingestion of KE does not affect endurance exercise performance. In contrast, co-ingestion of KE and BIC during the initial phase of an endurance event stimulates high-intensity exercise performance at the end of the race. As such, KE together with BIC ingestion appears to be a promising ergogenic strategy with potential relevance for various athletic populations including marathon runners, Ironman triathletes, and cross-country skiers.

Given our observation that KE does not impair high glycolytic flux during exercise, further studies should identify whether co-ingestion of KE and BIC may also increase exercise performance in short-term events (e.g. time-trials in road cycling, 400 m running). In addition, upcoming studies should investigate different dosing strategies as well as the underlying characteristics determining the variance in ßHB increments following KE (353), to optimize potential ergogenic effects. Furthermore, as previous studies indicated that a long-term increase in blood KB induced by a ketogenic diet enhances enzymes involved in the oxidation of KB and fatty acids (72, 241), it would be of high interest to investigate whether long-term ingestion of KE may induce similar responses, which may potentially result in further improvements in exercise performance.

Besides the beneficial effect of additional BIC intake compared to KE alone on endurance performance, BIC ingestion also counteracted the acute appetite-suppressing effect of KE post-exercise. This observation is not only relevant in an athletic population to optimize post-exercise recovery by allowing adequate food intake (5), but may also increase the therapeutic potential of ketosis in various disease settings (e.g. cancer, Alzheimer's disease, lymphedema) wherein the beneficial effects of KE (24, 42, 45) may be hampered by reductions in food intake (32, 38).

We observed that post-exercise ingestion of KE during endurance training overload counteracts the development of physiological and muscular maladaptations, and enhances endurance performance. This holds direct clues for athletes participating in either overload training or competition (e.g. grand tour cycling and training camps). In addition, the ability of KE to counteract these training-induced catabolic events, also suggests that KE may counteract other catabolic conditions including disease-induced cachexia or exercise-induced relative energy deficiency (RED-S) (98, 275). KE intake during training overload also resulted in a

small, but significant increase in bone mineral content (BMC). Given the long term decrease of BMC observed in cyclists (285) and its pivotal role in fracture risk (317), future research should address whether long-term KE intake may counteract age- or exercise-induced decreases in BMC or may increase BMC in osteoporotic patients.

The observation that KE is able to markedly stimulate muscle angiogenesis *in vivo* opens new perspectives to potentially counteract capillary rarefaction either induced by training cessation or by various diseases including peripheral arterial disease and chronic obstructive pulmonary disease (176, 177, 204, 239, 243, 284). In addition, future studies should also explore the potential of KE to stimulate de novo angiogenesis during rehabilitation following injury or disease.

We also reported that KE is able to counteract the exercise-induced rise in GDF15. Although the precise role and regulation of GDF15 is still elusive, recent studies observed that GDF15 levels progressively increase with aging, while others reported a clear association between elevated GDF15 and cardiovascular and all-cause mortality (22, 56, 383, 402). Therefore, future research should explore the potential implications of KE-induced suppression of GDF15 in various clinical conditions.

7. General conclusion

Recent data indicated that elevating blood KB by ingestion of KE, suppresses glycolytic flux during exercise, thereby sparing finite muscle glycogen stores and stimulating endurance performance. In contrast to this opinion, we observed that KE ingestion under conditions mimicking real world endurance cycling competitions, does not counteract muscle glycogen breakdown nor alters endurance performance. In addition, we reported that KE, by causing transient metabolic acidosis, results in reduced extracellular buffering capacity and elevated fatigue perception in the approach of the final stage of the race. Therefore, it is reasonable to assume that the potential of KE to enhance endurance exercise performance may be overruled by ketosis-induced acid-base dysregulation. Hence, we hypothesized that concomitant ingestion of BIC to counteract KE-induced acidosis may unlock the ergogenic potential of KE. According to our hypothesis, co-ingestion of KE and BIC fully counteracted ketoacidosis and resulted in a 5% performance improvement during a 15 min time-trial at the end of a ~3 h 15 min simulated cycling race. In addition to our studies investigating the acute effect of KE on endurance performance, we also hypothesized that KE ingestion post-exercise may negate endurance training-induced overreaching. We showed that KE intake indeed markedly blunted the occurrence of physiological symptoms implicated in the development of overreaching, and at the same time stimulated endurance exercise performance. Moreover, we observed that KE stimulated the formation of new capillaries in response to overload training, which holds direct clues for various clinical populations afflicted by capillary rarefaction. Taken together, our data indicate that co-ingestion of KE and BIC may evolve as an attractive approach to acutely enhance a 15-min time-trial performance at the end of a cycling race, while prolonged KE administration is a potent strategy to enhance training adaptations during endurance training overload.

ACKNOWLEDGEMENTS, PERSONAL CONTRIBUTIONS AND CONFLICT OF INTEREST

Acknowledgements and personal contributions.

Article 1: Conception and design of the study: Chiel Poffé and Peter Hespel. Biopsy sampling: Stijn Bogaerts. Data collection and biochemical analyses: Chiel Poffé and Monique Ramaekers. Analysis and interpretation of the data and manuscript drafting: Chiel Poffé and Peter Hespel. All authors critically evaluated the manuscript and approved for submission.

Article 2: Conception and design of the study: Chiel Poffé and Peter Hespel. Data collection: Chiel Poffé, Monique Ramaekers and Stijn Bogaerts. Analysis and interpretation of the data and manuscript drafting: Chiel Poffé and Peter Hespel. All authors critically evaluated the manuscript and approved for submission.

Article 3: Conception and design of the study: Chiel Poffé and Peter Hespel. Biopsy sampling: Ruud Van Thienen. Data collection and biochemical analyses: Chiel Poffé and Monique Ramaekers. Analysis and interpretation of the data and manuscript drafting: Chiel Poffé and Peter Hespel. All authors critically evaluated the manuscript and approved for submission.

Article 4: Manuscript drafting: Chiel Poffé and Peter Hespel. All authors (Chiel Poffé, Monique Ramaekers, Ruud Van Thienen and Peter Hespel) have read and approved the final version of this manuscript.

Article 5: Manuscript drafting: Chiel Poffé and Peter Hespel. All authors (Chiel Poffé, Monique Ramaekers, Ruud Van Thienen and Peter Hespel) have read and approved the final version of this manuscript.

Article 6: Manuscript drafting: Chiel Poffé and Peter Hespel. All authors (Chiel Poffé, Monique Ramaekers and Peter Hespel) have read and approved the final version of this manuscript.

Article 7: Conception and design of the study: Chiel Poffé and Peter Hespel. Data collection, biochemical and histological analyses: Chiel Poffé and Ruben Robberechts. Analysis and interpretation of the data and manuscript drafting: Chiel Poffé and Peter Hespel.

Medical supervision and muscle biopsy procedures: Ruud Van Thienen. All authors critically revised the manuscript and approved the final version of the manuscript.

Conflict of interest. The authors declare that they have no competing interests.

REFERENCES

1. **Abe T**, **Kitaoka Y**, **Kikuchi DM**, **Takeda K**, **Numata O**, **Takemasa T**. High-intensity interval training-induced metabolic adaptation coupled with an increase in Hif-1α and glycolytic protein expression. *J Appl Physiol* 119: 1297–302, 2015.
2. **Achten J**, **Halson SL**, **Moseley L**, **Rayson MP**, **Casey A**, **Jeukendrup AE**. Higher dietary carbohydrate content during intensified running training results in better maintenance of performance and mood state. *J Appl Physiol* 96: 1331–1340, 2004.
3. **Acworth I**, **Nicholass J**, **Morgan B**, **Newsholme EA**. Effect of sustained exercise on concentrations of plasma aromatic and branched-chain amino acids and brain amines. *Biochem Biophys Res Commun* 137: 149–153, 1986.
4. **Allan R**, **Mawhinney C**. Is the ice bath finally melting? Cold water immersion is no greater than active recovery upon local and systemic inflammatory cellular stress in humans. *J Physiol* 595: 1857–1858, 2017.
5. **American Dietetic Association**, **Dietitians of Canada**, **American College of Sports Medicine**, **Rodriguez N**, **Di Marco N**, **Langley S**. American College of Sports Medicine position stand. Nutrition and athletic performance. *Med Sci Sports Exerc* 41: 709–731, 2009.
6. **Andersen P**, **Henriksson J**. Capillary supply of the quadriceps femoris muscle of man: adaptive response to exercise. *J Physiol* 270: 677–690, 1977.
7. **Arase K**, **Fisler JS**, **Shargill NS**, **York DA**, **Bray GA**. Intracerebroventricular infusions of 3-OHB and insulin in a rat model of dietary obesity. *Am J Physiol - Regul Integr Comp Physiol* 255: 974–981, 1988.
8. **Armstrong LE**, **Vanheest JL**. The unknown mechanism of the overtraining syndrome Clues from depression and psychoneuroimmunology. *Sport Med* 32: 185–209, 2002.
9. **Aubry A**, **Hausswirth C**, **Louis J**, **Coutts AJ**, **Le Meur Y**. Functional overreaching: the key to peak performance during the taper? *Med Sci Sports Exerc* 46: 1769–1777, 2014.
10. **Balasse E**, **Couturier E**, **Franckson JRM**. Influence of sodium β-hydroxybutyrate on glucose and free fatty acid metabolism in normal dogs. *Diabetologia* 3: 488–493, 1967.
11. **Balasse E**, **Ooms HA**. Changes in the concentrations of glucose, free fatty acids, insulin and ketone bodies in the blood during sodium ß-hydroxybutyrate infusions in man. *Diabetologia* 4: 133–135, 1968.

12. **Balasse EO**, **Féry F**. Ketone body production and disposal: effects of fasting, diabetes, and exercise. *Diabetes Metab Rev* 5: 247–270, 1989.
13. **Barron JL**, **Noakes TD**, **Levy W**, **Smith C**, **Millar RP**. Hypothalamic dysfunction in overtrained athletes. *J Clin Endocrinol Metab* 60: 803–806, 1985.
14. **Bellenger CR**, **Karavirta L**, **Thomson RL**, **Robertson EY**, **Davison K**, **Buckley JD**. Contextualizing parasympathetic hyperactivity in functionally overreached athletes with perceptions of training tolerance. *Int J Sports Physiol Perform* 11: 685–692, 2016.
15. **Bellenger CR**, **Thomson RL**, **Robertson EY**, **Davison K**, **Nelson MJ**, **Karavirta L**, **Buckley JD**. The effect of functional overreaching on parameters of autonomic heart rate regulation. *Eur J Appl Physiol* 117: 541–550, 2017.
16. **Bellinger P**. Functional overreaching in endurance athletes: a necessity or cause for concern? [Epub ahead of print]. *Sport. Med.* (2020). doi: 10.1007/s40279-020-01269-w.
17. **Bellinger PM**, **Sabapathy S**, **Craven J**, **Arnold B**, **Minahan C**. Overreaching attenuates training-induced improvements in muscle oxidative capacity. *Med Sci Sport Exerc* 52: 77–85, 2019.
18. **Berthoud H-R**. The vagus nerve, food intake and obesity. *Regul Pept* 149: 15–25, 2008.
19. **Biolo G**, **Tipton KD**, **Klein S**, **Wolfe RR**. An abundant supply of amino acids enhances the metabolic effect of exercise on muscle protein. *Am J Physiol - Endocrinol Metab* 273: 122–129, 1997.
20. **Bosquet L**, **Merkari S**, **Arvisais D**, **Aubert AE**. Is heart rate a convenient tool to monitor overreaching? A systematic review of the literature. *Br J Sports Med* 42: 709–714, 2008.
21. **Breen L**, **Philp A**, **Witard OC**, **Jackman SR**, **Selby A**, **Smith K**, **Baar K**, **Tipton KD**. The influence of carbohydrate-protein co-ingestion following endurance exercise on myofibrillar and mitochondrial protein synthesis. *J Physiol* 589: 4011–4025, 2011.
22. **Brenière C**, **Méloux A**, **Pédard M**, **Marie C**, **Thouant P**, **Vergely C**, **Béjot Y**. Growth differentiation factor-15 (GDF-15) is associated with mortality in ischemic stroke patients treated with acute revascularization therapy. *Front Neurol* 10: 1–8, 2019.
23. **Broatch JR**, **Petersen A**, **Bishop DJ**. Postexercise cold water immersion benefits are not greater than the placebo effect. *Med Sci Sports Exerc* 46: 2139–2147, 2014.
24. **Broatch JR**, **Petersen A**, **Bishop DJ**. The influence of post-exercise cold-water immersion on adaptive responses to exercise: a review of the literature. *Sport Med* 48: 1369–1387, 2018.
25. **Brooks GA**. Governor recalled! Now what? *J Physiol* 568: 355, 2005.

