UNIVERSITY OF OXFORD
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The effects of a novel substrate on exercise energetics in elite athletes

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Doctor of Philosophy Thesis

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Thesis abstract

The physiological ketosis of starvation makes sound evolutionary sense, as ketone bodies have several thermodynamic advantages over other nutritional substrates, in addition to their actions to conserve protein and glucose stores. Utilising the body's metabolic responses to ketosis by delivering a novel nutritional source of ketone bodies, the work in this thesis explored the metabolic effects of ketosis on physical performance in humans. First, the pharmacokinetics and dosing requirements for ketone containing drink preparations were characterised in a population of athletes and healthy controls (n = 45). Using endurance exercise as a model of physiologic stress, the functional impact of ketosis during sustained high intensity effort was investigated in high performance athletes (n = 22). It was shown that nutritional ketosis improved performance in 18/22 athletes, who set 14 new best performances during 30 min of rowing. Furthermore, when ketones and glucose were delivered together, cycling performance was improved by 2% (n = 8) following 1.5 hours of fatiguing effort, compared with optimal carbohydrate intake. Blood D-β-hydroxybutyrate reached 3-5 mM following ketone drinks, equivalent to several days of total fasting, but rapidly decreased during exercise. It was found that higher physical workloads correlated with larger decreases in plasma ketone concentration (n = 8), consistent with their oxidation as respiratory fuels. Nutritional ketosis significantly altered fuel metabolism during exercise in elite athletes (n = 10), decreasing peripheral lipolysis, skeletal muscle glycolytic intermediates, blood lactate, and branched chain amino acid release. In conclusion this work suggests a new hierarchy of substrate preference during physical stress, whereby mimicking the physiology of starvation, the energetic consequences of oxidising ketones may significantly enhance athletic performance. The extrapolation of these findings may have therapeutic implications for patient populations where energetic demands are high, and deleterious switches in substrate selection occur.

(Abstract length: 294 words)

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Abbreviation

$\Delta G'$ Gibbs free energy (nonstandard conditions)

ΔG^o Gibbs free energy (standard conditions)

ΔH^o Heat of combustion (standard conditions)

AcAc Acetoacetate

ADH Alcohol dehydrogenase

ADP Adenosine diphosphate

ALDH Aldehyde dehydrogenase

AMPK Adenosine monophosphate kinase

ATP Adenosine triphosphate

AUC Area under curve

BCAA Branched chain amino acids

CCRF Clinical cardiovascular research facility

CD147 Immune complex with MCT

CHO Carbohydrate

C_{max} Maximal concentration (blood)

CNS Central Nervous System

CO_2 Carbon dioxide

CoA Co-enzyme A

CoQ Co-enzyme Q

C-OX Cytochrome oxidase

CPT1 Carnitine Palmitoyl CoA Transferase receptor 1 complex.

Cyt C Cytochrome C

DHAP Dihydroxyacetone phosphate

E Electrical potential (redox potential)

ECW Extracellular water

F Faraday constant (96.485 kJ / mol / V)

$FADH_2$ Flavin adenine dinucleotide

FFA Free fatty acid

GABA Gamma-amino butyric acid

GB Great Britain

GI Gastrointestinal

GLUT Glucose transporter

H^+ Proton

HCO_3 Bicarbonate

ICU Intensive care

K_m Michaelis Menten rate constant

LDH Lactate dehydrogenase

MCT Monocarboxylate transporter

MPC Pyruvate transporter

MR_c Cerebral metabolic ratio

NADH Nicotine adenine dinucleotide

NIH National Institute of Health

NMR	Nuclear magnetic resonance
NMR	Nuclear Magnetic Resonance
O_2	Oxygen
OCMR	Oxford Centre of Clinical Magnetic Resonance Imaging
PB	Personal best
PDC	Pyruvate dehydrogenase complex
PDH	Pyruvate dehydrogenase
P_i	Inorganic phosphate
Q	Reaction quotient
R	Universal gas constant
RER	Respiratory exchange ratio
RPE	Rating of perceived exertion
RPM	Revolutions per minute
RQ	Respiratory quotient
S	Entropy
SB	Seasons best
SEM	Standard error of the mean
SGLT	Sodium/glucose transporter
T	Temperature (Kelvin)
$T^{1/2}$	Half life (decay or elimination)
TAG	Triacylglycerol
TBW	Total body water
TCA	Tricyclic acid cycle
T_{Max}	Time (T) at maximal concentration
UCP	Uncoupling protein
UK	United Kingdom
VCO_2	Volume of carbon dioxide
$VO_{2\ max}$	Maximal volume of oxygen uptake (L/min)
W_{max}	Maximal work output (Watts)
βHB	D-β-Hydroxybutyrate

Declaration

I declare that the work presented in this thesis is my own including all of, but not limited to:

1. study design
2. ethical applications
3. subject recruitment
4. experimental trials
5. data analysis
6. data presentation (including figures and tables)

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1 Introduction

1.1 Diet, metabolism and human performance

An ancient Spanish proverb *'Diet cures more than the lancet'* suggests that the importance of diet in maintaining good health is an age old concept. Mechanisms by which the body uses the fuels we eat to sustain life, or in the case of excess, store the surplus energy, have fascinated generations of scientists. Carbohydrates, fat and protein are the fundamental sources of dietary energy. While these primary types of macronutrient are limited, the particular composition, and relative contribution to our calorific needs varies widely. Until recently, little was known of the metabolic systems that linked diet with human performance. In 1665, Hooke described the concept of the cell and proved its existence[1], paving the way for later examination of the biochemical reactions sustaining its function. In 1937, Krebs made arguably the most important breakthrough in biochemistry [2], describing a cycle of enzymatic reactions uniting dietary fuel combustion with cellular energy provision. This final common pathway for substrate metabolism has allowed the detailed study of the flow of energy transformation (energetics) from dietary sources to the 'energy currency' ATP.

Cell fuel preference is not fixed to one substrate, as noted in 1920 by Lindhard [3], but can change according to the energy demands of the body. Endurance exercise in particular provides an ideal method of testing the relationship between diet and human performance. Many have used this knowledge to describe the metabolic demands of exercise, and propose theories for the limitations to energetic performance. Yet much is still to be discovered in this area. Demand is growing in the applied fields of medicine, nutrition, and sport for optimal dietary strategies to enhance, or sustain, cellular energetics during exercise or in disease.

One such promising nutritional agent is a novel, edible form of dietary ketone. Ketone bodies are fuels endogenously produced by the body in response to a variety of physiological or pathological conditions. Ketone bodies, acetoacetate (AcAc) and D-β Hydroxybutyrate (βHB), are respiratory fuels which can be oxidised by most body tissues, and are formed in large quantities by the liver in response to low blood

glucose and insulin. Therefore an edible ketone allows, for the first time, a nutritional ketosis by means of a single drink.

1.2 Mitochondrial efficiency and substrate energy transduction

The energy currency adenosine triphosphate (ATP) is required to power all mammalian cells. Each active cell derives the majority of its chemical energy from the combustion of carbon substrates using oxygen. This process is highly regulated and occurs within the mitochondria, specialised organelles sensitive to the changing energy requirements of the cell. Mitochondria harness the latent energy within the carbon chains of dietary substrates by the controlled breaking of covalent bonds in specific sequences, which are better known as ‘metabolic pathways’.

The conservation of energy from the oxidation of carbon fuels is imparted to the mitochondria and is ultimately used to phosphorylate ADP to yield ATP. This process is performed by the electron transport chain, where electrons from the redox reactions of substrate oxidation are trapped to create an electrochemical gradient. Electrons travel down their electro chemical gradient via highly specialised protein channels in the mitochondrial inner membrane. This proton motive force is used to spin subunits of the F_1F_OATPase complex and generate ATP (Figure 1) [4, 5]. The end products of complete oxidation of carbon fuels are water and CO_2, which is subsequently exchanged for oxygen by the respiratory system.

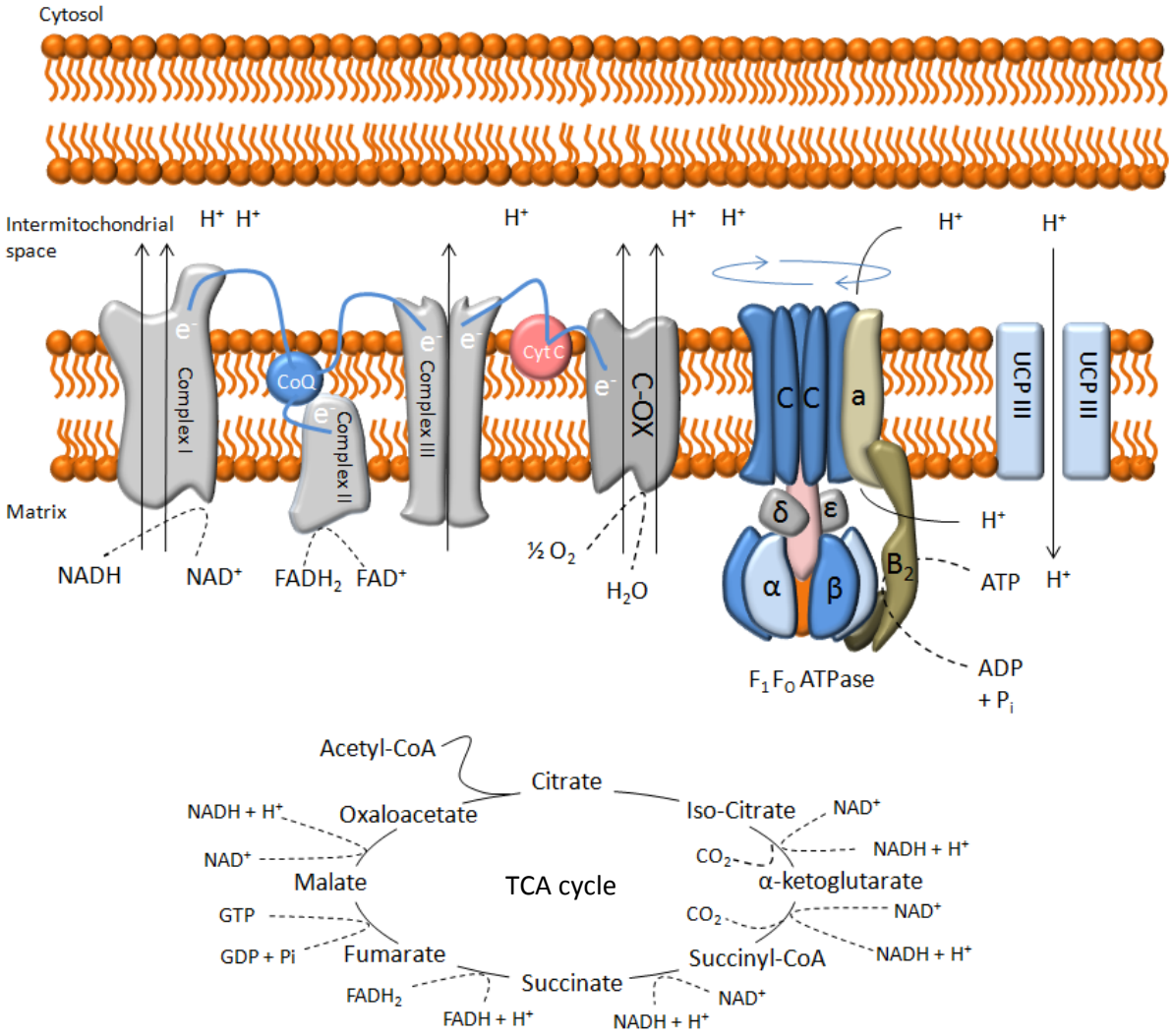


Figure 1 Mitochondrial energy transduction. This schematic shows the mechanism of the electron transport chain creating an electrochemical gradient across the inner-mitochondrial membrane, which is used to drive the F_1F_OATPase complex. The electrochemical gradient can be dissipated by uncoupling proteins (UCPIII) which release protons into the mitochondrial matrix without passing through the F_1F_OATPase, thereby decreasing mitochondrial efficiency. Formation of ATP from ADP + Pi via the F_1F_OATPase provides the energy to power cell function. Production of reducing equivalents, NADH, and $FADH_2$, to feed the electron transport chain is from the oxidation of carbon units by the TCA cycle under aerobic conditions.

Mitochondria are not however, perfect bomb calorimeters. Dissipation of the mitochondrial proton gradient via means other than the F_1F_OATPase complex is known as 'mitochondrial uncoupling' and is an important cause of metabolic inefficiency. Uncoupling occurs through specific channels in the inner mitochondrial membrane that allow the free passage of H^+ to the mitochondrial matrix without generating

ATP. Up to 20 % of whole body oxygen uptake is lost by uncoupled mitochondrial respiration at rest [6]. The magnitude of mitochondrial uncoupling may be important during physiologic stress, such as exercise, where energy demand is high and oxygen supply is limited. Losses in mitochondrial energy conversion may impair muscle contractile performance, and incur an additional oxygen cost [7].

1.3 Substrate selection as a determinant of energetic performance

Mitochondrial redox state is affected by the substrate oxidised. The electrochemical gradient created by the electron transport chain depends on a continuous supply of reducing equivalents in the form of NADH or $FADH_2$. These reducing equivalents undergo cyclical reduction and oxidation in an intimate relationship with the TCA Cycle and the complexes of the electron transport chain (Figure 1). The energy of the proton motive force generated by the pumping of protons from the mitochondrial matrix (via complexes 1, 3 and 4 of the electron transport chain), depends upon the difference in electrical potential between the (NAD^+/NADH) couple, and the co-enzyme Q couple [8, 9]. This can be expressed as:

$$\Delta G' = -n\ F\ \Delta E_{\frac{Site\ II}{Site\ I}}$$

The stored proton gradient is dissipated through the F_1F_OATPase, and used to form ATP. This gradient is the free energy of ATP hydrolysis ($\Delta G'_{ATP}$). Whilst the electron transport chain is in itself a remarkably efficient series of biochemical reactions [4], the free energy of ATP hydrolysis is not identical for all substrates. Ketone bodies are more chemically reduced than pyruvate, and result in an increased electron transport chain redox span through the reduction of the NAD^+ couple and oxidation of the co-enzyme Q couple. This, in turn, creates a greater $\Delta G'_{ATP}$ for the generation of ATP. In the working rat heart, this resulted in an increase in hydraulic efficiency of 28% during perfusion of ketone bodies compared with glucose alone [10].

The heat of combustion is of inherent importance when considering the potential impact of mitochondrial substrate selection on energetic performance. Pyruvate, the end product of glycolysis, has a lower heat of

combustion per C_2 unit than either βHB or palmitate, providing less potential energy to the electron transport chain.

Table 1 Heats of combustion of selected fuels

Substrate	**ΔHº kcal/mol**	**ΔHº kcal/mol C_2 units**
$C_{18}H_{32}O_2$ (Palmitate)	- 2384.8	-298
$C_4H_8O_3$ (β Hydroxybutyrate)	- 487.2	-243.6
$C_6H_{12}O_6$ (Glucose)	- 669.9	-223.6
$C_3H_6O_3$ (Lactate)	- 326.8	-217.9
$C_3H_4O_3$ (Pyruvate)	- 278.5	-185.7

Adapted from Veech et al [11].

Palmitate, despite its large potential energy, is not able to provide greater mitochondrial redox power. The reasons for this lie in the architecture of fatty acid metabolism by β-oxidation, and the changes in mitochondrial membrane uncoupling proteins in response to persistently elevated FFA. Only 50% of the reducing equivalents produced in the process of β-oxidation are able to donate electrons at complex I of the electron transport chain, whereas the remaining half are donated by $FADH_2$ at the flavoprotein site further 'downstream' at complex II [11]. This results in a reduced ATP yield per C_2 unit, and a loss of mitochondrial efficiency. The redox span of the respiratory chain is diminished during fat metabolism, as the Q couple is reduced, rather than oxidised, as with ketone metabolism. This decreases the potential difference between the matrix and inter-mitochondrial membrane space and therefore $\Delta G'_{ATP}$. Raised free fatty acids also increase the expression of mitochondrial uncoupling proteins [12], which impair mitochondrial efficiency by allowing the passage of protons into the matrix via non ATP generating pathways.

