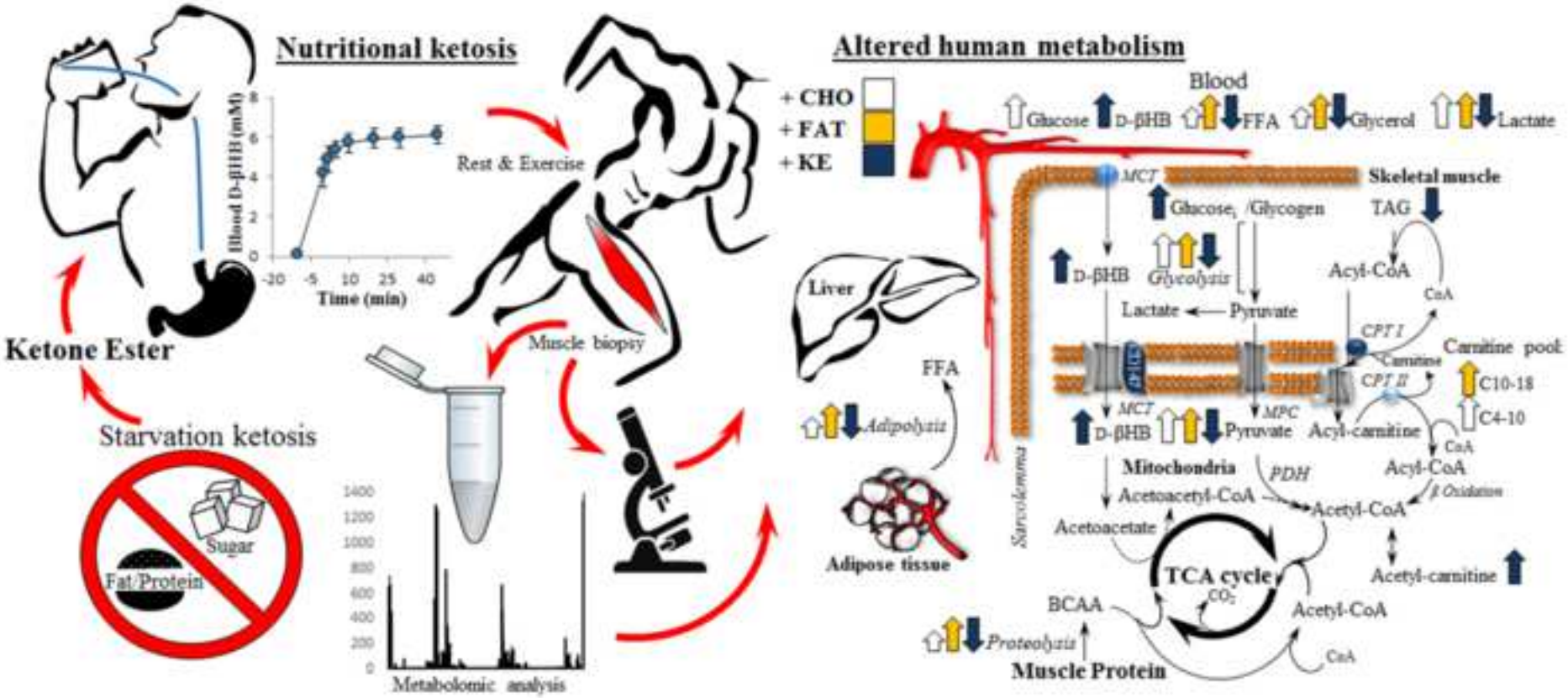
Graphical Abstract



Nutritional ketosis alters fuel preference and thereby endurance performance in athletes

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Summary:

Ketosis, the metabolic response to energy crisis, is a mechanism to sustain life by altering oxidative fuel selection. Often overlooked for its metabolic potential, ketosis is poorly understood outside of starvation or diabetic crisis. Thus we studied the biochemical advantages of ketosis in humans using a ketone ester-based form of nutrition without the unwanted milieu of endogenous ketone body production by caloric or carbohydrate restriction. In 5 separate studies of 39 high-performance athletes, we show how this unique metabolic state improves physical endurance by altering fuel competition for oxidative respiration. Ketosis decreased muscle glycolysis and plasma lactate concentrations, whilst providing an alternative substrate for oxidative phosphorylation. Ketosis increased intramuscular triacylglycerol oxidation during exercise, even in the presence of normal muscle glycogen, co-ingested carbohydrate and elevated insulin. These findings may hold clues to greater human potential, and a better understanding of fuel metabolism in health and disease**.**

Introduction:

Ketone body metabolism is a survival trait conserved in higher organisms to prolong life during an energy deficit or metabolic crisis. The misapprehension that physiological ketosis is an unwanted, or even life threatening complication of disease or starvation, has overshadowed the sound evolutionary purpose of ketone metabolism for many decades (VanItallie and Nufert 2003, Veech 2004). The advantages of ketone body metabolism during starvation are clear; providing an oxidizable carbon source to conserve precious glucose/gluconeogenic reserves, whilst simultaneously satisfying the specific fuel demands of the brain. Ketone bodies, when present, act not only as respiratory fuels to power oxidative phosphorylation, but as signals regulating the preferential oxidation and mobilization of fuel substrates (Robinson and Williamson 1980). The conservation of carbohydrate reserves in the form of glycogen and gluconeogenic skeletal muscle protein is a hallmark of starvation induced ketosis (Cahill 1970), dramatically increasing survival duration (Cahill Jr and Owen 1968, Felig, Owen et al. 1969). Ketosis may also provide thermodynamic advantages over other carbon substrates by increasing the free energy conserved in ATP (ΔG_{ATP}) by the oxidation of ketones during mitochondrial oxidative phosphorylation (Sato, Kashiwaya et al. 1995). The combination of improved energetic efficiency and fuel sparing is vitally important not only during famine, but could also provide clues to new methods of sustaining human performance, or restoring dysregulated substrate metabolism.

Produced continuously under normal physiological conditions, a significant increase in the ketone bodies, D-β-hydroxybutyrate (D-βHB) and acetoacetate (AcAc), rarely manifests in concentrations above 1 mM (Robinson and Williamson 1980). However, the production of ketone bodies increases rapidly in response to calorie deprivation or energy deficit such as starvation, prolonged exercise, and as part of the clinical manifestations of diseases, such as uncontrolled diabetes (Robinson and Williamson 1980).

As a fuel source, ketone bodies are readily oxidized by most body tissues (Robinson and Williamson 1980), the major exception being the liver due to its lack of the enzyme succinyl-CoA:3-ketoacid CoA transferase, which permits oxidative disposal of ketones in the TCA cycle. The favourable thermodynamic characteristics of ketone body oxidation and their regulatory role controlling the preferential use and release of other substrates, such as fat and glucose, may also have therapeutic utility for the treatment of disease (Veech 2004, Keene 2006).

Achieving ketosis by feeding D-βHB in an acid or salt form is not advisable due to the accompanying acid/salt load. To circumvent this, and the unwanted dietary restriction of adhering to a ketogenic diet, we generated an edible form of a ketone body by transesterifying ethyl *(R)*-3-hydroxybutyrate with *(R)*-1,3-butanediol using lipase (Figure 1, and Table S1). Previously we have shown the nutritional ingestion of this *(R)*-3-hydroxybutyl *(R)*-3-hydroxybutyrate ketone ester (KE) is a safe and effective way of elevating blood ketone levels (Clarke, Tchabanenko et al. 2012, Shivva, Cox et al. 2016), and provides a means of investigating human ketone metabolism independent of caloric or carbohydrate deficit.

In some ways, the metabolic demands of prolonged exercise parallel (albeit on much more rapid scale) the metabolic conditions pertinent to survival in starvation; it being well known that skeletal muscle fuel selection becomes inflexible as exercise intensity rises, placing a premium on carbohydrate reserves, resulting in an almost exclusive reliance on glycogen and blood glucose for its energy requirements (Romijn, Coyle et al. 1993, van Loon, Greenhaff et al. 2001). We reasoned that the combination of improved energetic efficiency and fuel sparing induced by ketosis is vitally important not just in famine, and that harnessing the metabolic actions of ketosis in nutritional form may provide a method of sustaining human physical performance (Cox and Clarke 2014).

Therefore we sought to determine the mechanisms governing skeletal muscle substrate metabolism during acute nutritional ketosis in exercising humans, as well as their effects on endurance performance in this unique metabolic state.

Results:

Exercise intensity alters the metabolism of nutritional ketosis (Study 1)

To determine whether exercise intensity altered the metabolism of diet derived ketosis we examined the effects of steady state exercise on the clearance of blood and urinary D-βHB in six male endurance athletes (Table S2). An identical amount of KE was consumed by athletes at rest, and during 45 min of cycling exercise (40% and 75% of W_{Max}) in a randomised cross over design (Figure 2A). Ingestion of a drink containing 573 mg/kg body weight of KE resulted in a rapid rise in circulating D-βHB from overnight fasted levels (0.1 mM) to ~3 mM after 10 min of rest. After the onset of exercise D-βHB concentrations were divergent, reaching new steady state concentrations after approximately 10 min, with high (75% W_{Max}) intensity exercise reducing D-βHB concentrations by 1.05 ± 0.2 mM compared to workloads of 40% W_{Max}, and by 3.1 ± 0.4 mM compared with resting conditions (Figure 2B). D-βHB area under curve (AUC) during 45 min of rest or exercise was significantly decreased with increasing exercise intensity (Figure 2C), and correlated closely with increasing oxygen consumption (Figure 2D). Indirect calorimetry equations were adjusted for ketone oxidation (Frayn 1983) (Supplemental methods) and used to calculate relative contributions of each substrate to total oxygen consumption during exercise at 40% and 75% W_{Max} (Figure 2E). D-βHB oxidation was estimated to account for 16-18% of total oxygen consumption during exercise.

Estimated D-βHB oxidation during steady state exercise increased from 0.35 g/min at 40% W_{Max} to ~0.5 g/min at 75% intensity (Figure 2F). Urinary elimination of D-βHB during exercise was negligible, ranging from 0.05 to 0.3 g (~0.2% of total ingested KE) over the entirety of the protocol, although did correlate positively with D-βHB AUC (Figure 2G).

The metabolic effects of nutritional substrate alteration during exercise (Study 2)

Each athlete (n=10, Table S3) completed three experimental trials consisting of 1 h of constant load cycling at 75% of W_{Max} in a randomised, single-blind, cross-over design (Figure 3A). Isocaloric drinks contained a minimum of 96% of their calories from the one substrate (Figure 3A, and Supplemental information). Subjects ingested 573 mg/kg BW of KE, isocaloric carbohydrate, or fat 15 min prior to the start of exercise, and 191 mg/kg BW KE 45 min into each 1 h trial. Resting blood ketone body kinetic profiles, using an identical protocol, were determined on a separate (non-exercising) study day.

Ingestion of a drink containing 573 mg/kg body weight of KE resulted in a rapid rise in circulating D-βHB from overnight fasted levels of 0.13 ± 0.1 mM to 3.5 ± 0.3 mM during 10 min of rest, where they remained throughout 1 h of exercise (Figure 3B). When no exercise was performed, plasma D-βHB concentrations increased to > 5 mM.

Lactate concentrations were the same at baseline for all conditions (Fig. 3C). However, after the onset of exercise, blood lactate concentrations were significantly lower on KE, resulting in average exercise lactate concentrations ~2-3 mM (~50%) lower than CHO, and lower than FAT at 30 and 45 min.

FFA concentrations were significantly higher at baseline on FAT after 24 h of high-fat low-carbohydrate meals (Figure 3D), remaining elevated throughout exercise compared with CHO or KE, reaching 0.85 mM at the end of exercise. FFA concentrations were lower than FAT at baseline before, and fell after CHO or KE ingestion. Ketosis suppressed the rise in FFA seen after 25 min of exercise compared with FAT and, to a lesser extent CHO. Exercise caused significant increases in plasma glycerol following both CHO and FAT ingestion (Figure 3E), but not after KE.

Plasma glucose concentrations were similar for all athletes at baseline, but increased significantly after consuming CHO (Figure 3F). Glucose fell during the first 10 min of exercise after CHO or KE, and was significantly lower after KE than either FAT or CHO intake within 5 min of exercise, remaining lower than FAT for much of the exercise protocol.

Plasma insulin concentrations were significantly elevated following CHO compared with FAT and KE (Figure 3G). Insulin concentrations peaked 10 min after the CHO drink, and fell to baseline levels after 25 min of exercise. There were no significant differences in insulin after FAT and KE intake. Gas exchange (RER) was higher on CHO, with values consistently close to unity on all arms (Table S4). There were no significant differences between FAT and KE.

Ketosis altered skeletal muscle metabolism at rest and during exercise

D-βHB and other metabolites were measured in skeletal muscle biopsies before and after cycling exercise (Supplemental Information). At rest, after KE intake, intramuscular concentrations of D-βHB were ~3 fold higher than after the ingestion of carbohydrate (CHO) or fat (FAT) (Figure 4A) and remained double the concentrations following either FAT or CHO after 1 h of exercise. Intramuscular glucose was increased pre-exercise following CHO vs. FAT and KE, but was significantly greater at the end of exercise on KE (Figure 4B). Pre-exercise muscle concentrations of the glycolytic intermediates, glyceraldehyde-3-phosphate, 2&3-phosphoglycerate and pyruvate, were significantly lower following KE consumption compared with CHO and FAT. Fructose-1,6-bisphosphate and 1,3-bisphosphoglycerate were similar at rest in all subjects (Figure 4C-G, S1).

Following exercise, concentrations of all measured muscle glycolytic intermediates were significantly lower after KE vs. CHO and FAT. The sum of glycolytic intermediates also decreased proportionately with increased intramuscular D-βHB concentration (Figure 4H). Taken together

these findings suggest that ketosis suppressed skeletal muscle glycolysis; explaining the lower blood lactate concentration described previously. Glycolytic intermediates were not different following FAT and CHO.

The drinks did not change free carnitine and acyl-carnitine concentrations before exercise, but after 1 h of high intensity exercise, free carnitine was lower and acetyl- and short chain C3-carnitines were higher following KE vs. CHO and FAT (Figure 4I, & S1), with a positive relationship between acetyl-carnitine/free carnitine ratio and D-βHB (Figure 4J). C8 and C10 carnitine derivatives were higher following the CHO drinks, whereas C16 and C18 longer chain acyl-carnitines were increased following FAT intake (Figure 4K, L & S1). The pool of TCA intermediates remained largely unaffected by substrate provision, both at rest and following exercise, albeit expanding ~2 fold with exercise. With the exception of increased oxaloacetate concentrations following FAT, and lower malate concentrations after CHO (Figure 4M&N), TCA metabolites were unchanged by altered nutritional substrate provision (Figure S1).

Branched chain amino acids (BCAA), leucine, isoleucine and valine, are mobilized during exercise as muscle energetic and anaplerotic demands increase (van Hall, van der Vusse et al. 1995). At rest, skeletal muscle BCAAs were significantly higher after FAT than CHO or KE (Figure S2A). During exercise, leucine + isoleucine increased, but were 50% lower following the ketone drink than CHO or FAT. The exercise-induced demand for anaplerotic substrates was reflected in the strong positive relationship between muscle leucine + isoleucine and muscle pyruvate (Figure S2B). Reducing glycolytic demand during exercise by increasing intramuscular D-βHB proportionately decreased leucine + isoleucine, and pyruvate (Figure S2C&D).

The effects of synergistic carbohydrate and ketone delivery on human substrate metabolism (Study 3)

The provision of carbohydrate with high ketone levels would never usually co-exist with an intact insulin axis, and is unique to this form of ketosis. In order to determine the metabolic effects of synergistic nutritional provision of KE and carbohydrate during exercise, each athlete (n=8, Table S5) completed three experimental trials consisting of 1 h of constant load cycling at 75% W_{Max} in a randomised, single-blind, cross-over design (Figure S3A). Alterations in plasma metabolites were highly reproducible, with ingestion of KE increasing D-βHB levels vs. CHO and B3 (Figure S3B) similar to Studies 1 and 2. To mimic the effects of ketone agonism of the nicotinic acid receptor (Taggart, Kero et al. 2005), but without the oxidizable carbon source, nicotinic acid (B3) was ingested as a control. Blood lactate concentrations were significantly decreased during exercise after KE+CHO vs. CHO and B3 (Figure S3C), with no differences observed between the latter. Plasma FFA concentration fell on all arms after administration of study drinks or B3. During exercise on CHO, FFA concentration rose in identical fashion to Study 2, significantly higher than KE+CHO or B3 (Figure S3D) after ~30 min, as would be expected.