26. **Brooks GA**. Bioenergetics of exercising humans. *Compr Physiol* 2: 537–562, 2012.
27. **Budgett R**, **Hiscock N**, **Arida R**, **Castell LM**. The effects of the 5-HT2C agonist mchlorophenylpiperazine on elite athletes with unexplained underperformance syndrome (overtraining). *Br J Sports Med* 44: 280–283, 2010.
28. **Burke LM**. Re-examining high-fat diets for sports performance: did we call the "nail in the coffin" too soon? *Sport Med* 45: 33–49, 2015.
29. **Burke LM**, **Angus DJ**, **Cox GR**, **Cummings NK**, **Febbraio MA**, **Gawthorn K**, **Hawley JA**, **Minehan M**, **Martin DT**, **Hargreaves M**. Effect of fat adaptation and carbohydrate restoration on metabolism and performance during prolonged cycling. *J Appl Physiol* 89: 2413–2421, 2000.
30. **Burke LM**, **Hawley JA**, **Angus DJ**, **Cox GR**, **Clark SA**, **Cummings NK**, **Desbrow B**, **Hargreaves M**. Adaptations to short-term high-fat diet persist during exercise despite high carbohydrate availability. *Med Sci Sports Exerc* 34: 83–91, 2002.
31. **Burke LM**, **Hawley JA**, **Wong SHS**, **Jeukendrup AE**. Carbohydrates for training and competition. *J Sports Sci* 29: 17–27, 2011.
32. **Burke LM**, **Pyne DB**. Bicarbonate loading to enhance training and competitive performance. *Int J Sports Physiol Perform* 2: 93–97, 2007.
33. **Burke LM**, **Ross ML**, **Garvican-Lewis LA**, **Welvaert M**, **Heikura IA**, **Forbes SG**, **Mirtschin JG**, **Cato LE**, **Strobel N**, **Sharma AP**, **Hawley JA**. Low carbohydrate, high fat diet impairs exercise economy and negates the performance benefit from intensified training in elite race walkers. *J Physiol* 595: 2785–2807, 2017.
34. **Buyse L**, **Decroix L**, **Timmermans N**, **Barbé K**, **Verrelst R**, **Meeusen R**. Improving the diagnosis of nonfunctional overreaching and overtraining syndrome. *Med Sci Sports Exerc* 51: 2524–2530, 2019.
35. **Cadegiani FA**, **Kater CE**. Hormonal aspects of overtraining syndrome: a systematic review. *BMC Sports Sci Med Rehabil* 9: 1–15, 2017.
36. **Cadegiani FA**, **Kater CE**. Hypothalamic-pituitary-adrenal (HPA) axis functioning in overtraining syndrome: findings from endocrine and metabolic responses on overtraining syndrome (EROS)-EROS-HPA axis. *Sport Med* 3: 1–11, 2017.
37. **Cadegiani FA**, **Kater CE**. Novel insights of overtraining syndrome discovered from the EROS study. *BMJ Open Sport Exerc Med* 5: 1–11, 2019.
38. **Cahill G**, **Felig P**, **Owen O**, **Wahren J**. Metabolic adaptation to prolonged starvation in man. *Nord Med* 83: 89, 1970.
39. **Cahill GF**. Starvation in man. *Clin Endocrinol Metab* 5: 397–415, 1976.

40. **Cahill GF**. Fuel metabolism in starvation. *Annu Rev Nutr* 26: 1–22, 2006.
41. **Cahill GF**, **Herrera MG**, **Morgan AP**, **Soeldner JS**, **Steinke J**, **Levy PL**, **Reichard GA**, **Kipnis DM**. Hormone-fuel interrelationships during fasting. *J Clin Invest* 45: 1751–1769, 1966.
42. **Cahill GFJ**, **Owen OE**. Starvation and survival. *Trans Am Clin Climatol Assoc* 79: 13–20, 1968.
43. **Cahill GFJ**, **Veech RL**. Ketoacids? Good medicine? *Trans Am Clin Climatol Assoc* 114: 143–149, 2003.
44. **Cairns SP**, **Lindinger MI**. Do multiple ionic interactions contribute to skeletal muscle fatigue? *J Physiol* 586: 4039–4054, 2008.
45. **Calbet JAL**, **González-Alonso J**, **Helge JW**, **Søndergaard H**, **Munch-Andersen T**, **Saltin B**, **Boushel R**. Central and peripheral hemodynamics in exercising humans: Leg vs arm exercise. *Scand J Med Sci Sport* 25: 144–157, 2015.
46. **Carey GB**. Mechanisms regulating adipocyte lipolysis. *Adv Exp Med Biol* 441: 157–170, 1998.
47. **Carr AJ**, **Hopkins WG**, **Gore CJ**. Effects of acute alkalosis and acidosis on performance. A meta-analysis. *Sport Med* 41: 801–814, 2011.
48. **Chambers MA**, **Moylan JS**, **Smith JD**, **Goodyear LJ**, **Reid MB**. Stretch-stimulated glucose uptake in skeletal muscle is mediated by reactive oxygen species and p38 MAP-kinase. *J Physiol* 587: 3363–3373, 2009.
49. **Churchward-Venne TA**, **Burd NA**, **Phillips SM**. Nutritional regulation of muscle protein synthesis with resistance exercise: Strategies to enhance anabolism. *Nutr Metab* 9: 40–47, 2012.
50. **Clarke K**, **Tchabanenko K**, **Pawlosky R**, **Carter E**, **Knight NS**, **Murray AJ**, **Cochlin LE**, **King MT**, **Wong AW**, **Roberts A**, **Robertson J**, **Veech RL**. Oral 28-day and developmental toxicity studies of (R)-3-hydroxybutyl (R)-3-hydroxybutyrate. *Regul Toxicol Pharmacol* 63: 196–208, 2012.
51. **Clarke K**, **Tchabanenko K**, **Pawlosky R**, **Carter E**, **Todd King M**, **Musa-Veloso K**, **Ho M**, **Roberts A**, **Robertson J**, **VanItallie TB**, **Veech RL**. Kinetics, safety and tolerability of (R)-3-hydroxybutyl (R)-3-hydroxybutyrate in healthy adult subjects. *Regul Toxicol Pharmacol* 63: 401–408, 2012.
52. **Coates AM**, **Millar PJ**, **Burr JF**. Blunted cardiac output from overtraining is related to increased arterial stiffness. *Med Sci Sports Exerc* 50: 2459–2464, 2018.
53. **Cohen PP**. Studies in ketogenesis. *J Biol Chem* 119: 333–346, 1937.

54. **Cohen PP**, **Stark IE**. Hepatic ketogenesis and ketolysis in different species. *J Biol Chem* 126: 97–107, 1938.

55. **Conley KE**, **Kemper WF**, **Crowther GJ**. Limits to sustainable muscle performance: interaction between glycolysis and oxidative phosphorylation. *J Exp Biol* 204: 3189–3194, 2001.

56. **Conte M**, **Ostan R**, **Fabbri C**, **Santoro A**, **Guidarelli G**, **Vitale G**, **Mari D**, **Sevini F**, **Capri M**, **Sandri M**, **Monti D**, **Franceschi C**, **Salvioli S**. Human aging and longevity are characterized by high levels of mitokines. *Journals Gerontol Ser A* 74: 600–607, 2019.

57. **Costa RJS**, **Jones GE**, **Lamb KL**, **Coleman R**, **Williams JHH**. The effects of a high carbohydrate diet on cortisol and salivary immunoglobulin A (s-IgA) during a period of increase exercise workload amongst olympic and ironman triathletes. *Int J Sports Med* 26: 880–885, 2005.

58. **Costill DL**, **Bowers R**, **Branam G**, **Sparks K**. Muscle glycogen utilization during prolonged exercise on successive days. *J Appl Physiol* 31: 834–838, 1971.

59. **Costill DL**, **Flynn MG**, **Kirwan JP**, **Houmard JA**, **Mitchell JB**, **Thomas R**, **Park SH**. Effects of repeated days of intensified training on muscle glycogen and swimming performance. *Med Sci Sport Exerc* 20: 249–254, 1988.

60. **Coutts AJ·**, **Wallace LK**, **Slattery KM**. Monitoring changes in performance, physiology, biochemistry, and psychology during overreaching and recovery in triathletes. *Int J Sports Med* 28: 125–134, 2007.

61. **Coutts AJ**, **Slattery KM**, **Wallace LK**. Practical tests for monitoring performance, fatigue and recovery in triathletes. *J Sci Med Sport* 10: 372–381, 2007.

62. **Cox PJ**, **Clarke K**. Acute nutritional ketosis: implications for exercise performance and metabolism. *Extrem Physiol Med* 3: 1–9, 2014.

63. **Cox PJ**, **Kirk T**, **Ashmore T**, **Willerton K**, **Evans R**, **Smith A**, **Murray AJ**, **Stubbs B**, **West J**, **McLure SW**, **King MT**, **Dodd MS**, **Holloway C**, **Neubauer S**, **Drawer S**, **Veech RL**, **Griffin JL**, **Clarke K**. Nutritional ketosis alters fuel preference and thereby endurance performance in athletes. *Cell Metab* 24: 256–268, 2016.

64. **Coyle EF**. Timing and method of increased carbohydrate intake to cope with heavy training, competition and recovery. *J Sports Sci* 9: 29–51, 1991.

65. **Coyle EF**. Physiological determinants of endurance exercise performance. *J Sci Med Sport* 2: 181–189, 1999.

66. **Coyle EF**, **Jeukendrup AE**, **Oseto MC**, **Hodgkinson BJ**, **Zderic TW**. Low-fat diet alters intramuscular substrates and reduces lipolysis and fat oxidation during exercise. *Am J Physiol - Endocrinol Metab* 280: E391–E398, 2001.

67. **Coyle EF**, **Jeukendrup AE**, **Wagenmakers AJM**, **Saris WHM**. Fatty acid oxidation is directly regulated by carbohydrate metabolism during exercise. *Am J Physiol - Endocrinol Metab* 273: E268–E275, 1997.

68. **Dalle S**, **De Smet S**, **Geuns W**, **Van Rompaye B**, **Hespel P**, **Koppo K**. Effect of stacked sodium bicarbonate loading on repeated all-out exercise. *Int J Sport Med* 40: 711–716, 2019.

69. **Das A**, **Huang GX**, **Bonkowski MS**, **Longchamp A**, **Li C**, **Schultz MB**, **Kim L**, **Osborne B**, **Joshi S**, **Lu Y**, **Treviño-villarreal JH**, **Kang M**, **Hung T**, **Lee B**, **Williams EO**, **Igarashi M**, **Mitchell JR**, **Wu LE**, **Turner N**, **Arany Z**, **Guarente L**, **Sinclair DA**. Impairment of an endothelial NAD+-H2S signaling network is a reversible cause of vascular aging. *Cell* 173: 74–89, 2018.

70. **Davis JA**. Anaerobic threshold: review of the concept and directions for future research. *Med. Sci. Sports Exerc.* 17: 6–21, 1985.

71. **De Bock K**, **Richter EA**, **Russell AP**, **Eijnde BO**, **Derave W**, **Ramaekers M**, **Koninckx E**, **Léger B**, **Verhaeghe J**, **Hespel P**. Exercise in the fasted state facilitates fibre type-specific intramyocellular lipid breakdown and stimulates glycogen resynthesis in humans. *J Physiol* 564: 649–660, 2005.

72. **De Feyter HM**, **Behar KL**, **Rao JU**, **Madden-Hennessey K**, **Ip KL**, **Hyder F**, **Drewes LR**, **Geschwind J-F**, **De Graaf RA**, **Rothman DL**. A ketogenic diet increases transport and oxidation of ketone bodies in RG2 and 9L gliomas without affecting tumor growth. *Neuro Oncol* 18: 1079–1087, 2016.

73. **De Oliveira EP**, **Burini RC**, **Jeukendrup A**. Gastrointestinal complaints during exercise: prevalence, etiology, and nutritional recommendations. *Sport Med* 44: 79–85, 2014.

74. **De Smet S**, **Van Thienen R**, **Deldicque L**, **James R**, **Sale C**, **Bishop DJ**, **Hespel P**. Nitrate intake promotes shift in muscle fiber type composition during sprint interval training in hypoxia. *Front Physiol* 7: 1–11, 2016.

75. **Dearlove DJ**, **Faull OK**, **Clarke K**. Context is key: exogenous ketosis and athletic performance. *Curr Opin Physiol* 10: 81–89, 2019.

76. **Dearlove DJ**, **Faull OK**, **Rolls E**, **Clarke K**, **Cox PJ**. Nutritional ketoacidosis during incremental exercise in healthy athletes. *Front Physiol* 10: 1–6, 2019.

77. **Debold EP**, **Beck SE**, **Warshaw DM**. Effect of low pH on single skeletal muscle myosin mechanics and kinetics. *Am J Physiol - Cell Physiol* 295: 173–179, 2008.

78. **Derave W**, **Lund S**, **Holman GD**, **Wojtaszewski J**, **Pedersen O**, **Richter EA**. Contraction-stimulated muscle glucose transport and GLUT-4 surface content are dependent on glycogen content. *Am J Physiol - Endocrinol Metab* 277: 1103–1110, 1999.

79. **Desrochers S**, **Dubreuil P**, **Brunet J**, **Jette M**, **David F**, **Landau BR**, **Brunengraber H**. Metabolism of (R,S)-1,3-butanediol acetoacetate esters, potential parenteral and enteral nutrients in conscious pigs. *Am J Physiol - Endocrinol Metab* 268: 660–667, 1995.

80. **Dietrich MO**, **Horvath TL**. Limitations in anti-obesity drug development: the critical role of hunger-promoting neurons. *Nat Rev Drug Discov* 11: 675–691, 2012.

81. **Dupuy O**, **Douzi W**, **Theurot D**, **Bosquet L**, **Dugué B**. An evidence-based approach for choosing post-exercise recovery techniques to reduce markers of muscle damage, soreness, fatigue, and inflammation: a systematic review with meta-analysis. *Front Physiol* 9: 1–15, 2018.

82. **Dupuy O**, **Lussier M**, **Fraser S**, **Bherer L**, **Audiffren M**, **Bosquet L**. Effect of overreaching on cognitive performance and related cardiac autonomic control. *Scand J Med Sci Sport* 24: 234–242, 2014.

83. **Dyck DJ**, **Peters SJ**, **Wendling PS**, **Chesley A**, **Hultman E**, **Spriet LL**. Regulation of muscle glycogen phosphorylase activity during intense aerobic cycling with elevated FFA. *Am J Physiol - Endocrinol Metab* 270: E116–E125, 1996.

84. **Dyck DJ**, **Putman CT**, **Heigenhauser GJF**, **Hultman E**, **Spriet LL**. Regulation of fat-carbohydrate interaction in skeletal muscle during intense aerobic cycling. *Am J Physiol - Endocrinol Metab* 265: E852, 1993.

85. **Ebbeling CB**, **Swain JF**, **Feldman HA**, **Wong WW**, **Hachey DL**, **Garcia-Lago E**, **Ludwig DS**. Effects of dietary composition on energy expenditure during weight-loss maintenance. *JAMA* 307: 2627–2634, 2012.

86. **Egan B**, **Carson BP**, **Garcia-Roves PM**, **Chibalin A V.**, **Sarsfield FM**, **Barron N**, **McCaffrey N**, **Moyna NM**, **Zierath JR**, **O'Gorman DJ**. Exercise intensity-dependent regulation of peroxisome proliferator-activated receptor γ coactivator-1α mRNA abundance is associated with differential activation of upstream signalling kinases in human skeletal muscle. *J Physiol* 588: 1779–1790, 2010.

87. **Egan B**, **D'Agostino DP**. Fueling performance: ketones enter the mix. *Cell Metab* 24: 373–375, 2016.

88. **Egan B**, **Zierath JR**. Exercise metabolism and the molecular regulation of skeletal muscle adaptation. *Cell Metab.* 17: 162–184, 2013.