1.4 Exercise as a model of physiological stress

The onset of acute exercise triggers a rapid increase in demand for substrate, and oxygen (mediated via an increase in cardiac output), with metabolic rate raised approximately 10-fold above resting conditions during high intensity exercise [6]. Depending on the intensity of exercise, durations of physical effort may

last for minutes, hours, or even days, placing large metabolic, structural, and cognitive demands on body systems to sustain this output. Similar changes occur in many clinical disease states with high energy requirements, elevated cardiac output, and limited tissue oxygen supply, characteristic of high dependency care, surgery, or medical emergencies. The functional demands of exercise can be used to quantify responses to treatment, or as a diagnostic tool to identify factors limiting physical capacity [13]. Exercise therefore provides an ideal tool for the examination of human physical capacity and its controlling factors, under reproducible conditions.

1.5 Fuel selection in exercise

Effective energy delivery in the form of oxidisable substrates is at the core of homeostatic regulation during exercise. Muscle, heart and brain demand great quantities of energy to power exercise and control essential responses to this stressful stimulus. Despite their common requirement for fuel, each of these organs differs in their ability to use particular substrates and their degree of reliance on circulating supply vs. intracellular stores during exercise.

1.6 Skeletal muscle

Muscular fuel selection is under the influence of many factors, including local tissue energetic environment and systemic homeostasis. Physiological responses to workload and duration of exercise have shaped our understanding of muscle metabolism. Muscle cells have a remarkable capacity for adaption to metabolic stressors, which in the context of endurance exercise, centre on optimal fuel selection and oxidative efficiency. Athletic training results in many alterations in enzymatic function, the detailed description of which is outside the scope of this thesis, but well described in articles by Hurley et al [14], Leblanc et al [15], and reviewed by Holloszy et al [16]. Metabolic regulation of fuel metabolism may change profoundly following a period of exercise training, featuring a marked upregulation in the enzymes responsible for either glycolysis or fat oxidation [17], or occasionally both. Mitochondria too become more plentiful, seeking to harness a greater tissue oxygen fraction. Tissue vascularity

increases[18], as does cell sensitivity to circulating hormones including insulin, all of which enable a greater capacity to utilise circulating fuel supplies [19].

At the mitochondrial level, the exact mechanisms of substrate selection are still to be fully elucidated. The limiting steps in fuel oxidation by the mitochondria are different for each substrate, and are likely heavily influenced by intermediate metabolites of parallel pathways. Early theory to explain the dynamic balance between carbohydrate and fat metabolism was put forward by Randle et al in 1963 [20]. The capacity of the mitochondria to alter its preferential fuel selection was initially recognised by the apparent decrease in glycolysis in the presence of increased free fatty acids (FFA) at rest. This was explained by Randle in his glucose-free fatty acid cycle hypothesis. This suggested carbohydrate oxidation could be regulated by the fatty acid induced suppression of pyruvate dehydrogenase complex (PDC) activation by a high acetyl-CoA/CoA ratio. This in turn elevates citrate concentrations inhibiting glycolytic flux at the level of phosphofructokinase. This theory does not seem to hold true during exercise, as several authors have noted [21, 22]. The major arguments against the glucose-FFA cycle are the finding that increasing exercise workloads cause a marked switch in substrate preference towards carbohydrates, irrespective of the FFA concentration [21], and that fatty acid oxidation is not limited by FFA availability [23, 24].

One theory for the altered mitochondrial fuel preference of carbohydrate over fat with increasing exercise intensity may be due to high glycolytic rates inhibiting carnitine palmitoyl CoA transferase (CPT1). Elevated flux through the glycolysis pathway can lead to accumulation of acetyl-CoA as fatty acid transport is saturated. The accumulated acetyl-CoA is metabolised to malonyl CoA which inhibits CPT1 in vitro. However, multiple studies of rat [25] and human muscle [26, 27] have failed to find evidence for the elevation of malonyl CoA concentration during exercise.

High intensity exercise could also cause a shortage of muscle carnitine, essential for the transport of fatty acid groups across the mitochondrial membranes. This shortage could potentially limit the rate of fat oxidation and explain a switch towards carbohydrate reliance for energy production at high intensities.

The accumulation of acetyl groups during heavy exercise can be buffered by free carnitine, when pyruvate dehydrogenase complex (PDC) flux is high. This apparent ability to act as reservoir for acetyl groups markedly reduces the availability of free carnitine, and therefore rates of fat oxidation during exercise, as reviewed by Wall et al [28].

Explaining the limitations in mitochondrial fatty acid oxidation with incremental load is only one piece of a complex puzzle. Muscle glycogen stores provide the most important source of carbohydrate to fuel exercise, and exhaustion of this supply leads to fatigue and impaired performance. These stores are rapidly depleted once workloads of ~70 % $VO_{2\ max}$ are exceeded [29, 30]. This threshold is accompanied by a decline in fatty acid oxidation, meaning that any substrate oxidised in this domain of exercise intensity could have a glycogen sparing effect. In this instance, increased uptake and oxidation from circulating blood glucose may partially contribute to the carbohydrate requirements of working muscle. However, the magnitude of the circulating glucose contribution to total energy expenditure is small (10-15%) and therefore can only delay inevitable fatigue [22].

Conventional literature on muscle fuel selection mechanisms has not considered the role of ketone bodies as a major fuel source, although the latter are well known to be metabolised by skeletal muscle. Ketone bodies have a similar respiratory quotient (RQ) to that of glucose (AcAc = 1.0, βHB = 0.89) if completely oxidised [31], and do not rely on glucose transporter (GLUT) or CPT1 transport to enter cell or mitochondrial spaces, unlike carbohydrates or fat. Ketones, by virtue of their evolutionary role to conserve carbohydrate stores, may exert an inhibiting control over glycolysis, whilst themselves being substituted as a fuel. Pathways of the major mitochondrial fuels are schematically presented in Figure 2.

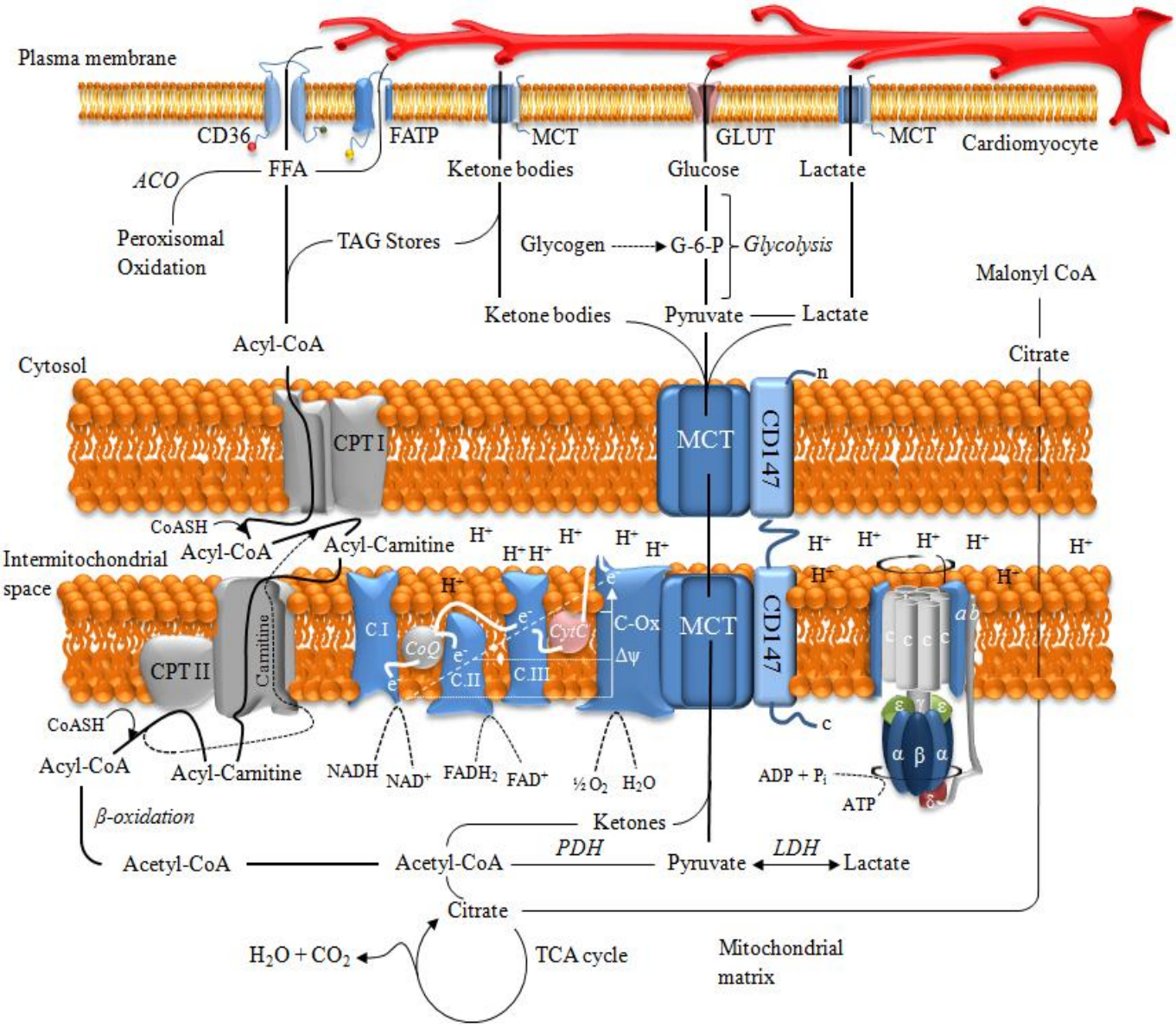


Figure 2 Mechanisms of fuel metabolism and mitochondrial substrate transport in a human muscle cell. *Left to right:* Long chain fatty acids are esterified to carnitine, and transported into the mitochondrial matrix via the carnitine shuttle. This is facilitated by Carnitine Palmitoyl Transferase 1 complex (CPT1). CPT1 and phosphofructokinase (Phos-Fruc Kinase) activity are under negative feedback from translocated citrate from the mitochondrial matrix, via the tricarboxylate transporter. Mitochondrial electron transport chain complexes pump protons into the inter-mitochondrial space, to create an electrochemical gradient. Electrons are ferried between complexes by Co enzyme Q (CoQ) and Cytochrome C (Cyt C). Ketones bodies, lactate and pyruvate can be transported into the matrix by the monocarboxylate transporter (MCT1 + CD147) which is intimately associated with the terminal complex of the electron transport chain, Cytochrome Oxidase complex (C-OX). Once in the mitochondrial matrix, substrates from all three pathways are metabolised to Acetyl-CoA and oxidised in the Krebs cycle.

1.7 Heart

Myocardial fuel selection has many similarities to that of skeletal muscle, but has a greater capacity to scavenge circulating metabolites. Studies of human myocardial energetics are limited due to the invasive nature of the procedures required, or require specialised techniques [32]. Canine, porcine, and rodent models have been extensively studied however, and provide the basis for our understanding of myocardial energetics.

Under normal conditions, the principle substrate for myocardial oxidation is FFA [33]. Fatty acids may provide up to 95% of the ATP requirements of the non-ischaemic heart. Increased blood glucose and insulin cause a shift in substrate preference away from fats, associated with a significant improvement in myocardial oxygen consumption (MVO_2) for a given left ventricular power output [34]. The reverse is true when the heart is perfused with increasing concentrations of triacylglycerol (TAG) or FFA, resulting in an additional oxygen cost of between 25% and 48% for the same work output compared to glucose and insulin infusion [35]. The loss of myocardial efficiency when metabolising fat has been attributed to reductions in mitochondrial electron transport chain coupling, and the increased stoichiometric oxygen requirement to oxidise fat [36]. Deleterious substrate selection may also be a feature of many myocardial diseases, intimately linking energetic performance and mortality [36, 37].

During acute onset exercise, contractile power and MVO_2 can increase 4 to 6 fold above resting levels. Fatty acid oxidation does rise when cardiac workload increases, but the relative change in carbohydrate oxidation, and that of other glucose equivalents (e.g. lactate) is much greater [38, 39]. The extent of substrate switching towards glycolytic fuels, is in part, proportional to their arterial concentration [39]. Increases in circulating FFA concentration occur during prolonged exercise, resulting in a greater rate of FFA uptake and β oxidation. Lactate concentrations may also become markedly elevated during exercise, and can be utilised by the heart, with a concomitant fall in FFA oxidation [39, 40]. A similar situation is true with the administration of nicotinic acid, which suppresses FFA release from peripheral adipocytes,

and therefore arterial FFA concentrations. This results in a greatly reduced myocardial FFA uptake, and a reliance on carbohydrates and lactate for fuel [41].

Ketone bodies, when present in elevated concentrations, are preferentially metabolised over other fuels, accounting for 83% of myocardial CO_2 production in an in-vivo canine heart [42]. Several studies have shown that ketones are oxidised in preference to FFA [43, 44], while more recently others have examined the effect of ketone bodies on mitochondrial energetics with striking results. In an isolated perfused rat heart, cardiac hydraulic work increased by 28% following the infusion of ketone bodies, with a decrease in oxygen uptake compared to glucose alone. Ketones also exert control over glucose oxidation, resulting in decreased rates of glycolysis [45, 46].

1.8 Brain

The metabolic requirements of the brain are large, with the brain consuming 20% of total body oxygen uptake at rest, and 10-15% of cardiac output dedicated to brain perfusion [47]. The average human brain weighs 1.5 kg and consumes 100-150 g of glucose per day at rest [48]. Early research (1930-1946) into brain metabolism noted that the respiratory quotient (RQ) across the brain was close to unity (1.0), indicating glucose as the primary fuel source [49]. Further experiments in animal models and humans showed that 87% of the CO_2 produced by the brain could be accounted for by the oxidation of glucose, 11% by lactate, and ~1-2% by pyruvate under normal conditions [50]. Further evidence for the brain's reliance on blood glucose to maintain neural function can be observed in induced hypoglycaemia. Lowering blood glucose with insulin caused a decrease in brain oxygen consumption, whereas peripheral skeletal muscle maintained the same respiratory rate by the oxidation of fats and other fuels [51]. Prolonged hypoglycaemia and low insulin levels stimulate ketogenesis, with ketones being the only alternative fuels to glucose that can be metabolised in significant quantities by the brain at rest [52]. Thus, ketones have evolved to provide the brain with fuel during long periods of starvation, where exhaustion of body glucose and protein stores would be the inevitable result of maintaining glucose supply to the brain [52, 53].

Cerebral glucose metabolism is therefore central to the effective function of one of the most highly metabolically active organs of the body [54]. However, this narrow palate of possible energy supply places the brain under significant energetic strain during exercise because prolonged exercise causes blood glucose to fall, as skeletal muscle demands increase and exhaustion of liver glycogen stores ensue. Exercise also causes a profound stimulatory effect on neural processing, as inputs from proprioceptive, pain, sensory and motor afferents, as well as higher cortical inputs, require simultaneous co-ordination [55-57]. The stimulating effect of exercise on neural energy demand can be represented by a change in cerebral metabolic ratio (MR_c) [56], which is calculated from the relationship of cerebral oxygen consumption relative to its substrate:

Cerebral metabolic ratio (MR_c) = Uptake of O_2 / glucose + ½ lactate

Cerebral metabolic ratio falls dramatically with exercise from resting levels of ~6 to as low as 3 during severe exercise [58]. In curious parallel with skeletal muscle, which switches preferential fuel selection with increasing demand, brain MR_c remains stable at low intensity exercise, but sharply falls during exercise above the 'moderately hard' perception of effort (Borg scale 15/20 RPE) [59, 60]. A distinct nadir can be seen in MR_c at this point, which correlates well with the increasing mental effort required to sustain exercise. Increasing physical workloads past this threshold results in a proportional decrease in brain MR_c, and an increased mental effort [61]. These findings suggest that the 'will' to continue exercise is related to the energetic conditions of the brain and intimately with neural fuel selection.