Plasma glucose remained virtually unaltered by vitamin B3 consumption; however CHO and KE+CHO conditions resulted in transient decreases in plasma glucose on initiation of exercise, which returned to pre-exercising concentrations after 35-45 min (Figure S3E). Alterations in plasma glucose can be explained by the increases in plasma insulin following carbohydrate containing drinks on KE+CHO and CHO (Figure S3F). No changes in plasma insulin were observed after B3 ingestion, which remained low throughout exercise. No differences in plasma insulin concentration were observed between KE+CHO and CHO conditions. Gas exchange (RER) was similar between all three arms, with values consistently around unity (Table S6).

Synergistic substrate delivery alters human skeletal muscle metabolism

202 At rest, following KE+CHO ingestion, intramuscular concentrations of D-βHB were ~7 fold higher
203 than after the ingestion of CHO or vitamin B3 (Figure 5A), and >5 fold at the end of exercise.
204 Consumption of drinks containing carbohydrate resulted in significant increases in intramuscular
205 total hexose (carbohydrate) concentration at rest. However, following 1 h of exercise at 75% W_{Max},
206 hexose concentrations were significantly higher on KE+CHO vs. CHO or B3 reflecting preserved
207 intramuscular carbohydrate stores (Figure 5B). Average plasma lactate concentration during
208 exercise negatively correlated with end exercise intramuscular hexose (Figure 5C), whilst
209 intramuscular hexose concentrations at the end of exercise correlated positively with free carnitine
210 (Figure 5D). Intramuscular glutamine concentrations were increased on KE+CHO vs B3 and CHO
211 (Figure 5E). No correlation was found between blood D-βHB and intramuscular D-βHB (Figure
212 S4A), in keeping with selective trans-sarcolemmal transport by monocarboxylate transporters
213 (MCT) (Halestrap and Meredith 2004). A strongly positive correlation (r = 0.72, p < 0.05) was
214 found between intramuscular D-βHB concentration and intramuscular hexose at the end of
215 exercise on KE+CHO (Figure S4B).

216 *Alterations in carnitine metabolism*

217 Free carnitine concentrations were elevated on KE+CHO vs. B3 at rest and significantly greater
218 than both CHO and B3 after exercise at 75% W_{Max} for 60 min (Figure 5F). Acetyl-carnitine/free
219 carnitine ratio was elevated on KE+CHO vs. CHO or B3 at rest likely reflecting alterations in
220 acetyl-CoA/CoA ratio. After exercise however the reverse was observed with a pronounced
221 increase in ratio on CHO and B3 but not on KE+CHO where a decrease occurred (Figure 5G).
222 Commensurate with these changes, an increase in acetyl-carnitine was observed on KE+CHO at
223 rest vs. CHO and B3. However after exercise no differences in acetyl-carnitine were observed
224 between nutritional conditions (Figure 5H). Dramatic increases in C_4-OH carnitine ('keto-
225 carnitine') levels were observed following KE+CHO both at rest and after exercise (Figure 5I)

likely reflecting buffered intra-mitochondrial ketone, and a strongly positive relationship (r =0.93, p<0.01) was observed between C_4-OH-carnitine concentration and acetyl-carnitine on KE+CHO (Figure 5J).

The effect of nutritional ketosis on intramuscular fat and glycogen stores in prolonged exercise (Study 4)

Having demonstrated the actions of acute nutritional ketosis on skeletal muscle energy metabolism we sought to determine whether these changes resulted in altered intramuscular stores of fat and glycogen during prolonged (2 h) exercise (Figure 6A).

Alterations in plasma metabolites were highly reproducible between study participants (n=7, Table S7) and comparable with those in Studies 1-3. Ingestion of ketone ester increased D-βHB levels from 0.1 mM after an overnight fast to 2.2 mM (p<0.01) following KE+CHO ingestion (Figure 6B). Blood D-βHB concentration continued to slowly increase throughout exercise with regular ingestion of drinks, reaching 3.2 ± 0.2 mM after 2 h of exercise. Similar profiles in blood AcAc were observed (Figure S5A). D-βHB concentration remained unchanged on CHO throughout exercise (0.1 ± 0.05 mM, p<0.01 vs. KE+CHO). Blood lactate concentrations were significantly decreased during exercise on KE+CHO vs. CHO (Figure 6C). Plasma glucose concentrations were, on average ~1-2 mM higher on CHO following ingestion of high carbohydrate containing drinks (Figure 6D). Plasma FFA concentration fell progressively on KE+CHO over the course of the study. Similar alterations were observed on CHO at rest. However during exercise FFA concentration was significantly higher than KE+CHO after 2 h. (Figure 6E). No significant differences were observed in plasma insulin or cortisol (Figure S5B&C). In contrast to the previous studies involving shorter and higher intensity exercise, respiratory exchange ratios were consistently lower for much of the 2 h exercise duration on KE+CHO studies vs. CHO (Table S8) suggesting greater lipid oxidation.

Intramuscular triacylglycerol (IMTG) content was not significantly different between nutritional conditions at baseline. However, after 2 h of exercise at 70% $VO_{2\ Max}$ intramuscular lipids fell by 24% during KE+CHO, but only 1% on CHO ($p<0.01$) (Figure 6F). Intramuscular glycogen content was not significantly different between nutritional conditions with all athletes demonstrating a high level (dark staining) of intramuscular glycogen before exercise (Figure 6G). As expected after 2 h of exercise, glycogen concentrations fell on both arms with reductions in dark PAS staining and proportionate increases in moderate and light staining intensities. The degree of change was most marked on CHO where significantly more glycogen deposits appeared moderate, light or were no longer visible vs. KE+CHO ($p<0.05$).

The effect of nutritional ketosis on endurance exercise performance (Study 5).

Finally, to determine whether exercise performance could be altered by the metabolic changes arising from nutritional provision of carbohydrate and KE, we examined the effects of steady state exercise and time trial performance in (n=8) highly trained endurance athletes (Table S9). Study participants completed two blinded bicycle exercise trials following an overnight fast, consisting of 1 h steady state workload at 75% W_{Max} followed by a blinded 30 min time trial (TT) for maximum distance (Figure 7A, & Supplemental information). Ingestion of a drink containing 573 mg/kg body weight of KE resulted in a rapid rise in circulating D-βHB from overnight fasted levels to ~2 mM after 20 min. Ketone concentrations remained elevated throughout subsequent exercise with a fall in concentration on initiation of exercise at 75% W_{Max} workload, after which blood concentration rose reaching a new approximate steady state after 30 min, where they remained for the rest of the protocol.

In almost identical fashion to studies 3 and 4, blood lactate concentrations increased during exercise, but were ~1.5-2 mM lower on KE+CHO vs. CHO (Figure 7C). Blood glucose was raised

following ingestion of both drinks at rest, but fell during the first 10 min of exercise (Figure 7D). Glucose concentrations were lower on KE+CHO vs. CHO during the first 25 min but were similar by 1 h. KE+CHO significantly suppressed the exercise induced rise in FFA seen after 25 min of exercise vs. CHO (Figure 7E). No significant differences in gas exchange parameters were detected during the 1 h constant load exercise (Table S10)**.**

Time trial performance following 1 h of high intensity exercise was significantly improved in KE+CHO vs. CHO conditions. Athletes cycled on average 411 +/- 162 m further ($p<0.05$) over 30 min on KE+CHO vs. CHO equating to a mean performance improvement of 2%. Pooled and individual TT performances are shown in Figure 7F&G. The metabolic changes arising from altered nutritional substrate provision during exercise are summarised in Figure 7H.

Discussion:

In common with many disease conditions, the possible range of oxidizable carbon sources to power exercise becomes highly selective, favouring glucose as energetic demands increase (Romijn, Coyle et al. 1993, van Loon, Greenhaff et al. 2001). Here we show how a nutritional source of ketone bodies alters conventional muscle fuel metabolism and physical performance, alone and in combination with nutritional carbohydrates. This physiological state operates in stark contrast to that of endogenous ketosis, where replete glucose reserves, an intact insulin axis, and elevated ketone bodies would never usually coexist.

Ketosis alters the hierarchy of skeletal muscle substrate metabolism

Substrate metabolism in the normal human body is flexible, our bodies having evolved to utilize different fuel sources depending on their availability (Randle, Garland et al. 1963). During exercise, energy expenditure increases dramatically above resting levels, with rapid turnover of mobilized fuels required to keep pace with ATP demand (Spriet and Peters 1998). Usually, as exercise intensity increases, mitochondrial oxidation of fatty acids reaches a ceiling, shifting the burden of energy provision to carbohydrate so that glycolytic supply of pyruvate is the major carbon source for oxidation during heavy exercise (Romijn, Coyle et al. 1993, van Loon, Greenhaff et al. 2001). Despite the stimulation of sustained exercise here, the elevated circulating ketone concentrations significantly decreased human skeletal muscle glycolytic intermediates, including pyruvate. Remarkably, this suppression of glycolysis occurred despite physical workloads that would normally be highly glycolytic (~75% W_{Max}).

Conversely, the same exercise overrode inhibition of glycolysis by fatty acids, in agreement with evidence suggesting that the glucose-FFA cycle (Randle, Garland et al. 1963) does not operate during intense exercise. Rather, we suggest that ketone metabolism may hold hierarchical preference over carbohydrate and fat metabolism, even during conditions that strongly favour

carbohydrate oxidation, such as heavy exercise. In essence, ketosis allows substrate competition for respiration during exercise that is not observed in their absence. In support of this theory, intramuscular D-βHB and acetyl-carnitine levels were raised ~3-7 fold by KE ingestion, whilst glycolytic intermediates were decreased without altering the pool of TCA cycle metabolites during exercise. This suggests that ketones and fats were oxidized as an alternative to pyruvate, easing the reliance on glycolysis to provide acetyl-CoA to the TCA cycle. Furthermore, ketosis reduced intramuscular BCAA concentrations, supporting previous evidence that ketosis tightly regulates glycolysis (and therefore pyruvate), ultimately reducing the requirement for BCAA deamination (Thompson and Wu 1991). Such metabolic effects have a sound evolutionary basis, limiting the catabolism of carbohydrates and skeletal muscle protein for gluconeogenesis in starvation.

Taken together these findings support a mechanism whereby ketosis alters substrate signalling, oxidation, and energy transduction in working muscle, free of the confounding effects of elevated FFA, and reduced carbohydrate reserves that occur with endogenous ketosis (Phinney, Bistrian et al. 1983, Phinney, Bistrian et al. 1983).

Ketosis, the physiological replacement for glucose

We have shown here how nutritional ketosis enables equivalent physiological function to that of glucose, but *via* very different metabolic actions. Preservation of physiological function is very much in keeping with survival metabolism, where maintenance of homeostasis during conditions of altered fuel availability is vital (Cahill Jr and Owen 1968). Ample evidence during starvation (Hagenfeldt and Wahren 1971, Fery and Balasse 1983), and during high fat diets (Phinney, Bistrian et al. 1983), suggests that ketone oxidation by skeletal muscle is minimal following the transition from fed, to starvation states (Fery and Balasse 1983); conditions where glycogen is exhausted and FFA oxidation predominates. The observations that 'starved' skeletal muscle does not utilise significant quantities of ketone bodies are in contrast to our findings in this post-

absorptive (glycogen replete) state that ketone body oxidation may account for ~10-18% of the total oxygen consumption during exercise; values in close agreement with radio-isotope studies of exercising man (Balasse, Fery et al. 1978). Furthermore the permissive link between the supply of carbohydrate to sustain anaplerosis, and thus TCA flux, during rat heart perfusion with acetoacetate is well known (Russell and Taegtmeyer 1991, Russell and Taegtmeyer 1991). As such it seems that ketone bodies may 'burn in the flame of carbohydrates', whereupon the ensuing cataplerosis (and exhaustion of muscle glycogen) in 'starved' muscle may limit ketone body oxidation to preserve a circulating substrate for the brain; in keeping with evolutionary biology.

Substrate competition for respiratory oxidation

We have demonstrated how ketosis alters the hierarchy of fuel selection, restoring substrate competition for respiration where fatty acid oxidation cannot conventionally keep pace with TCA flux. In support of previous work in rodents (Sato, Kashiwaya et al. 1995, Kashiwaya, King et al. 1997) (Ruderman, Saha et al. 1999) we have provided evidence of a synergistic action between carbohydrates and ketone bodies, accentuating vital elements of the major fuel pathways known to influence muscular energy transduction. In comparison to carbohydrate consumption alone (CHO), nutritional ketosis from KE+CHO consumption dramatically increased human skeletal muscle IMTAG oxidation. Remarkably, this occurred despite highly glycolytic workloads and with increased concentrations of glucose and insulin, as both drinks contained significant quantities of carbohydrate. Conversely, feeding isocaloric carbohydrates during the same exercise demonstrated no appreciable oxidation of IMTAG. Inhibition of lipolysis via nicotinic acid receptor agonism (Taggart, Kero et al. 2005) could conceivably reduce circulating FFA availability, thus increasing IMTAG oxidation. However this seems unlikely, as providing nicotinic acid, with no oxidizable carbon source, only increased the reliance on glycolysis for energy provision in Study 3; similar to the findings of Bergstrom (Bergstrom, Hultman et al. 1969). Furthermore FFA levels

following carbohydrate and KE ingestion are suppressed, and any small (<0.1 mM) differences in circulating FFA cannot account for the magnitude of change in the intramuscular lipids observed. It is tempting to suggest that a greater capacity to oxidize fatty-acid derived carbon moieties during ketosis could power improvements in exercise capacity (as shown here) where exhaustion of glycogen reserves limit physical endurance. However further work is required to confirm this. Skeletal muscle adaptions to exercise training may have influenced the changes in substrate preference observed here, and it remains to be seen whether similar changes occur in untrained individuals.

Randle cycle revisited?

The promotion of intramuscular lipid oxidation during ketosis makes sound teleological sense; in effect signalling an 'energetic crisis' in the organism (Robinson and Williamson 1980, Newman and Verdin 2014), conserving glucose by forcing skeletal muscle to shift substrate oxidation to more ample fat reserves. Unlike glucose or fat, acetyl group production from ketone bodies is independent of both PDH and CPT transporters (Halestrap and Meredith 2004), with the increased acetyl-carnitine concentrations observed during ketosis representing an increase in acetyl-CoA production from ketones or fat, rather than glycolysis (Sato, Kashiwaya et al. 1995, Kashiwaya, King et al. 1997). As was proposed by Randle (Randle, Garland et al. 1963, Randle 1998), feedback inhibition of glycolysis by a high acetyl-CoA/CoA ratio, or $NADH/NAD^+$ ratio during ketosis could account for the observed decrease in glycolytic intermediates and preserved intramuscular carbohydrate stores; as has been reported in rodent muscle (Maizels, Ruderman et al. 1977), and heart (Williamson and Krebs 1961, Sato, Kashiwaya et al. 1995). Ketones may have improved the efficiency of either the carnitine transport of acyl-CoA or β-oxidation, resulting in greater acyl-group oxidation. KE ingestion resulted in profound differences in carnitine species, increasing free carnitine during exercise when fed with carbohydrate. As suggested by Wall *et al*

(Stephens, Constantin-Teodosiu et al. 2007, Wall, Stephens et al. 2011) (who observed an improvement in physical performance with greater free carnitine availability) the matching of TCA flux with acetyl-CoA supply may have been improved, rendering oxidative ATP production more efficient. Ketosis may also augment (or mimic) the physiological actions of carbohydrate and insulin (Kashiwaya, King et al. 1997), increasing ketone body disposal in preference to glucose or fat. Such metabolic actions suggest a plausible mechanism to allow the rapid clearance of ketone bodies on re-feeding following starvation; thus restoring conventional fuel metabolism. Similar findings have been shown during hyper-insulinaemic clamp and ketone salt infusions in man (Keller, Lustenberger et al. 1988). However the exact mechanism of how ketones promoted skeletal muscle fatty acid oxidation during conditions in which glucose is conventionally preferred, and in the presence of an intact insulin axis is unknown.

Altered athletic performance

In some ways the demands of endurance exercise parallel (albeit on much more rapid scale) the metabolic constraints pertinent to survival in starvation; placing a premium on glucose reserves and effective oxidative respiration. We have shown here the benefit of inducing ketosis, and how the combination of metabolic alterations achieved by nutritional ketosis may create a potentially advantageous physiological state, distinctly different from that of endogenous ketosis (Cahill 1970). Athletic adaptions to harness greater circulating fuels for combustion (including ketones) are well known (Johnson and Walton 1972, Winder, Baldwin et al. 1974), making athletes ideally placed to capitalize on altered substrate provision. However it remains unclear whether similar changes to those shown here can occur in untrained individuals.