89. **Egger F**, **Meyer T**, **Such U**, **Hecksteden A**. Effects of sodium bicarbonate on high-intensity endurance performance in cyclists: a double-blind, randomized cross-over trial. *PLoS One* 9: 1–15, 2014.

90. **Elamin M**, **Ruskin DN**, **Masino SA**, **Sacchetti P**. Ketone-based metabolic therapy: is increased nad+ a primary mechanism? *Front Mol Neurosci* 10: 1–9, 2017.

91. **Ensinger H**, **Weichel T**, **Lindner KH**, **Grunert A**, **Ahnefeld FW**. Effects of norepinephrine, epinephrine, and dopamine infusions on oxygen consumption in volunteers. *Crit Care Med* 21: 1502–1508, 1993.

92. **Ensinger H**, **Weichel T**, **Lindner KH**, **Grünert A**, **Georgieff M**. Are the effects of noradrenaline, adrenaline and dopamine infusions on VO2 and metabolism transient? *Intensive Care Med* 21: 50–56, 1995.

93. **Esler M**, **Jennings G**, **Korner P**, **Willett IAN**, **Dudley F**, **Hasking G**, **Anderson W**. Assessment of human sympathetic nervous system activity from measurements of norepinephrine spillover. *Hypertension* 11: 3–20, 1988.

94. **Ettema G**, **Loras HW**. Efficiency in cycling: a review. *Eur J Appl Physiol* 106: 1–14, 2009.

95. **Evans M**, **Cogan KE**, **Egan B**. Metabolism of ketone bodies during exercise and training: physiological basis for exogenous supplementation. *J Physiol* 595: 2857–2871, 2017.

96. **Evans M**, **Egan B**. Intermittent running and cognitive performance after ketone ester ingestion. *Med Sci Sport Exerc* 50: 2330–2338, 2018.

97. **Evans M**, **McSwiney FT**, **Brady AJ**, **Egan B**. No benefit of ingestion of a ketone monoester supplement on 10-km running performance. *Med Sci Sports Exerc* 51: 2506–2515, 2019.

98. **Evans WJ**, **Morley JE**, **Argilés J**, **Bales C**, **Baracos V**, **Guttridge D**, **Jatoi A**, **Kalantar-Zadeh K**, **Lochs H**, **Mantovani G**, **Marks D**, **Mitch WE**, **Muscaritoli M**, **Najand A**, **Ponikowski P**, **Rossi Fanelli F**, **Schambelan M**, **Schols A**, **Schuster M**, **Thomas D**, **Wolfe R**, **Anker SD**. Cachexia: a new definition. *Clin Nutr* 27: 793–799, 2008.

99. **Faull OK**, **Dearlove DJ**, **Clarke K**, **Cox PJ**. Beyond RPE: the perception of exercise under normal and ketotic conditions. *Front Physiol* 10: 1–10, 2019.

100. **Febbraio MA**, **Lambert DL**, **Starkie RL**, **Proietto J**, **Hargreaves M**. Effect of epinephrine on muscle glycogenolysis during exercise in trained men. *J Appl Physiol* 84: 465–470, 1998.

101. **Felig P**, **Owen OE**, **Wahren J**, **Cahill GF**. Amino acid metabolism during prolonged starvation. *J Clin Invest* 48: 584–594, 1969.

102. **Ferraresso RLP**, **Buscariolli De Oliveira R**, **MacEdo DV**, **Alessandro Soares Nunes L**, **Brenzikofer R**, **Damas D**, **Hohl R**. Interaction between overtraining and the interindividual variability may (not) trigger muscle oxidative stress and cardiomyocyte apoptosis in rats. *Oxid Med Cell Longev* 2012: 1–11, 2012.

103. **Fery F**, **Balasse EO**. Ketone body turnover during and after exercise in overnight-fasted and starved humans. *Am J Physiol - Endocrinol Metab* 8: E318–E325, 1983.

104. **Fery F**, **Balasse EO**. Effect of exercise on the disposal of infused ketone bodies in humans. *J Clin Endocrinol Metab* 67: 245–250, 1988.

105. **Fisher JP**. Autonomic control of the heart during exercise in humans: role of skeletal muscle afferents. *Exp Physiol* 99: 300–305, 2014.

106. **Flier JS**. What's in a name? In search of leptin's physiological role. *J Clin Endocrinol Metab* 83: 1407–1413, 1998.

107. **Foster C**. Monitoring training in athletes with reference to overtraining syndrome. *Med Sci Sports Exerc* 30: 1164–1168, 1998.

108. **Foster C**, **Lehmann M**. Overtraining syndrome. In: *Running injuries*, edited by Guten GN. Saunders, Philadelphia: 1997, p. 173–188.

109. **Freeman JM**, **Vining EPG**. Intractable epilepsy. *Epilepsia* 33: 1132–1136, 1992.

110. **Fries S**, **Ricci M**, **Russell C**, **Laferrère B**. Regulation of leptin production in humans. *J Nutr* 130: 3127–3131, 2000.

111. **Fry AC**, **Kraemer WJ**, **Van Borselen F**, **Lynch JM**, **Triplett NT**, **Koziris LP**, **Fleck SJ**, **Fleck Cate SJ**. Catecholamine responses to short-term high-intensity resistance exercise overtraining. *J Appl Physiol* 77: 941–946, 1994.

112. **Fry AC**, **Schilling BK**, **Weiss LW**, **Chiu LZF**. B2 -Adrenergic receptor downregulation and performance decrements during high-intensity resistance exercise overtraining. *J Appl Physiol* 101: 1664–1672, 2006.

113. **Fry RW**, **Morton AR**, **Keast D**. Overtraining in athletes. *Sport Med* 12: 32–65, 1991.

114. **Fukao T**, **Lopaschuk GD**, **Mitchell GA**. Pathways and control of ketone body metabolism: on the fringe of lipid biochemistry. *Prostaglandins Leukot. Essent. Fat. Acids* 70: 243–251, 2004.

115. **Fuller JT**, **Bellenger CR**, **Thewlis D**, **Arnold J**, **Thomson RL**, **Tsiros MD**, **Robertson EY**, **Buckley JD**. Tracking performance changes with running-stride variability when athletes are functionally overreached. *Int J Sports Physiol Perform* 12: 357–363, 2017.

116. **Gaitanos GC**, **Williams C**, **Boobis LH**, **Brooks S**. Human muscle metabolism during intermittent maximal exercise. *J Appl Physiol* 75: 712–719, 1993.

117. **García-Caballero M**, **Zecchin A**, **Souffreau J**, **Truong A-CK**, **Teuwen L-A**, **Vermaelen W**, **Martín-Pérez R**, **de Zeeuw P**, **Bouché A**, **Vinckier S**, **Cornelissen I**, **Eelen G**, **Ghesquière B**, **Mazzone M**, **Dewerchin M**, **Carmeliet P**. Role and therapeutic potential of dietary ketone bodies in lymph vessel growth. *Nat Metab* 1: 666–675, 2019.

118. **Gerhardt J**. Diabetes mellitus und aceton. *Wiener Medizinische Press* 6: 672, 1865.

119. **Ghiarone T**, **Andrade-Souza VA**, **Learsi SK**, **Tomazini F**, **Ataide-Silva T**, **Sansonio A**, **Fernandes MP**, **Saraiva KL**, **Figueiredo RCBQ**, **Tourneur Y**, **Kuang J**, **Lima-Silva AE**, **Bishop DJ**. Twice-a-day training improves mitochondrial efficiency, but not mitochondrial biogenesis, compared with once-daily training. *J Appl Physiol* 127: 713–725, 2019.

120. **Gibson AA**, **Seimon R V.**, **Lee CMY**, **Ayre J**, **Franklin J**, **Markovic TP**, **Caterson ID**, **Sainsbury A**. Do ketogenic diets really suppress appetite? A systematic review and meta-analysis. *Obes Rev* 16, 2015.

121. **Gil CI**, **Ost M**, **Kasch J**, **Schumann S**, **Heider S**, **Klaus S**. Role of GDF15 in active lifestyle induced metabolic adaptations and acute exercise response in mice. *Sci Rep* 9: 1–9, 2019.

122. **Glatz JFC**, **Luiken JJFP**, **Bonen A**. Membrane fatty acid transporters as regulators of lipid metabolism: implications for metabolic disease. *Physiol Rev* 90: 367–417, 2010.

123. **Gleeson M**. Biochemical and immunological responses to overtraining. *J Sport Sci Med* 1: 31–41, 2002.

124. **Gleeson M**, **Bishop NC**. Modification of immune responses to exercise by carbohydrate, glutamine and anti-oxidant supplements. *Immunol Cell Biol* 78: 554–561, 2000.

125. **Goedecke JH**, **Christie C**, **Wilson G**, **Dennis SC**, **Noakes TD**, **Hopkins WG**, **Lambert E V.** Metabolic adaptations to a high-fat diet in endurance cyclists. *Metabolism* 48: 1509–1517, 1999.

126. **Gomez-Merino D**, **Béquet F**, **Berthelot M**, **Chennaoui M**, **Guezennec CY**. Site-dependent effects of an acute intensive exercise on extracellular 5-HT and 5-HIAA levels in rat brain. *Neurosci Lett* 301: 143–146, 2001.
127. **Gough LA**, **Deb SK**, **Sparks SA**, **McNaughton LR**. Sodium bicarbonate improves 4 km time trial cycling performance when individualised to time to peak blood bicarbonate in trained male cyclists. *J Sports Sci* 36: 1705–1712, 2018.
128. **Grabacka M**, **Pierzchalska M**, **Dean M**, **Reiss K**. Regulation of ketone body metabolism and the role of PPARα. *Int J Mol Sci* 17, 2016.
129. **Greenham G**, **Buckley JD**, **Garrett J**, **Eston R**, **Norton K**. Biomarkers of physiological responses to periods of intensified, non-resistance-based exercise training in well-trained male athletes: a systematic review and meta-analysis. *Sport Med* 48: 2517–2548, 2018.
130. **Guthrie JP**, **Jordan F**. Amine-catalyzed decarboxylation of acetoacetic acid. The rate constant for decarboxylation of a ß-imino acid. *J Am Chem Soc* 94: 9136–9141, 1972.
131. **Guzmán M**, **Geelen MJH**. Regulation of fatty acid oxidation in mammalian liver. *Biochim Biophys Acta* 1167: 227–241, 1993.
132. **Hackney AC**, **Lane AR**, **Register-Mihalik J**, **O'Leary CB**. Endurance exercise training and male sexual libido. *Med Sci Sports Exerc* 49: 1383–1388, 2017.
133. **Hagenfeldt L**, **Wahren J**. Human forearm muscle metabolism during exercise. *Scand J Clin Lab Invest* 21: 314–320, 1968.
134. **Halson SL**. Monitoring training load to understand fatigue in athletes. *Sport Med* 44: 139–147, 2014.
135. **Halson SL**. Sleep in elite athletes and nutritional interventions to enhance sleep. *Sport Med* 44: 13–23, 2014.
136. **Halson SL**, **Bartram J**, **West N**, **Stephens J**, **Argus CK**, **Driller MW**, **Sargent C**, **Lastella M**, **Hopkins WG**, **Martin DT**. Does hydrotherapy help or hinder adaptation to training in competitive cyclists? *Med Sci Sports Exerc* 46: 1631–1639, 2014.
137. **Halson SL**, **Bridge MW**, **Meeusen R**, **Busschaert B**, **Gleeson M**, **Jones DA**, **Jeukendrup AE**. Time course of performance changes and fatigue markers during intensified training in trained cyclists. *J Appl Physiol* 93: 947–956, 2002.
138. **Halson SL**, **Jeukendrup AE**. Does overtraining exist? An analysis of overreaching and overtraining research. *Sport Med* 34: 967–981, 2004.

139. **Halson SL**, **Lancaster GI**, **Achten J**, **Gleeson M**, **Jeukendrup AE**. Effects of carbohydrate supplementation on performance and carbohydrate oxidation after intensified cycling training. *J Appl Physiol* 97: 1245–1253, 2004.

140. **Hargreaves M**, **Richter EA**. Regulation of skeletal muscle glycogenolysis during exercise. *Can J Sport Sci* 13: 197–203, 1988.

141. **Hargreaves M**, **Spriet LL**. Exercise metabolism: fuels for the fire. In: *The biology of exercise*, edited by Zierath J, Joyner M, Hawley J. New York: Cold Spring Harbor Laboratory Press, 2017, p. 57–71.

142. **Harvey KL**, **Holcomb LE**, **Kolwicz SC**. Ketogenic diets and performance. *Nutrients* 11: 2296–2311, 2019.

143. **Hausswirth C**, **Louis J**, **Aubry A**, **Bonnet G**, **Duffield R**, **Le Meur Y**. Evidence of disturbed sleep and increased illness in overreached endurance athletes. *Med Sci Sport Exerc* 46: 1036–1045, 2014.

144. **Havemann L**, **West SJ**, **Goedecke JH**, **Macdonald IA**, **St Clair Gibson A**, **Noakes TD**, **Lambert E V.** Fat adaptation followed by carbohydrate loading compromises high-intensity sprint performance. *J Appl Physiol* 100: 194–202, 2006.

145. **Hawley JA**, **Burke LM**, **Phillips SM**, **Spriet LL**, **Hawley JA**. Nutritional modulation of training-induced skeletal muscle adaptations. *J Appl Physiol* 110: 834–845, 2011.

146. **Hawley JA**, **Hargreaves M**, **Joyner MJ**, **Zierath JR**. Integrative biology of exercise. *Cell* 159: 738–749, 2014.

147. **Hawley JA**, **Leckey JJ**. Carbohydrate dependence during prolonged, intense endurance exercise. *Sport Med* 45: 5–12, 2015.

148. **Haworth S**, **Brotherton AM**. Correcting metabolic acidosis leads to an increase in dietary protein intake in patients with established chronic kidney disease. *J Hum Nutr Diet* 24: 285, 2011.

149. **Hearris MA**, **Hammond KM**, **Fell JM**, **Morton JP**. Regulation of muscle glycogen metabolism during exercise: implications for endurance performance and training adaptations. *Nutrients* 10: 1–21, 2018.