Recently the role of lactate in supplying glucose equivalents to the brain during exercise has received particular attention [62, 63]. Elevated blood lactate levels occur during strenuous exercise, and can contribute significantly to the overall fuel supply selected by the brain [64]. In fact, it has been suggested that cerebral uptake of lactate in molar amounts may match or exceed that of glucose during severe intensity exercise, when blood lactate concentrations are markedly elevated (~15 mM) [65, 66].

Brain glycogen represents the only significant neural store of available energy [67]. In relative terms, the size of these stores is considerably less (10 %) than those contained in skeletal muscle [67, 68]. However, since glycogen stores are confined to astrocytes [69] that occupy less than half the brain's volume, cellular concentrations do in fact approach those of skeletal muscle. During prolonged exercise, brain glycogen levels fall, both globally and in regions of the brain associated with a greater metabolic demand [70]. Glycogen stores may therefore be a vital reserve of fuel to power neural function, and depletion of these stores may result in impaired energetics and limit brain performance.

Ketone body metabolism in the brain has been studied for decades, where ketones are well known to be a major fuel source under certain physiological conditions [71]. Brain uptake of ketone bodies is proportional to their plasma concentration, and dramatically reduces cerebral reliance on glucose for fuel. During fasting, ketone bodies are elevated to 5-7 mM, which can contribute greater than 60% of the total energy requirements of the brain [72].

1.9 A novel nutritional ketone substrate

Previously, a controlled physiological ketosis required adherence to a low carbohydrate, high fat 'ketogenic diet', starvation, or the intravenous infusion of the salt of acetoacetate or D-β-hydroxybutyrate. All of these methods are unpleasant, impractical, or have potentially harmful side effects [73]. One solution to this problem is to create an ester link between a ketone body and an alcohol, such as 1,3-butanediol, that itself undergoes metabolism to a ketone via hepatic conversion. This ester bond can be easily broken by gut or tissue esterases to release both components without the need for a salt or acid (Figure 3).

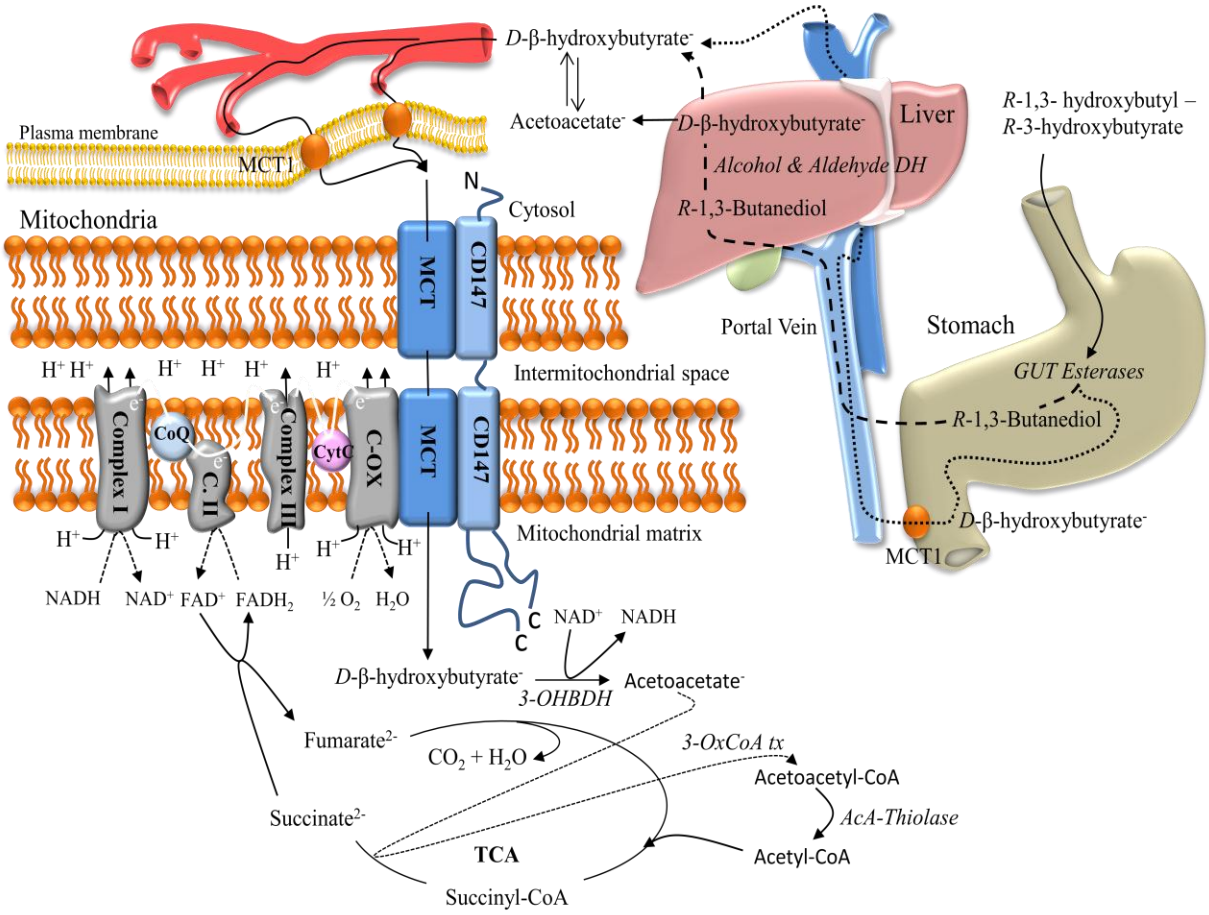


Figure 3 Oral ketone ester metabolism. Ingested *R*-1,3-hydroxybutyl-*R*-3-hydroxybutyrate ketone ester is hydrolysed in the gut by nonspecific gut esterases, which cleave the ester into R-1,3-butanediol and D-β-hydroxybutyrate components. Both metabolites are absorbed into the portal circulation with R-1,3-butanediol undergoing first pass metabolism in the liver to form D-β-hydroxybutyrate. D-β-hydroxybutyrate is subsequently released into the circulating blood compartment, and is transported into tissues via the plasma membrane monocarboxylate transporter. Once inside the mitochondrion they provide an alternative fuel for oxidative phosphorylation.

1.9.1 Ketone ester metabolism and circulating ketosis

The first practical ketone ester synthesised to be hydrolysed in plasma, free of a sodium salt load, and effectively induce a rapid circulating ketonaemia was described by Birkhahn et al [74, 75] in the late 1970's using a monoester of glycerol and acetoacetate (monacetoacetin) delivered parenterally in rats. Prior to this, the butyl alcohol, RS-1, 3-butanediol, was known to produce an artificial ketosis via oxidation in the liver, resulting in the release of the ketone body, βHB, as described for isolated liver

mitochondria [76], rats [77], and humans [78]. Two iso-enantiomers of βHB were produced from the administration of a racemic mix of RS-1,3-butanediol, with the *S* enantiomer an unnatural physiological substance [76], although it is readily oxidised by body tissues [79-81]. In 1995, the administration of both oral and parenteral ketone esters containing RS-1,3-butanediol, and either βHB, or acetoacetate, was described in pigs by Desrochers [82, 83], similar to the ketone monoester used in this thesis. It should be noted that the preparation and chemical manufacture of the R-1,3-butanediol-R-3-hydroxybutyrate monoester used in this work, was designed to prevent the production of the non-physiological *S* enantiomer of βHB following hydrolysis. This was ensured by using only the pure *R* isoform of 1,3-butanediol in the initial reaction process (Appendix 9.1).

Esters containing both 1,3-butanediol and ketone bodies (acetoacetate or βHB) taken orally are rapidly hydrolysed (cleaved at the ester bond) in the proximal alimentary tract by gut esterases. Liberated βHB and 1,3-butanediol are rapidly absorbed (most likely similar to ethanol via gastric and proximal small intestine mucosa [84]) into the portal venous system to undergo further oxidation in the liver. The fates of the two basic compounds differ due to the inability of the liver to utilise ketone bodies as a fuel source [85]. Enterically absorbed βHB is released freely into circulating blood, whereas 1,3-butanediol undergoes a series of oxidation steps in the liver via alcohol and aldehyde dehydrogenase to produce D-β-hydroxybutyrate.

1.9.2 Ketone metabolism

The body's handling of ketone bodies can be divided into endogenous production and utilisation. Most published literature has focussed on the mechanisms of ketone body production during various physiological or pathological states. The first definitive description of ketosis was in a Bradshawe lecture given by Dreschfeld to the Royal College of Physicians in 1886 [86]. He described the pathological findings of diabetic ketoacidosis, and referred to the notes of Kussmaul several years before. He also described the two principle ketone bodies and their association with severe ill health in this context.

Table 2 Physiological ketosis in heath and disease

Physiological condition	**Ketosis (mM)**	**Reference/review**
Fed	0.05 - 0.1	Balasse et al [87]
Starvation 12-24 hours	0.3 - 0.5	Fery et al [88]
Starvation 3-10 days	3 - 6	Cahill[52]
Starvation 2-4 weeks	5 - 8	Cahill et al[53]
Exercise (prolonged)	Up to 2	Koeslag et al [89]
Pregnancy (labour)	0.5 - 3	Patterson et al [90]
Pregnancy 48 hour fast	4 - 6	Robinson et al [91]
High fat diet (long-term)	4- 8	Gilbert et al [92]
Neonate	0.5 – 1.5	Bougneres et al [93]
Pathology		
Diabetic ketoacidosis (severe)	20 - 30	Alberti et al [94] Laffel [95]
Alcoholic ketosis	1.5-5.7	Wrenn & Halperin et al [96, 97]
Salicylate poisoning	Up to 1-4	Bartels et al [98]
Isopropyl Alcohol poisoning	Up to 8.5	Lovejoy et al [99]

Ketosis denotes total whole blood values of circulating ketone where reported.

Endogenous production of ketone bodies occurs in various circumstances (Table 2), and is the result of either depletion of glucose stores, elevated circulating free fatty acid (FFA) concentration, or an inability to metabolise circulating glucose, as in diabetes. In these conditions, ketone bodies are synthesised by the liver from circulating FFA liberated from peripheral adipose stores via lipolysis. In this process, fat stores of triacylglycerol (TAG) are cleaved into smaller non-esterified subunits, which are released into the bloodstream. Lipolysis is under the feedback control of many endocrine processes, and is principally mediated by catecholamine stimulation and insulin receptor agonism [100, 101]. Ketone bodies also exert a powerful negative feedback on peripheral adipolysis, with elevated ketone bodies acting on the nicotinic acid receptor in adipose tissue to suppress the release of FFA [102], and therefore regulate hepatic ketone production.

Hepatic ketone synthesis involves the complex sensing of multiple substrate pathways, with a cascade of enzymatic regulation between ketone production, carbohydrate (glucose) and fatty acid availability. Hepatic sensing of carbohydrates, flux through glycogenolysis pathways, and its relationship with

subsequent ketone body production has long been appreciated[1]. A clear reciprocal relationship between rates of lipogenesis and rates of ketogenesis in isolated hepatocytes can be observed. Malonyl-CoA concentration is hypothesised to control the rate of fatty acyl CoA entry into the mitochondrion, and therefore the generation of ketone bodies from acetoacetyl CoA, acting as a metabolic regulator between pathways. The mechanism for malonyl CoA's proposed role in the regulation of lipid metabolism was proposed in the 1970's [103]. This theory suggested that malonyl CoA which, as an intermediate of the lipogenesis pathway inhibits carnitine palmitoyl transferase 1 (CPT1) and therefore entry of long chain fats into the mitochondrion. Subsequent elevations in acyl-CoA in the cytosol provide greater substrate for the manufacture of TAG, which is subsequently stored in cells.

Ketogenesis is under tight feedback control, and is sensitive to both feeding state (hepatic glycogen) and to energy demand (flux). The overall control of ketone production is shared by both peripheral and hepatic mechanisms, which together respond to the changing energy status of the body [104].

In contrast to ketogenesis, comparatively less is known about ketone utilisation. The principle role of ketones in peripheral tissues is that of an oxidisable fuel source. However, this view may be simplistic, as ketones act as potent biochemical signals that have wide reaching consequences for fuel homeostasis and organ function [91, 105, 106].

The pathway for ketone body oxidation involves the distribution of acetoacetate and βHB in circulating plasma. The volume of distribution of these ketone bodies is small (0.1 - 0.2 L/kg bodyweight [107, 108]) indicating primary dissolution within the blood fluid compartment and low lipid solubility. Thus primary carriage to tissues is in the most part blood flow mediated. Once distributed by the capillary network, transport across the outer plasma membrane is facilitated by monocarboxylate transporters (MCT). This family of transporter proteins were only recently discovered and characterised [109] (1990's - present), where traditional thinking suggested concentration dependant diffusion into the intracellular space [110].

[1] Ketone pathophysiology and biochemistry comprehensively reviewed by Laffel (Diabetes Metabolism Res Rev, 1999. **15**: p. 412-26).

Currently, 14 types of discrete MCT proteins have been described [111]. MCT subtypes vary greatly both in subcellular location [112], and in the tissues where they are primary expressed [110, 113]. The importance of MCT expression cannot be overemphasised, as the low lipid solubility of ketone bodies makes facilitated membrane transport specific for each tissue. A wide range of Km values exist for ketone bodies amongst the MCT family [110, 114], and again give an insight into the functional relevance to each tissue of the transporter proteins. The MCT proteins are not, however, specific to ketone body transport. Many other monocarboxylates are also transported across these channels, the most important of which is lactate [115, 116]. Several authors have described the cell-cell lactate shuttle hypothesis that suggests that MCT exchange of lactate between cells may enable its use as an oxidative fuel by cells experiencing different metabolic demand [117-119]. This theory, despite ignoring the potential of ketones to be used as fuel under identical circumstances, may provide a plausible model for the understanding of ketone body transport and subcellular oxidation, given that the mechanism for membrane transport is identical.

Considerable plasticity in MCT expression has already been described. Several authors have documented that endurance athletes have significantly higher concentrations of skeletal muscle MCT as a result of exercise training [120-122]. MCT expression has also been shown to increase after a single bout of strenuous exercise, suggesting that trained endurance athletes may be ideally placed to benefit from supplemental ketones as an energy supply.

Once inside the plasma membrane, βHB may be metabolised in one of two possible fates. Ketones are directly transported across the inner mitochondrial membrane (MCT facilitated) and oxidised, or converted to butyryl CoA in the cytosol, and stored as lipid. In the former, mitochondrial βHB is converted to acetoacetate by β-hydroxybutyrate dehydrogenase, then a CoA group added by oxo acid-CoA transferase to form acetoacetyl CoA. Finally, acetyl CoA is formed via acetoacetyl CoA Thiolase, and oxidised in the TCA Cycle.

1.10 High performance athletes as models of fuel metabolism

In many respects, some of the most refined examples of human physiology and metabolism are to be found in the highly trained, athletic cohorts of competitive sport. In particular, endurance sports involving great muscular recruitment and high levels of aerobic fitness induce mitochondrial [123, 124] and muscular adaptations [125-127] making such athletes ideal to study fuel metabolism.

Aerobic endurance training increases mitochondrial oxidative capacity, and increases selection of fatty acids for fuel at a given exercise intensity [128-130]. Increased expression of proteins involved in plasma membrane transport [131-134], and β-oxidation of fats are also well described in athletic cohorts [135]. Lactate metabolism is also highly upregulated [136, 137], with blood lactate profiles showing significantly reduced circulating concentrations for identical workloads after a period of aerobic training [131, 138-140].

Athletes therefore represent a useful model to test fuel metabolism, with predictable responses to exercise stress, and a well-characterised metabolic phenotype.

1.11 Wider applications of ketosis

Elevated ketone body concentrations may confer therapeutic benefits in several pathological conditions. High fat ‘ketogenic’ diets have been used since the 1920’s for the treatment of intractable paediatric epilepsy [141, 142]. Currently, this dietary approach is still the only beneficial therapy available to children who suffer recurrent seizures despite optimal medications. This form of diet is unpleasant, and long term adherence is a constant challenge for carers and patients alike [143, 144].

The mechanism for the protective elevation of the seizure threshold during ketosis is, as yet, still to be elucidated. One theory is that ketone bodies have a membrane stabilising effect within neural tissue, and this is possibly linked to actions at the GABA receptor [145, 146]. Other theories have suggested that decreased glucose levels or an increase in the mitochondrial free energy of ATP hydrolysis is responsible

for the therapeutic effects of metabolising ketones [11]. However, conclusive evidence for these theories is still lacking.