In study 5 bicycle time trial performance was ~2% greater following KE+CHO vs. CHO, representing a modest increase in physical capacity in these highly trained athletes, despite

significant changes in muscular metabolism. These findings support evidence that the ceiling for human performance is not purely constrained by muscular energetics (Noakes 2011). However, ketosis may not be advantageous in physiological conditions which rely almost solely on anaerobic glycolysis, or extremely high glycolytic flux for ATP production, such as sprint or short duration exercise. Furthermore, highly glycolytic exercise may even be impaired if ketone body oxidation restricts glycolysis by negative feedback, either by an increase in $NADH/NAD^+$ or acetyl-CoA/CoA ratio. Therefore the utility of nutritional ketosis appears more suited to metabolic conditions were dysregulated substrate selection exists, and where incremental improvements in energy transduction, or carbohydrate preservation, may translate to significant increases in muscular endurance. Further work to determine the factors influencing human performance in ketosis, including the role of cerebral metabolism, is already underway.

Conclusion:

We have demonstrated the metabolic effects of elevated circulating ketone bodies as a fuel and biological signal, to create a unique physiological condition. Ketosis may reinstate the operation of substrate competition for respiration, whilst improving oxidative energy transduction under certain conditions, such as endurance exercise. Consequently, nutritional ketosis may help to unlock greater human metabolic potential.

Experimental procedures:

Subjects and screening

Endurance athletes (n = 39) participated in a series of studies investigating the effects of nutritional ketosis on resting and exercise metabolism (See supplemental information Figure S6 for diagrammatic overview). Ethics approvals for all human studies were granted in accordance with Oxfordshire Regional Ethics Committee (OXREC) and NHS national research ethics service (NRES) requirements. The trials were deemed not to constitute clinical trials, and were not registered as such. Written informed consent was obtained from all participants following an explanation of the risks associated with participation, and all testing conformed to the standards of ethical practice as outlined in the declaration of Helsinki. Prior to their inclusion, athletes completed a confidential medical questionnaire and received a resting ECG before exercise testing. Participants were asked not to perform strenuous exercise within 48 h of each test, to refrain from alcohol and caffeine for 24 h and to consume an identical pre-testing meal the night before every test. Water intake was provided *ad libitum* to each participant. In all studies comparing the effects of nutritional substrates, drink allocation was concealed and the trials were conducted in a randomized, single-blind, cross-over fashion. A double randomization method was used; the order of drink allocation was determined using a random number generator, and the order of participation was determined by participant enrolment.

General study design (Study 1)

To determine whether exercise intensity altered the metabolism of diet derived ketosis we examined the effects of steady state exercise on the clearance of blood and urinary D-βHB in six male endurance athletes (Table S2). An identical amount of KE (573 mg/kg BW) was consumed

by athletes at rest, and during 45 min of cycling exercise 40% and 75% of W_{Max} in a randomised cross over designed trial (Figure 2A) with 1 week between trials.

General study design (Study 2)

In order to compare the metabolic alterations arising from the provision of ketones as an alternative fuel during the same physical workload, male athletes (n=10) (Table S3) undertook a 3 way cross over study of fixed intensity cycling at 75% W_{Max} for 1 h. Before each test, athletes consumed a taste matched, isocaloric flavored beverage containing ≥96% of calories from carbohydrate (dextrose = CHO), KE (573 mg/kg BW), or Fat (FAT). Blood and respiratory gas samples were collected at regular intervals throughout exercise (Figure 3A). Muscle biopsy was performed before and after exercise on all participants.

General study design (Study 3)

In order to investigate the metabolic alterations arising from the synergistic combination of fuel substrates at rest and during the same physical workload, male athletes (n = 8) (Table S4) undertook a 3 way cross-over study of fixed intensity cycling at 75% W_{Max} for 1 h. Before each test, athletes consumed a taste matched, isocaloric flavored beverage containing 60% of calories from carbohydrate (dextrose), and KE (573 mg/kg BW) = KE+CHO, or a mixture of carbohydrates (CHO). On the third arm no calories were provided in the beverage, and vitamin B3 (1000 mg = B3) was ingested as a control to mimic the effects of ketone agonism of the nicotinic acid receptor, but without the oxidizable carbon source. Blood and respiratory gas samples were collected at regular intervals throughout exercise (Figure S3A). Muscle biopsy was performed before and after exercise on all participants.

General study design (study 4)

To investigate the effects of ketosis on intramuscular fuel reserves during prolonged exercise a further study of n = 7 male athletes (Table S7) undertook a 2-way cross over study of fixed intensity at 70% $VO_{2\ Max}$ for 2 h. All participants consumed a taste matched, isocaloric flavoured beverage containing 60% of calories from carbohydrate (dextrose) and 40% from KE = KE+CHO, or a mixture of carbohydrates (CHO). 50% of the total KE (573 mg/kg BW) was ingested at baseline, with the remaining 50% ingested as equal aliquots at 30 min, 1 h and 90 min during exercise. Blood and respiratory gas samples were collected at regular intervals throughout exercise (Figure 6A). Muscle biopsy was performed before and after exercise on all participants.

General study design (study 5)

To determine the effect of altered substrate metabolism on human physical performance, study participants (n=6 male, n=2 female) (Table S9) completed two blinded exercise trials following an overnight fast, consisting of 1 h steady state workload at 75% W_{Max} followed by a blinded 30 min time trial for maximum distance. Before each test, athletes consumed a drink containing either ketone and dextrose, or carbohydrates alone, in randomised order (Figure 7). Athletes completed all trials on identical bike set up dimensions (SRM training systems™, Germany), with no external stimuli. Blood and pulmonary gas measurements were collected during the first 1 h fixed workload period; however athletes were left free of distractions throughout the time trial, with a blood sample obtained immediately after the completion of the time trial. Athletes were blinded to work output, heart rate and cadence during the 30 min time trial, and only elapsed time was visible to athletes.

Baseline testing and workload prescription

All participants undertook a stepped (25 W/3 min) incremental exercise test to exhaustion on an electronically braked bicycle ergometer (Ergoline, Germany) for the determination of $VO_{2\ Max}$

(Cortex Biophysik, Germany) and W_{Max} at least 1 week prior to the start of each trial (Supplemental Information). The same ergometer was used for subsequent exercise tests.

Substrate drinks

In Studies 1 and 2, participants ingested drinks containing >96% of total calories from a single dietary fuel substrate as carbohydrate, KE, or long chain fat (Supplemental Information). In Studies 3 and 4 participants' ingested drinks containing isocaloric quantities of carbohydrate + KE, or 1:1:2 mixtures of dextrose, fructose, and maltodextrin. In both latter studies a minimum of 1.2 g/min of carbohydrate supply was ensured during exercise trials to allow comparisons according to evidence based 'optimal carbohydrate feeding strategy' (Jeukendrup and Jentjens 2000, Jentjens, Achten et al. 2004). In Studies 3-5, drinks were prepared that contained KE as 40% of calories, with the remainder made up from carbohydrate (dextrose). The dose response, determined previously (Clarke, Tchabanenko et al. 2012, Shivva, Cox et al. 2016), showed that 500 mg of KE/kg body weight produced blood D-βHB concentrations of ~3 mM after 30-60 min. All drinks were taste, color and volume matched (Supplemental Information).

Pulmonary gas exchange and blood sampling:

Respiratory gas collections (Cortex Biophysik, Germany) were obtained at identical times during exercise as blood was sampled (Supplemental Information). Blood samples (2 ml) were obtained via a venous catheter inserted percutaneously into an antecubital vein (Supplemental Information). Samples were immediately stored on ice, centrifuged (3600 rpm for 10 min) and stored at -80ºC until further analysis. Glucose, FFA, triglycerides, D-βHB and lactate were assayed using a commercial automated bench-top analyzer (ABX Pentra, France). Glycerol and insulin assays were performed using ELISA kits (Mercodia, Sweden). Acetoacetate was assayed using enzymatic methods (Bergmeyer and Gawehn 1974).

Muscle biopsy

Muscle tissue was collected using percutaneous needle biopsies from the lower third of the vastus lateralis muscle (Bard Monopty™, USA). Samples were obtained from new incisions at rest and immediately following exercise. Tissue was frozen immediately in liquid nitrogen and stored at -80 ºC until further analysis.

Metabolite extraction from skeletal muscle

Metabolites were extracted from approximately 100 mg tissue using a modified Folch method (Le Belle 2002). The aqueous and organic fractions were separated and split into 2 identical volumes to allow multiple analyses (supplemental information). Histological analyses were performed using staining and confocal microscopy methods described previously (Gollnick, Armstrong et al. 1973, Halkjaer-Kristensen and Ingemann-Hansen 1979, Koopman, Schaart et al. 2001) (Supplemental Information).

^{1}H-NMR analysis of aqueous metabolites

Half of the aqueous fraction (~25 mg wet weight tissue) was dried under nitrogen, and resuspended in 600 µL D_2O containing 0.09% w/v NaCl (Sigma), 0.01% w/v NaN_3 (Sigma) and 0.25 mM deuterated sodium-3-trimethylsilylproprionate (NaTMSP-2,2,3,3-D4, Cambridge Isotope Laboratories, USA) as a chemical shift reference. Samples were analysed on a Bruker NMR spectrometer interfaced with an 11.8 Tesla superconducting magnet at 310K using a ^{1}H-NOESY 1D pulse sequence with 128 scans. Data were integrated using fixed integral sizes of 0.02 ppm within 1D Spec Manager (v12, Advanced Chemistry Development, Canada).

Carnitine analysis

Half the aqueous fractions were combined with half the organic fraction, and 200 µL acyl-carnitine standard containing eight deuterated species was added (Cambridge Isotope Laboratories, Inc.). Samples were dried under nitrogen and butylated with 3 M butanolic-HCl (Sigma). Samples were resuspended in 200 µL acetonitrile containing 0.1% v/v formic acid (Sigma) and analysed using multiple reaction monitoring on a Waters Quattro Premiere XE triple quadrupolar mass spectrometer. Chromatograms were integrated using QuanLynx v4.1 (Waters Ltd, UK).

Statistics:

Results are expressed as means ± SEM and significance was established *a priori* at $p < 0.05$. All clinical and laboratory data were analysed for all subjects (Supplemental Information). Statistical analysis was performed using SPSS (V21, USA). For the human trials containing paired data with 3 arms, repeated measures ANOVA was performed following initial tests to ensure sphericity assumptions were not violated, and then corrected with additional post-hoc Tukey corrections for multiple comparisons where appropriate (Supplemental Information). Cycling performance results were paired comparisons containing two arms, with comparisons performed using a two tailed paired T-test. Correlations were tested using a two-tailed Pearson's test.

Contributions:

Study design: PC, TK, KC

Conducting studies: PC, TK, AS, SM, BS, SD, CH, SN, RV, MTK

Analysis: PC, TA, TK, CW, JW, BS, JG, AM, MD, SM, RE

Manuscript preparation: PC, KC

Manuscript editing: All

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Competing interests declaration:

The intellectual property, and patents covering the uses of ketone bodies and esters are owned by BTG Ltd, The University of Oxford, the NIH and TdeltaS Ltd. Should royalties ever accrue from these patents, RV, KC, AM, MTK and PC as named inventors may receive a share of royalties as determined by the terms of the respective institutions. KC is director of TdeltaS™, a spin out company of the University of Oxford, to develop and commercialize products based on the ketone ester. BS, TK, and SM are employees of TdeltaS Ltd.

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678 **Figure legends:**

679 ***Figure 1:*** **Proposed ketone ester metabolism**

680 Ingested KE is hydrolysed in the small intestine by nonspecific gut esterases (Van Gelder, Shafiee
681 et al. 2000), which cleave it into D-β-hydroxybutyrate (D-βHB) and (*R*)-1,3-butanediol. Both
682 metabolites are absorbed into the portal circulation, with butanediol undergoing first pass
683 metabolism in the liver to form D-βHB (Desrochers, David et al. 1992). D-βHB is subsequently
684 released into the circulating blood, to be transported into muscle cytosol and mitochondria via the
685 monocarboxylate transporters (MCTs) (Halestrap and Meredith 2004). Glucose is transported
686 across the sarcolemma by GLUTs, and FFAs are transported by the FAT/CD36 transporters.
687 Once inside the mitochondrial matrix, all substrates are metabolised to acetyl-CoA and oxidised
688 in the TCA cycle.

689

Figure 2: Effects of exercise on D-βHB metabolism (Study 1)

Incremental exercise intensity increases the clearance of blood ketosis (lowering blood ketone concentrations) following the consumption of identical quantities of KE.

A. Study protocol and interventions

B. D-βHB concentrations at rest and exercise at 40% and 75% W_{Max}

C. D-βHB AUC during rest and exercise at 40% and 75% W_{Max}

D. D-βHB AUC vs. oxygen consumption

E. Calculated contribution of D-βHB oxidation to total O_2 consumption in exercise.

F. Calculated oxidation of D-βHB (g/min) at 40% and 75% W_{Max}

G. D-βHB AUC vs. urinary elimination during exercise

All data are means ± SEM.

Figure 3: Effects of dietary substrates on plasma metabolites during exercise (Study 2)

Acute nutritional substrate provision before exercise resulted in significant alterations in circulating metabolite concentrations, with KE ingestion increasing blood ketone levels, whilst reducing blood lactate, and circulating fats.

A. Study protocol and interventions

B. D-βHB concentrations

C. Plasma lactate concentrations

D. Plasma FFA concentrations

E. Plasma glycerol concentrations

F. Plasma glucose concentrations

G. Plasma insulin concentrations

All data are means ± SEM. $^{\S}p<0.05$ exercise vs. resting, †$p<0.05$ KE vs. FAT, *$p<0.05$ KE vs. CHO, ‡$p<0.05$ CHO vs. FAT.

Figure 4: Metabolic effects of dietary substrates on human skeletal muscle metabolism before and after exercise (Study 2)

The effects of carbohydrate, fat and KE ingestion on skeletal muscle metabolism pre (Pre) and post (Post) cycling exercise for 1 h at 75% W_{Max}. Glycolytic and TCA cycle intermediates are expressed relative to carbohydrate (CHO).

A. D-βHB concentrations
B. Intramuscular glucose concentrations
C. Fru-1,6-P2: fructose-1,6-bisphophosphate concentrations
D. GAP: glyceraldehyde-3-phosphate concentrations
E. 1,3-BisPG: 1,3-bisphosphoglycerate concentrations
F. 2&3-PG: 2- and 3-phosphoglycerate concentrations
G. Pyruvate concentrations
H. Σ Glycolytic intermediates vs. D-βHB concentrations
I. Acetyl carnitine concentrations
J. Acetyl carnitine/free carnitine ratio vs. D-βHB concentrations
K. C8-canitine concentrations
L. C18:1 Carnitine concentrations
M. Malate concentrations
N. Oxaloacetate concentrations

†p<0.05 KE vs. FAT. *p<0.05 KE vs. CHO, ‡ p<0.05 FAT vs. CHO. All data are means ± SEM. G-6-P: glucose-6-phosphate, Fru-6-P: fructose-6-phosphate, PEP: phosphoenolpyruvate. Arbitrary units; AU, Normalised Units (NU).

Figure 5: Metabolic effects of dietary substrates on human skeletal muscle metabolism before and after exercise (Study 3)

Nutritional substrate provision significantly altered the major pathways of muscular energy transduction, with KE ingestion increasing total carbohydrate levels, and shifting the carnitine axis.

A. Intramuscular D-βHB concentrations

B. Intramuscular Hexose concentrations

C. End exercise intramuscular hexose vs. mean plasma lactate during exercise

D. End exercise intramuscular hexose vs. free carnitine

E. Intramuscular glutamine concentrations

F. Intramuscular free carnitine concentrations

G. Intramuscular acetyl-carnitine/free carnitine ratio

H. Intramuscular acetyl-carnitine concentrations

I. Intramuscular C_4-OH carnitine concentrations

J. Intramuscular C_4-OH carnitine vs. acetyl-carnitine

All data are means ± SEM. †p<0.05 KET vs. B3, *p<0.05 KET vs. CHO, ‡ p<0.05 B3 vs. CHO.

TCA: Tri-carboxylic acid cycle, PDH: Pyruvate dehydrogenase, Succ-CoA: Succinyl-CoA, CACT: Carnitine-acylcarnitine translocase, CPT I and II: carnitine palmitoyltransferase, normalised units; NU, arbitrary units; AU.