150. **Hecksteden A**, **Skorski S**, **Schwindling S**, **Hammes D**, **Pfeiffer M**, **Kellmann M**, **Ferrauti A**, **Meyer T**, **Lucia A**. Blood-borne markers of fatigue in competitive athletes – Results from simulated training camps. *PLoS One* 11: 1–13, 2016.

151. **Hedelin R**, **Wiklund U**, **Bjerle P**, **Henriksson-Larsen K**. Cardiac autonomic imbalance in an overtrained athlete. *Med Sci Sports Exerc* 32: 1531–1533, 2000.

152. **Helge JW**. A high carbohydrate diet remains the evidence based choice for elite athletes to optimise performance. *J Physiol* 595: 2775, 2017.

153. **Helge JW**, **Watt PW**, **Richter EA**, **Rennie MJ**, **Kiens B**. Fat utilization during exercise: adaptation to a fat-rich diet increases utilization of plasma fatty acids and very low density lipoprotein-triacylglycerol in humans. *J Physiol* 537: 1009–1020, 2001.

154. **Hellsten Y**, **Nyberg M**, **Jensen LG**, **Mortensen SP**. Vasodilator interactions in skeletal muscle blood flow regulation. *J Physiol* 590: 6297–6305, 2012.

155. **Hendrickse P**, **Degens H**. The role of the microcirculation in muscle function and plasticity. *J Muscle Res Cell Motil* 40: 127–140, 2019.

156. **Henríquez-Olguin C**, **Knudsen JR**, **Raun SH**, **Li Z**, **Dalbram E**, **Treebak JT**, **Sylow L**, **Holmdahl R**, **Richter EA**, **Jaimovich E**, **Jensen TE**. Cytosolic ROS production by NADPH oxidase 2 regulates muscle glucose uptake during exercise. *Nat Commun* 10: 1–11, 2019.

157. **Hermansen L**, **Hultman E**, **Saltin B**. Muscle glycogen during prolonged severe exercise. *Acta Physiol Scand* 71: 129–139, 1967.

158. **Hespel P**, **Richter EA**. Glucose uptake and transport in contracting, perfused rat muscle with different pre-contraction glycogen concentrations. *J Physiol* 427: 347–359, 1990.

159. **Hespel P**, **Richter EA**. Mechanism linking glycogen concentration and glycogenolytic rate in perfused contracting rat skeletal muscle. *Biochem J* 284: 777–780, 1992.

160. **Heymann E**. Carboxylesterases and amidases. In: *Enzymatic Basis of Detoxication. biochemical Pharmacology and Toxicology*, edited by Jakoby W. New York: Academic Press, 1980, p. 291–323.

161. **Higgins TR**, **Heazlewood IT**, **Climstein M**. A random control trial of contrast baths and ice baths for recovery during competition in U/20 rugby union. *J Strength Cond Res* 25: 1046–1051, 2011.

162. **Hirschfeld F**. Beobachtungen über die acentonurie und das coma diabeticum. *Z Klin Med* 28: 176–209, 1895.

163. **Hoier B**, **Nordsborg N**, **Andersen S**, **Jensen L**, **Nybo L**, **Bangsbo J**, **Hellsten Y**. Pro- and anti-angiogenic factors in human skeletal muscle in response to acute exercise and training. *J Physiol* 590: 595–606, 2012.

164. **Høier B**, **Rufener N**, **Bojsen-Møller J**, **Bangsbo J**, **Hellsten Y**. The effect of passive movement training on angiogenic factors and capillary growth in human skeletal muscle. *J Physiol* 588: 3833–3845, 2010.

165. **Holdsworth DA**, **Cox PJ**, **Kirk T**, **Stradling H**, **Impey SG**, **Clarke K**. A ketone ester drink increases postexercise muscle glycogen synthesis in humans. *Med Sci Sport Exerc* 49: 1789–1795, 2017.

166. **Hollidge-Horvat MG**, **Parolin ML**, **Wong D**, **Jones NL**, **Heigenhauser GJF**. Effect of induced metabolic alkalosis on human skeletal muscle metabolism during exercise. *Am J Physiol - Endocrinol Metab* 278: 647–658, 2000.

167. **Holloszy JO**, **Kohrt WM**. Regulation of carbohydrate and fat metabolism during and after exercise. *Annu Rev Nutr* 16: 121–138, 1996.

168. **Holloway TM**, **Snijders T**, **Van Kranenburg J**, **Van Loon LJC**, **Verdijk LB**. Temporal response of angiogenesis and hypertrophy to resistance training in young men. *Med Sci Sports Exerc* 50: 36–45, 2018.

169. **Hooper SL**, **Mackinnon LT**, **Howard A**, **Gordon RD**, **Bachmann AW**. Markers for monitoring overtraining and recovery. *Med Sci Sport Exerc* 27: 106–112, 1995.

170. **Hoppeler H**, **Kayar SR**. Capillarity and oxidative capacity of muscles. *Am J Physiol* 3: 113–116, 1988.

171. **Horowitz JF**, **Mora-Rodriguez R**, **Byerley LO**, **Coyle EF**. Lipolytic suppression following carbohydrate ingestion limits fat oxidation during exercise. *Am J Physiol - Endocrinol Metab* 273: E768–E775, 1997.

172. **Howald H**, **Hoppeler H**, **Claassen H**, **Mathieu O**, **Straub R**. Influences of endurance training on the ultrastructural composition of the different muscle fiber types in humans. *Pflügers Arch - Eur J Physiol* 403: 369–376, 1985.

173. **Hudlicka O**, **Brown M**, **Egginton S**. Angiogenesis in skeletal and cardiac muscle. *Physiol Rev* 72: 369–417, 1992.

174. **Hue L**, **Taegtmeyer H**. The Randle cycle revisited: a new head for an old hat. *Am J Physiol - Endocrinol Metab* 297: 578–591, 2009.

175. **Hugo SE**, **Cruz-Garcia L**, **Karanth S**, **Anderson RM**, **Stainier DYR**, **Schlegel A**. A monocarboxylate transporter required for hepatocyte secretion of ketone bodies during fasting. *Genes Dev* 26: 282–293, 2012.

176. **Hüttemann M**, **Lee I**, **Malek MH**. (−)-Epicatechin maintains endurance training adaptation in mice after 14 days of detraining. *FASEB J* 26: 1413–1422, 2012.

177. **Inai T**, **Mancuso M**, **Hashizume H**, **Baffert F**, **Haskell A**, **Baluk P**, **Hu-Lowe DD**, **Shalinsky DR**, **Thurston G**, **Yancopoulos GD**, **McDonald DM**. Inhibition of vascular endothelial growth factor (VEGF) signaling in cancer causes loss of endothelial fenestrations, regression of tumor vessels, and appearance of basement membrane ghosts. *Am J Pathol* 165: 35–52, 2004.

178. **Isales CM**, **Min L**, **Hoffman WH**. Acetoacetate and β-hydroxybutyrate differentially regulate endothelin-1 and vascular endothelial growth factor in mouse brain microvascular endothelial cells. *J Diabetes Complications* 13: 91–97, 1999.

179. **Israel S**. The problems of overtraining with reference to performance physiology and internal medicine [in German]. *Med Sport* 16: 1–12, 1976.

180. **James S**, **Kjerulf Greer B**. Influence of exogenous β-hydroxybutyrate on walking economy and rating of perceived exertion. *J Diet Suppl* 16: 463–469, 2019.

181. **Jansson E**, **Esbjörnsson M**, **Holm I**, **Jacobs I**. Increase in the proportion of fast-twitch muscle fibres by sprint training in males. *Acta Physiol Scand* 140: 359–363, 1990.

182. **Jensen L**, **Bangsbo J**, **Hellsten Y**. Effect of high intensity training on capillarization and presence of angiogenic factors in human skeletal muscle. *J Physiol* 557: 571–582, 2004.

183. **Jensen TE**, **Richter EA**. Regulation of glucose and glycogen metabolism during and after exercise. *J Physiol* 590: 1069–1076, 2012.

184. **Jensen TE**, **Sylow L**, **Rose AJ**, **Madsen AB**, **Angin Y**, **Maarbjerg SJ**, **Richter EA**. Contraction-stimulated glucose transport in muscle is controlled by AMPK and mechanical stress but not sarcoplasmatic reticulum Ca2+ release. *Mol Metab* 3: 742–753, 2014.

185. **Jeppesen J**, **Kiens B**. Regulation and limitations to fatty acid oxidation during exercise. *J Physiol* 590: 1059–1068, 2012.

186. **Jeukendrup A**, **Saris WHM**, **Brouns F**, **Kester ADM**. A new validated endurance performance test. *Med Sci Sports Exerc* 2: 266–270, 1996.

187. **Jeukendrup AE**. Carbohydrate intake during exercise and performance. *Nutrition* 20: 669–677, 2004.

188. **Jeukendrup AE**, **Hesselink MKC**. Overtraining-what do lactate curves tell us? *Br J Sp Med* 28: 239–240, 1994.

189. **Johnen H**, **Lin S**, **Kuffner T**, **Brown DA**, **Wang V**, **Tsai W**, **Bauskin AR**, **Wu L**, **Pankhurst G**, **Jiang L**, **Junankar S**, **Hunter M**, **Fairlie D**, **Lee NJ**, **Enriquez RF**, **Baldock PA**, **Corey E**, **Apple FS**, **Murakami MM**, **Lin E-J**, **Wang C**, **During MJ**, **Sainsbury A**, **Herzog H**, **Breit SN**. Tumor-induced anorexia and weight loss are mediated by the TGF-b superfamily cytokine MIC-1. *Nat Med* 13: 1333–1340, 2007.

190. **Johnson RH**, **Walton JL**. Fitness, fatness, and post-exercise ketosis. *Lancet* 297: 566–568, 1971.

191. **Johnson RH**, **Walton JL**. The effect of exercise upon acetoacetate metabolism in athletes and non-athletes. *Q J Exp Physiol Cogn Med Sci* 57: 73–79, 1972.

192. **Johnson RH**, **Walton JL**, **Krebs HA**, **Williamson DH**. Metabolic fuels during and after severe exercise in athletes and non-athletes. *Lancet* 2: 452–455, 1969.

193. **Jordy AB**, **Kiens B**. Regulation of exercise-induced lipid metabolism in skeletal muscle. *Exp Physiol* 99: 1586–1592, 2014.

194. **Jowett M**, **Quastel JH**. Fat metabolism III. The formation and breakdown of acetoacetic acid in animal tissues. *Biochem J* 29: 2181–2191, 1935.

195. **Joyner MJ**, **Casey DP**. Regulation of increased blood flow (hyperemia) to muscles during exercise: a hierarchy of competing physiological needs. *Physiol Rev* 95: 549–601, 2015.

196. **Karlsson J**, **Saltin B**. Diet, muscle glycogen, and endurance performance. *J Appl Physiol* 31: 203–206, 1971.

197. **Karwi QG**, **Uddin GM**, **Ho KL**, **Lopaschuk GD**. Loss of metabolic flexibility in the failing heart. *Front Cardiovas Med* 5: 1–19, 2018.

198. **Kesl SL**, **Poff AM**, **Ward NP**, **Fiorelli TN**, **Ari C**, **Van Putten AJ**, **Sherwood JW**, **Arnold P**, **D'Agostino DP**. Effects of exogenous ketone supplementation on blood ketone, glucose, triglyceride, and lipoprotein levels in Sprague-Dawley rats. *Nutr Metab (Lond)* 13: 9, 2016.

199. **Kety SS**. The general metabolism of the brain in vivo. In: *The metabolism of the nervous system*, edited by Richter D. London: Pergamon Press, 1957, p. 221.

200. **Kiens B**, **Essen-Gustavsson B**, **Christensen NJ**, **Saltin B**. Skeletal muscle substrate utilization during submaximal exercise in man: effect of endurance training. *J Physiol* 469: 459–478, 1993.

201. **Kiens B**, **Roemen THM**, **Van Der Vusse GJ**. Muscular long-chain fatty acid content during graded exercise in humans. *Am J Physiol - Endocrinol Metab* 276: 352–357, 1999.

202. **Killer SC**, **Svendsen IS**, **Jeukendrup AE**, **Gleeson M**. Evidence of disturbed sleep and mood state in well-trained athletes during short-term intensified training with and without a high carbohydrate nutritional intervention. *J Sports Sci* 35: 1402–1410, 2017.
203. **Kimura I**, **Inoue D**, **Maeda T**, **Hara T**, **Ichimura A**, **Miyauchi S**, **Kobayashi M**, **Hirasawa A**, **Tsujimoto G**, **Lefkowitz RJ**. Short-chain fatty acids and ketones directly regulate sympathetic nervous system via G protein-coupled receptor 41 (GPR41). *PNAS* 108: 8030–8035, 2011.
204. **Kissane RW**, **Egginton S**. Exercise-mediated angiogenesis. *Curr Opin Physiol* 10: 193–201, 2019.
205. **Kleinert M**, **Clemmensen C**, **Sjøberg KA**, **Carl CS**, **Fuglsbjerg Jeppesen J**, **Wojtaszewski JFP**, **Kiens B**, **Richter EA**. Exercise increases circulating GDF15 in humans. *Mol Metab* 9: 187–191, 2018.
206. **Klip A**. The many ways to regulate glucose transporter 4. *Appl Physiol Nutr Metab* 34: 481–487, 2009.
207. **Klok MD**, **Jakobsdottir S**, **Drent ML**. The role of leptin and ghrelin in the regulation of food intake and body weight in humans: a review. *Obes Rev* 8: 21–34, 2007.
208. **Koeslag JH**, **Noakes TD**, **Sloan AW**. Post-exercise ketosis. *J Physiol* 301: 79–90, 1980.
209. **Koh HJ**, **Toyoda T**, **Fujii N**, **Jung MM**, **Rathod A**, **Middelbeek RJW**, **Lessard SJ**, **Treebak JT**, **Tsuchihara K**, **Esumi H**, **Richter EA**, **Wojtaszewski JFP**, **Hirshman MF**, **Goodyear LJ**. Sucrose nonfermenting AMPK-related kinase (SNARK) mediates contraction-stimulated glucose transport in mouse skeletal muscle. *Proc Natl Acad Sci U S A* 107: 15541–15546, 2010.
210. **Koninckx E**, **Van Leemputte M**, **Hespel P**. Effect of a novel pedal design on maximal power output and mechanical efficiency in well-trained cyclists. *J Sport Sci* 26: 1015–1023, 2008.
211. **Koninckx E**, **Van Leemputte M**, **Hespel P**. Effect of isokinetic cycling versus weight training on maximal power output and endurance performance in cycling. *Eur J Appl Physiol* 109: 699–708, 2010.
212. **Korevaar DA**, **Cohen J**, **McInnes MD**. Ketone ester supplementation in endurance athletes: a miracle drink or spin? *J Physiol* 597: 4407–4408, 2019.
213. **Koul PB**. Diabetic ketoacidosis: A current appraisal of pathophysiology and management. *Clin Pediatr (Phila)* 48: 135–144, 2009.
214. **Koutedakis Y**, **Sharp NCC**. Seasonal variations of injury and overtraining in elite athletes. *Clin J Sport Med* 8: 18–21, 1998.