Ketones have been successfully used to treat several inherited diseases of fatty acid metabolism, namely fatty acyl CoA dehydrogenase deficiency [147]. In this condition, the enzyme deficiency results in an inability to metabolise fatty acids via β-oxidation, resulting in a reliance on glucose or amino acids for energy requirements. The oral supplementation of ketone salts resulted in a relatively low level of ketosis (0.4 mM) which was sufficient to improve cardiac function and the MRI appearances of brain leukodystrophy [148].

1.12 This thesis

Inferences relating to cell metabolism and function at rest are of limited utility in understanding processes that limit human performance. From an evolutionary perspective, we are not designed to be sedentary, with the excesses of modern lifestyle evident in obesity and diabetes epidemics [149, 150]. In pathologic conditions, the stimulus of exercise or illness challenging homeostasis, and the capacity to withstand this insult, is of utmost relevance to the patient and clinician alike.

Ketone bodies have many potentially interesting theoretical applications in human health and disease. The physiologic basis of ketones is their impact on mitochondrial function and fuel supply during energetic stress. The biochemical breakthrough of an edible ketone allows the testing of some of these hypotheses. However, the new physiologic conditions induced following the consumption of this nutritional form of ketosis required characterisation in humans before further work could progress.

The work in this thesis explored the physiological effects of exogenous nutritional ketone bodies on human substrate, and exercise metabolism.

2 General methods

2.1 Ethical considerations and principles

Ethical approval

All studies were reviewed and given favourable ethical opinions by the regional Oxford Research Ethics Review Committee (OXREC) as part of the following applications:

1. 10/H0604/3
2. 10/H0605/10
3. 11/SC/0223
4. 07/Q1605/37

All testing was performed according to the principles of ethical research practice, as described in the declaration of Helsinki 2008 [151]. Cardiopulmonary exercise testing was performed according to the guidance contained within the American Thoracic Society position statement 2003[13] for cardiopulmonary exercise physiology.

Consent

All study subjects were provided with detailed study information, protocol description and study timeline more than 24 hours before the consent process. All subjects provided written informed consent before any participation in this research, and were free to withdraw from further participation at any time.

2.2 Subjects

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 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.

2.2.1 Eligibility criteria

- Age 18-40 years

- Currently undertaking endurance training > 12 hours 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.

2.2.2 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

2.3 Body Composition

All subjects were weighed at baseline and during the following trials; with body composition recorded using a non-invasive electrical bio-impedance device (bodystat™, Douglas, Isle of Mann). This device calculated body composition based on a two compartment model algorithm, with the assumption that the impedance of a biological tissue comprises two components, the resistance and the reactance [152, 153]. By measuring the impedance at 50 kHz and 200 kHz and by applying predictive equations, it is possible to estimate both extra-cellular water (ECW) and total body water (TBW).

2.4 Pulmonary gas exchange

Pulmonary gas exchange analysis was used to determine both time resolved kinetics, and 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 offloaded at the end of each test and time weighted into 10 second averages, and pooled over 2 min 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.

2.5 Incremental load physical testing

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 (Monark 928e Vansbro, Sweden) following personalised saddle, and handlebar adjustments. Subjects commenced all trials 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 [154]:

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

Ear prick capillary blood samples were obtained at the completion of each 3 min increment during the maximal trial to determine lactate kinetic profiles, and anaerobic thresholds (example in Figure 4).

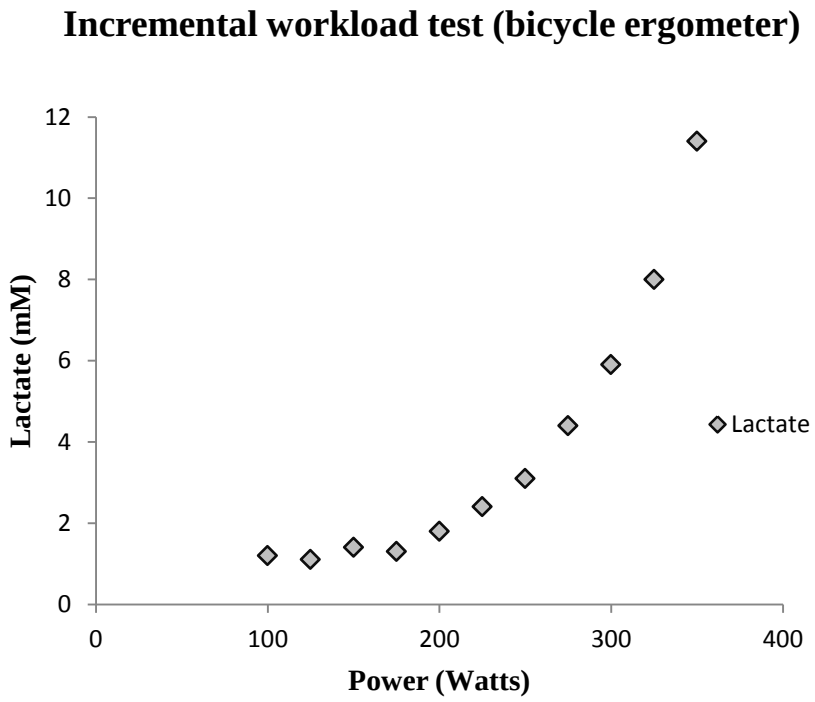


Figure 4 Example of a lactate profile from an incremental exercise test to volitional fatigue in a study participant. Lactate profiles were used to inform exercise domains and guide workloads for subsequent tests.

Determination of the anaerobic threshold

Anaerobic or ventilatory thresholds were determined for each trial from gas exchange measurements obtained during incremental maximal tests using the v-slope method [155, 156], as well as concomitant blood lactate profiles.

Determination of maximal oxygen uptake

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.

2.6 Blood sampling

IV cannulation

During stationary bicycle ergometer or rowing trials, subjects had a single 22 G (BD Venflon™, Becton Dickinson infusion therapy, Sweden) 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™, Becton Dickinson infusion therapy, Sweden) was placed on the catheter hub to allow repeated blood draws.

During exercise, blood samples were obtained via the 3 way tap (1-2 ml) and the catheter flushed with a small, 1-2 ml, prime of sterile isotonic (0.9%) saline (BD, Plymouth, UK) to prevent coagulation [157].

Storage of samples

Blood samples obtained during exercise were immediately transferred to cooled 4 ml blood tubes containing ethylenediaminetetraacetic acid (EDTA) (BD Vacutainer, Becton Dickinson, Plymouth UK), and immersed in an ice bath at + 4ºC until the end of the test. On completion of the exercise trial, all

samples were immediately centrifuged at 3600 rpm for 9 min at + 4ºC to separate cells from plasma. Plasma was subsequently pipetted in duplicate eppendorf tubes, rapidly frozen and stored at -80ºC until further analysis.

Capillary samples

Capillary blood samples (5-150 µL) were obtained at serial points during exercise, or during blood ketone kinetics at rest via an arterialised ear or fingertip, using a sterile lancet technique (Accu-chek™). Droplet capillary samples were subsequently immediately analysed using handheld analysers for lactate (Lactate Pro, Arkray, Japan), D-β-hydroxybutyrate, and glucose. The validity, accuracy, and reproducibility of these hand held analysers has been published previously [158-162].

2.7 Blood analysis

Direct assay

Frozen serum samples were thawed at room temperature for 1 hour, divided into aliquots and analysed in triplicate for determination of glucose, FFA, triglyceride, β-OH-Butyrate, and lactate using a commercial automated bench-top analyser (ABX Pentra 400, Montpellier, France). Samples were compared to internal standards, and calibrations performed before and after every batch of samples.

Elisa

Elisa assays were manually performed using commercially available assay kits for insulin (Mercodia, Sweden), and glycerol (Cayman Chemicals, USA).

2.8 Muscle biopsy

Muscle tissue was collected via percutaneous needle biopsy technique [163], 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 chlorhexidine spray (Hydrex®, 0.5% chlorhexidine, 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.

2.9 Muscle analysis

Triple quadrupolar-mass spectrometry (conducted at Cambridge University)

Metabolites were extracted from muscle by separating metabolites into soluble fractions using the water/chloroform/methanol technique [164, 165]. This method required the aliquoting of 600 µL of 2:1 chloroform/methanol into 2 ml eppendorf tubes containing approximately 100 mg of tissue, before lysis of the tissue for 20 min at 25 Hz in a tissue lyser (Qiagen, UK). The resulting homogenate was centrifuged for 25 min at 13000 rpm after the addition of a further 200 µL of water and chloroform. The separated aqueous and organic fractions were removed and stored on ice. This process was repeated twice to increase the extraction yield, before drying down the aqueous and organic fractions overnight in a fume hood. The dried fractions were reconstituted in 200 µL of acetonitrile and frozen at -80º C until further analysis. All samples were subsequently analysed using a Quattro™ triple quadrupolar mass spectrometer (Waters Ltd, Hertfordshire, UK).

Further methods detailing the running of specific analyses are contained within Appendix 9.2.

2.10 Statistics

Statistical analysis was performed using SPSS (V17, Chicago, USA) for all treatments. Statistical significance was determined as $p < 0.05$. All data were checked for normality of distribution prior to statistical analysis. All data were paired comparisons, and in the case of repeated trials containing more than two arms, an ANOVA for repeated measures was performed following initial tests to ensure that

sphericity assumptions were not violated, and corrected with additional post hoc Tukey, or Sidak corrections where appropriate.

2.11 WADA and banned substances

This ketone monoester was declared to the world anti-Doping agency (WADA) for an opinion on its legality for use in competition by athletes. The use of this ester was deemed by WADA to not constitute a banned substance. A copy of this communication (letter) is included in the Appendix 9.3.

3 Pharmacokinetics of D-β-hydroxybutyrate-R-1,3-butanediol

3.1 Thesis context

The oral ketone ester, D-β-hydroxybutyrate-R-1,3-butanediol, is the first of its kind to be studied in human subjects. Critical to the safe and effective oral delivery of this novel macronutrient was an understanding of its pharmacokinetic and dynamic characteristics. The aim of the work in this chapter was to determine the blood concentration kinetics of βHB following single administration of drinks containing ketone ester.

3.2 Introduction

Nutritional approaches to achieve ketosis have been used for decades as therapeutic interventions for weight-loss[166, 167], refractory epilepsy [168-170] and rare metabolic deficiencies [171]. Methods to produce a dietary ketosis, such as the Atkin's diet, have significant limitations [172], with ketosis coming at the cost of elevated blood lipids and depleted glycogen stores [173-175]. However esters analogous to that used here, were developed and tested in porcine and canine models from 1970-1999. The primary objective of this research was to develop a feasible alternative method of caloric delivery for parenteral or enteral nutrition.

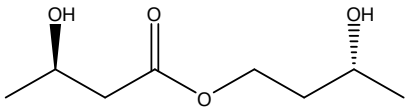
3.2.1 Chemical production

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% purity of raw (D-β-hydroxybutyrate-R 1,3-Butanediol) monoester (Appendix 9.1 for further details).

3.2.2 Physical properties of the ketone monoester

The physical properties of the ketone monoester are outlined in Table 3.

Table 3 Chemical and physical properties of D-β-hydroxybutyrate-R-1,3-butanediol monoester

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, acrid smell
Boiling point	145°C at 1.8 Torr
State at room temperature	Liquid
Density	1.0731 g/mL at 22°C

3.3 Methods

Drink preparations

In order to deliver the ketone ester in edible form the pure ketone ester was dissolved into two different drink preparations, designed to control for different factors within the respective study designs.

1. A chocolate flavoured milkshake drink containing 5.2% (by weight) of the ketone ester. In % K cals, it contained 20% protein, 45% carbohydrate, 5% fat and 30% ketone ester.
2. A citrus flavoured sports water (base constituent derived from a commercially available low calorie drink, V-spark™, Glaceau, UK).

Due to the strongly bitter taste of the pure ketone ester, the choice of drink formulations required extensive in-house development. The chocolate milkshake preparation (powder) was dissolved with water at a ratio of 1:1.5, and mixed in a food blender for 2 min before administration.

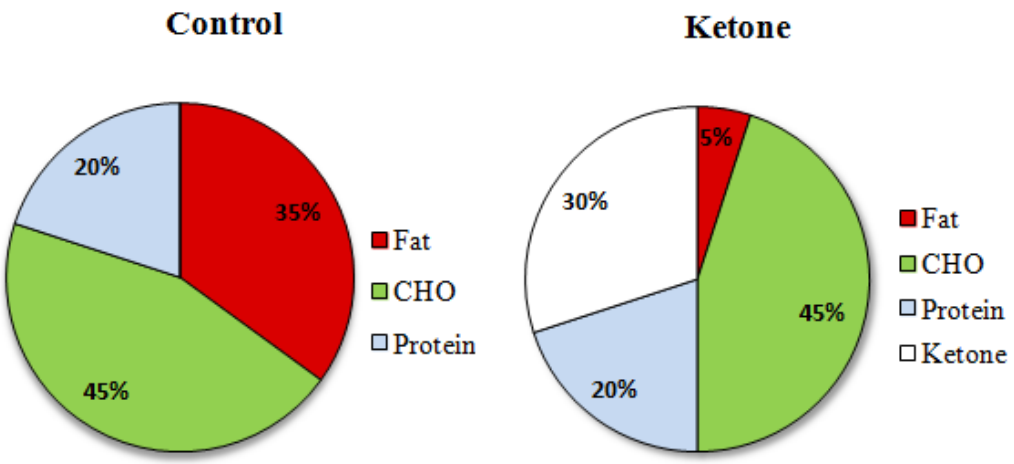


Figure 5 Calorie composition of preparation 1 (milkshake drink). Fat = long chain triglyceride, CHO = Carbohydrate. Control drink (left) and Ketone drink (right).

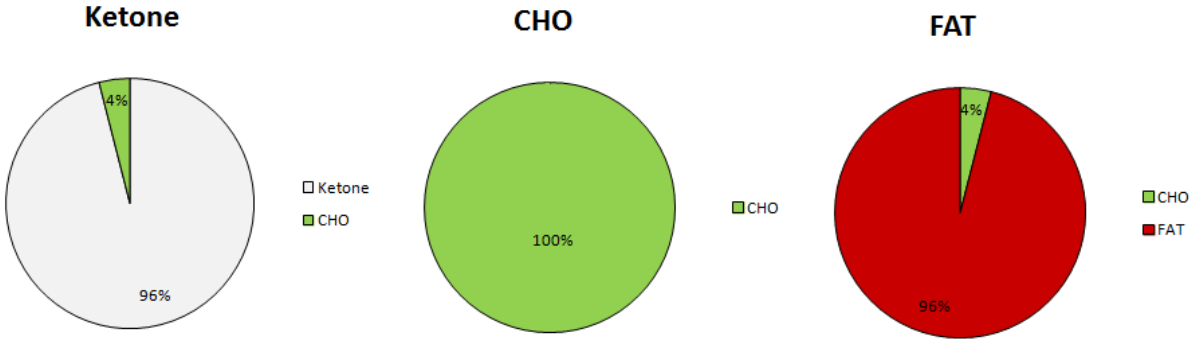


Figure 6 Sports water (preparation 2) calorie composition (left) and control drinks (middle and right) used in subsequent studies CHO = Carbohydrate, and Fat.

Subjects and protocols

Healthy athletes (n = 25) and normal controls (n = 20) ingested body weight adjusted doses of ketone monoester, with serial measurement of blood βHB according to methods previously described. Pharmacokinetic profiles were established in the course of study design and methods for subsequent exercise trials. A subset of n = 6 healthy athletes ingesting a 291 mg/kg dose of ketone ester also completed a symptom based questionnaire detailing systemic and gastrointestinal effects (see Appendix 9.5 for questionnaire) before and throughout ketosis.

Subjects were studied in the post absorptive state after an 8-12 hour fast. Blood βHB was measured every 15 min until complete elimination. Subjects were permitted water ad libitum during this time, and remained sedentary throughout the time course of measurements. Subjects were permitted a maximum of 5 min to ingest the respective preparations containing the ketone monoester, to reduce bias in gut absorption.