Figure 6: The effects of ketosis on intramuscular fat and carbohydrate fuel reserves during prolonged exercise (Study 4)

Combined provision of nutritional ketosis with carbohydrate ingestion increased intramuscular triacylglycerol breakdown whilst preserving muscle glycogen during sustained endurance exercise.

A. Study protocol, and interventions

B. Plasma D-βHB concentrations

C. Plasma lactate concentrations

D. Plasma glucose concentrations

E. Plasma FFA concentrations

F. Intramuscular triacylglycerol (IMTAG) levels (expressed as a % change during exercise)

G. Intramuscular glycogen (PAS stain intensity).

All data are means ± SEM. †$p<0.05$ KE+CHO vs. CHO.

Figure 7: The effects of altered fuel metabolism on human physical performance

Combined provision of nutritional ketosis with carbohydrate ingestion to fuel exercise altered fuel metabolism, and increased bicycle time-trial performance after 1 h of fatiguing exercise.

A. Study protocol, and interventions

B. Plasma D-βHB concentrations

C. Plasma lactate concentrations

D. Plasma glucose concentrations

E. Plasma FFA concentrations

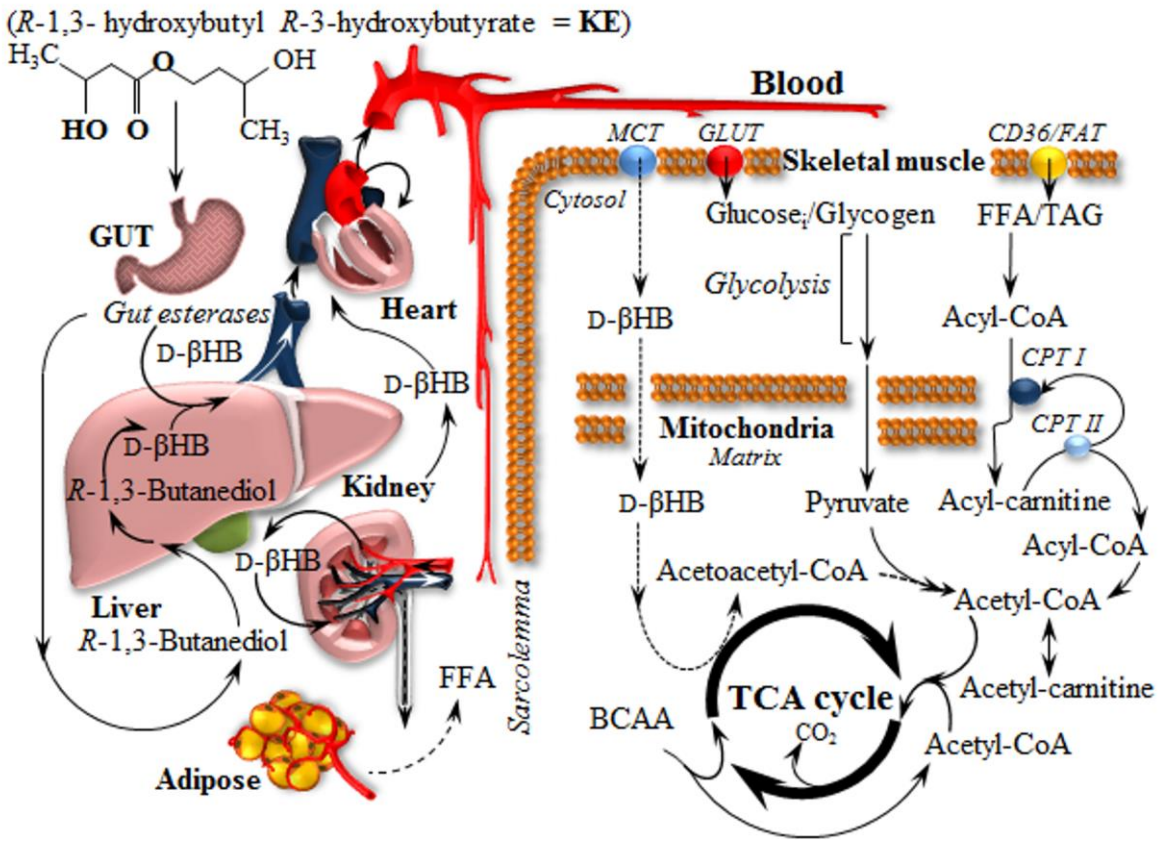
F. Time trial (TT) performance (expressed as % vs. CHO)

G. Bland Altman plot of TT performance.

H. Summary of the changes in the major fuel pathways involved in skeletal muscle energy transduction during exercise following nutritional ingestion of fat (FAT), carbohydrate (CHO) and KE.

All data are means ± SEM. †$p<0.05$ KE+CHO vs. CHO.

Figure 1


(R-1,3- hydroxybutyl R-3-hydroxybutyrate = KE)
H3C
O
OH
HO O
CH3
Blood
MCT
GLUT
CD36/FAT
Skeletal muscle
Cytosol
Glucosei/Glycogen
FFA/TAG
GUT
Glycolysis
Gut esterases
Heart
D-βHB
Acyl-CoA
CPT I
CPT II
Mitochondria
Matrix
R-1,3-Butanediol
Kidney
Pyruvate
Acyl-carnitine
Acyl-CoA
Acetoacetyl-CoA
Acetyl-CoA
Liver
R-1,3-Butanediol
FFA
Sarcolemma
TCA cycle
Acetyl-carnitine
BCAA
CO2
Acetyl-CoA
Adipose

782 **Figure 2**

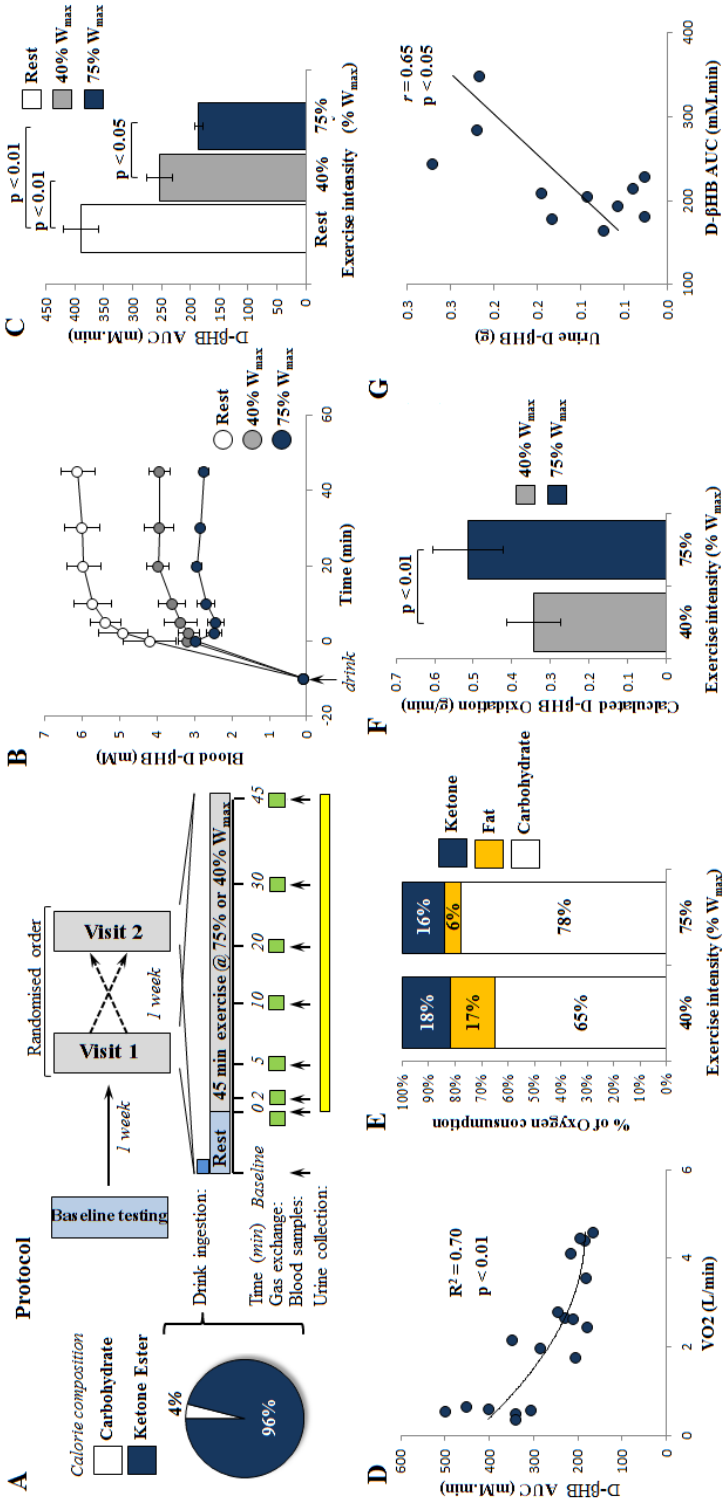
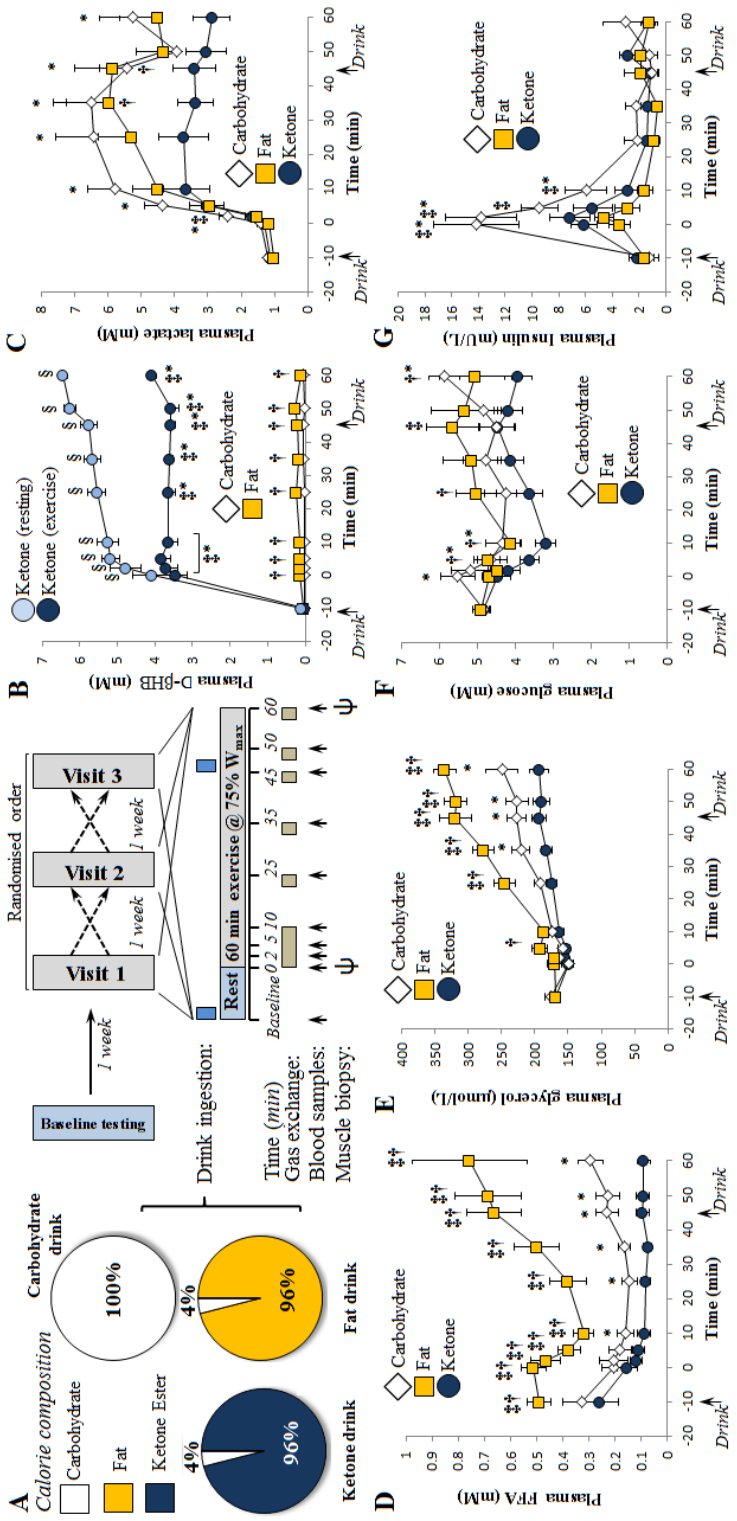
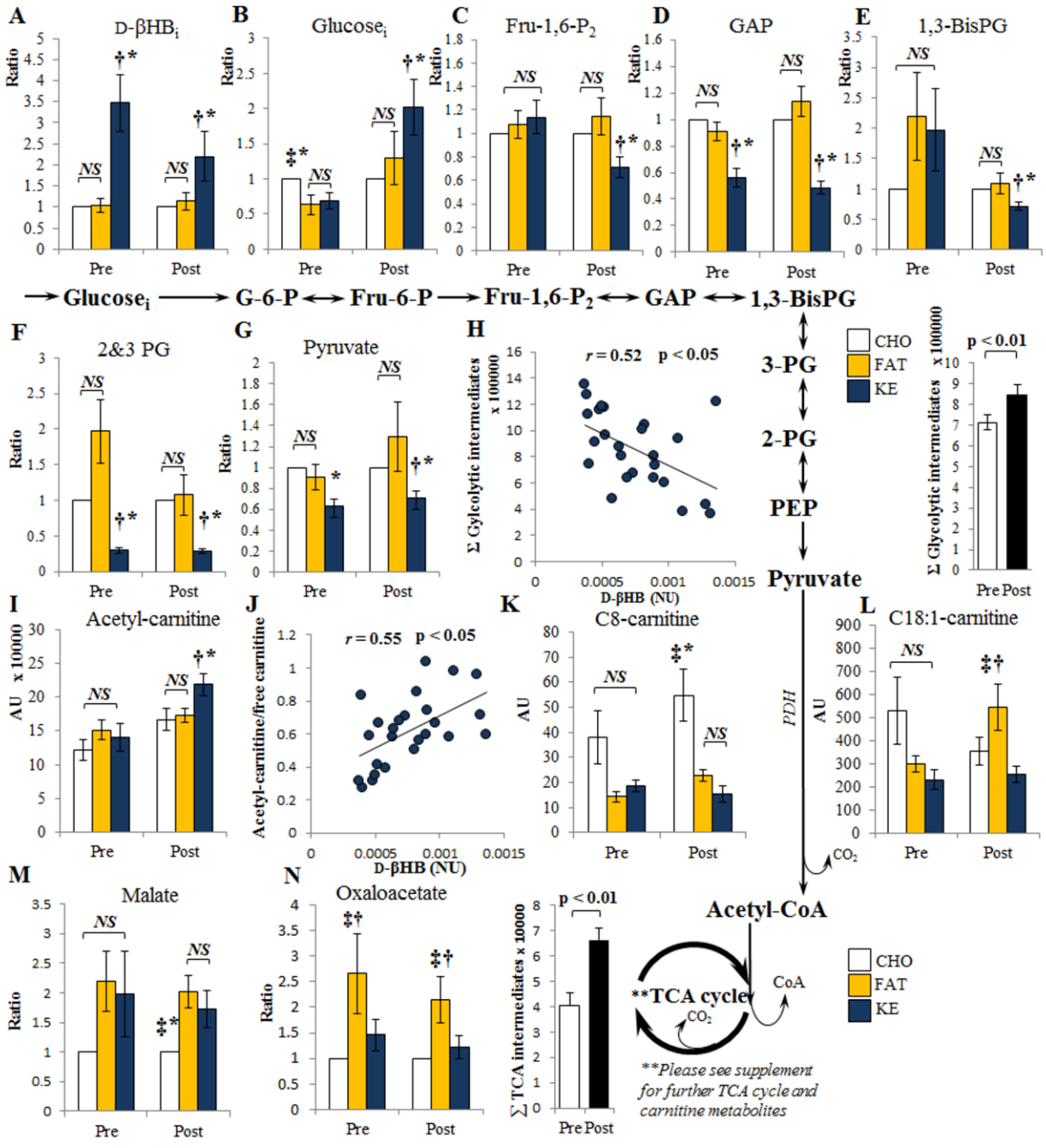