215. **Kreher J**. Diagnosis and prevention of overtraining syndrome: an opinion on education strategies. *Open Access J Sport Med* 7: 115–122, 2016.

216. **Kreher**, **Schwartz**. Overtraining syndrome: a practical guide. *Sports Health* 4: 128–138, 2012.

217. **Kreider RB**, **Fry AC**, **O'Toole ML**. *Overtraining in sport*. 1st ed. Champaign, IL: Human Kinetics, 1997.

218. **Krock LP**, **Hartung GH**. Influence of post-exercise activity on plasma catecholamines, blood pressure and heart rate in normal subjects. *Clin Auton Res* 2: 89–97, 1992.

219. **Kuipers H**, **Keizer HA**. Overtraining in elite athletes, review and directions for the future. *Sport Med* 6: 79–92, 1988.

220. **Laeger T**, **Metges CC**, **Kuhla B**. Role of β-hydroxybutyric acid in the central regulation of energy balance. *Appetite* 54: 450–455, 2010.

221. **Laeger T**, **Pöhland R**, **Metges CC**, **Kuhla B**. The ketone body β-hydroxybutyric acid influences agouti-related peptide expression via AMP-activated protein kinase in hypothalamic GT1-7 cells. *J Endocrinol* 213: 193–203, 2012.

222. **Laffel L**. Ketone bodies: a review of physiology, pathophysiology and application of monitoring to diabetes. *Diabetes Metab Res Rev* 15: 412–426, 1999.

223. **Lambert E V.**, **Speechly DP**, **Dennis SC**, **Noakes TD**. Enhanced endurance in trained cyclists during moderate intensity exercise following 2 weeks adaptation to a high fat diet. *Eur J Appl Physiol Occup Physiol* 69: 287–293, 1994.

224. **Langfort J**, **Ploug T**, **Ihlemann J**, **Saldo M**, **Holm C**, **Galbo H**. Expression of hormone-sensitive lipase and its regulation by adrenaline in skeletal muscle. *Biochem J* 340: 459–465, 1999.

225. **Lardy HA**, **Hansen RG**, **Philips PH**. The metabolism of bovine epididymal spermatozoa. *Arch Biochem* 6: 41–51, 1945.

226. **Lardy HA**, **Philips PH**. Studies of fat and carbohydrate oxidation in mammalian spermatozoa. *Arch Biochem* 6: 53–61, 1945.

227. **Laurens C**, **Parmar A**, **Murphy E**, **Carper D**, **Lair B**, **Maes P**, **Vion J**, **Boulet N**, **Fontaine C**, **Marquès M**, **Larrouy D**, **Harant I**, **Thalamas C**, **Montastier E**, **Caspar-Bauguil S**, **Bourlier V**, **Tavernier G**, **Grolleau J-L**, **Bouloumié A**, **Langin D**, **Viguerie N**, **Bertile F**, **Blanc S**, **de Glisezinski I**, **O'Gorman D**, **Moro C**. Growth and differentiation factor 15 is secreted by skeletal muscle during exercise and promotes lipolysis in humans. *JCI Insight* 5: 1–14, 2020.

228. **Le Meur Y**, **Hausswirth C**, **Natta F**, **Couturier A**, **Bignet F**, **Vidal PP**. A multidisciplinary approach to overreaching detection in endurance trained athletes. *J Appl Physiol* 114: 411–420, 2013.

229. **Le Meur Y**, **Louis J**, **Aubry A**, **Guéneron J**, **Pichon A**, **Schaal K**, **Corcuff J-B**, **Hatem SN**, **Isnard R**, **Hausswirth C**, **Meur LY**. Maximal exercise limitation in functionally overreached triathletes: role of cardiac adrenergic stimulation. *J Appl Physiol* 117: 214–222, 2014.

230. **Le Meur Y**, **Pichon A**, **Schaal K**, **Schmitt L**, **Louis J**, **Gueneron J**, **Vidal PP**, **Hausswirth C**. Evidence of parasympathetic hyperactivity in functionally overreached athletes. *Med Sci Sports Exerc* 45: 2061–2071, 2013.

231. **Le Roux CW**, **Neary NM**, **Halsey TJ**, **Small CJ**, **Martinez-Isla AM**, **Ghatei MA**, **Theodorou NA**, **Bloom SR**. Ghrelin does not stimulate food intake in patients with surgical procedures involving vagotomy. *J Clin Endocrinol Metab* 90: 4521–4524, 2005.

232. **Leckey JJ**, **Ross ML**, **Quod M**, **Hawley JA**, **Burke LM**. Ketone diester ingestion impairs time-trial performance in professional cyclists. *Front Physiol* 8: 1–10, 2017.

233. **Leeder J**, **Gissane C**, **Van Someren K**, **Gregson W**, **Howatson G**. Cold water immersion and recovery from strenuous exercise: a meta-analysis. *Br J Sports Med* 46: 233–240, 2012.

234. **Lehmann, M; Foster, C; Keul J**. Overtraining in endurance athletes: a brief review. *Med Sci Sports Exerc* 25: 854–862, 1993.

235. **Lehmann M**, **Foster C**, **Dickhuth H-H**, **Gastmann U**. Autonomic imbalance hypothesis and overtraining syndrome. *Med Sci Sport Exerc* 30: 1140–1145, 1998.

236. **Lehmann M**, **Foster C**, **Gastmann U**, **Keizer H**, **Steinacker JM**. Definition, types, symptoms, findings, underlying mechanisms, and frequency of overtraining and overtraining syndrome. In: *Overload, Performance Incompetence, and Regeneration in Sport*, edited by Lehmann M, Foster C, Gastmann U, Keizer H, Steinacker JM. Boston, MA: Springer US, 2007, p. 1–6.

237. **Lepers R**, **Hausswirth C**, **Maffiuletti N**, **Brisswalter J**, **Van Hoecke J**. Evidence of neuromuscular fatigue after prolonged cycling exercise. *Med Sci Sports Exerc* 32: 1880–1886, 2000.

238. **Liekens S**, **De Clercq E**, **Neyts J**. Angiogenesis: regulators and clinical applications. *Biochem Pharmacol* 61: 253–270, 2001.

239. **Lloyd PG**, **Prior BM**, **Li H**, **Yang HT**, **Terjung RL**. VEGF receptor antagonism blocks arteriogenesis, but only partially inhibits angiogenesis, in skeletal muscle of exercise-trained rats. *Am J Physiol - Hear Circ Physiol* 288: H759-68, 2005.

240. **Lowry OH**, **Passoneau JV**. A flexible system of enzymatic analysis. In: *Academic Press, New York, NY*. 1972.

241. **Ma S**, **Huang Q**, **Tominaga T**, **Liu C**, **Suzuki K**. An 8-Week ketogenic diet alternated interleukin-6, ketolytic and lipolytic gene expression, and enhanced exercise capacity in mice. *Nutrients* 10, 2018.

242. **Macia L**, **Wang V**, **Tsai W**, **Nguyen AD**, **Johnen H**, **Kuffner T**, **Shi Y-C**, **Lin S**, **Herzog H**, **Brown DA**, **Breit SN**, **Sainsbury A**, **Aguila MB**. Macrophage Inhibitory Cytokine 1 (MIC-1/GDF15) decreases food intake, body weight and improves glucose tolerance in mice on normal & obesogenic diets. *PLoS One* 7: 1–8, 2012.

243. **Malek MH**, **Olfert IM**, **Esposito F**. Detraining losses of skeletal muscle capillarization are associated with vascular endothelial growth factor protein expression in rats. *Exp Physiol* 95: 359–368, 2010.

244. **Margolis LM**, **O'fallon KS**. Utility of ketone supplementation to enhance physical performance: a systematic review. *Adv. Nutr.* .

245. **Margonis K**, **Fatouros IG**, **Jamurtas AZ**, **Nikolaidis MG**, **Douroudos I**, **Chatzinikolaou A**, **Mitrakou A**, **Mastorakos G**, **Papassotiriou I**, **Taxildaris K**, **Kouretas D**. Oxidative stress biomarkers responses to physical overtraining: implications for diagnosis. *Free Radic Biol Med* 43: 901–910, 2007.

246. **Matos NF**, **Winsley RJ**, **Williams CA**. Prevalence of nonfunctional overreaching/overtraining in young english athletes. *Med Sci Sports Exerc* 43: 1287–1294, 2011.

247. **Maughan RJ**, **Depiesse F**, **Geyer H**. The use of dietary supplements by athletes. *J Sports Sci* 25: 103–113, 2007.

248. **McConell GK**. It's well and truly time to stop stating that AMPK regulates glucose uptake and fat oxidation during exercise. *Am J Physiol Endocrinol Metab* 318: 564–567, 2020.

249. **McConell GK**, **Fabris S**, **Proietto J**, **Hargreaves M**. Effect of carbohydrate ingestion on glucose kinetics during exercise. *J Appl Physiol* 77: 1537–1541, 1994.

250. **McConell GK**, **Lee-Young RS**, **Chen ZP**, **Stepto NK**, **Huynh NN**, **Stephens TJ**, **Canny BJ**, **Kemp BE**. Short-term exercise training in humans reduces AMPK signalling during prolonged exercise independent of muscle glycogen. *J Physiol* 568: 665–676, 2005.

251. **McConell GK**, **Rattigan S**, **Lee-Young RS**, **Wadley GD**, **Merry TL**. Skeletal muscle nitric oxide signaling and exercise: A focus on glucose metabolism. *Am J Physiol - Endocrinol Metab* 303: 301–307, 2012.

252. **McGarry J**. Ketogenesis. In: *Ellenberg & Rifkin's Diabetes Mellitus*, edited by Porte D, Sherwin R. New York: McGraw-Hill, 1996, p. 19–28.

253. **McGarry JD**, **Foster DW**. Hormonal control of ketogenesis. Biochemical considerations. *Arch Intern Med* 137: 495–501, 1977.

254. **McGarry JD**, **Woeltje KF**, **Kuwajima M**, **Foster DW**. Regulation of ketogenesis and the renaissance of carnitine palmitoyltransferase. *Diabetes Metab Rev* 5: 271–284, 1989.

255. **McGee SL**, **Fairlie E**, **Garnham AP**, **Hargreaves M**. Exercise-induced histone modifications in human skeletal muscle. *J Physiol* 587: 5951–5958, 2009.

256. **McGuire LC**, **Cruickshank AM**, **Munro PT**. Alcoholic ketoacidosis. *Emerg Med J* 23: 417–420, 2006.

257. **McNaughton L**, **Curtin R**, **Goodman G**, **Perry D**, **Turner B**, **Showell C**. Anaerobic work and power output during cycle ergometer exercise: effects of bicarbonate loading. *J Sports Sci* 9: 151–160, 1991.

258. **Meeusen R**, **Duclos M**, **Foster C**, **Fry A**, **Gleeson M**, **Nieman D**, **Raglin J**, **Rietjens G**, **Steinacker J**, **Urhausen A**. Prevention, diagnosis, and treatment of the overtraining syndrome: joint consensus statement of the European College of Sport Science and the American College of Sports Medicine. *Med Sci Sports Exerc* 45: 186–205, 2013.

259. **Meeusen R**, **Duclos M**, **Gleeson M**, **Rietjens G**, **Steinacker J**, **Urhausen A**. Prevention, diagnosis and treatment of the overtraining syndrome. *Eur J Sport Sci* 6: 1–14, 2006.

260. **Meeusen R**, **Piacentini AMF**, **Busschaert AB**, **Buyse L**, **De Schutter AG**, **Stray-Gundersen AJ**. Hormonal responses in athletes: the use of a two bout exercise protocol to detect subtle differences in (over)training status. *Eur J Appl Physiol* 91: 140–146, 2004.

261. **Meeusen R**, **Piacentini MF**, **Van Den Eynde S**, **Magnus L**, **De Meirleir K**. Exercise performance is not influenced by a 5-HT reuptake inhibitor. *Int J Sports Med* 22: 329–336, 2001.

262. **Meeusen R**, **Watson P**. Amino acids and the brain: do they play a role in "central fatigue"? *Int J Sport Nutr Exerc Metab* 17: 37–46, 2007.

263. **Meeusen R**, **Watson P**, **Hasegawa H**, **Roelands B**, **Piacentini MF**. Central fatigue: the serotonin hypothesis and beyond. *Sport. Med.* 36 Springer: 881–909, 2006.

264. **Meeusen R**, **Watson P**, **Hasegawa H**, **Roelands B**, **Piacentini MF**, **Meeusen R**, **Watson P**, **Hasegawa H**, **Piacentini MF**. Brain neurotransmitters in fatigue and overtraining. *Appl Physiol Nutr Metab* 32: 857–864, 2007.

265. **Merrill GF**, **Kurth EJ**, **Hardie DG**, **Winder WW**. AICA riboside increases AMP-activated protein kinase, fatty acid oxidation, and glucose uptake in rat muscle. *Am J Physiol - Endocrinol Metab* 273: 1107–1112, 1997.

266. **Merry TL**, **Ristow M**. Do antioxidant supplements interfere with skeletal muscle adaptation to exercise training? *J Physiol* 594: 5135–5147, 2016.

267. **Mikkelsen KH**, **Seifert T**, **Secher NH**, **Grøndal T**, **Van Hall G**. Systemic, cerebral and skeletal muscle ketone body and energy metabolism during acute hyper-D-β-hydroxybutyratemia in post-absorptive healthy males. *J Clin Endocrinol Metab* 100: 636–643, 2015.