Blood sampling and analysis

Venous, and capillary blood samples were obtained according to methods previously described (General Methods 2.6 and 2.7).

Body composition analysis

Body composition analysis was performed using a Bodystat® bio-impedance device (General Methods, 2.3).

Analysis of data and statistics

Statistical significance was determined as $p < 0.05$. Comparisons between groups were performed using one way ANOVA with post hoc Sidak correction (SPSS V17, USA). Pharmacokinetic parameters were calculated from raw data, either by direct observation to determine maximum values, or in the case of time constants for rates of appearance (r), individual data sets were analysed using an iterative least sum of squares fitting procedure (Microsoft solver™, USA) fitted to a mono-exponential function of the form:

$$f(x) = A(1 - e^{-\frac{t}{r}})$$

Where A is the amplitude constant, r is the time constant, and t is time in minutes.

Area under the curve (AUC) was determined using a modified Simpson's rule [176, 177]. Data are expressed as the means +/- SEM unless otherwise stated, and the coefficient of variation (CV) is also reported for relevant pharmacological parameters.

3.4 Results

3.4.1 βHB kinetics (preparation 1)

The pharmacokinetic profile of the acute ingestion of a milkshake drink containing 500 mg/kg ketone monoester was performed in 10 healthy subjects. Blood βHB concentrations increased rapidly following the ingestion of the drink preparation, reaching a maximal concentration of 3.3 +/- 0.17 mM after an average of ~1 hour. Individual kinetic responses are presented in Table 4.

Table 4 Pharmacokinetics and anthropometric data from individual subjects (drink preparation I)

Morphology				**Parameter**				
Dose: **500mg/kg**								
Subject	Sex	Weight (kg)	Fat %	C_{Max} (mM)	T_{Max} (min)	*r* (min)	T_{Elim} (min)	AUC (mM.min)
1	M	84.1	15.0	3.3	45	14.2	150	272
2	F	72.8	19.9	3.9	90	26.5	300	580
3	M	84.3	17.6	2.8	90	12.7	270	371
4	M	77.0	8.5	3.1	45	19.5	300	515
5	M	61.1	9.2	2.2	60	9.8	270	344
6	M	65.9	6.5	3.6	90	24.1	300	578
7	M	77.0	14.0	3.2	60	15.5	240	299
8	M	82.0	22.3	3.6	75	31.6	255	468
9	F	68.9	18.5	4.1	75	18.3	270	584
10	M	68.4	14.5	3.3	45	11.1	255	457
Mean		74.2	14.6	3.31	67.5	18.3	261	447
+/- SE		2.5	1.6	0.17	6.02	2.26	14	38
CV %				16.5	28.2	39.0	17	27

Kinetic parameters of blood βHB following single dose ingestion (fasted state)

Inter-individual variation in kinetic responses ranged between ~17% and 39% with maximal concentrations (C_{Max}) having the lowest inter-individual variation. Time taken for complete elimination of circulating βHB varied between 150 min and 300 min (average = 261+/-14 min).

3.4.2 Effect of body composition on blood kinetic profiles

There were no significant correlations between body morphological or demographic characteristics and peak blood βHB concentrations, or kinetic profiles for drink preparation 1 (Figure 7).

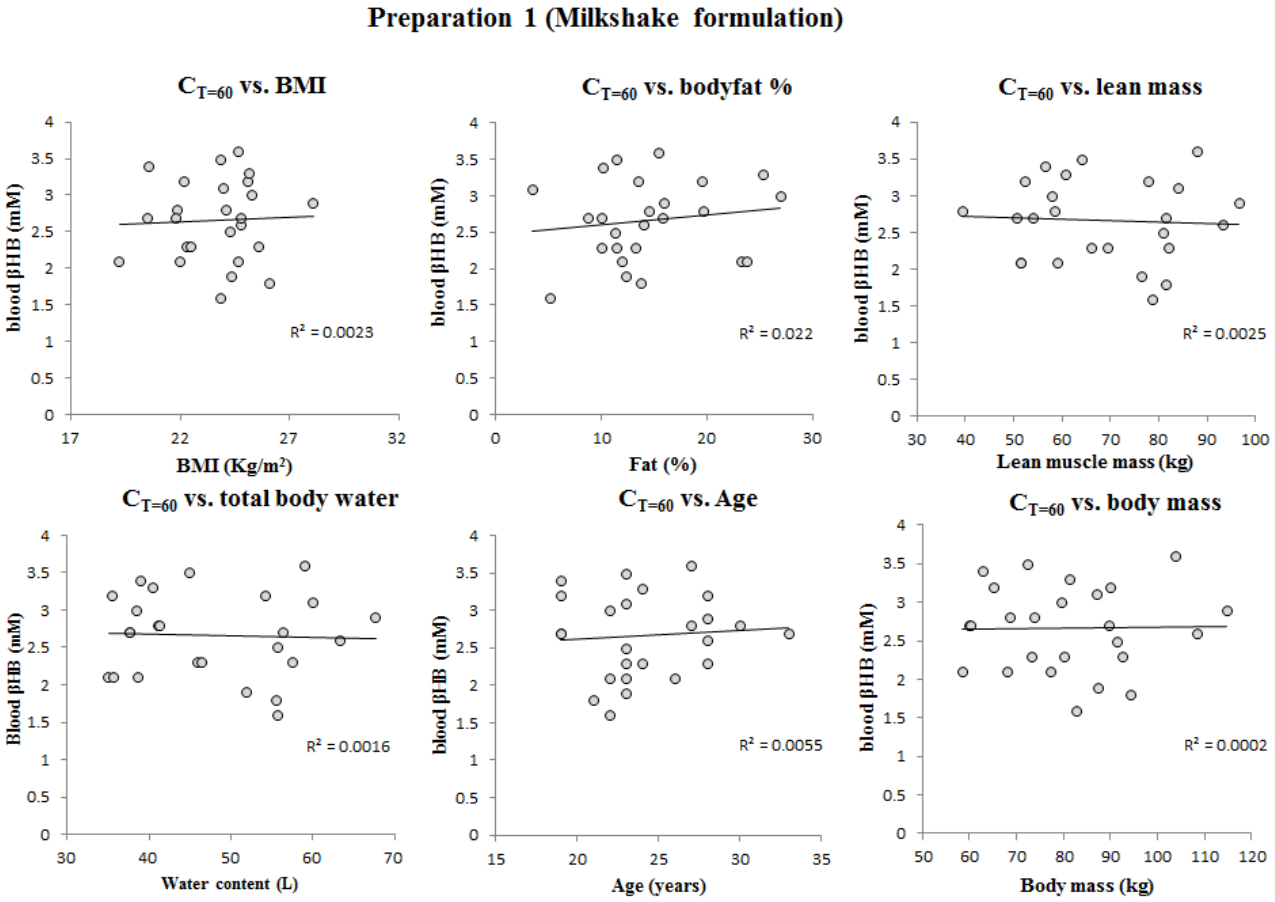


Figure 7 Graphical representation of the relationship between morphological variables and blood ketone concentration 60 min after the ingestion of the milkshake preparation ($C_{T=60}$). Data is from resting values obtained during chapter 4 (n=26). BMI = Body Mass Index. C_T = concentration at t = 60 min.

No significant correlation (Using Pearson's correlation coefficient) was observed between $C_{T=60}$ and BMI, body fat %, lean mass, total body water, age, or body mass.

3.4.3 βHB kinetics (preparation 2)

Similar kinetic profiles to the milkshake drink were observed over a range of different doses following the ingestion of the sports water preparation. Individual kinetic profiles are presented in tables 5 and 6. Ingestion of 4 increasing doses (192, 291, 395, and 573 mg/kg) of ketone ester all resulted in significant

elevations in blood βHB. In comparison to the milkshake preparation, an equivalent C_{Max} (~3.2 mM, p = 0.86 between preparations) was achieved after only 291 mg/kg dose of ketone ester, rather than the 500 mg/kg administered in the milkshake preparation. Furthermore, time to reach this maximal blood concentration (T_{Max}) was halved with this preparation (35 min vs. 67 min, $p < 0.05$ one way ANOVA).

3.4.4 Effect of dose on blood kinetic profiles

Increasing oral ketone dose resulted in a linear proportional increase ($R^2 = 0.9893$ $p < 0.001$, Figure 8) in peak plasma βHB concentration (C_{Max}). An increasing dose also resulted in a non-linear increase in AUC and progressively longer elimination time from the bloodstream. An increase in the time taken to reach peak blood concentrations was also observed with increasing dose (Figure 9).

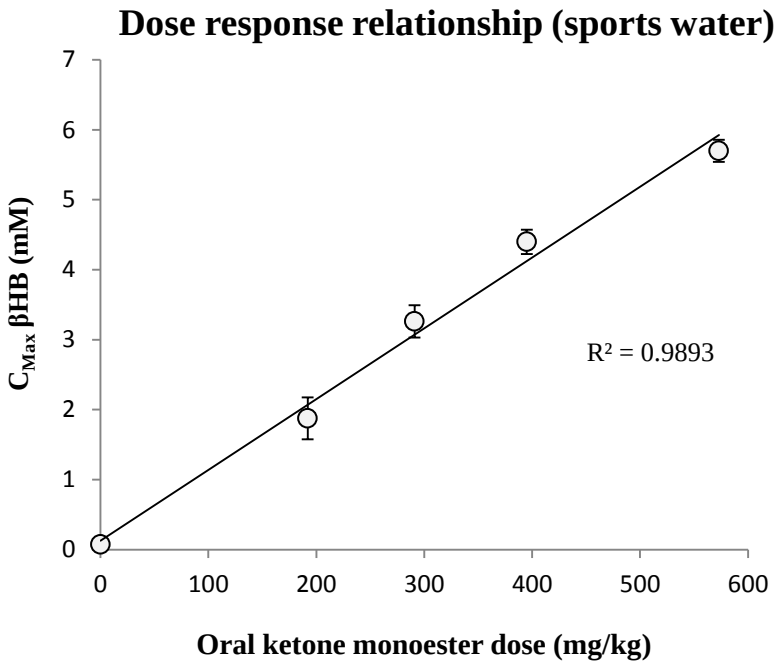


Figure 8 Dose / response relationship between blood βHB concentration and oral dose of ketone monoester. Each data point corresponds to the average value +/- SEM of C_{Max} presented in table 5 and 6. $R^2 = 0.989$ ($p < 0.001$ two tailed Pearson's correlation).

Table 5 Pharmacokinetics of drink preparation II

Morphology				**Parameter**				
Dose: **573mg/kg**								
Subject	Sex	Weight (kg)	Fat %	C_{Max} (mM)	T_{Max} (min)	*r* (min)	T_{Elim} (min)	AUC (mM.min)
1	F	67.0	20.9	4.3	60	15.3	300	1061.3
2	F	64.9	24.6	7.1	60	13.0	375	2164.5
3	F	60.0	19.2	4.9	60	15.1	300	1145.3
4	M	83.2	5.8	6.0	30	6.7	270	1117.5
5	F	66.4	19.5	6.3	90	15.8	300	1446.0
6	M	73.2	16.9	5.1	60	12.9	315	1016.3
7	M	71.1	6.0	6.2	75	14.0	300	1459.5
8	M	76.8	5.5	5.0	60	14.5	315	1026.0
9	M	86.1	21.8	6.4	90	29.9	420	2186.3
Mean		72.1	15.6	5.70	65.0	15.3	322	1402.5
+/- SE		2.9	2.5	0.30	6.12	2.0	15.5	156.1
CV %				15.9	28.3	40.2	14.4	33.4
Dose: **395mg/kg**								
Subject	Sex	Weight (kg)	Fat %	C_{Max} (mM)	T_{Max} (min)	*r* (min)	T_{Elim} (min)	AUC (mM.min)
1	M	87.1	8.6	5.0	30	7.0	225	468.8
2	F	53.4	12.5	3.8	45	8.9	285	435.0
3	M	63.5	16.2	5.0	60	7.7	300	680.3
4	F	52.1	14.5	3.5	45	4.4	225	380.3
5	F	64.4	21.3	5.0	105	12.8	225	684.8
6	F	72.1	16.9	3.4	60	12.8	240	405.0
7	M	84.8	17.1	4.2	30	6.4	240	422.3
8	M	78.0	27.3	5.3	60	5.9	225	530.3
Mean		69.4	16.8	4.40	54.4	8.2	246	500.8
+/- SE		4.7	2.0	0.27	8.5	1.1	10.5	42.7
CV %				17.3	44.0	37.8	12.2	24.2

Kinetic parameters of blood βHB following single dose ingestion (fasted state)

Table 6 Pharmacokinetics of drink preparation II (Continued)

Morphology				**Parameter**				
Dose: **291mg/kg**								
Subject	Sex	Weight (kg)	Fat %	C_{Max} (mM)	T_{Max} (min)	*r* (min)	T_{Elim} (min)	AUC (mM.min)
1	M	89.1	8.3	4.1	30	12	210	300.8
2	F	58.9	7.8	3.4	30	2.3	225	287.3
3	M	74.1	16.9	3.2	15	14.5	285	306.0
4	F	78.0	27.3	3.6	30	6.4	240	421.5
5	F	54.6	13.2	2.1	75	5.1	240	225.0
6	M	95.8	5.5	3.6	30	3.1	255	395.3
7	M	109.5	15.6	2.5	30	6.6	210	228.8
8	M	72.1	10.1	3.6	30	9.9	255	388.5
Mean		79.0	13.1	3.26	35.63	7.49	240	319.1
+/- SE		6.5	2.5	0.23	8.0	1.52	9.0	26.6
CV %				20.1	51.2	57.4	10.5	23.5
Dose: **192mg/kg**								
Subject	Sex	Weight (kg)	Fat %	C_{Max} (mM)	T_{Max} (min)	*r* (min)	T_{Elim} (min)	AUC (mM.min)
1	F	64.5	14.6	2.5	20	2.9	150	216.5
2	M	87.4	13.5	1.4	25	9.4	135	140.5
3	M	86.8	17.3	2.2	25	9.9	165	283.5
4	M	83.7	16.2	1.4	30	18.4	135	144.5
Mean		80.6	15.4	1.88	25.0	10.3	146	196.3
+/- SE		5.4	0.84	0.28	2.04	3.17	14.3	33.9
CV %				29.9	16.3	62.3	9.8	34.5

Kinetic parameters of blood βHB following single dose ingestion (fasted state)

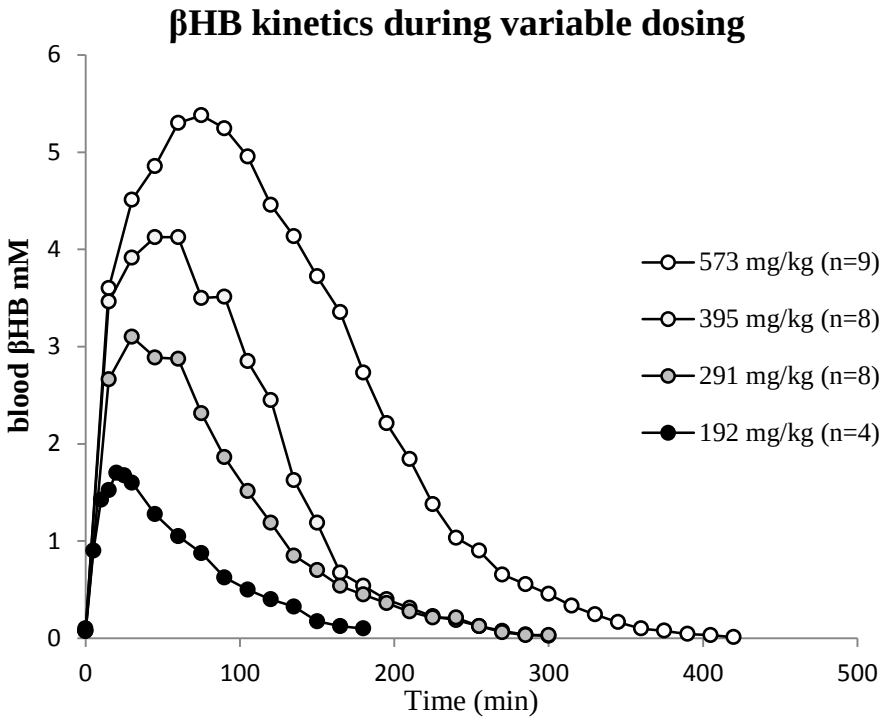


Figure 9 Blood profile of βHB following a single acute administration of various doses of ketone monoester (preparation 2). Error bars are omitted for data clarity. Individual data is presented in tables 5 and 6.