A
Protocol
Baseline testing
1 week
Randomised order
Visit 1
Visit 2
1 week
Rest
45 min exercise @ 75% or 40% Wmax
Drink ingestion:
Time (min) Baseline
Gas exchange:
Blood samples:
Urine collection:
Calorie composition
Carbohydrate
Ketone Ester
4%
96%
B
Blood D-βHB (mM)
Time (min)
drink
Rest
40% Wmax
75% Wmax
C
D-βHB AUC (mM.min)
p < 0.01
p < 0.01
p < 0.05
Rest
40%
75%
Exercise intensity (% Wmax)
D
D-βHB AUC (mM.min)
R² = 0.70
p < 0.01
VO2 (L/min)
E
% of Oxygen consumption
Ketone
Fat
Carbohydrate
18%
17%
65%
16%
6%
78%
40%
75%
Exercise intensity (% Wmax)
F
Calculated D-βHB Oxidation (g/min)
p < 0.01
40% Wmax
75% Wmax
40%
75%
Exercise intensity (% Wmax)
G
Urine D-βHB (g)
r = 0.65
p < 0.05
D-βHB AUC (mM.min)

Figure 3

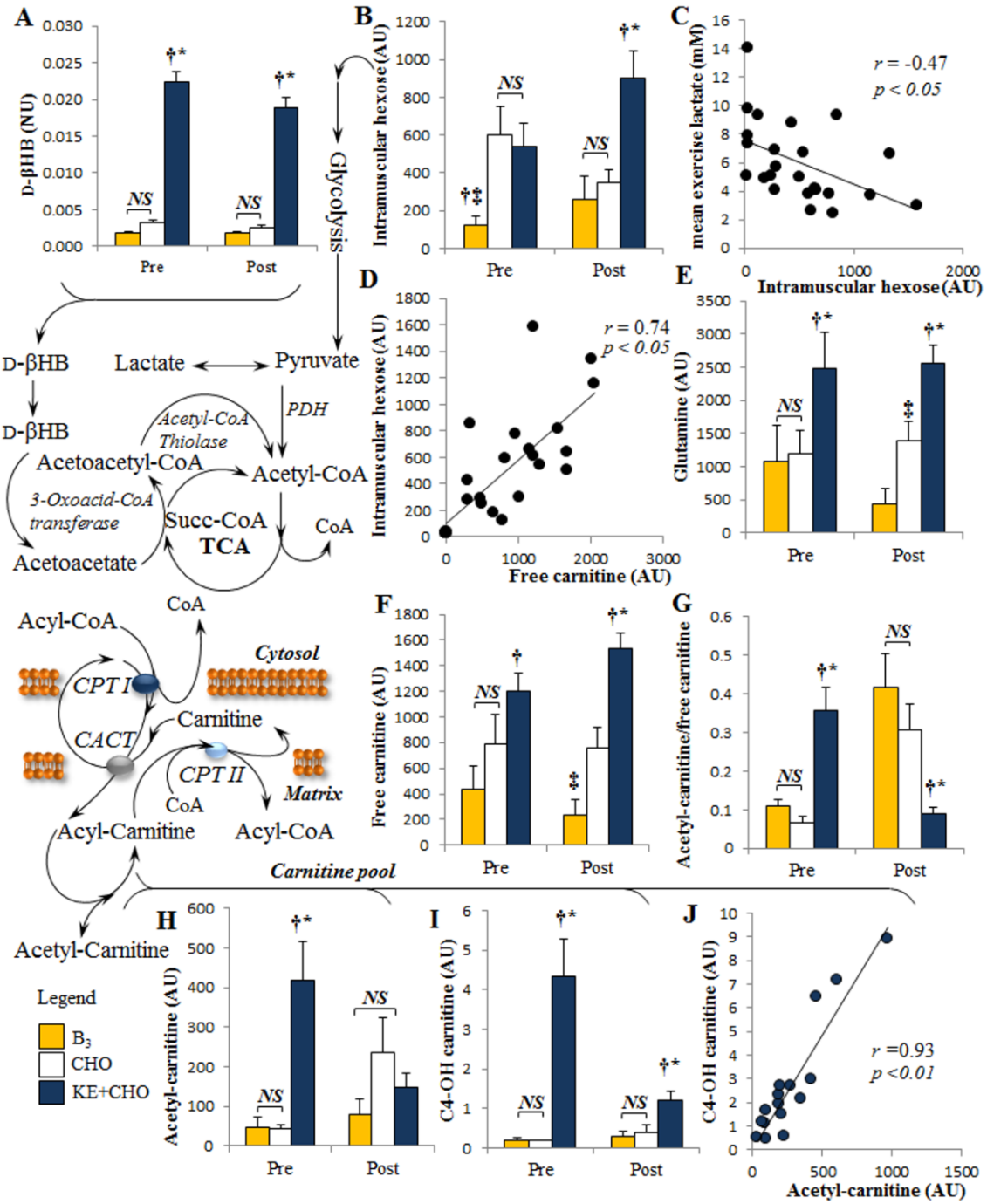

A
Calorie composition
Carbohydrate
Fat
Ketone Ester
Carbohydrate drink
100%
4%
96%
Ketone drink
4%
96%
Fat drink
Baseline testing
1 week
Randomised order
Visit 1
Visit 2
Visit 3
1 week
1 week
Drink ingestion:
Time (min)
Gas exchange:
Blood samples:
Muscle biopsy:
Rest
60 min exercise @ 75% Wmax
Baseline 0 2 5 10 25 35 45 50 60
B
Plasma D-βHB (mM)
Ketone (resting)
Ketone (exercise)
Carbohydrate
Fat
Time (min)
Drink
C
Plasma lactate (mM)
Carbohydrate
Fat
Ketone
Time (min)
Drink
D
Plasma FFA (mM)
Carbohydrate
Fat
Ketone
Time (min)
Drink
E
Plasma glycerol (μmol/L)
Carbohydrate
Fat
Ketone
Time (min)
Drink
F
Plasma glucose (mM)
Carbohydrate
Fat
Ketone
Time (min)
Drink
G
Plasma Insulin (mU/L)
Carbohydrate
Fat
Ketone
Time (min)
Drink

783 **Figure 4**



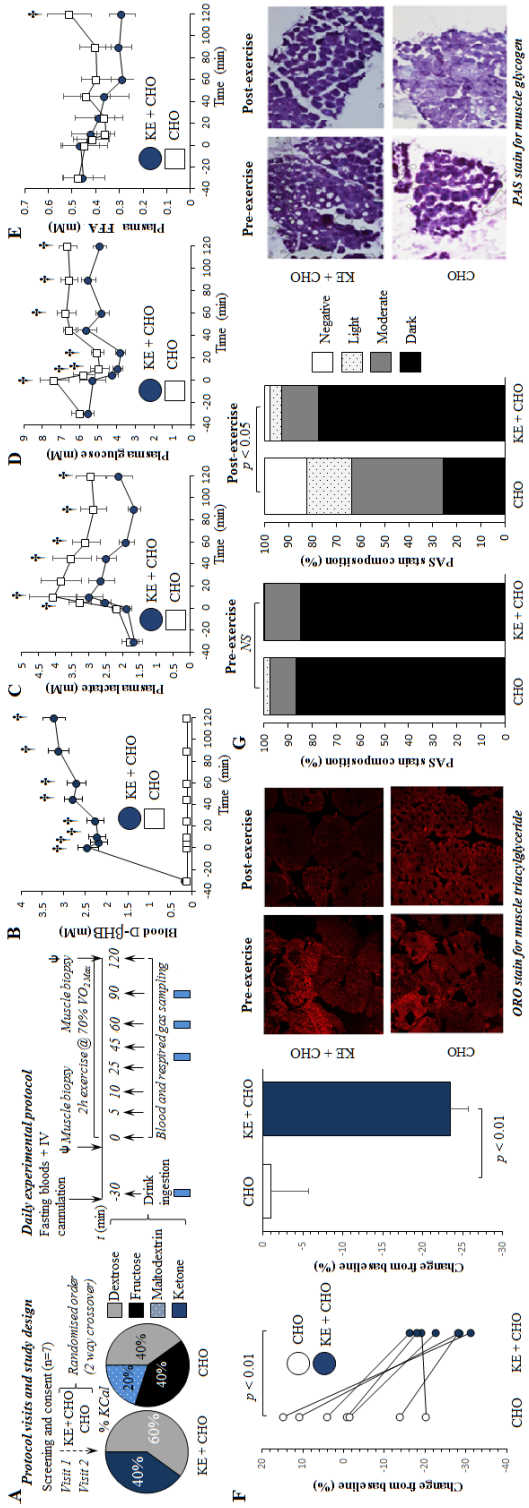
784

Figure 5

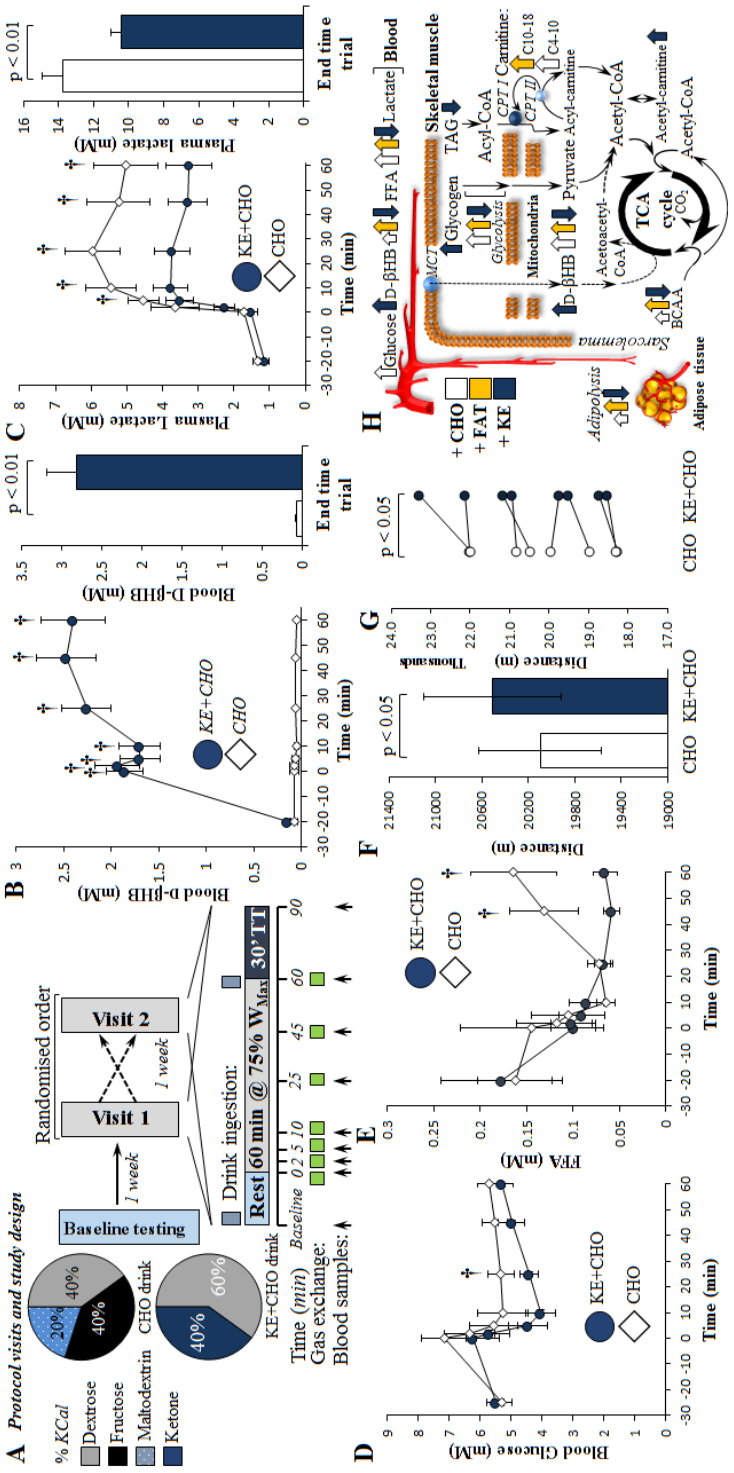

A
D-βHIB (NU)
Pre
Post
NS
†*
Glycolysis
D-βHB
Lactate
Pyruvate
PDH
Acetyl-CoA Thiolase
Acetoacetyl-CoA
Acetyl-CoA
3-Oxoacid-CoA transferase
Succ-CoA TCA
CoA
Acetoacetate
B
Intramuscular hexose (AU)
†‡
C
mean exercise lactate (mM)
r = -0.47
p < 0.05
Intramuscular hexose (AU)
D
r = 0.74
p < 0.05
Free carnitine (AU)
E
Glutamine (AU)
‡
F
Free carnitine (AU)
†
G
Acetyl-carnitine/free carnitine
Acyl-CoA
Cytosol
CPT I
CACT
Carnitine
CPT II
Matrix
Acyl-Carnitine
Carnitine pool
Acetyl-Carnitine
Legend
B3
CHO
KE+CHO
H
Acetyl-carnitine (AU)
I
C4-OH carnitine (AU)
J
r =0.93
p <0.01
Acetyl-carnitine (AU)

785

Figure 6



786 **Figure 7**


A
Protocol visits and study design
% KCal
Dextrose
Fructose
Maltodextrin
Ketone
20%
40%
40%
CHO drink
40%
60%
KE+CHO drink
Baseline testing
1 week
Randomised order
Visit 2
Visit 1
1 week
Drink ingestion:
Rest
60 min @ 75% WMax
30' TT
Time (min)
Baseline
0 2 5 10
25
45
60
90
Gas exchange:
Blood samples:
B
Blood D-βHB (mM)
KE+CHO
CHO
Time (min)
C
Plasma Lactate (mM)
KE+CHO
CHO
Time (min)
p < 0.01
End time trial
Blood D-βHB (mM)
p < 0.01
End time trial
Plasma Lactate (mM)
p < 0.01
End time trial
D
Blood Glucose (mM)
KE+CHO
CHO
Time (min)
E
FFA (mM)
KE+CHO
CHO
Time (min)
F
Distance (m)
p < 0.05
CHO
KE+CHO
G
Distance (m)
Thousands
p < 0.05
CHO
KE+CHO
H
Glucose
D-βHB
FFA
Lactate
Blood
Skeletal muscle
TAG
Glycogen
Glycolysis
Acyl-CoA
CPT I
Carnitine:
C10-18
C4-10
CPT II
Mitochondria
Pyruvate
Acyl-carnitine
Acetoacetyl-CoA
Acetyl-CoA
TCA cycle
CO2
Acetyl-carnitine
Acetyl-CoA
MCT
Sarcolemma
BCAA
Adipolysis
Adipose tissue
+ CHO
+ FAT
+ KE
p < 0.05
CHO
KE+CHO

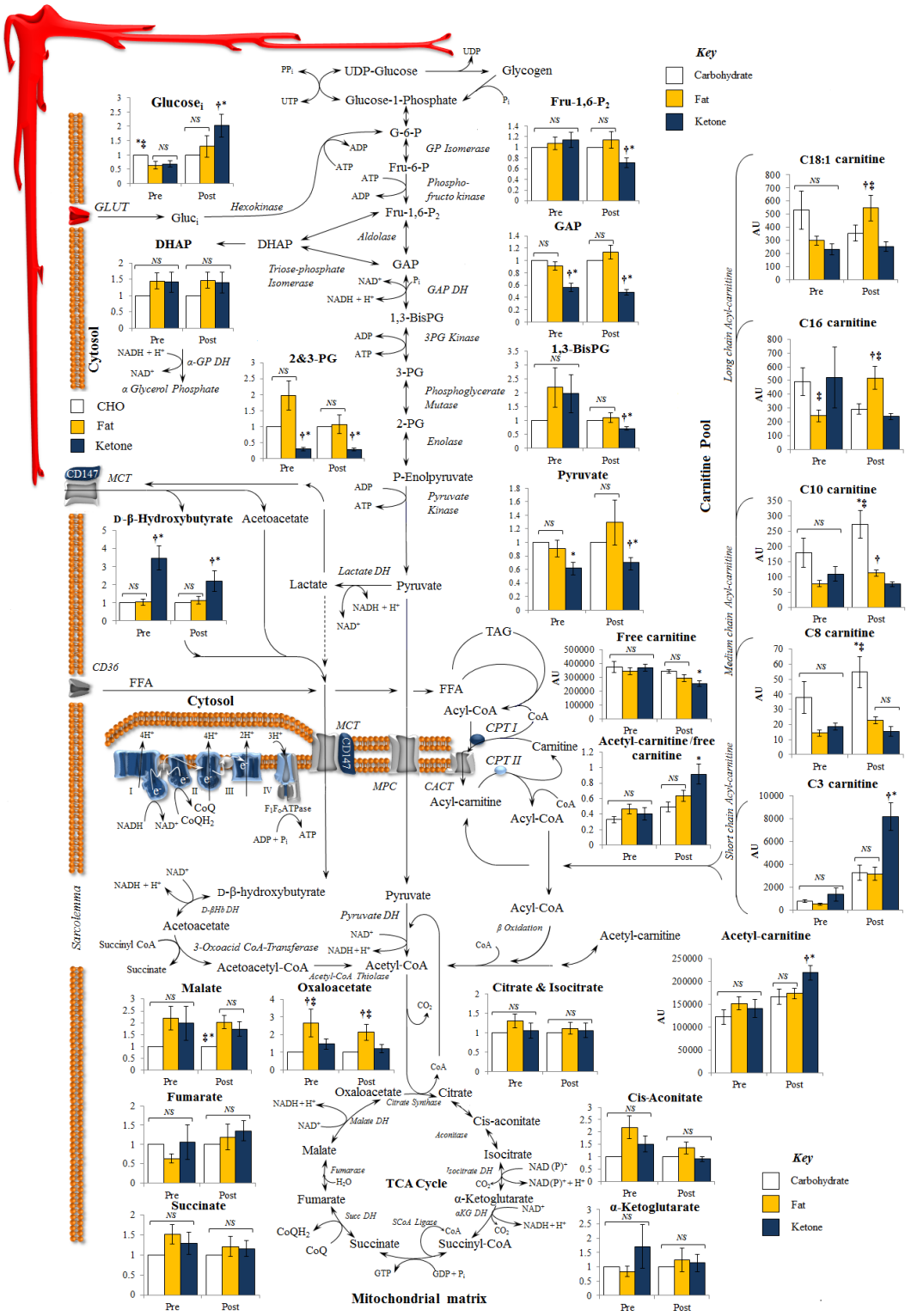
Cox et al 2016

Nutritional ketosis alters fuel preference and thereby endurance performance in athletes.