268. **Milkiewicz M**, **Ispanovic E**, **Doyle JL**, **Haas TL**. Regulators of angiogenesis and strategies for their therapeutic manipulation. *Int J Biochem Cell Biol* 38: 333–357, 2006.

269. **Millet GP**, **Vleck VE**, **Bentley DJ**. Physiological differences between cycling and running: lessons from triathletes. *Sport Med* 39: 179–206, 2009.

270. **Millet GY**, **Lepers R**. Alterations of neuromuscular function after prolonged running, cycling and skiing exercises. *Sport Med* 34: 105–116, 2004.

271. **Millet GY**, **Lepers R**, **Maffiuletti NA**, **Babault N**, **Martin V**, **Lattier G**. Alterations of neuromuscular function after an ultramarathon. *J Appl Physiol* 92: 486–492, 2002.

272. **Millet GY**, **Martin V**, **Lattier G**, **Ballay Y**. Mechanisms contributing to knee extensor strength loss after prolonged running exercise. *J Appl Physiol* 94: 193–198, 2003.

273. **Mitchell GA**, **Kassovska-Bratinova S**, **Boukaftane Y**, **Robert MF**, **Wang SP**, **Ashmarina L**, **Lambert M**, **Lapierre P**, **Potier E**. Medical aspects of ketone body metabolism. *Clin Investig Med* 18: 193–216, 1995.

274. **Morgan W**, **O'Connor P**, **Sparling P**, **Pate R**. Psychological characterization of the elite female distance runner. *Int J Sports Med* 08: S124–S131, 1987.

275. **Mountjoy M**, **Sundgot-Borgen J**, **Burke L**, **Carter S**, **Constantini N**, **Lebrun C**, **Meyer N**, **Sherman R**, **Steffen K**, **Budgett R**, **Ljungqvist A**. The IOC consensus statement: beyond the Female Athlete Triad-Relative Energy Deficiency in Sport (RED-S). *Br J Sports Med* 48: 491–497, 2014.

276. **Müller TD, Nogueiras R, Andermann ML, Andrews ZB, Anker SD, Argente J, Batterham RL, Tschöp MH, et al.** Ghrelin. *Mol Metab* 4: 437–460, 2015.

277. **Newman JC**, **Verdin E**. B-hydroxybutyrate: much more than a metabolite. *Diabetes Res Clin Pract* 106: 173–181, 2014.

278. **Newman JC**, **Verdin E**. β-Hydroxybutyrate: a signaling metabolite. *Annu Rev Nutr* 37: 51–76, 2017.

279. **Newsholme EA**, **Acworth I**, **Blomstrand E**. Amino acids, brain neurotransmitters and a function link between muscle and brain that is important in sustained exercise. In: *Advances in myochemistry*, edited by Benzi G. London, UK: John Libbey Eurotext, 1987, p. 127–133.

280. **Newsholme EA**, **Parry-Billings M**, **McAndrew N**, **Budgett R**. A biochemical mechanism to explain some characteristics of overtraining. In: *Advances in Nutrition and Top Sport*, edited by Brouns F. Karger Publishers, p. 79–93.

281. **Noakes T**, **Volek JS**, **Phinney SD**. Low-carbohydrate diets for athletes: What evidence? *Br. J. Sports Med.* 48 BMJ Publishing Group: 1077–1078, 2014.

282. **O'Malley T**, **Myette-Cote E**, **Durrer C**, **Little JP**. Nutritional ketone salts increase fat oxidation but impair high-intensity exercise performance in healthy adult males. *Appl Physiol Nutr Metab* 42: 1031–1035, 2017.

283. **Odland LM**, **Heigenhauser GJF**, **Spriet LL**. Effects of high fat provision on muscle PDH activation and malonyl-CoA content in moderate exercise. *J Appl Physiol* 89: 2352–2358, 2000.

284. **Olenich SA**, **Audet GN**, **Roberts KA**, **Olfert IM**. Effects of detraining on the temporal expression of positive and negative angioregulatory proteins in skeletal muscle of mice. *J Physiol* 592: 3325–3338, 2014.

285. **Olmedillas H**, **González-Agüero A**, **Moreno LA**, **Casajus JA**, **Vicente-Rodríguez G**. Cycling and bone health: a systematic review. *BMC Med* 10: 1–10, 2012.

286. **Opie LH**. *Heart physiology from cell to circulation*. 4th ed. Baltimore: Lippincott Williams & Wilkins, 2003.

287. **Owen OE**, **Morgan AP**, **Kemp HG**, **Sullivan JM**, **Herrera MG**, **Cahill GF**. Brain metabolism during fasting. *J Clin Invest* 46: 1589–1595, 1967.

288. **Ozaki N**, **Ringe B**, **Gubernatis G**, **Takada Y**, **Yamaguchi T**, **Yamaoka Y**, **Oellerich M**, **Ozawa K**, **Pichlmayr R**. Changes in energy substrates in relation to arterial ketone body ratio after human orthotopic liver transplantation. *Surgery* 113: 403–409, 1993.

289. **Palmer GS**, **Borghouts LB**, **Noakes TD**, **Hawley JA**. Metabolic and performance responses to constant-load vs. variable-intensity exercise in trained cyclists. *J Appl Physiol* 87: 1186–1196, 1999.

290. **Pandit R**, **Beerens S**, **Adan RAH**. Role of leptin in energy expenditure: the hypothalamic perspective. *Am J Physiol Regul Integr Comp Physiol* 312: 938–947, 2017.

291. **Pannier JL**, **Bouckaert JJ**, **Lefebvre RA**. The antiserotonin agent pizotifen does not increase endurance performance in humans. *Eur J Appl Physiol Occup Physiol* 72: 175–178, 1995.

292. **Paoli A**. Ketogenic diet for obesity: friend or foe? *Int. J. Environ. Res. Public Health* 11: 2014.

293. **Paoli A**, **Bianco A**, **Grimaldi KA**. The ketogenic diet and sport: a possible marriage? *Exerc Sport Sci Rev* 43, 2015.

294. **Parolin ML**, **Chesley A**, **Matsos MP**, **Spriet LL**, **Jones NL**, **Heigenhauser GJF**. Regulation of skeletal muscle glycogen phosphorylase and PDH during maximal intermittent exercise. *Am J Physiol - Endocrinol Metab* 277: E890–E900, 1999.

295. **Patel S**, **Alvarez-guaita A**, **Melvin A**, **Coll AP**, **Savage DB**, **Rahilly SO**. GDF15 provides an endocrine signal of nutritional stress in mice and humans. *Cell Metab* 29: 707–718, 2019.

296. **Paulus MP**, **Stein MB**. An insular view of anxiety. *Biol Psychiatry* 60: 383–387, 2006.

297. **Pernow B**, **Saltin B**. Availability of substrates and capacity for prolonged heavy exercise in man. *J Appl Physiol* 31: 416–422, 1971.

298. **Persson BEH**, **Sterky GCG**. Effect of prolonged fasting and ketogenic diet on levels of blood lipids and ketones in obese children. *Acta Paediatr* 55: 153–162, 1966.

299. **Peters EM**, **Goetzsche JM**, **Grobbelaar B**, **Noakes TD**. Vitamin C supplementation reduces the incidence of postrace symptoms of upper-respiratory-tract infection in ultramarathon runners. *Am J Clin Nutr* 57: 170–174, 1993.

300. **Peters JP**, **Van Slyke DD**. *Quantitative clinical chemistry: interpretations*. Volume 1,. Baltimore: Williams & Wilkins, 1946.

301. **Petibois C**, **Cazorla G**, **Poortmans JR**, **Déléris G**. Biochemical aspects of overtraining in endurance sports: the metabolism alteration process syndrome. *Sport. Med.* 33: 83–94, 2003.

302. **Pfeiffer B**, **Stellingwerff T**, **Hodgson AB**, **Randell R**, **Pöttgen K**, **Res P**, **Jeukendrup AE**. Nutritional intake and gastrointestinal problems during competitive endurance events. *Med Sci Sports Exerc* 44: 344–351, 2012.

303. **Phinney SD**, **Bistrian BR**, **Evans WJ**, **Gervino E**, **Blackburn GL**. The human metabolic response to chronic ketosis without caloric restriction: preservation of submaximal exercise capability with reduced carbohydrate oxidation. *Metabolism* 32: 769–776, 1983.

304. **Pichot V**, **Busso T**, **Roche F**, **Garet M**, **Costes F**, **Duverney D**, **Lacour JR**, **Barthélémy JC**. Autonomic adaptations to intensive and overload training periods: a laboratory study. *Med Sci Sports Exerc* 34: 1660–1666, 2002.

305. **Pinckaers PJM**, **Churchward-Venne TA**, **Bailey D**, **van Loon LJC**. Ketone bodies and exercise performance: the next magic bullet or merely hype? *Sport Med* 47: 383–391, 2017.

306. **Poffé C**, **Ramaekers M**, **Van Thienen R**, **Hespel P**. Ketone ester supplementation blunts overreaching symptoms during endurance training overload. *J Physiol* 597: 3009–3027, 2019.

307. **Post RM**, **Goodwin FK**. Simulated behavior states: an approach to specificity in psychobiological research. *Biol Psychiatry* 7: 237–54, 1973.

308. **Prins PJ**, **Noakes TD**, **Welton GL**, **Haley SJ**, **Esbenshade NJ**, **Atwell AD**, **Scott KE**, **Abraham J**, **Raabe AS**, **Buxton JD**, **Ault DL**. High rates of fat oxidation induced by a low-carbohydrate, high-fat diet, do not impair 5-km running performance in competitive recreational athletes. *J Sport Sci Med* 18: 738–750, 2019.

309. **Quant PA**, **Tubbs PK**, **Brand MD**. Glucagon activates mitochondrial 3-hydroxy-3-methylglutaryl-CoA synthase in vivo by decreasing the extent of succinylation of the enzyme. *Eur J Biochem* 187: 169–174, 1990.

310. **Racinais S**, **Girard O**, **Micallef JP**, **Perrey S**. Failed excitability of spinal motoneurons induced by prolonged running exercise. *J Neurophysiol* 97: 596–603, 2007.

311. **Raglin J**, **Sawamura S**, **Alexiou S**, **Hassmen P**, **Kentta G**. Training practices and staleness in 13-18-year-old swimmers: a cross-cultural study. *Pediatr Exerc Sci* 12: 61–70, 2000.

312. **Randle, P. J., Garland, P. B., Hales, C. N., Newsholme EA**. The glucose fatty-acid cycle its role in insulin sensitivity and the metabolic disturbances of diabetes mellitus. *Lancet* 281: 785–789, 1963.

313. **Randle PJ**. Fuel selection in animals. *Biochem Soc Trans* 14: 799–806, 1986.

314. **Randle PJ**, **Newsholme EA**, **Garland PB**. Regulation of glucose uptake by muscle. 8. Effects of fatty acids, ketone bodies and pyruvate, and of alloxan-diabetes and starvation, on the uptake and metabolic fate of glucose in rat heart and diaphragm muscles. *Biochem J* 93: 652–665, 1964.

315. **Richter EA**, **Hargreaves M**. Exercise, GLUT4, and skeletal muscle glucose uptake. *Physiol Rev* 93: 993–1017, 2013.

316. **Richter EA**, **Mikines KJ**, **Galbo H**, **Kiens B**. Effect of exercise on insulin action in human skeletal muscle. *J Appl Physiol* 66: 876–885, 1989.

317. **Rizzoli R**, **Bianchi ML**, **Garabédian M**, **Mckay HA**, **Moreno LA**. Maximizing bone mineral mass gain during growth for the prevention of fractures in the adolescents and the elderly. *Bone* 46: 294–305, 2010.

318. **Robergs R**, **Hutchinson K**, **Hendee S**, **Madden S**, **Siegler J**. Influence of pre-exercise acidosis and alkalosis on the kinetics of acid-base recovery following intense exercise. *Int J Sport Nutr Exerc Metab* 15: 59–74, 2005.

319. **Robinson AM**, **Williamson DH**. Physiological roles of ketone bodies as substrates and signals in mammalian tissues. *Physiol Rev* 60: 143–187, 1980.

320. **Rodger S**, **Plews D**, **Laursen P**, **Driller M**. Oral β-hydroxybutyrate salt fails to improve 4-minute cycling performance following submaximal exercise. *J Sci Cycl* 6: 26–31, 2017.

321. **Roepstorff C**, **Vistisen B**, **Roepstorff K**, **Kiens B**. Regulation of plasma long-chain fatty acid oxidation in relation to uptake in human skeletal muscle during exercise. *Am J Physiol - Endocrinol Metab* 287: 696–705, 2004.

322. **Rojas-Morales P**, **Tapia E**, **Pedraza-Chaverri J**. B-Hydroxybutyrate: a signaling metabolite in starvation response? *Cell. Signal.* 28: 2016.

323. **Romijn JA**, **Coyle EF**, **Sidossis LS**, **Gastaldelli A**, **Horowitz JF**, **Endert E**, **Wolfe RR**. Regulation of endogenous fat and carbohydrate metabolism in relation to exercise intensity and duration. *Am J Physiol - Endocrinol Metab* 265: 380–391, 1993.

324. **Romijn JA**, **Coyle EF**, **Sidossis LS**, **Zhang XJ**, **Wolfe RR**. Relationship between fatty acid delivery and fatty acid oxidation during strenuous exercise. *J Appl Physiol* 79: 1939–1945, 1995.

325. **Rose AJ**, **Richter EA**. Skeletal muscle glucose uptake during exercise: how is it regulated? *Physiology* : 260–270, 2005.

326. **Sarafoglou K**, **Hoffman GF**, **Roth KS**. *Pediatric endocrinology and inborn errors of metabolism*. 2nd ed. New York City: McGraw-Hill Education, 2017.

327. **Sass JO**. Inborn errors of ketogenesis and ketone body utilization. *J Inherit Metab Dis* 35: 23–28, 2012.

328. **Sato K**, **Kashiwaya Y**, **Keon C**, **Tsuchiya N**, **King MT**, **Radda G**, **Chance B**, **Clarke K**, **Veech RL**. Insulin, ketone bodies, and mitochondrial energy transduction. *FASEB J* 9: 651–658, 1995.

329. **Scott BE**, **Laursen PB**, **James LJ**, **Boxer B**, **Chandler Z**, **Lam E**, **Gascoyne T**, **Messenger J**, **Mears SA**. The effect of 1,3-butanediol and carbohydrate supplementation on running performance. *J Sci Med Sport* 22: 702–706, 2019.