3.4.5 Tolerance and symptom profile during resting ketosis

A subset of n = 6 performed subjective symptom reporting during a standard 395 mg/kg dose of ketone monoester preparation 2. Individual descriptive characteristics are presented in Table 7. Subjects were all free of symptoms prior to the administration of ketone drinks; however in 4/6 participant's mild systemic symptoms were reported almost immediately following the ingestion of nutritional ketones. The types of symptoms were consistent between subjects who reported systemic upset, and consisted of headache, nausea, or dizziness. None of the reported symptoms were graded as more than mild-moderate (see Appendix 9.5 for scale), and no symptoms were reported at all in two study participants.

Table 7 Systemic and GI symptom scores following 291 mg/kg of preparation 2 (n=6).

Time	**Symptoms**					
	Subject 1	*Subject 2*	*Subject 3*	*Subject 4*	*Subject 5*	*Subject 6*
Pre	Nil	Nil	Nil	Nil	Nil	Nil
15 min	Nil	N1, D1	N2, D1,H1	D3,H3	Nil	Nil
30 min	D1	N1, D1	N2, D2,H1	D3,H3	Nil	Nil
45 min	D1,N1	N1	N1,D1,H2	D3,H2	Nil	Nil
60 min	D1	N1	N1,D1	D2,H3	Nil	Nil
75 min	D1	N1,D1	N1,D1,H1	D2,H3	Nil	Nil
90 min	D2	N1,D1	N1,D1,	D2,H3	Nil	Nil
120 min	D3	D2	D1	D1,H3	Nil	Nil
150 min	D4	Nil	Nil	H2	Nil	Nil
180 min	D5	Nil	Nil	H2	Nil	Nil
210 min	Nil	Nil	Nil	H2	Nil	
240 min	Nil	Nil	Nil	H2	Nil	

Data expressed as discrete variables from subjective rating scale of symptoms (see appendix) numerically rated on a 1-8 severity scale where 1 = mild, 8 = unbearable. N = nausea, D = dizziness, H = headache, Nil = no reported symptoms.

3.5 Discussion

Oral administration of this ketone monoester is an effective means of raising blood ketone levels, similar to those seen in many 'physiological' states, such as fasting. The ketone ester resulted in rapid increases in blood βHB in a dose dependent fashion. No evidence for an effect of body composition on the metabolic handling of this ester was found in this work.

Ketone ester pharmacokinetics

Pharmacokinetic modeling in this instance provides a number of challenges. Firstly the two components of this ester (once cleaved) have distinctly different metabolic journeys, although the resultant effect was the formation of βHB in blood, and subsequent oxidation as a respiratory fuel (Figure 10). Upon ester hydrolysis, free R-1,3-butanediol is released which is rapidly absorbed via gastric mucosa, whereas βHB may require membrane transport by intestinal monocarboxylate transporters (MCT) further down the gastrointestinal tract.

MCT expression on the luminal surface throughout the intestinal tract is principally isoform MCT 1, which increases in expression distally from the jejunum to colon in humans [178]. However, it

appears that different MCT isoforms (MCT 4 and 5) predominate on the basolateral membrane, each possessing distinct kinetic properties [178]. This suggests a complex affinity and distribution relationship for intestinal βHB absorption. The dissimilar absorption sites for each molecular subunit of ketone ester may well contribute to the inter-individual variability in plasma kinetic profiles observed earlier.

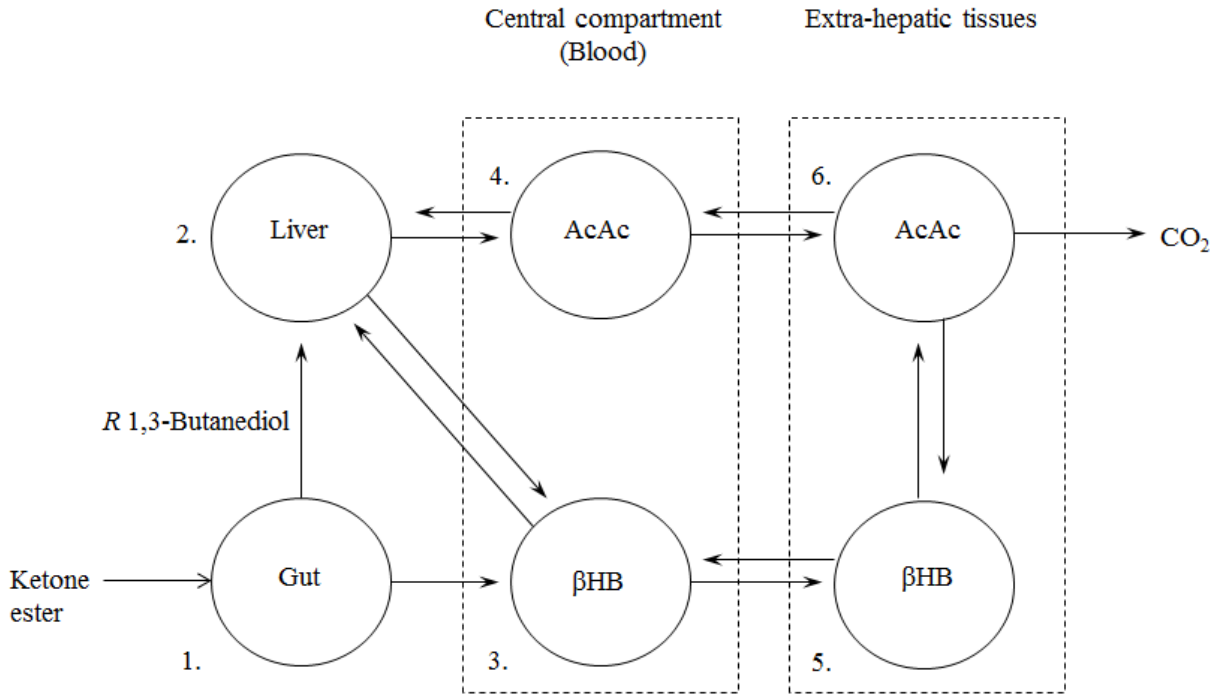


Figure 10 Proposed 6-compartmental model of ketone ester metabolism.

1,3-Butanediol, once diffused into the portal system, is oxidized in the liver by a series of dehydrogenase enzymes in identical fashion to ethanol [179, 180]. Multiple isoforms of alcohol dehydrogenase and aldehyde dehydrogenase have been identified, and result in disparate kinetic properties in the handling of ethanol [181]. The same can be observed in the varying inter-individual C_{Max} profiles following an identical dose of ketone ester. These kinetic constraints are important for the understanding of the potential therapeutic window of ketosis and for tolerance.

Symptoms and tolerance

The doses of ketone monoester employed here were targeted to achieve a blood concentration similar to that seen in a number of physiological states, and did not seek to investigate the upper limits of

possible ketosis via this method, nor of overdose. However, even during lower concentrations of ketosis, mild systemic symptoms such as nausea, dizziness, and headache were reported by some individuals. The exact cause of these findings is unknown, however oral administration of ketone salts in similar doses to those required to reach a modest physiological ketosis (0.3-0.5M) have been well described in the context of paediatric epilepsy and other metabolic conditions [11, 182]. The tolerance for the βHB or AcAc fractions delivered in this manner are seemingly free of any adverse effects, but the salt load and modest ketosis have precluded widespread clinical application. The tolerance profiles for 1,3-butanediol however, may not be as benign. It is apparent from literature reports on 1,3-butanediol that circulating concentrations in excess of 15 mM result in sedation and lethargy in pigs, with similar findings following the ingestion of other short chain alcohols. The association between the potency of alcoholic compounds to cause neurological impairment, and their lipid permeability is well described [183]. In the case of 1,3-butanediol, published literature suggests reduced lipid permeability than that of ethanol, attributable to the additional carbon linkage. The decreased lipid permeability of butanediol should theoretically exhibit a reduced neurological symptom profile compared with ethanol at equivalent blood concentrations. Principally conducted in rodents, these studies however, were unable to assess subtle variations in psychological state, cognitive processing and neuromuscular co-ordination relevant to humans; impairment of which are all features of even mild ethanol intoxication. Additionally conflicting literature evidence regarding the inebriating potency of 1,3-butanediol and a lack of formal studies in humans suggests cautious dosing considerations should be taken. This is particularly relevant for doses aiming to achieve supra-physiological ketone concentrations where acute ingestion of more than 40-50 g of 1,3-butanediol may be required. Whilst such concentrations are lower than those already studied in pigs, and rodents without adverse effect, the bioequivalent effect in humans is not known. In relative terms, the maximum legal blood ethanol level for operating a motor vehicle in the UK is 17.3 mM [184, 185]. However effects of ethanol on neuromotor co-ordination and cognitive function have been reported at blood concentrations below 6 mM. Regardless of any relative differences in sedating or inebriating potential between ethanol and 1,3-butanediol, any such effects are undesirable. Whilst low levels of blood alcohol (~ 2 mM) are unlikely to have any detectible effect, athletic performance may be

detrimentally affected at higher blood concentrations [186, 187]. Over the longer term, multiple toxicology[188-190], and teratogenicity [191] studies, as well as reviews by the FDA suggest 1,3-butanediol is safe for human consumption, and can be found in many foods as a bacteriostatic food additive or flavoring [192]. Study of chronic 1,3-butanediol administration in man was performed in 1973[78] without adverse effects when contributing 5% of daily calorie intake.

From this work and published findings [193, 194] it can be seen that while safe and effective for humans this ester may be accompanied by mild systemic upsets, possibly as a result of low levels of circulating 1,3-butanediol. These effects are not present in all subjects, and further work should aim to address the acute toxicity profile of supra physiological doses of ketone ester.

3.6 Limitations

It was not possible to track the functional time courses of the 1,3-butanediol component of this ester or its metabolic intermediates during this study. Stable isotope labeling would be required to determine the relative contributions of each half of the monoester with respect to their rates of appearance and time course. Furthermore, detailed compartmental analysis of this ester was not achievable in this study design, and much work needs to be done to determine correct pool models in light of the known complications of non-steady state acetoacetate/βHB equilibrium under many conditions [195]. However the generation of a working model for further compartmental analysis is conceivable based on current knowledge (Figure 10).

Repeatability studies were not performed in the course of this work, and may be useful to determine the intra-individual variation following the administration of nutritional ketone bodies from this ketone ester.

Effect of drink preparation on ketone kinetics

Further characterization of the effects of varying tonicity, volume of suspensions, and viscosity may be useful in understanding the inter-subject variability seen in this study. As already observed, significant differences were evident in the kinetic profiles between milkshake and sports water preparations.

Multiple dosing and chronic ingestion

Multiple doses of ketone ester will be required to achieve a pseudo steady state ketosis, and was not systematically investigated in the course of this work. This may provide additional challenges to achieve consistent effects. Firstly, due to the different absorptive sites of ketone ester, inevitable changes in splanchnic flow[196], meal composition [197], and even diurnal changes in gut transit time [198, 199] may complicate dosing requirements. Secondly, the rapid absorption and metabolism of βHB using this method will require large cumulative doses to maintain elevated plasma concentrations. Future work should aim to characterize the effect of multiple doses to achieve steady state blood ketosis.

3.7 Conclusions

This method of nutritional ketone body administration allows an effective means of providing a rapid ketosis in humans.

4 Nutritional ketosis and elite endurance performance

4.1 Thesis Context

Over the last century our understanding of the fundamental processes underlying human performance has expanded greatly. At the intersection of elite sport and substrate metabolism lies the potential to investigate the processes that define the limits of human physiology. Natural human competitive instinct and the advent of the modern Olympic Games have spawned and nurtured a fascination with high performance sport, with an estimated audience of 4 billion for the 2012 London Olympics [200]. This chapter explored the effects of a nutritional ketosis on elite athletic performance.

4.2 Introduction

The acute alteration of dietary macronutrients has long been known to influence physical performance [201]. The unifying mechanism for the generation of useful mechanical work from carbon-derived dietary fuels under aerobic conditions was first reported by Krebs [2]. He described a sequence of biochemical reactions culminating in the oxidation of carbon fuels to CO_2 with the creation of a mitochondrial electrochemical gradient used to phosphorylate ADP to ATP. Much work has been done to elucidate the cellular mechanisms regulating fuel supply during physical demands such as exercise or other physiologic insults. The preferential selection of fuels for oxidation is known to be in dynamic equilibrium, with the balance shifting rapidly in response to energy demand [202]. Substrate preference is also clearly influenced by the availability and supply of carbon substrates, as described by Randle and colleagues in their 'Glucose-Fatty acid cycle'. This conceptualised the interdependence of substrate supply and demand, in an effort to explain how pyruvate entry into the TCA cycle could be influenced by fatty acid availability. This theory suggested that oxidation of carbohydrates could be regulated by the fatty acid induced suppression of pyruvate dehydrogenase complex (PDC) activation by high acetyl-CoA/CoA ratio, and elevated citrate concentrations inhibiting forward glycolytic flux at the level of phosphofructokinase.

However the control of fuel selection during exercise does not seem to obey the conventional rules of the 'Randle cycle', as discussed in Chapter 1. Rather, the physiological demands of exercise are met

by an increased reliance on carbohydrate oxidation [21]. In starvation, the conservation of glucose and carbohydrate stores are of utmost importance and it is here that a frequently overlooked quirk of substrate metabolism occurs. In response to high energy demand and low blood glucose and insulin, our body produces ketones in the form of acetoacetate and D-β-hydroxybutyrate (βHB). These 4 carbon substrates are an evolutionary adaption to preserve carbohydrate stores, and provide an efficient fuel to sustain the energy requirements of brain, heart, muscle, and other organs. The much popularised Atkins diet sought to induce a nutritional ketosis through the manipulation of macronutrient intake [203]. However this diet has many metabolic disadvantages for the competitive athlete. Depletion of glycogen stores, and raised free fatty acids (FFA) during the dietary regime create a milieu of suboptimal cellular conditions leading to impaired physical performance [7, 204]. However, a novel method of inducing a rapid ketosis without the need for any alterations in macronutrient intake is now possible in humans. Administering an oral ketone mono-ester avoids a salt or acid load previously required to induce a similar magnitude of ketosis [205]. This method may therefore provide a nutritional form of ketone body to fuel exercise, a physiological state known to place great demand on fuel selection and energetic efficiency. To test the interaction between nutritional substrates and exercise performance with and without additional ketones, a cohort of high performance endurance athletes completed blinded maximal exercise tests of different durations following a meal replacement drink.

4.3 Methods

Subjects and recruitment

Athletes were recruited according to the qualification criteria defined by GB rowing for national trials. Both male and female athletes of all weight categories were included in the trial, with every effort made to recruit athletes of sub-elite or elite calibre. All athletes were healthy, with no previous history of medical illness. Athletes had been training continuously for at least 3 months prior to enrolment, with all athletes regularly performing these tests within their training and competition schedules.

A baseline medical questionnaire, physical examination, body composition analysis and ECG were performed before any exercise testing (Table 8). A baseline blood test was performed to exclude other possible medical causes that may influence performance, including anaemia [206], iron deficiency[206], electrolyte disturbances[207, 208], or other haematological abnormalities [209].