Supplemental information For: *Cell metabolism*

Supplemental figures:

Figure S1



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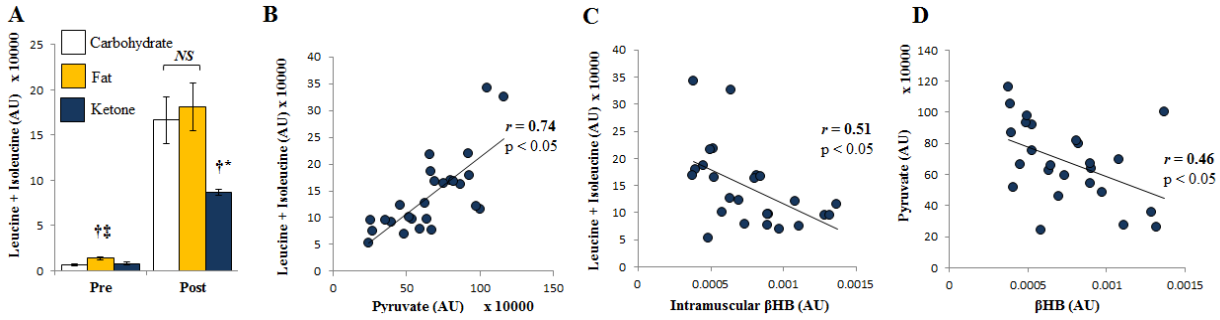
Nutritional ketosis alters fuel preference and thereby endurance performance in athletes.

Supplemental information For: *Cell metabolism*

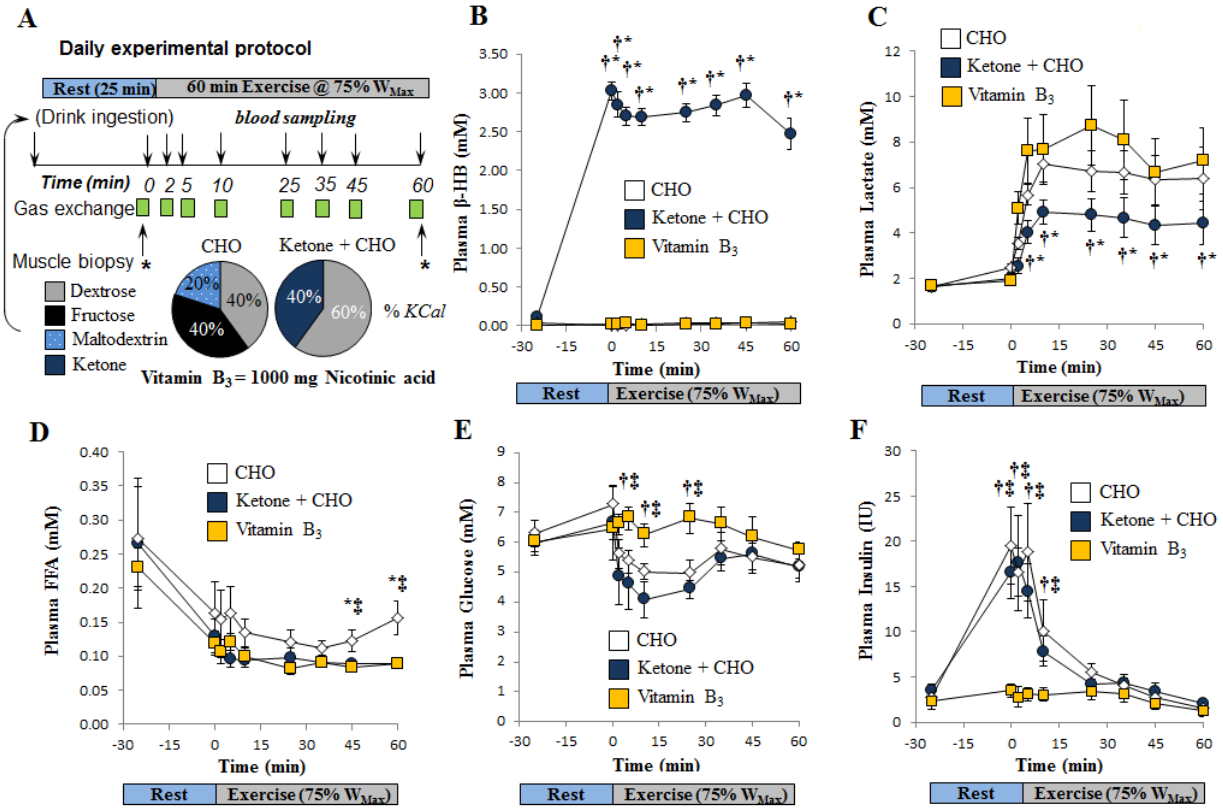
3 **Figure S2**



Nutritional ketosis alters fuel preference and thereby endurance performance in athletes.

Supplemental information For: *Cell metabolism*

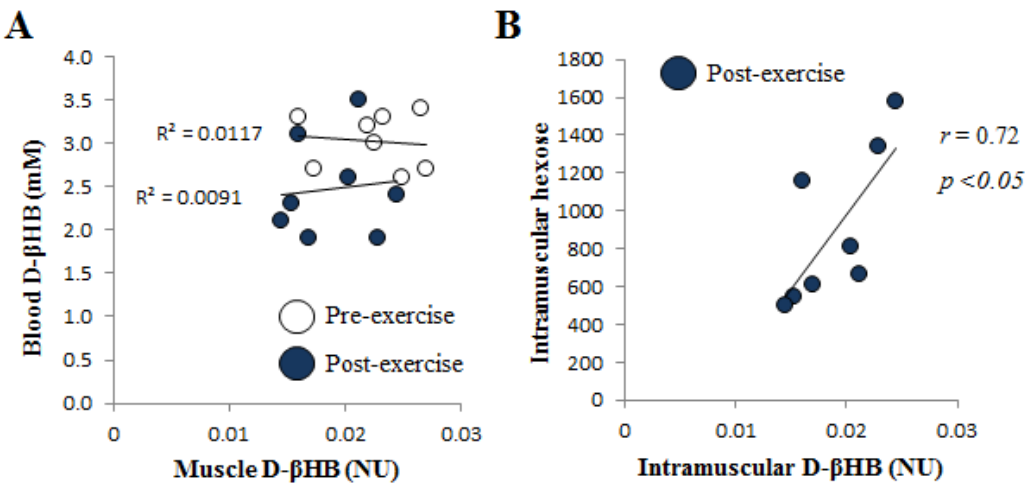
4 **Figure S3**



Nutritional ketosis alters fuel preference and thereby endurance performance in athletes.

Supplemental information For: *Cell metabolism*

Figure S4



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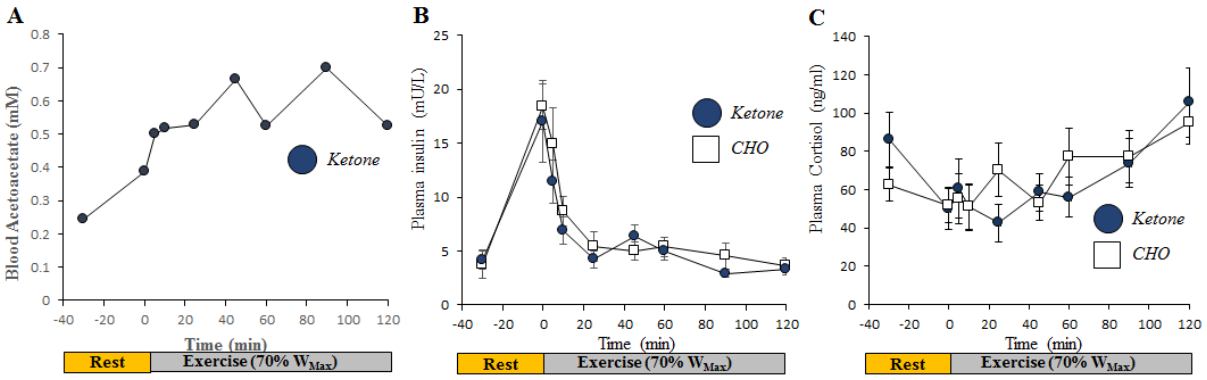
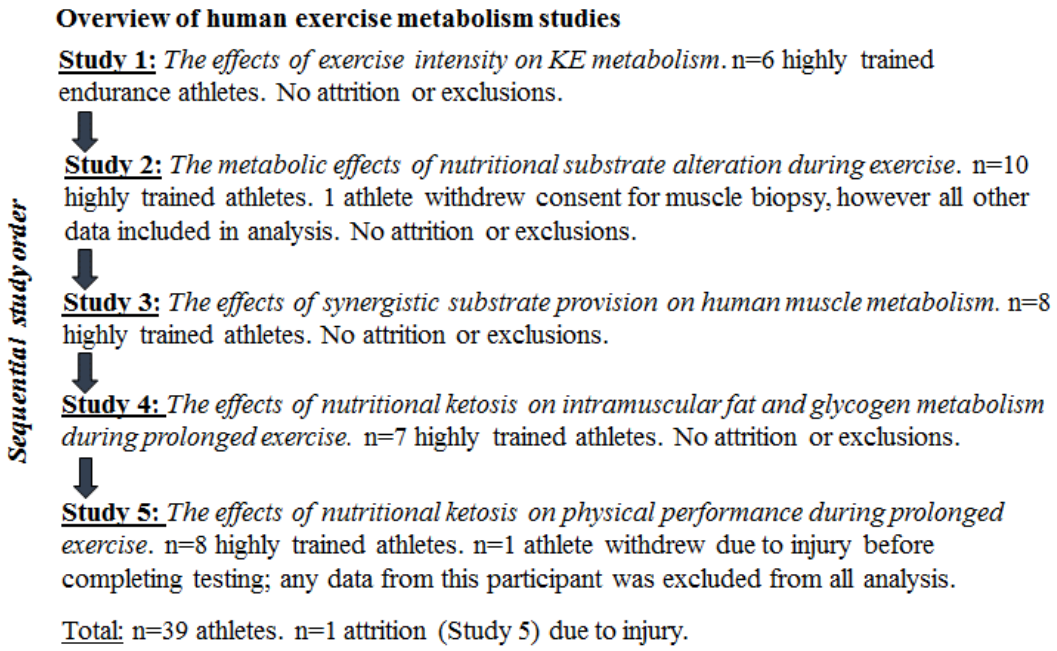
Nutritional ketosis alters fuel preference and thereby endurance performance in athletes.

Supplemental information For: *Cell metabolism*

6 **Figure S5**



7 **Figure S6**


Overview of human exercise metabolism studies
Study 1: The effects of exercise intensity on KE metabolism. n=6 highly trained endurance athletes. No attrition or exclusions.
Study 2: The metabolic effects of nutritional substrate alteration during exercise. n=10 highly trained athletes. 1 athlete withdrew consent for muscle biopsy, however all other data included in analysis. No attrition or exclusions.
Study 3: The effects of synergistic substrate provision on human muscle metabolism. n=8 highly trained athletes. No attrition or exclusions.
Study 4: The effects of nutritional ketosis on intramuscular fat and glycogen metabolism during prolonged exercise. n=7 highly trained athletes. No attrition or exclusions.
Study 5: The effects of nutritional ketosis on physical performance during prolonged exercise. n=8 highly trained athletes. n=1 athlete withdrew due to injury before completing testing; any data from this participant was excluded from all analysis.
Total: n=39 athletes. n=1 attrition (Study 5) due to injury.
Sequential study order

Supplemental tables:

Table S1. Physical properties of KE

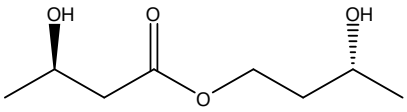
Monoester	Physical property
Chemical name	D-β-hydroxybutyrate-R 1,3-Butanediol Monoester
Molecular formula	$C_8H_{16}O_4$
Molecular weight	176
Physical structure	
Appearance	Colourless, Viscous Oil
Taste	Extremely bitter
Boiling point	145°C at 1.8 Torr
State at room temperature	Liquid
Density	1.0731 g/mL at 22°C

Table S2.

Anthropometric characteristics (Study 1)

Athlete (n=6 male)	**Mean (+/- SE)**
Age (yr)	28.8 (+/- 0.3)
Height (m)	1.89 (+/- 0.06)
Weight (kg)	89.7 (+/- 8.0)
$VO_{2\ Max}$ (L/min)	5.35 (+/- 0.3)
W_{Max} (W)	386 (+/- 19)
BMI (kg/m^2)	24.6 (+/- 0.6)

12 **Table S3**.

13 Anthropometric characteristics (Study 2)

Athlete (n=10 male)	**Mean (+/- SEM)**
Age (yr)	27.6 (+/- 1.6)
Height (m)	1.91 (+/- 0.04)
Weight (kg)	91.3 (+/- 5.2)
$VO_{2\ Max}$ (L/min)	5.35 (+/- 0.2)
W_{Max} (W)	392 (+/- 12)
BMI (kg/m^2)	24.8 (+/- 0.7)

Table S4.

Gas exchange obtained during rest and exercise in study 2

Time	VO_2 (L/min)			RER		
	KE	*CHO*	*FAT*	*KE*	*CHO*	*FAT*
Rest	0.53 ± 0.12	0.54 ± 0.14	0.51 ± 0.14	0.98 ± 0.05	1.02 ± 0.04	0.94 ± 0.06
2min	3.39 ± 0.11	3.35 ± 0.12	3.17 ± 0.15	0.88 ± 0.03	0.96 ± 0.03‡*	0.85 ± 0.03
5 min	3.72 ± 0.08	3.77 ± 0.12	3.67 ± 0.15	0.99 ± 0.02	1.03 ± 0.02‡*	0.98 ± 0.02
10 min	3.82 ± 0.09	3.88 ± 0.12	3.75 ± 0.16	0.97 ± 0.01	0.99 ± 0.01	0.96 ± 0.02
25 min	3.81 ± 0.11	3.83 ± 0.12	3.72 ± 0.13	0.96 ± 0.02	0.98 ± 0.02‡	0.94 ± 0.02
35 min	3.75 ± 0.11	3.87 ± 0.11	3.70 ± 0.16	0.95 ± 0.01	0.97 ± 0.02	0.93 ± 0.02
45 min	3.88 ± 0.13	3.95 ± 0.13	3.61 ±0.17	0.94 ± 0.01	0.95 ± 0.01	0.93 ± 0.02
50 min	3.80 ± 0.12	3.82 ± 0.13	3.77 ± 0.16	0.93 ± 0.01	0.95 ± 0.01‡	0.90 ± 0.02
60 min	3.87 ± 0.14	4.05 ± 0.14	3.85 ± 0.12	0.95 ± 0.02	0.95 ± 0.01	0.91 ± 0.02

Data are expressed as mean ± SEM. ‡ $p<0.05$ fat vs. carbohydrate and * $p < 0.05$ KE vs. carbohydrate.