330. **Seidner G**, **Garci Alvarez M**, **Yeh J**, **O'Driscoll KR**, **Klepper J**, **Stump TS**, **Wang D**, **Spinner NB**, **Birnbaum MJ**, **DeVivo D**. GLUT-1 deficiency syndrome caused by haploinsufficiency of the blood-brain barrier hexose carrier. *Nat Genet* 18: 188–191, 1998.

331. **Shaw DM**, **Merien F**, **Braakhuis A**, **Maunder ED**, **Dulson DK**. Effect of a ketogenic diet on submaximal exercise capacity and efficiency in runners. *Med Sci Sports Exerc* 51: 2135–2146, 2019.

332. **Shaw DM**, **Merien F**, **Braakhuis A**, **Plews D**, **Laursen P**, **Dulson DK**. The effect of 1,3-butanediol on cycling time-trial performance. *Int J Sport Nutr Exerc Metab* 29: 466–473, 2019.

333. **Shimazu T**, **Hirschey MD**, **Newman J**, **He W**, **Shirakawa K**, **Le Moan N**, **Grueter CA**, **Lim H**, **Saunders LR**, **Stevens RD**, **Newgard CB**, **Farese R V**, **de Cabo R**, **Ulrich S**, **Akassoglou K**, **Verdin E**. Suppression of oxidative stress by β-hydroxybutyrate, an endogenous histone deacetylase inhibitor. *Science* 339, 2013.

334. **Sidossis LS**, **Stuart CA**, **Shulman GI**, **Lopaschuk GD**, **Wolfe RR**. Glucose plus insulin regulate fat oxidation by controlling the rate of fatty acid entry into the mitochondria. *J Clin Invest* 98: 2244–2250, 1996.

335. **Siegl A**, **Kösel EM**, **Tam N**, **Koschnick S**, **Langerak NG**, **Skorski S**, **Meyer T**, **Lamberts RP**. Submaximal markers of fatigue and overreaching; implications for monitoring athletes. *Int J Sports Med* 38: 675–682, 2017.

336. **Siegler JC**, **Marshall PWM**, **Bishop D**, **Shaw G**, **Green S**. Mechanistic insights into the efficacy of sodium bicarbonate supplementation to improve athletic performance. *Sport Med* 2: 1–13, 2016.

337. **Simpson IA**, **Appel NM**, **Hokari M**, **Oki GD**, **Holman GD**, **Maher F**, **Koehler-Stec EM**, **Vannucci SJ**, **Smith QR**. Blood-brain barrier glucose transporter: effects of hypo- and hyperglycemia revisited. *J Neurochem* 72: 238–247, 1999.

338. **Simpson NS**, **Gibbs EL**, **Matheson GO**. Optimizing sleep to maximize performance: implications and recommendations for elite athletes. *Scand. J. Med. Sci. Sport.* 27 Blackwell Munksgaard: 266–274, 2017.

339. **Škorjanc D**, **Jaschinski F**, **Heine G**, **Pette D**. Sequential increases in capillarization and mitochondrial enzymes in low-frequency-stimulated rabbit muscle. *Am J Physiol - Cell Physiol* 274: 810–818, 1998.

340. **Snyder A**. Overtraining and glycogen depletion hypothesis. *Med Sci Sport Exerc* 30: 1146–1150, 1998.

341. **Snyder AC**, **Kuipers H**, **Cheng B**, **Servais R**, **Fransen E**. Overtraining following intensified training with normal muscle glycogen. *Med Sci Sport Exerc* 22: 1063–1070, 1995.

342. **Sokoloff L**. Metabolism of the central nervous system in vivo. In: *Handbook of Physiology*. Baltimore: Waverly Press Inc, 1960, p. 1843.

343. **Soto-Mota A**, **Norwitz NG**, **Clarke K**. Why a d-β-hydroxybutyrate monoester? *Biochem Soc Trans* 48: 51–59, 2020.

344. **Soto-Mota A**, **Vansant H**, **Evans RD**, **Clarke K**. Safety and tolerability of sustained exogenous ketosis using ketone monoester drinks for 28 days in healthy adults. *Regul Toxicol Pharmacol* 109: 1–5, 2019.

345. **Spriet LL**. Anaerobic metabolism in human skeletal muscle during short-term, intense activity. *Can J Physiol Pharmacol* 70: 157–165, 1992.

346. **Spriet LL**. New insights into the interaction of carbohydrate and fat metabolism during exercise. *Sport Med* 44: 87–96, 2014.

347. **Spriet LL**, **Lindinger MI**, **Heigenhauser GJF**, **Jones NL**. Effects of alkalosis on skeletal muscle metabolism and performance during exercise. *Am J Physiol - Regul Integr Comp Physiol* 251: 833–839, 1986.

348. **Spriet LL**, **Matsos G**, **Peters SJ**, **Heigenhauser GJ**, **Jones NL**. Effects of acidosis on rat muscle metabolism and performance during heavy exercise. *Am J Physiol - Cell Physiol* 248: 337–347, 1985.

349. **Srivastava S**, **Kashiwaya Y**, **King MT**, **Baxa U**, **Tam J**, **Niu G**, **Chen X**, **Clarke K**, **Veech RL**. Mitochondrial biogenesis and increased uncoupling protein 1 in brown adipose tissue of mice fed a ketone ester diet. *FASEB J* 26: 2351–2362, 2012.

350. **Strålfors P**, **Olsson H**, **Belfrage P**. Hormone-sensitive lipase. In: *The Enzymes*, edited by Boyer P, Krass E. New York: Academic Press, 1987, p. 144–177.

351. **Strüder HK**, **Hollmann W**, **Platen P**, **Donike M**, **Gotzmann A**, **Weber K**. Influence of paroxetine, branched-chain amino acids and tyrosine on neuroendocrine system responses and fatigue in humans. *Horm Metab Res* 30: 188–94, 1998.

352. **Stubbs BJ**, **Cox PJ**, **Evans RD**, **Cyranka M**, **Clarke K**, **de Wet H**. A ketone ester drink lowers human ghrelin and appetite. *Obesity* 26: 269–273, 2018.

353. **Stubbs BJ**, **Cox PJ**, **Evans RD**, **Santer P**, **Miller JJ**, **Faull OK**, **Magor-Elliott S**, **Hiyama S**, **Stirling M**, **Clarke K**. On the metabolism of exogenous ketones in humans. *Front Physiol* 8: 848, 2017.

354. **Stubbs BJ**, **Cox PJ**, **Kirk T**, **Evans RD**, **Clarke K**. Gastrointestinal effects of exogenous ketone drinks are infrequent, mild, and vary according to ketone compound and dose. *Int J Sport Nutr Exerc Metab* 29: 596–603, 2019.

355. **Stubbs BJ**, **Koutnik AP**, **Poff AM**, **Ford KM**, **D'Agostino DP**. Commentary: ketone diester ingestion impairs time trial performance in professional cyclists. *Front Physiol* 9: 279, 2018.

356. **Suárez Y**, **Sessa WC**. MicroRNAs as novel regulators of angiogenesis. *Circ Res* 104: 442–454, 2009.

357. **Svendsen IS**, **Killer SC**, **Carter JM**, **Randell RK**, **Asker ·**, **Jeukendrup E**, **Gleeson · Michael**. Impact of intensified training and carbohydrate supplementation on immunity and markers of overreaching in highly trained cyclists. *Eur J Appl Physiol* 116: 867–877, 2016.

358. **Sylow L**, **Jensen TE**, **Kleinert M**, **Højlund K**, **Kiens B**, **Wojtaszewski J**, **Prats C**, **Schjerling P**, **Richter EA**. Rac1 signaling is required for insulin-stimulated glucose uptake and is dysregulated in insulin-resistant murine and human skeletal muscle. *Diabetes* 62: 1865–1875, 2013.

359. **Sylow L**, **Kleinert M**, **Richter EA**, **Jensen TE**. Exercise-stimulated glucose uptake — regulation and implications for glycaemic control. *Nat Rev Endocrinol* 13: 133–148, 2016.

360. **Sylow L**, **Nielsen IL**, **Kleinert M**, **Møller LLV**, **Ploug T**, **Schjerling P**, **Bilan PJ**, **Klip A**, **Jensen TE**, **Richter EA**. Rac1 governs exercise-stimulated glucose uptake in skeletal muscle through regulation of GLUT4 translocation in mice. *J Physiol* 594: 4997–5008, 2016.

361. **Taggart AKP**, **Kero J**, **Gan X**, **Cai TQ**, **Cheng K**, **Ippolito M**, **Ren N**, **Kaplan R**, **Wu K**, **Wu TJ**, **Jin L**, **Liaw C**, **Chen R**, **Richman J**, **Connolly D**, **Offermanns S**, **Wright SD**, **Waters MG**. (D)-β-hydroxybutyrate inhibits adipocyte lipolysis via the nicotinic acid receptor PUMA-G. *J Biol Chem* 280: 26649–26652, 2005.

362. **Tanskanen M**, **Atalay M**, **Uusitalo A**. Altered oxidative stress in overtrained athletes. *J Sports Sci* 28: 309–317, 2010.

363. **Ten Haaf T**, **Foster C**, **Meeusen R**, **Roelands B**, **Piacentini MF**, **Van Staveren S**, **Koenderman L**, **De Koning JJ**. Submaximal heart rate seems inadequate to prescribe and monitor intensified training. *Eur J Appl Physiol* 19: 1082–1091, 2019.

364. **Ten Haaf T**, **Van Staveren S**, **Oudenhoven E**, **Piacentini MF**, **Meeusen R**, **Roelands B**, **Koenderman L**, **Daanen HAM**, **Foster C**, **De Koning JJ**. Prediction of functional overreaching from subjective fatigue and readiness to train after only 3 days of cycling. *Int J Sports Physiol Perform* 12: 87–94, 2017.

365. **Thio LL**, **Erbayat-Altay E**, **Rensing N**, **Yamada KA**. Leptin contributes to slower weight gain in juvenile rodents on a ketogenic diet. *Pediatr Res* 60: 413–417, 2006.

366. **Thomas L**, **Busso T**. A theoretical study of taper characteristics to optimize performance. *Med Sci Sport Exerc* 37: 1615–1621, 2005.

367. **Thomas LK**, **Ittmann M**, **Cooper C**. The role of leucine in ketogenesis in starved rats. *Biochem J* 204: 399–403, 1982.

368. **Thomsen HH**, **Rittig N**, **Johannsen M**, **Møller AB**, **Jørgensen JO**, **Jessen N**, **Møller N**. Effects of 3-hydroxybutyrate and free fatty acids on muscle protein kinetics and signaling during LPS-induced inflammation in humans: anticatabolic impact of ketone bodies. *Am J Clin Nutr* 108: 1–11, 2018.

369. **Tipton MJ**, **Collier N**, **Massey H**, **Corbett J**, **Harper M**. Cold water immersion: kill or cure? *Exp Physiol* 102: 1335–1355, 2017.

370. **Todd KS**, **Butterfield GA**, **Calloway DH**. Nitrogen balance in men with adequate and deficient energy intake at three levels of work. *J Nutr* 114: 2107–2118, 1984.

371. **Urhausen A**, **Kindermann W**. Diagnosis of overtraining. What tools do we have? *Sport Med* 32: 95–102, 2002.

372. **van Loon LJC**, **Greenhaff PL**, **Constantin-Teodosiu D**, **Saris WHM**, **Wagenmakers AJM**. The effects of increasing exercise intensity on muscle fuel utilisation in humans. *J Physiol* 536: 295–304, 2001.

373. **Van Proeyen K**, **Szlufcik K**, **Nielens H**, **Ramaekers M**, **Hespel P**. Beneficial metabolic adaptations due to endurance exercise training in the fasted state. *J Appl Physiol* 110: 236–245, 2011.

374. **Vandoorne T**, **De Smet S**, **Ramaekers M**, **Van Thienen R**, **De Bock K**, **Clarke K**, **Hespel P**. Intake of a ketone ester drink during recovery from exercise promotes mTORC1 signaling but not glycogen resynthesis in human muscle. *Front Physiol* 8: 1–12, 2017.

375. **VanItallie T**, **Nonas RD**, **Di Rocco A**, **Boyar K**, **Hyams K**, **Heymsfield SB**. Treatment of Parkinson disease with diet-induced hyperketonemia: a feasibility study. *Neurology* 64: 728–730, 2005.

376. **Vanitallie TB**. Biomarkers, ketone bodies, and the prevention of Alzheimer's disease. *Metabolism* 64: 51–57, 2015.

377. **VanItallie TB**, **Nufert TH**. Ketones: metabolism's ugly duckling. *Nutr Rev* 61: 327–341, 2003.

378. **Veech RL**. The therapeutic implications of ketone bodies: the effects of ketone bodies in pathological conditions: ketosis, ketogenic diet, redox states, insulin resistance, and mitochondrial metabolism. *Prostaglandins, Leukot Essent Fat Acids* 70: 309–319, 2004.

379. **Veech RL**, **Chance B**, **Kashiwaya Y**, **Lardy H a**, **Cahill GF**. Ketone bodies, potential therapeutic uses. *IUBMB Life* 51: 241–247, 2001.

380. **Volek JS**, **Freidenreich DJ**, **Saenz C**, **Kunces LJ**, **Creighton BC**, **Bartley JM**, **Davitt PM**, **Munoz CX**, **Anderson JM**, **Maresh CM**, **Lee EC**, **Schuenke MD**, **Aerni G**, **Kraemer WJ**, **Phinney SD**. Metabolic characteristics of keto-adapted ultra-endurance runners. *Metabolism* 65, 2016.

381. **Volek JS**, **Noakes T**, **Phinney SD**. Rethinking fat as a fuel for endurance exercise. *Eur J Sport Sci* 15: 13–20, 2015.

382. **Volek JS**, **Phinney SD**. *The art and science of low carbohydrate performance*. Miami, FL: Beyond Obesity LLC, 2012.