Table 8: Physical characteristics of study athletes

Athlete	***HWM***	***LWM***	***HWW***	***LWW***
Age (yr)	24.8 (+/-1.0)	24.2 (+/-1.6)	24.7 (+/-1.2)	20.4 (+/-0.9)
Height (m)	1.95 (+/-2.4)	1.81 (+/-2.7)	1.80 (+/-1.7)	1.72 (+/-1.8)
Weight (kg)	96.1 (+/-3.0)	75.2 (+/-2.6)	77.8 (+/-1.7)	63.0 (+/-1.9)
Fat %	12.5 (+/-1.1)	10.1 (+/-1.5)	23.7 (+/-1.9)	16.1 (+/-2.9)
Lean mass (kg)	83.8 (+/-1.9)	67.8 (+/-3.4)	56.8 (+/-4.2)	52.8 (+/-1.3)
BMI (kg/m^2)	25.3 (+/-0.4)	22.9 (+/-0.4)	24.0 (+/-0.8)	21.5 (+/-1.0)
Hb (g/L)	148.2 (+/-0.3)	152.3 (+/-0.2)	137.5 (+/-0.5)	127.0 (+/-0.2)
2 km PB (min:s)	6:04 (+/-3.0)	6:24 (+/-2.0)	7:05 (+/-6.0)	7:20 (+/-4.0)

HWM: Heavy weight male; ***LWM*:** Light weight male; ***HWW***: Heavy weight women; ***LWW***: Light weight women

4.3.1 Experimental design and performance trial

Subjects presented to the OCMR department, at the John Radcliffe Hospital, Oxford, following an overnight fast of at least 8 hours. Testing was performed at identical times of the day to reduce the effect of diurnal patterns on performance. Athletes were asked to consume an identical pre-testing meal the night before, as they would before a major competition, and to repeat this before every visit. Athletes were asked not to perform strenuous exercise in the 48 hours prior to each test, and to refrain from alcohol for 24 hours. Every effort was made to ensure that athletes were tested within the same macrocycle of training, and to standardise the timing of testing within a training week.

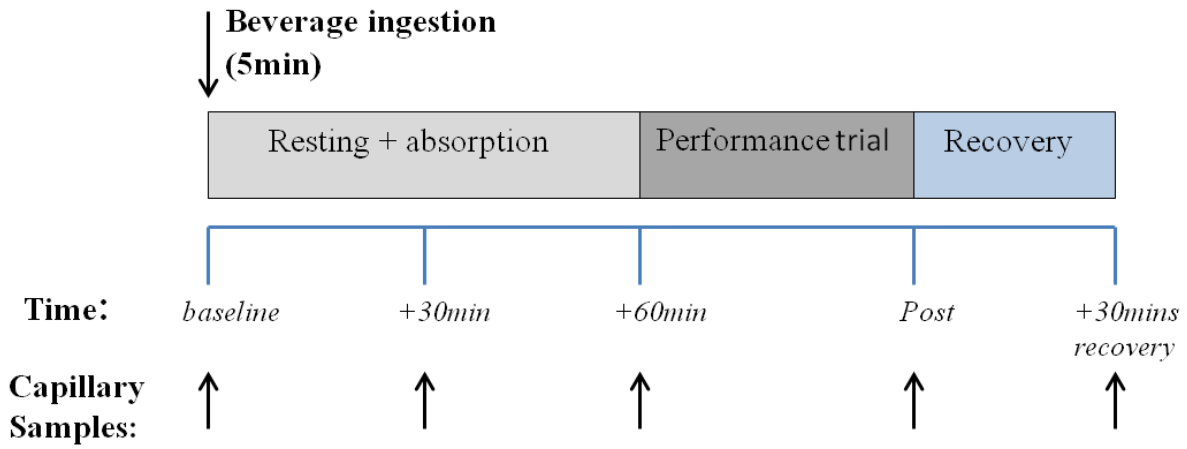


Figure 11 Schematic of study protocol for each day of testing; showing time points for drink ingestion, blood collection and performance trials. This protocol was repeated for control and ketone drinks.

On arrival subjects were weighed and their body composition recorded, before being escorted to a private room in the testing facility. Participants then ingested a body-weight adjusted dose of ketone or control drink over 5 min, and remained undisturbed for 60 min except for capillary blood samples at 30 min and 1 hour post ingestion (Figure 11).

All performance tests were conducted using a Concept II rowing ergometer (Model D, Nottingham, UK), which participants all used as part of everyday training, so required no familiarisation to the testing apparatus. Testing was performed in a private area free of distractions, and open to free air flow. All tests were performed in ambient conditions between 18 and 21º C, with additional cooling provided by floor mounted fans to minimise thermal stress. Athletes were tested individually in the presence of the same study investigator on each occasion. Verbal encouragement was provided if requested, otherwise testing was performed in silence. If music was requested by the athletes, an identical playlist was ensured on both trials. All capillary blood samples were obtained within 30 sec of exercise completion.

Performance data were automatically collected and stored in the Concept II ergometer (PM3 device) and offloaded in 5 min intervals during 30 min tests, or 500 m during 2 km trials. Data were stored on a data chip and transferred to a study laptop for analysis. Real time data were displayed on a screen in

front of the athlete to ensure adherence to stroke rates, with time or distance remaining, and 500 m split time also displayed. Athletes were forbidden from using the pacing boat function.

Drink preparation and dosing

Each athlete received 2.083 g/kg body weight of raw ketone drink powder mixed at a ratio of 1:1.5 parts water in a food blender for 2 min. The active ketone drink contained the raw ketone ester at a dose calculated to achieve a peak ketonaemia of 3 mM of β-hydroxybutyrate, equivalent to several days of total fasting. Control drinks were identical in taste, viscosity and colour to ketone, with the calorific equivalent of ketone replaced with long chain triglyceride in the placebo preparations. As a powder the compositions of milkshakes (% dry weight) were as follows: carbohydrate 45%, ketone 30%, protein 20%, and fat (5% for ketone drink or 35% for control). All subjects were blinded to drink allocation, and consumed drinks in a 5 min time interval, after which subjects rested for 60 min to allow digestion and minimise gastric distress.

Blood sampling

Capillary blood samples (10-50 µL) were obtained via an arterialised finger prick (Accu-chek™), and immediately analysed for glucose, βHB, and lactate using handheld monitors (Optium Xceed®, Abbott, UK and lactate Pro, Arkray, Japan).

Resting βHB kinetics

To evaluate the effects of exercise on the profile of blood ketone concentration, n = 7 representative age and sex matched athletic controls (n = 6 male and n = 1 female) were selected to undertake identical administration and absorption regimes of ketone containing beverage during resting sedentary conditions to enable comparison of resting vs. exercise changes in blood ketone concentration.

Statistics

Performance outcomes (distance in m over 30 min, and seconds to complete 2 km trial) were analysed using paired Student t-tests (two tailed), once a normal distribution of data was determined.

Comparisons of exercise vs. control blood ketone kinetics were made with a one way repeated measures ANOVA, with significant effects analysed using Tukey Post hoc correction.

Data evaluation was performed using SPSS (Version 17, Chicago, USA) with statistical significance set at $p < 0.05$. All data are presented as means +/- standard error of the mean, and represent the pooled averages of n = 22 for 30 min trial and n = 13 for 2 km time trial.

Two subjects in total were excluded from all analysis. One participant was excluded on the basis of intercurrent illness, and another due to vomiting during the 30 min trial with subsequent mechanical interference with testing apparatus. Neither result, if included would have altered overall study findings.

4.4 Results

4.4.1 30 min performance

A significant performance improvement was observed following ketone ingestion in the longer 30 min duration performance trial. Overall, 18/22 athletes achieved an improved performance while metabolising ketones, with an average gain in performance of 0.7% (Figure 12 and 13). In total, five athletes achieved new personal best performances (PB), while a further 9 achieved a new season's best performance (SB). Only one athlete in the control group achieved a new best performance. During ketosis, athletes rowed a mean distance of 51.2 +/-13 m ($p < 0.001$) further than the control arm (Figure 14 for Bland Altman plot of individual performances). There were no effects of trial order on performance outcome, with an average improvement of 55 +/- 26 m, and 47 +/- 7 m for those randomised to the ketone arm first, or the control respectively.

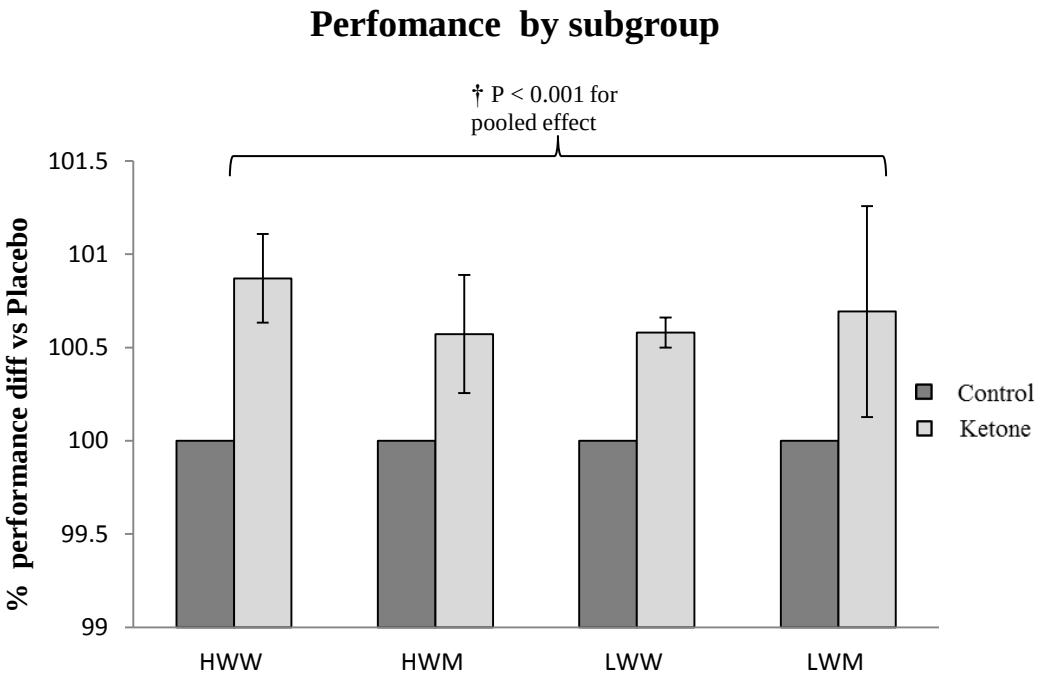


Figure 12 Performance effect for 30 min trial by subgroup of weight category relative to control (expressed as %). † Pooled effect $p < 0.001$ (two tailed T-test). Data expressed as means +/- SE.

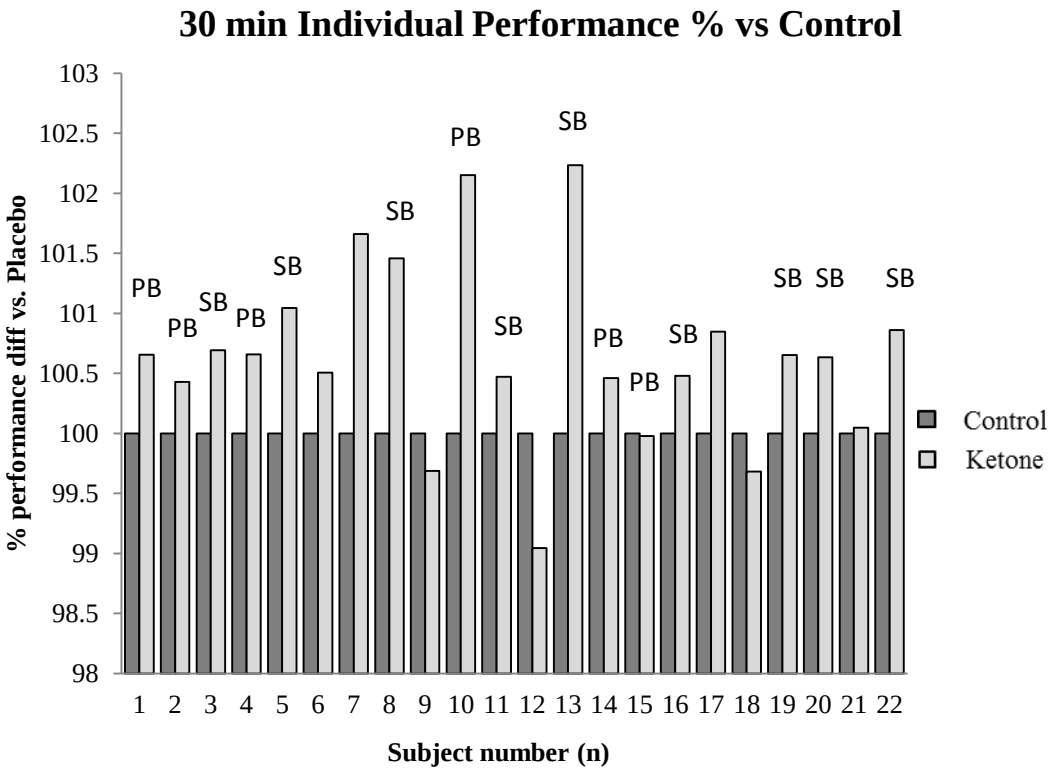


Figure 13 Individual performances relative to control (expressed as a % of control), **PB** Personal best performance, **SB** Seasons best performance.

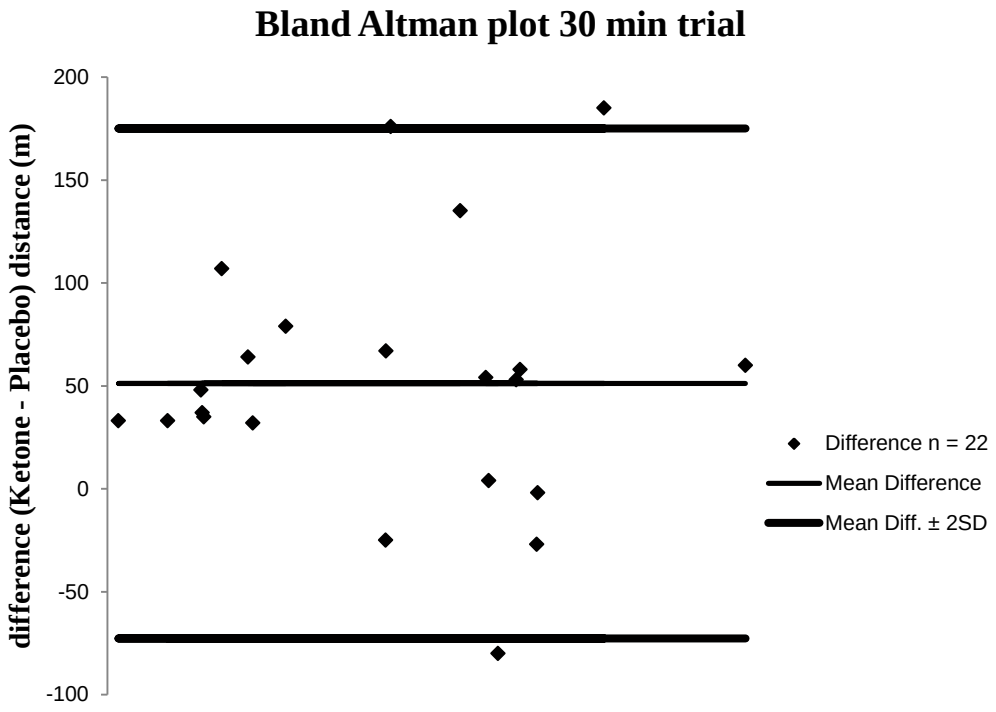


Figure 14 Bland Altman plot of individual performances over 30 min trials expressed as differences in raw distance travelled (m). Mean difference between ketone and control of 51.2m ($p < 0.001$ two tailed T-test). Horizontal scale omitted to protect the identity of athletes.

4.4.2 Power outputs

Power outputs during the 30 min test were almost identical during the first third of the test. However as the duration of exercise increased, power output between ketone and control arms became divergent (Figure 15). Power outputs were significantly greater during ketosis in the middle ($p < 0.05$) and final ($p < 0.01$) thirds of the time trial compared with control, corresponding to average increases of 4.9 +/- 1.6 and 9.5 +/- 2.6 W respectively. Power outputs followed similar profiles in both groups, with the lowest power outputs recorded during the middle third of the test ($p < 0.05$ for both arms). In the last part of the 30 min trial, athletes on the control arm were unable to achieve the levels of work performed in the initial part of the trial ($p < 0.05$), whereas power outputs during ketosis were not significantly different.