Table S5

Anthropometric characteristics (Study 3)

Athlete (n=8 male)	Mean (+/- SE)
Age (yr)	27.4 (+/- 1.0)
Height (m)	1.85 (+/- 0.04)
Weight (kg)	79.8 (+/- 4.7)
$VO_{2\ Max}$ (L/min)	5.15 (+/- 0.2)
W_{Max} (W)	387 (+/- 19)
BMI (kg/m^2)	23.1 (+/- 0.6)

Table S6.

Gas exchange data obtained during rest and exercise in Study 3

Time	VO_2 (L/min)			RER		
	KE+CHO	*CHO*	*B3*	*KE+CHO*	*CHO*	*B3*
Rest	0.59 +/-0.06	0.56 +/-0.05	0.61 +/-0.06	1.00 +/- 0.03	1.04 +/- 0.04	0.93 +/- 0.06
2min	3.89 +/- 0.16	3.89 +/- 0.15	3.76 +/- 0.18	0.99 +/- 0.03	1.04 +/- 0.03*	1.09 +/- 0.04†
5 min	4.07 +/- 0.14	4.04 +/- 0.16	4.06 +/- 0.19	0.99 +/- 0.02	1.01 +/- 0.02	1.03 +/- 0.02
10 min	4.10 +/- 0.16	4.06 +/- 0.13	4.06 +/- 0.16	0.96 +/- 0.01	0.99 +/- 0.01	1.01 +/- 0.02
25 min	4.05 +/- 0.13	4.19 +/- 0.16	4.00 +/- 0.15	0.97 +/- 0.02	0.99 +/- 0.02	1.02 +/- 0.02
35 min	4.10 +/- 0.13	4.18 +/- 0.16	4.05 +/- 0.17	0.96 +/- 0.02	0.97 +/- 0.02	1.01 +/- 0.03
45 min	4.18 +/- 0.13	4.20 +/- 0.16	4.08 +/- 0.17	0.96 +/- 0.02	0.96 +/- 0.01	1.00 +/- 0.04
60 min	4.21 +/- 0.12	4.22 +/- 0.16	4.20 +/- 0.20	0.96 +/- 0.02	0.97 +/- 0.02	1.00 +/- 0.03

Data expressed as mean +/- SEM. † $p < 0.05$ between KE+CHO and B3.* $p < 0.05$ between KE+CHO and CHO**.**

Table S7.

Anthropometric characteristics (Study 4)

Athlete (n=7 male)	**Mean (+/- SE)**
Age (yr)	29.4 (+/- 1.0)
Height (m)	1.85 (+/- 0.04)
Weight (kg)	79.8 (+/- 4.7)
$VO_{2\ Max}$ (L/min)	4.85 (+/- 0.2)
W_{Max} (W)	387 (+/- 19)
BMI (kg/m^2)	23.1 (+/- 0.6)

Table S8. Gas exchange data obtained during exercise in Study 4

Time	VO_2 (L/min)		RER	
	KE+CHO	*CHO*	*KE+CHO*	*CHO*
Rest	0.56 +/- 0.08	0.57 +/- 0.07	0.98 +/- 0.03	0.98 +/- 0.05
5min	3.49 +/- 0.17	3.34 +/- 0.14	0.94 +/- 0.03	0.99 +/- 0.02
10 min	3.38 +/- 0.17	3.32 +/- 0.18	0.94 +/- 0.01†	0.99 +/- 0.02
25 min	3.45 +/- 0.17	3.28 +/- 0.19	0.93 +/- 0.01†	0.98 +/- 0.01
45 min	3.49 +/- 0.19	3.35 +/- 0.19	0.91 +/- 0.01†	0.96 +/- 0.01
60 min	3.51 +/- 0.13	3.35 +/- 0.20	0.91 +/- 0.01†	0.98+/- 0.01
90 min	3.54 +/- 0.19	3.46 +/- 0.20	0.92 +/- 0.01†	0.98 +/- 0.01
120 min	3.63 +/- 0.19	3.49 +/- 0.20	0.92 +/- 0.01	0.96 +/- 0.01

Data expressed as mean +/- SEM. († $p < 0.05$).

28 **Table S9**. Anthropometric characteristics (Study 5)

Athlete (n=6 male, n=2 female)	Mean (+/- SE)
Age	29.4 (+/- 1.0)
Height (m)	1.87 (+/- 0.04)
Weight (Kg)	84.9 (+/- 5.2)
$VO_{2\ Max}$ (L/min) male	5.37 (+/- 0.3)
$VO_{2\ Max}$ (L/min) female	3.30 (+/- 0.1)
W_{max} (W) male	404 (+/- 12)
W_{max} (W) female	282 (+/- 14)
BMI (kg/m^2)	24.3 (+/- 0.9)

29 **Table S10**. Gas exchange data obtained during exercise in Study 5

Time	VO_2 (L/min)		RER	
	KE+CHO	*CHO*	*KE+CHO*	*CHO*
Rest	0.52 +/- 0.05	0.58 +/- 0.07	1.00 +/- 0.04	1.02 +/- 0.06
2min	3.63 +/- 0.27	3.54 +/- 0.24	1.05 +/- 0.03	1.05 +/- 0.02
5 min	3.76 +/- 0.27	3.93 +/- 0.28	1.02 +/- 0.01	1.03 +/- 0.02
10 min	3.91 +/- 0.27	3.98 +/- 0.29	1.02 +/- 0.01	1.00 +/- 0.01
25 min	3.99 +/- 0.29	3.95 +/- 0.29	1.00 +/- 0.01	1.01 +/- 0.01
45 min	4.11 +/- 0.33	4.04 +/- 0.27	0.98 +/- 0.01	0.99 +/- 0.01
60 min	4.08 +/- 0.29	4.09 +/- 0.27	0.98 +/- 0.01	0.98 +/- 0.01

30

Nutritional ketosis alters fuel preference and thereby endurance performance in athletes.

Supplemental information For: *Cell metabolism*

Supplemental figure legends:

Figure S1: The effects of dietary substrate alteration on skeletal muscle fuel metabolism (Study 2)

The effects of carbohydrate, fat and ketone drinks on skeletal muscle metabolism pre (Pre) and post (Post) cycling exercise for 1 h at 75% W_{Max}. Glycolytic and TCA cycle intermediates are expressed relative to carbohydrate (CHO), except carnitine values are relative to each other. All data are means ± SEM. †$p<0.05$ KE vs. FAT. *$p<0.05$ KE vs. CHO, ‡ $p<0.05$ FAT vs. CHO.

$Gluc_i$: intracellular glucose, G-6-P: glucose-6-phosphate, Fru-6-P: fructose-6-phosphate, Fru-1,6-P2: fructose-1,6-bisphophosphate, DHAP: dihydroxyacetone phosphate, GAP: glyceraldehyde-3-phosphate, 1,3-BisPG: 1,3-bisphosphoglycerate, 2&3-PG: 2- and 3-phosphoglycerate, P-Enolpyruvate: phosphoenolpyruvate, GP-Isomerase: glucose phosphate isomerase, LDH: lactate dehydrogenase, MCT : monocarboxylate transporter, CPT: carnitine palmitoyltransferase, βHBDH: D-β-hydroxybutyrate dehydrogenase, αKG-DH: α-ketoglutarate dehydrogenase. Arbitrary units; AU.

Figure S2: The effects of dietary fuels on skeletal muscle BCAA and relationships to intramuscular ketone bodies (Study 2)

A. Intramuscular isoleucine + leucine before (Pre), and after exercise (Post)

B. Intramuscular isoleucine + leucine vs. intramuscular pyruvate

C. Intramuscular isoleucine + leucine vs. intramuscular D-βHB concentrations

D. Intramuscular pyruvate vs. D-βHB concentrations

All data are means ± SEM. †$p<0.05$ KE vs. FAT, *$p<0.05$ CHO vs. KE, ‡ $p<0.05$ CHO vs. FAT

AU (Arbitrary units).

Figure S3: Effects of dietary substrates on plasma metabolites during exercise (Study 3)

E. Study protocol, showing baseline testing, cross-over design, cycling exercise with study interventions, and substrate drink calorie composition (% Kcal)

F. D-βHB concentrations

G. Plasma lactate concentrations

H. Plasma FFA concentrations

I. Plasma glucose concentrations

J. Plasma Insulin concentrations

All data are means ± SEM. †$p<0.05$ KE+CHO vs. B3, *$p<0.05$ KE+CHO vs. CHO, ‡ $p<0.05$ B3 vs. CHO.

Figure S4: Skeletal muscle relationships to intramuscular and circulating ketone bodies (Study 3)

A. Blood D-βHB vs. intramuscular D-βHB concentrations

B. Intramuscular hexose vs. intramuscular D-βHB concentrations.

NU (Normalised units).

Figure S5: Plasma metabolites (Study 4)

A. Plasma acetoacetate concentrations

B. Plasma insulin concentrations

C. Plasma cortisol concentrations

68 ***Figure S6: Study overview diagram***

Supplemental experimental procedures:

Effect of exercise intensity on the metabolism of D-βHB (Study 1):

Six high performance athletes from endurance sports were recruited to take part in this study (anthropometric characteristics Table S2). All participants undertook a stepped (25 W/3 min) incremental exercise test to exhaustion on an electronically braked bicycle ergometer (Ergoline, Germany) for the determination of $VO_{2\,Max}$ and W_{Max} at least 1 week prior to the first trial. The same ergometer was used for subsequent exercise tests, which were completed by all athletes.

Drink preparation:

All athletes consumed a drink containing 96% of ketone ester as total calories before each trial, and dosed at 573mg/kg body weight for each athlete.

Urine collection:

Athletes were asked to completely empty their bladder immediately prior to the ingestion of KE, and once again immediately after the completion of the 45 min exercise period. Urine was collected into 3 L containers (Simport Plastics Ltd, Beloeil, Canada). Total urine volume was measured and 2 ml aliquots frozen at -80ºC until further analysis. Urinary D-βHB was assayed using a commercial automated bench-top analyzer (ABX Pentra, Montpellier, France). Total D-βHB elimination was calculated as n (mol) = C x V where C represents mol. concentration of D-βHB, and V the total volume of urine collected.

Indirect calorimetry and corrections for ketone oxidation:

Total substrate oxidation equations derived from indirect calorimetry do not account for the oxidation of ketone bodies under conventional conditions. Therefore corrections to the calculated total substrate oxidation rates during ketosis were undertaken according to the methods described by Frayn (Frayn 1983). Briefly, differences in blood D-βHB AUC calculated at rest, and during exercise conditions were used to determine the oxidation (mol) of circulating D-βHB, assuming a volume of distribution of 0.2 L/kg body weight (Beylot, Beaufrere et al. 1986). Total oxygen consumption, and carbon dioxide production resulting from ketone oxidation were therefore adjusted assuming 1 mol of gas occupies a volume of 22.4 L, and the stoichiometry of D-βHB oxidation requires 4.5 mol of oxygen per mol utilised as fuel (Frayn 1983). Conventional substrate oxidation equations (Frayn 1983, Peronnet and Massicotte 1991) were subsequently used to determine relative contributions of fat and carbohydrate to total oxygen consumption.

The metabolic effects of nutritional substrate alteration during exercise (Study 2):

Ten male high performance athletes from endurance sports were recruited to take part in this study (anthropomorphic characteristics in Table S3). One athlete withdrew consent for muscle biopsy, but all remaining collected data were included in the analyses.

Drink preparation:

Drinks were isocaloric in energy (mean calorific value 337 ± 15 Kcal) and taste matched using sweeteners (Neotame™, NutraSweet, USA) or bitter additive (Symrise, product number 648352, UK) to ensure blinding. Substrate calories were bodyweight-adjusted, and dosed to ensure a minimum carbohydrate delivery of 1.2 g/min of exercise (Jeukendrup 2004, Jeukendrup 2008) on the carbohydrate arm. Drinks were made up from commercially available sports water (Glaceau, UK), and matched for tonicity (13% solutions for all arms). All drinks contained a minimum of 96% of their energy from a sole substrate, as carbohydrate (maltodextrin:fructose, 5:1, Gu Gels, Berkeley, USA), long chain triglyceride (Calogen™, Zoetermeer, Netherlands), or KE. Subjects ingested 75% of each drink (total volume 393 ± 23 ml) 15 min prior to the start of exercise, over a 5 min time interval. At 45 min, athletes paused for 1 min to ingest the remaining 25% of the drink as a 'top up' for the final 15 min of exercise. During the study, subjects were allowed water *ad libitum.*

Standardised diets:

All athletes completed a 3 day diet diary in a representative training week to determine their individual average calorie intake. This calorie requirement was then used to determine meal plans overseen by a study dietician, with athletes instructed to adhere to the same diet/calorie intake for 24 hours before each study visit. Prior to the FAT trial, athletes consumed an isocaloric high fat low carbohydrate diet for 24 hours instead of their habitual diet in order to raise circulating FFA. Athletes received individual meal plans, and consumed diets consisting of approximately (in %kcals)

70% fat, 25% protein, and ~5% carbohydrate similar to previous work from our laboratory (Edwards, Murray et al. 2011).

The effects of synergistic carbohydrate and ketone delivery on human substrate metabolism (Study 3):

In order to investigate the metabolic alterations arising from the synergistic combination of fuel substrates at rest and during the same physical workload, male athletes (n = 8) (Table S5) undertook a 3 way cross-over study of fixed intensity cycling at 75% W_{max} for 1 h.
Dink preparation: In all studies a minimum of 1.2 g/min of carbohydrate supply was ensured so as not to feed isocaloric carbohydrates at a 'disadvantage' according to evidence based nutritional guidance for exercise performance. Drinks were prepared that contained *(R)*-3-hydroxybutyl *(R)*-3-hydroxybutyrate as 40% of calories, with the remainder made up from carbohydrate (dextrose). Additional matched calories from either ketone, or 'multiple transportable carbohydrates' were provided to allow comparisons according to evidence based 'optimal feeding strategy'. In order to mimic the physiological action of ketone bodies on the nicotinic acid receptor, but not to provide an oxidisable fuel source for muscle, participants ingested 1000 mg of nicotinic acid (vitamin B3) as a control. Calorie, tonicity and taste matching was performed as previously described in Study 2.

The effect of nutritional ketosis on intramuscular fat and glycogen oxidation in prolonged exercise (Study 4)

To investigate the effects of ketosis on intramuscular fuel reserves during prolonged exercise a further study of n = 7 male athletes (Table S7) undertook a 2-way cross over study of fixed intensity at 70% $VO_{2\ max}$ for 2 h.
Dink preparation: Identical composition drinks to study 3 were ingested by participants, with drinks were prepared that contained KE (*(R)*-3-hydroxybutyl *(R)*-3-hydroxybutyrate) as 40% of calories, with the remainder made up from carbohydrate (dextrose). On the CHO arm, calories derived from 'multiple transportable carbohydrates' were provided as 1:1:2 mixtures of dextrose, fructose, and maltodextrin. 50% of the total drink (573 mg/kg BW) was ingested at baseline, with the remaining 50% ingested as equal aliquots at 30 min, 60 min and 90 min during the exercise trial. This method of administering nutritional calories was chosen to maximize the contributions of ingested carbohydrate to energy production.
Calorie, tonicity and taste matching was performed as previously described in Study 2.

The effect of nutritional ketosis on human physical performance during prolonged exercise (Study 5).

To determine the effect of altered nutritional substrate metabolism on human physical performance, study participants (n=6 male, n=2 female)) (Table S9) completed two blinded exercise trials following an overnight fast, consisting of 60 min steady state workload at 75% W_{Max} followed by a blinded 30 min time trial (TT) for maximum distance. Before each test, athletes consumed a drink containing either ketone and dextrose, or carbohydrates alone, in randomised order. Athletes completed all trials on identical bike set up dimensions (SRM training systems, Germany), with no external stimuli. Blood and pulmonary gas measurements were collected during the first 60 min fixed workload period; however athletes were left free of distractions throughout the time trial, with a blood sample obtained immediately after the completion of the time trial. Athletes were blinded to work output, heart rate and cadence during the 30 min time trial, and only elapsed time was visible to athletes. No muscle biopsy was performed in this study.
Dink preparation: Identical composition drinks to study 3 and 4 were ingested by participants. Drinks were prepared that contained KE (*(R)*-3-hydroxybutyl *(R)*-3-hydroxybutyrate) as 40% of calories, with the remainder made up from carbohydrate (dextrose). On the CHO arm, calories derived from 'multiple transportable carbohydrates' were provided as 1:1:2 mixtures of dextrose, fructose, and maltodextrin. 50% of the total drink (573 mg/kg BW) was ingested at baseline, with the remaining 50% ingested as equal aliquots at 30 min, and before the TT at 60 min.
Calorie, tonicity and taste matching was performed as previously described in Study 2.