383. **Walter J**, **Nestelberger T**, **Boeddinghaus J**, **Twerenbold R**, **Croton L**, **Badertscher P**, **Wildi K**, **Wussler D**, **du Fay de Lavallaz J**, **Mueller C**, **Giménez MR**, **Puelacher C**, **Kozhuharov N**, **Morawiec B**, **Rentsch K**, **Miró Ò**, **Martin-Sanchez FJ**, **Muzyk P**, **Geigy N**, **Keller DI**, **Reichlin T**, **Lohrmann J**, **Biskup E**, **Kloos W**, **López B**, **Inostroza CF**, **Adrada ER**, **Kawecki D**, **Nowalany-Kozielska E**, **Parenica J**, **Meissner K**, **Kulangara C**, **Mahfouz R**, **von Eckardstein A**. Growth differentiation factor-15 and all-cause mortality in patients with suspected myocardial infarction. *Int J Cardiol* 292: 241–245, 2019.

384. **Wang-Wei Tsai V**, **Macia L**, **Johnen H**, **Kuffner T**, **Manadhar R**, **Beck Jørgensen S**, **Ki Michelle Lee-Ng K**, **Ping Zhang H**, **Wu L**, **Peter Marquis C**, **Jiang L**, **Husaini Y**, **Lin S**, **Herzog H**, **Brown DA**, **Sainsbury A**, **Breit SN**, **Morrison C**. TGF-b superfamily cytokine MIC-1/GDF15 is a physiological appetite and body weight regulator. *PLoS One* 8: 1–10, 2013.

385. **Waters RE**, **Rotevatn S**, **Li P**, **Annex BH**, **Yan Z**. Voluntary running induces fiber type-specific angiogenesis in mouse skeletal muscle. *Am J Physiol - Cell Physiol* 287: 1342–1348, 2004.

386. **Watt MJ**, **Heigenhauser GJF**, **O'Neill M**, **Spriet LL**. Hormone-sensitive lipase activity and fatty acyl-CoA content in human skeletal muscle during prolonged exercise. *J Appl Physiol* 95: 314–321, 2003.

387. **Watt MJ**, **Holmes AG**, **Steinberg GR**, **Mesa JL**, **Kemp BE**, **Febbraio MA**. Reduced plasma FFA availability increases net triacylglycerol degradation, but not GPAT or HSL activity, in human skeletal muscle. *Am J Physiol - Endocrinol Metab* 287: E120–E127, 2004.

388. **Watt MJ**, **Spriet LL**. Triacylglycerol lipases and metabolic control: implications for health and disease. *Am J Physiol - Endocrinol Metab* 299: 162–168, 2010.

389. **Westerblad BYH**, **Allen DG**. Changes of intracellular pH due to repetitive stimulation of single fibres from mouse skeletal muscle. *J Physiol* 449: 49–71, 1992.

390. **Westerblad H**. Acidosis is not a significant cause of skeletal muscle fatigue. *Med Sci Sports Exerc* 48: 2339–2342, 2016.

391. **White GE**, **Wells GD**. Cold-water immersion and other forms of cryotherapy: physiological changes potentially affecting recovery from high-intensity exercise. *Extrem Physiol Med* 2: 1, 2013.

392. **Wilson WM**, **Marsden CA**. In vivo measurement of extracellular serotonin in the ventral hippocampus during treadmill running. *Behav Pharmacol* 7: 101–104, 1996.

393. **Winder W**, **Baldwin K**, **Holloszy J**. Exercise-induced increase in the capacity of rat skeletal muscle to oxidize ketones. *Can J Physiol Pharmacol* 53: 86–91, 1975.

394. **Winder WW**, **Baldwin KM**, **Holloszy JO**. Enzymes involved in ketone utilization in different types of muscle: adaptation to exercise. *Eur J Biochem* 47: 461–467, 1974.

395. **Witard OC**, **Jackman SR**, **Kies AK**, **Jeukendrup AE**, **Tipton KD**. Effect of increased dietary protein on tolerance to intensified training. *Med Sci Sports Exerc* 43: 598–607, 2011.

396. **Witard OC**, **Turner JE**, **Jackman SR**, **Kies AK**, **Jeukendrup AE**, **Bosch JA**, **Tipton KD**. High dietary protein restores overreaching induced impairments in leukocyte trafficking and reduces the incidence of upper respiratory tract infection in elite cyclists. *Brain Behav Immun* 39: 211–219, 2014.

397. **Wojtaszewski JFP**, **Jorgenson SB**, **Hellsten Y**, **Hardie DG**, **Richter EA**. Glycogen-dependent effects of 5-aminoimidazole-4-carboxamide (AICA)-riboside on AMP-activated protein kinase and glycogen synthase activities in rat skeletal muscle. *Diabetes* 51: 284–292, 2002.

398. **Woods AL**, **Garvican-Lewis LA**, **Lundy B**, **Rice AJ**, **Thompson KG**. New approaches to determine fatigue in elite athletes during intensified training: Resting metabolic rate and pacing profile. *PLoS One* 12: 1–17, 2017.

399. **Woods AL**, **Rice AJ**, **Garvican-Lewis LA**, **Wallett AM**, **Lundy B**, **Rogers MA**, **Welvaert M**, **Halson S**, **McKune A**, **Thompson KG**. The effects of intensified training on resting metabolic rate (RMR), body composition and performance in trained cyclists. *PLoS One* 13: 1–24, 2018.

400. **Wright DC**, **Hucker KA**, **Holloszy JO**, **Han DH**. Ca2+ and AMPK both mediate stimulation of glucose transport by muscle contractions. *Diabetes* 53: 330–335, 2004.

401. **Wu X**, **Miao D**, **Liu Z**, **Liu K**, **Zhang B**, **Li J**, **Li Y**, **Qi J**. β-hydroxybutyrate antagonizes aortic endothelial injury by promoting generation of VEGF in diabetic rats. *Tissue Cell* 64: 101345, 2020.

402. **Xie S**, **Lu L**, **Liu L**. Growth differentiation factor-15 and the risk of cardiovascular diseases and all-cause mortality: a meta-analysis of prospective studies. *Clin Cardiol* 42: 513–523, 2019.

403. **Xin L**, **Ipek Ö**, **Beaumont M**, **Shevlyakova M**, **Christinat N**, **Masoodi M**, **Greenberg N**, **Gruetter R**, **Cuenoud B**. Nutritional ketosis increases NAD+/NADH ratio in healthy human brain: an in vivo study by 31P-MRS. *Front Nutr* 5: 1–8, 2018.

404. **Yamane M**, **Teruya H**, **Nakano M**, **Ogai R**, **Ohnishi N**, **Kosaka M**. Post-exercise leg and forearm flexor muscle cooling in humans attenuates endurance and resistance training effects on muscle performance and on circulatory adaptation. *Eur J Appl Physiol* 96: 572–580, 2006.

405. **Youm Y-H**, **Nguyen KY**, **Grant RW**, **Goldberg EL**, **Bodogai M**, **Kim D**, **D 'agostino D**, **Planavsky N**, **Lupfer C**, **Kanneganti TD**, **Kang S**, **Horvath TL**, **Fahmy TM**, **Crawford PA**, **Biragyn A**, **Alnemri E**, **Dixit VD**. Ketone body β-hydroxybutyrate blocks the NLRP3 inflammasome-mediated inflammatory disease. *Nat Med* 21: 263–269, 2015.

406. **Zhang M**, **Sun W**, **Qian J**, **Tang Y**. Fasting exacerbates hepatic growth differentiation factor 15 to promote fatty acid β-oxidation and ketogenesis via activating XBP1 signaling in liver. *Redox Biol* 16: 87–96, 2018.

407. **Zheng ZH**, **Anderstam B**, **Yu X**, **Qureshi AR**, **Heimbürger O**, **Lindholm B**. Bicarbonate-based peritoneal dialysis solution has less effect on ingestive behavior than lactate-based peritoneal dialysis solution. *Perit Dial Int* 29: 656–663, 2009.

408. **Zisman A**, **Peroni OD**, **Abel ED**, **Michael MD**, **Mauvais-Jarvis F**, **Lowell BB**, **Wojtaszewski JFP**, **Hirshman MF**, **Virkamaki A**, **Goodyear LJ**, **Kahn CR**, **Kahn BB**. Targeted disruption of the glucose transporter 4 selectively in muscle causes insulin resistance and glucose intolerance. *Nat Med* 6: 4–8, 2000.

409. **Zou X**, **Meng J**, **Li L**, **Han W**, **Li C**, **Zhong R**, **Miao X**, **Cai J**, **Zhang Y**, **Zhu D**. Acetoacetate accelerates muscle regeneration and ameliorates muscular dystrophy in mice. *J Biol Chem* 291: 2181–2195, 2016.

APPENDICES

Appositions - Bijstellingen

1. Doceren wordt meer en meer als een noodzakelijke bijkomstigheid gezien aan universiteiten. Dit terwijl kwalitatief onderwijs een cruciale bron van inspiratie vormt voor komende generaties.

2. Wetenschappelijk onderzoek legt meer en meer de nadruk op het geïsoleerd bestuderen van fundamentele processen en mechanismen. Er zou meer aandacht en waardering moeten zijn voor onderzoeksdesigns die de reële contextuele omstandigheden in rekening brengen.

3. Een recente casestudy schatte de ecologische voetafdruk van een PhD-project aan de KU Leuven op meer dan 20 ton CO_2 equivalent, wat overeenkomt met ruim 100 heen- en terugvluchten tussen Brussel en Parijs. Desondanks worden ecologie en duurzaamheid nauwelijks in overweging genomen bij het financiëren, uitvoeren en verspreiden van onderzoek. Onderzoeksinstellingen zouden net een voortrekkersrol moeten opnemen door bewustwording te creëren bij onderzoekers en hen te stimuleren CO_2-reducerende maatregelen te nemen. Zo is het ontbreken van een fietsvergoeding en het financiëel promoten van verre (> 50 km) dienstverplaatsingen met de auto een grote lacune in het duurzaamheidsbeleid van de KU Leuven.

Professional career and publication list

Chiel Poffé

EDUCATION

PhD curriculum in Biomedical Sciences, 2016-… - Exercise Physiology Research Group, KU Leuven. Dissertation: *The role of ketone bodies in physiological and muscular adaptations to exercise and training.* Promotor: Professor Peter Hespel

Master in Physical education and movement sciences, 2014-2016 – KU Leuven

Bachelor in Physical education and movement sciences, 2011-2014 – KU Leuven

PUBLICATIONS

Tanjeko AT, Serré J, Wüst R, Mpaka Messa GA, **Poffé C**, Swaminathan A, Maes K, Troosters T, Degens H & Gayan-Ramirez G (2020). Two weeks smoking cessation reverses cigarette smoke-induced skeletal muscle atrophy and mitochondrial dysfunction in mice. *Nicotine Tob Res*.

Poffé C, Ramaekers M & Hespel P (2019). Reply from Chiel Poffé, Monique Ramaekers and Peter Hespel. *J Physiol* 597, 5309-5310.

Poffé C, Ramaekers M, Van Thienen R & Hespel P (2019). Reply from Chiel Poffé, Monique Ramaekers, Ruud Van Thienen and Peter Hespel. *J Physiol* 597, 4679-4680.

Poffé C, Ramaekers M, Van Thienen R & Hespel P (2019). Reply from Chiel Poffé, Monique Ramaekers, Ruud Van Thienen and Peter Hespel. *J Physiol* 597, 4409-4410.

Poffé C, Ramaekers M, Van Thienen R & Hespel P (2019). Ketone ester supplementation blunts overreaching symptoms during endurance training overload. *J Physiol* 597, 3009-3027.

Poffé C, Dalle S, Kainz H, Berardi E & Hespel P (2018). A noninterfering system to measure in-cage spontaneous physical activity in mice. *J Appl Physiol* 125, 263-270.

De Smet S, D'Hulst G, **Poffé C**, Van Thienen R, Berardi E & Hespel P (2018). High-intensity interval training in hypoxia does not affect muscle HIF responses to acute hypoxia in humans. *EJAP* 118, 847-862.

In revision

Poffé C, Robberechts R, Van Thienen R & Hespel P. Exogenous ketosis enhances the angiogenic response to exercise.

Poffé C, Ramaekers M, Bogaerts S & Hespel P. Exogenous ketosis impacts neither performance nor muscle glycogen breakdown in prolonged endurance exercise.

Poffé C, Ramaekers M, Bogaerts S & Hespel P. Oral bicarbonate unlocks the ergogenic effect of exogenous ketosis in endurance exercise.

Dalle S, **Poffé C**, Hiroux C, Suhr F, Deldicque L & Koppo K. Ibuprofen treatment affects inflammatory and anabolic signaling, but not skeletal muscle regeneration following a

cardiotoxin-induced injury.

Dalle S, Hiroux C, **Poffé C**, Ramaekers M, Deldicque L & Koppo K. Cardiotoxin-induced skeletal muscle injury elicits profound changes in anabolic and stress signaling, and muscle fiber type composition.

In preparation

Poffé C, Pelgrim K, Jansen P & Hespel P. Haematological and hormonal dysregulations to endurance training overload in world-class cyclist during the Tour de France.

Poffé C, Ramaekers M & Hespel P. Exogenous ketosis impairs 30 min time-trial performance independent of bicarbonate supplementation.

CONFERENCE PRESENTATIONS

2019. Annual Congress of the European College of Sport Sciences – Prague, Czech Republic. Oral presentation – Ketone ester supplementation blunts overreaching symptoms and stimulates performance during endurance training overload.

2019. Symposium of the association of research into Hereditary Metabolic Diseases (ESN) – Utrecht, The Netherlands. Oral presentation (invited) – Ketone bodies for elite athletes.

2017. Vereniging voor de Kinesiologie – Ghent, Belgium. Oral presentation – Nutritional ketosis as an additional stimulus for post-exercise muscle protein synthesis.

SCIENTIFIC OUTREACH

2020. 1st sport medical symposium – SMC Zottegem, Belgium. Invited keynote: Ketones: the new superfuel.

2019. 11th sports congress – Ghent, Belgium. Invited keynote: Ketone intake as a nutritional strategy to increase endurance exercise performance.

2019. Poffé C & Hespel P. Editorial – Ketones: the new superfuel for athletes? *Ortho-Rheumato* 17, 1-2.

TEACHING EXPERIENCE

2016-…: Teaching assistant practical sessions Exercise Physiology

3rd bachelor Physical Education and Movement Sciences (B-KUL-L00B5B)

2nd bachelor Rehabilitation Sciences and Physiotherapy (B-KUL-L02A0A)