Participants and eligibility

Target populations for all studies were highly trained elite and sub-elite athletes currently participating in endurance sports requiring a large aerobic training base. All athletes were recruited from professional/semi-professional sporting clubs in the UK. Only athletes who were at a steady state phase of training were eligible for inclusion, so as not to be

influenced by early or late season shifts in specific conditioning. All study participants were tested within the same macrocycle of training and at the same time within a training week. Participants presented following an overnight fast and testing was at the same time of day (starting at 8 am) to reduce the effect of diurnal patterns on subsequent measurements. All participants were healthy non-smokers, not on medication and with no history of major illness. Testing was conducted at the John Radcliffe hospital, Oxford, UK.

Eligibility criteria

- Age 18-40 years
- Currently undertaking aerobic endurance training > 12 h per week for the past 3 months
- Physical injury and illness free
- Elite or sub elite performers in the endurance sports of rowing, cycling, or triathlon currently resident in the UK.

Exclusion criteria

- Any previous history of cardiovascular, neuromuscular, endocrine or neurological illness, or any other medical condition requiring medication.
- Smoking (of any description)
- Pregnancy or current breastfeeding, or female athletes not taking the oral contraceptive pill (due to the variability in hormonal patterns and substrate levels with different parts of the menstrual cycle)
- Inability or loss of ability to give informed consent
- Alcohol in the 24 hours prior to any study visit

Body composition analyses were performed using a bio-impedance Bodystat® 1500 Body Composition Analyser (Douglas, Isle of Man). Anthropometric characteristics for each study (1-5) are shown.

Determination of maximal workload on bicycle ergometer

Baseline testing to determine workloads for subsequent prescriptive steady state exercise, and to characterise study populations was performed on a stationary bicycle ergometer (Ergoline, Germany) following personalised saddle, and handlebar adjustments. Subjects commenced all tests in the post-absorptive state following an overnight fast of 8-12 hours, and were allowed water *ad libitum* prior to the start of the test to ensure adequate hydration. No standardised warm up was undertaken; instead workloads were initiated at 100 watts (low intensity) to allow a controlled progressive lead in to the maximal test.
Wattage was fixed in the rpm independent mode of the ergometer for all testing to ensure constant workloads, and increased by 25-35 watts every 3 min until volitional fatigue, with maximal workload (W_{max}) calculated according to the formula:

$$W_{Max} = \text{Watts}_{\text{Step N-1}} + (\text{time elapsed}/180\text{ s} \times \text{Wattage Increment})$$

Maximal oxygen uptake was defined as the average value of oxygen consumed at peak effort over 20 seconds. Furthermore, the attainment of two of the following criteria was required:

1. VO_2 did not increase with greater intensity, resulting in a plateau showing less than a 0.2 L/min increase in oxygen uptake.
2. Heart rate within 10 beats per min of age predicted maximum (220- age)
3. Respiratory exchange ratio greater than 1.10
4. Physical exhaustion

$VO_{2\,max}$ was in all cases expressed as volume in L/min.

Pulmonary gas exchange and blood sampling:

Pulmonary gas exchange analysis was used to quantify volumes of oxygen and carbon dioxide via indirect calorimetry. All data were collected in real time, and displayed online (Metasoft®, V7.9.1, Germany) on a dedicated study

computer connected to the indirect calorimetry system (Metalyzer 3BR2, Cortex, Germany). Breath by breath data were subsequently time weighted into 10 second averages, and pooled to calculate a grand average over this interval.

Calibrations and apparatus

Prior to every use, thorough calibration of the indirect calorimetry system was performed. Two point calibrations for gas concentration (ambient air vs. standardised calibrant gas, 17% O_2/5% CO_2) were performed before every test and adjusted for barometric pressure. A known 3L volume syringe (Hans Rudolph, Germany) was used to calibrate gas flow through the pneumotachograph turbine. Real-time changes in temperature were adjusted automatically by a housed temperature sensor within the pneumotachograph casing. The pneumotachograph and gas sampling assembly was attached to snug fitting face masks (Hans Rudolph V2®, Cranlea, UK) sized appropriately for each participant.

Respiratory gas collections (Metalyser 3BR2, Cortex Biophysik), obtained at the same times during exercise as blood was sampled, were performed continuously for 3 min prior to the onset of exercise. Thereafter respired gases were collected for 2 min intervals at identical time points to blood sampling.

Participants had a single 22 G (BD Venflon™, UK) venous catheter inserted percutaneously into their non-dominant forearm under aseptic conditions. Following insertion of the catheter, a small (2 ml) resting baseline sample of blood was taken in all trials, and a 3 way tap connecter (BD Connecta™, UK) was placed on the catheter hub to allow repeated blood draws.

During exercise, blood samples were obtained via the 3 way tap (2 ml) and the catheter flushed with a small, 1-2 ml, prime of sterile isotonic (0.9%) saline (BD, Plymouth, UK) to prevent coagulation. Blood samples obtained during exercise were immediately transferred to cooled 4 ml blood tubes containing ethylenediaminetetraacetic acid (EDTA) (BD Vacutainer, UK), and immersed in an ice bath at + 4ºC until the end of the test.

Ketone ester

This ketone ester has been designated as GRAS by the FDA allowing it to be used as a foodstuff in the USA. The chemical and physical properties of the KE are shown in Table S1.The safety and toxicology of this ester has been published previously (Clarke, Tchabanenko et al. 2012, Clarke, Tchabanenko et al. 2012). Prior to all studies involving competitive athletes, approval was obtained from the world anti-doping agency (WADA) who confirmed this nutritional ketone ester did not constitute a banned substance for use by athletes. Ketone ester was produced via the trans-esterification of the two major reagents R-1,3-butanediol (R-1,3-hydroxybutyl) and ethyl-β-hydroxybutyrate using enzymatic catalysts in a patented process to produce a > 99.8% purity of raw (D-β-hydroxybutyrate-R 1,3-Butanediol) monoester. Each batch of raw monoester was quality tested for impurities before use according to strict food grade standards as set out by the FDA. Smaller aliquots of raw ester were individually tested for microbial contamination (IFN, Reading, UK). All ketone ester was stored at or below room temperature in air tight containers to minimize contamination or exposure to environmental esterases.

Skeletal muscle biopsy:

Muscle tissue was collected via percutaneous needle biopsy technique, modified for use with a biopsy gun (Bard Monopty™, Bard biopsy systems, USA), from the lower third of the Vastus Lateralis muscle. All samples were obtained under aseptic conditions following skin cleaning with 0.5% chlorhexidine spray (Hydrex®, Ecolab Ltd, UK). Following this, 1-2 ml of local anaesthetic (1% Lidocaine hydrochloride without adrenaline, Hameln Pharmaceuticals, Gloucester, UK) was infiltrated into the subcutaneous layer, then a small incision was made in both the dermis and deep fascia to allow percutaneous passing of the biopsy gun. Biopsy samples were obtained from new incisions at each time point with the direction of the needle angulated away from the previous pass to ensure undisturbed fibres were sampled on each occasion. Four passes were made on each sampling time point, with approximately 20-30 mg of muscle tissue obtained on each pass, stored separately in labelled eppendorf tubes. Once removed, tissue was immediately frozen in liquid nitrogen and stored at -80º C until further processing.

Metabolite extraction from skeletal muscle

Metabolites were double-extracted from approximately 100 mg tissue using a modified Folch method (Le Belle 2002). The aqueous and organic fractions were separated and further split into 2 identical volumes to allow multiple analyses.

Nutritional ketosis alters fuel preference and thereby endurance performance in athletes.

Supplemental information For: *Cell metabolism*

^{1}H-NMR analysis of aqueous metabolites

Half of the aqueous fraction (~25 mg wet weight tissue) was dried under nitrogen, and resuspended in 600 µL D_2O containing 0.09% w/v NaCl (Sigma), 0.01% w/v NaN_3 (Sigma) and 0.25 mM deuterated sodium-3-trimethylsilylproprionate (NaTMSP-2,2,3,3-D4, Cambridge Isotope Laboratories, Inc.) as a chemical shift reference. Samples were analysed on a Bruker NMR spectrometer interfaced with an 11.8 Tesla superconducting magnet at 310K using a ^{1}H-NOESY 1D pulse sequence with 128 scans. Data were integrated using fixed integral sizes of 0.02 ppm within 1D Spec Manager (v12, Advanced Chemistry Development, Inc.Canada).

Carnitine analysis

Half the aqueous fraction was combined with half the organic fraction, and 200 µL acyl-carnitine standard containing eight deuterated species was added (Cambridge Isotope Laboratories, Inc. USA). Samples were dried under nitrogen and butylated with 3 M butanolic-HCl (Sigma). Samples were dried once more before resuspension in 200 µL acetonitrile containing 0.1% v/v formic acid (Sigma). Samples were analysed using multiple reaction monitoring on a Waters Quattro Premiere XE triple quadrupolar mass spectrometer and chromatograms integrated using QuanLynx v4.1 (Waters Ltd, Hertfordshire, UK).

TCA cycle and glycolytic intermediate analysis

Tissue extracts from the carnitine analysis were recovered, dried and resuspended in 50:50 water:acetonitrile containing universally-labelled glutamate as internal standard (Cambridge Isotope Laboratories Inc.). Metabolites were identified using a mass scan from *m/z* 50-1200 on a Waters Xevo G2 quadrupolar time-of-flight (qTOF) mass spectrometer. Tricarboxylic acid cycle intermediates and pyruvate were detected in butylated form and identified using fragmentation data from the high-energy function. Remaining glycolytic intermediates were detected underivatised and identified using fragmentation data. Chromatograms were integrated using QuanLynx v4.1 (Waters).

Lipid staining and quantification:

Histological staining techniques for intra-myocellular triglyceride (IMTG) obtained from muscle biopsy specimens were performed using the modified methods of Koopman, et al. (Koopman, Schaart et al. 2001). Muscle tissue (~30 mg) was embedded into Tissue-Tek™ (Sakura Finetek Europe, Zoeterwoude, the Netherlands) on dry ice, before sectioning with a Cryostat Microtome (Leica CM3050 S) into serial sections of 4 µm. Samples were chemically fixed for 1 hour using 3.7% formaldehyde solution (diluted in deionised water). Excess formaldehyde was removed by rinsing three times for 30 seconds in deionised water. Sections were then immersed in a working solution of ORO for 30 min. Samples were further rinsed and covered with a coverslip using 10% glycerol in phosphate-buffered saline (PBS). Image acquisition of samples was carried out immediately. Image analysis was performed using an Olympus Fluoview FV1200 microscope. ORO stained sections were examined using a Texas red excitation filter (540–580 nm). Images were analysed with ImageJ™ software to quantify the size and density of the lipid droplets giving results as the mean grey value.

PAS staining for muscle glycogen:

All steps were carried out at room temperature, using a protocol developed from the Periodic acid-Schiff (PAS) staining system kit (Sigma-Aldrich) and (Halkjaer-Kristensen and Ingemann-Hansen 1979). Samples were fixed for 5 minutes in Formalin-Ethanol Fixative Solution, and then subsequently rinsed under running tap water for 1 min. Slides were immersed in Periodic acid solution for 5 minutes, and repeatedly rinsed in distilled water. Schiff's reagent was then applied for 15 minutes before washing under running tap water for 5 minutes. Slides were counterstained in hematoxylin solution for 90 seconds and then rinsed under running tap water for 15–30 seconds. Sections acting as a negative control were pre-incubated with porcine pancreas α-amylase (Sigma-Aldrich) for 30 minutes. Images were obtained with a DSS1 Nikon Slide Scanner (x10magnification). Blinded, randomized, analysis of n=200 individual muscle fibers was performed on each muscle sample, and graded according to their PAS staining intensity according to the methods of Gollnick and Saltin et al (Gollnick, Armstrong et al. 1973). All slides were reviewed by an experienced histopathologist.

Statistics:

Results are expressed as means ± SEM and significance was taken at $p < 0.05$. One subject declined to have skeletal muscle biopsies in study 2, otherwise all clinical and laboratory data were analysed for all subjects (no attrition or exclusions) with the exception of *^{1}H-NMR* muscle biopsy data from one athlete on the post exercise fat arm (Study 2) excluded as an outlier (> 4 SD from the group mean). Statistical analysis was performed using SPSS (V21, Chicago, USA). For the human trials containing paired data with 3 arms, repeated measures ANOVA was performed following initial tests to ensure sphericity assumptions were not violated, and then corrected with additional post-hoc Tukey corrections for multiple comparisons where appropriate. Paired data were compared using two tailed student-t tests. Correlations were tested using a two-tailed Pearson's test.

Sample size estimates:

Study 1:

No *a-priori* data on the influence of exercise on the kinetics of oral KE was available prior to this study. Previous data obtained from resting studies demonstrated a blood D-βHB concentration of 3 +/- 0.2 mM is reliably obtained following the ingestion of 500 mg/kg KE (Clarke, Tchabanenko et al. 2012). Furthermore previous infusions of radiolabeled ketone salts during moderate exercise demonstrated a 1-2 mM fall in blood concentration, and that 10-18% of VCO_2 production was attributable to ketone oxidation (Fery and Balasse 1986). Therefore to detect a 1 mM fall in circulating blood ketosis from a mean pre-exercise value of 3 mM, with SD of 0.2 mM with 80% power ($\alpha = 0.05$) would require n=6 participants to reject the null hypothesis in a 2 tailed 3-way cross over design.

Study 2.

No *a-priori* data on the influence of ketosis on human skeletal muscle metabolism was available prior to this study. Therefore we estimated sample size based on the previous work conducted by Sato et al who provided glucose and a mixture of glucose and ketones in the working perfused rodent heart (to determine the energetic implications of altered substrate metabolism) (Sato, Kashiwaya et al. 1995). In this work it was shown the addition of ketones to working muscle reduced glycolytic intermediates by 2-3 fold whilst sustaining contractile function (Sato, Kashiwaya et al. 1995). Therefore we proposed that to detect a (conservative estimate) of 20% change in human skeletal muscle pyruvate (as a surrogate marker of glycolysis) with a SD of 12.5% with 80% power ($\alpha = 0.05$) would require n=10 participants to reject the null hypothesis in a 2 tailed 3-way cross over design.

Study 3.

Data obtained in study 2 demonstrated a ~40 % higher intramuscular glycose concentration after KE (450 +/- 115) vs. CHO (275 +/-112) in n=10 athletes at the end of 1 h of exercise consistent with the downstream reductions in glycolytic intermediates observed. We reasoned that the addition of 60% of calories to the KE drink would reduce this difference if supplementary oral glucose could restore conventional muscle fuel preference for glucose. Therefore we estimated that to observe a 40% change in intramuscular carbohydrate content after 1 h of exercise between KE+CHO vs. carbohydrate with 80% power ($\alpha = 0.05$) would require a sample size of n=8 athletes to reject the null hypothesis in a 2 tailed 3-way cross over design.

Study 4.

In study 2 and 3 we demonstrated a significant reduction in intramuscular glucose metabolism during exercise following KE ingestion vs. carbohydrate. We reasoned that this cumulative reduction in muscle glucose use would result in a sparing of total muscle glycogen content and a decrease in total intramuscular triacylglycerol levels. We validated the microscopy methods previously described to determine intra-muscular ORO signal intensity in needle biopsy specimens of 25-35 mg with mean values of 375 +/- 50 (AU) (n=8). We estimated that to detect a 24% change in triacylglycerol content published by (Wendling, Peters et al. 1996) to represent a 'meaningful' change in lipid content during exercise, with a SD of 13%, and 80% power ($\alpha = 0.05$) would require a sample size of n=7 athletes in a 2-tailed t-test to reject the null hypothesis.

Study 5.

No *a-priori* data on the effect of nutritional ketosis on cycling performance were known before this study; however the high reliability (CV ~1-1.5%) of repeated bicycle ergometer trials in well trained athletes has been published previously (Currell and Jeukendrup 2008). Therefore to detect a 2% difference in performance with 80% power ($\alpha = 0.05$) would require a sample size of n=8 athletes to reject the null hypothesis in a 2 tailed paired T-test.

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