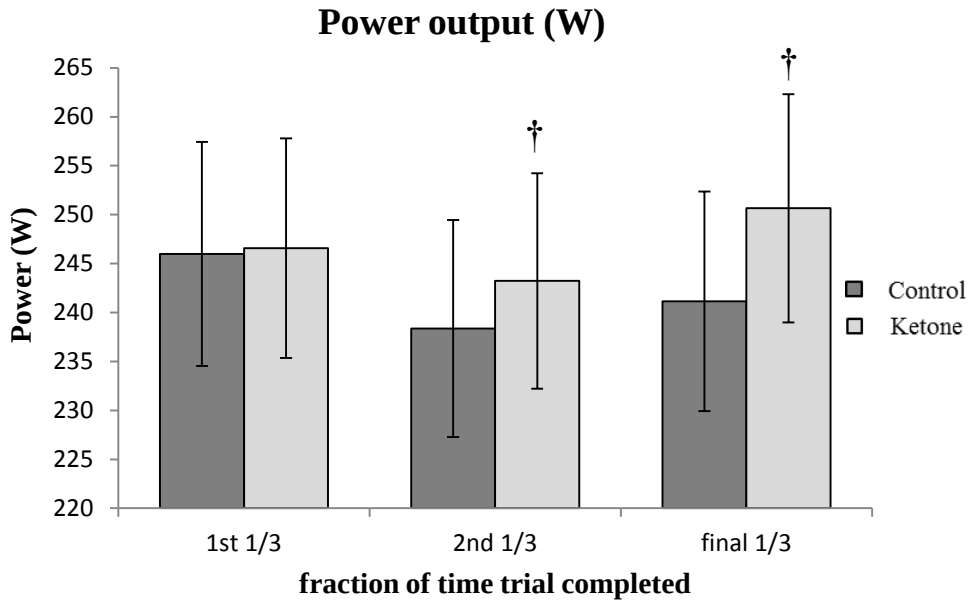


Figure 15 Power outputs during 30 min test pooled for thirds of the time trial completed. † $p < 0.05$ control vs. ketone arm. Data are expressed as means +/- SE.

4.4.3 Blood metabolites

Following the ingestion of the ketone containing beverage, blood levels of circulating βHB increased ~30 fold above overnight fasted levels, reaching 2.65 +/- 0.13 mM 60 min after ingestion (Figure 16). No differences in the absorptive profiles of blood βHB were seen between the athletes and the 7 athletic controls during resting conditions. However, blood βHB concentrations fell to 40% of pre-exercise concentration on completion of the 30 min trial ($p < 0.001$), and significantly less than non-exercising controls at comparative time points ($p < 0.001$). Blood ketone concentrations during the recovery phase 30 min after the end of exercise continued to fall ($p < 0.05$), most likely representing the on-going metabolism of ketones. Ketone concentrations were still markedly elevated ($p < 0.01$) relative to control at the completion of the performance trial, indicating that circulating ketones were elevated for the duration of the test, and had not been exhausted early in the trial.

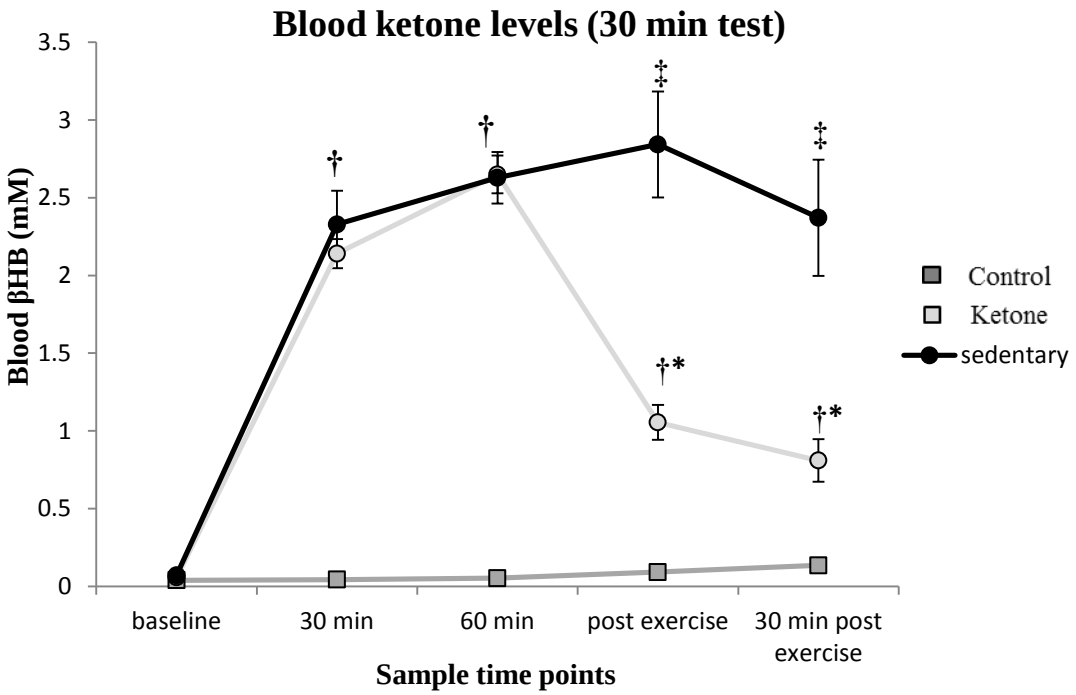


Figure 16 Changes in blood levels of βHB over the duration of each exercise trial. † $p < 0.01$ ketone vs. control for all time points.‡ $p < 0.05$ between sedentary and exercise conditions. * $p < 0.05$ vs. pre exercise values. Data expressed as mean +/- SEM.

Blood glucose concentrations were elevated above fasting levels 30 min following the ingestion of drinks on both arms, but were significantly higher in the control arm at 60 min ($p < 0.05$). Post exercise blood glucose concentrations increased in all subjects at the end of exercise and after 30 min of recovery (Figure 17). There was no statistical difference in blood glucose concentration at the end of exercise; however blood glucose was significantly lower ($p < 0.001$) in the ketone arm 30 min after the completion of the performance trial.

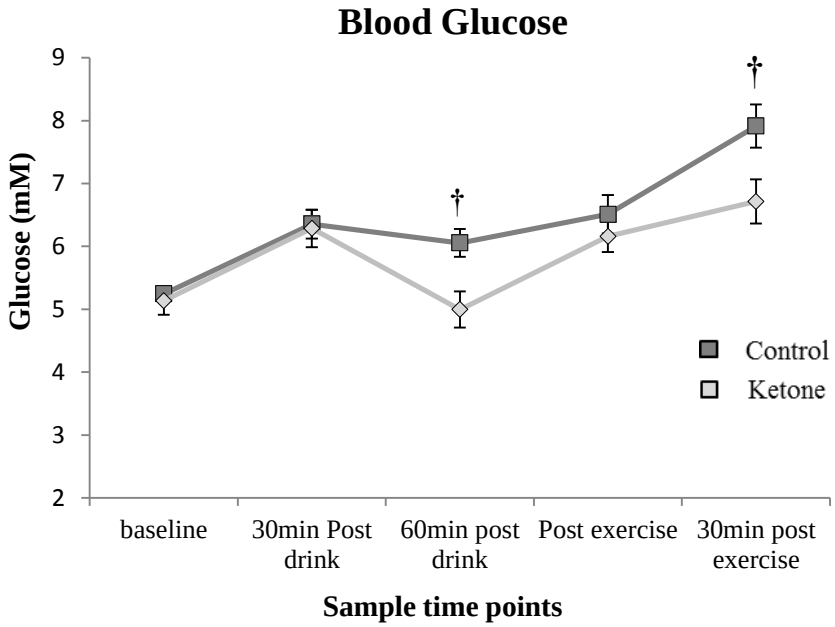


Figure 17 Blood glucose profiles during resting, post absorption, exercise and recovery on ketone and control drinks. Glucose concentrations were higher on control vs. ketone trials † $p < 0.05$.

There were no significant differences between ketone and control arms in blood lactate at any time point during the trials. Blood lactate concentrations at the end of exercise were markedly elevated compared to starting values on both arms (~15 mM), indicative of the 'all out' nature of this maximal trial. After 30 min of recovery lactate concentrations remained elevated relative to baseline, but were not statistically different between ketone or control arms (Figure 18).

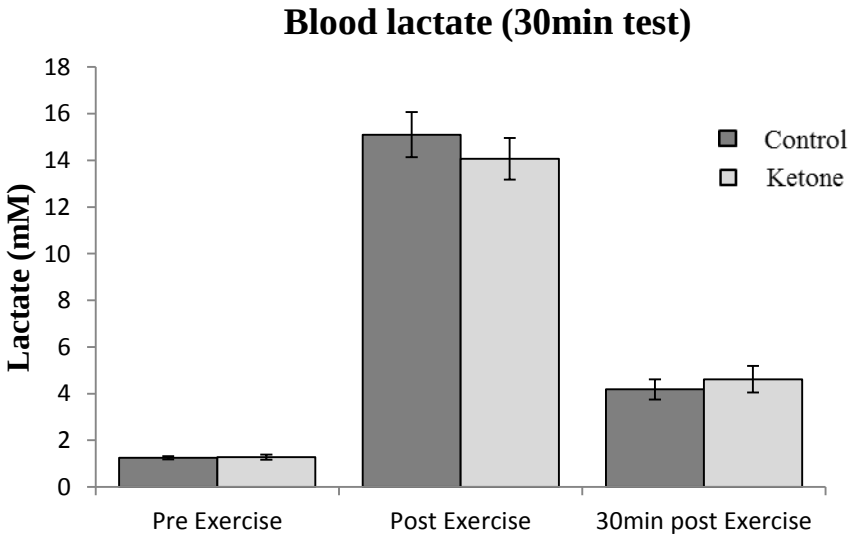


Figure 18 Blood lactate profiles before, immediately afterwards, and after 30 min of recovery. No significant differences were observed between arms at each time point. Data expressed as means +/- SE.

4.4.4 2km time trial

No differences in performance between control and ketone arms were observed during 2 km time trial tests (p = 0.22 Figures 19 and 20).

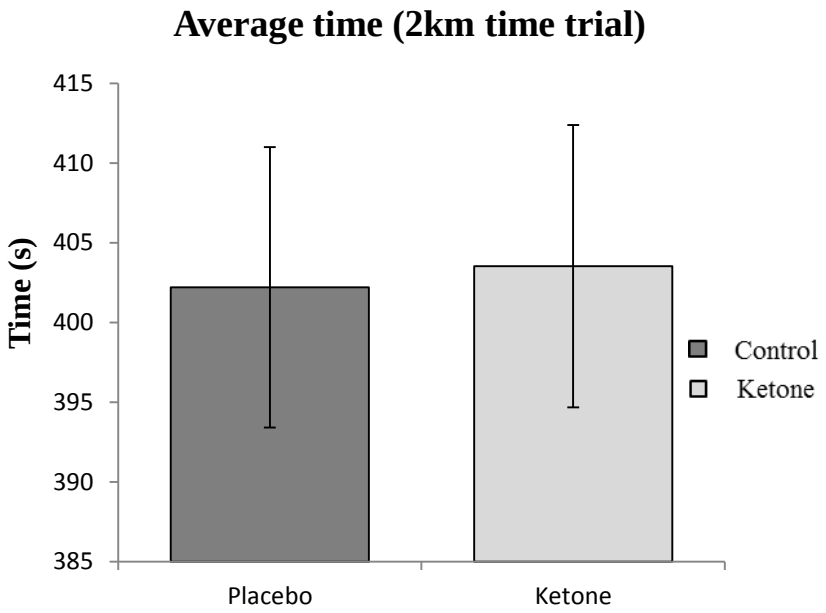


Figure 19 2 km time trial performances (pooled n = 13) were not different between control and ketone arms. Data are expressed as the mean +/- SE.

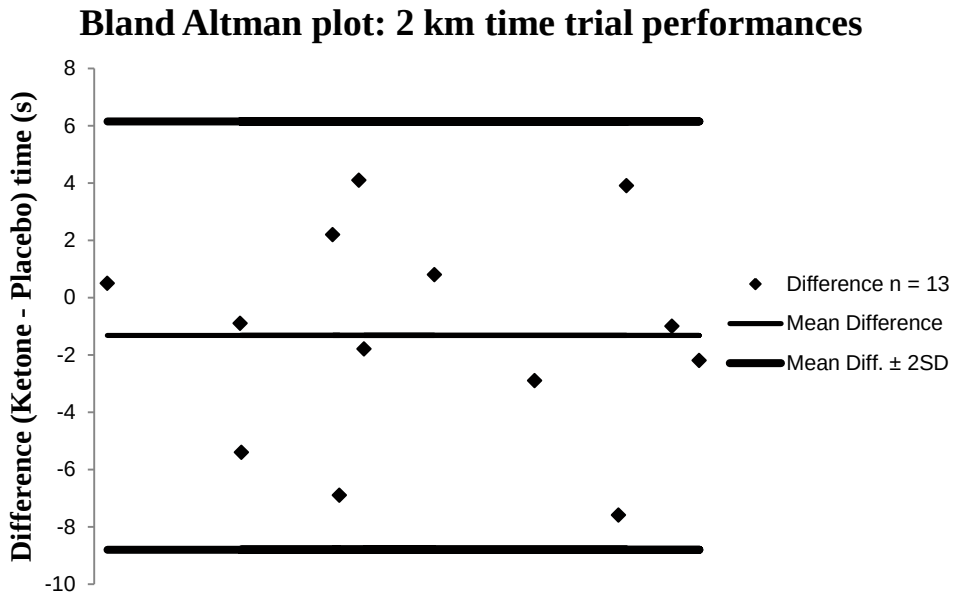


Figure 20 Bland Altman plot of 2 km rowing times. X axis omitted to protect athlete identity. No significant differences were observed between ketone and control, mean difference 1.32 sec +/- 1.08.

4.5 Discussion

This study showed for the first time, the oral administration of a ketone ester, and the efficacy of this method to induce an acute nutritional ketonaemia during exercise. Blood levels of βHB rose significantly to approximately 3 mM in all subjects following the ingestion of the ketone ester, and was rapidly metabolised during exercise. This degradation during exercise, and the well appreciated capacity of tissues to metabolise ketones, suggests that ketone bodies were used as a substrate to power exercise. The selection of ketone bodies as a substrate during exercise improved physical performance when the duration of exercise was of sufficient length.

Comparison of the power output profiles between ketone and control arms showed a significantly greater work output through the middle and final thirds of exercise during ketosis. This finding raises speculation of the mechanisms responsible for this performance enhancing effect. Generally speaking, the effects of ketosis may influence physical performance by affecting any one, or a combination of the following organ systems:

a) Skeletal muscle
b) Cardiovascular system (particularly cardiac pump function)
c) Central nervous system

Skeletal muscle

Alterations in skeletal muscle fuel selection may have reduced contractile fatigue, resulting in improved physical performance. The supply of nutritional ketones may have either improved energy transduction at the mitochondrial level by increasing the efficiency of ATP production, or increased the free energy available to power ATP generation. Both mechanisms have been shown to operate in the isolated perfused rat heart [210], and may well augment skeletal muscle performance during heavy sustained endurance effort.

Cardiac function:

High performance athletes are well known to have marked alterations in cardiac function and morphology that enables the athletic heart to maintain circulatory function during great demand [211,

212]. Myocardial fuel selection has received considerable attention as a potential therapeutic target to improve function in some disease states [37]. Similar to skeletal muscle, myocardial substrate selection shifts from FFA towards glucose as hydraulic workloads increase. Myocardial adaptation to exercise training, particularly changes in substrate metabolism, are complex and poorly understood [213]. It is generally accepted however that chronic high intensity exercise training leads to mitochondrial and enzymatic alterations, which improve energetic efficiency [213], and oxidative preference for carbohydrate [214, 215]. These training-related adaptations in the 'athlete's heart' and a known ability of the myocardium to oxidise ketones, may have improved myocardial contractile performance. Although myocardial function was not assessed formally, no changes in heart rate were observed between arms in this trial.

Central nervous system and performance

Another possible explanation for our findings could be an influence of central nervous system (CNS) governance over pacing strategy and perceived physical stress during performance trials [216]. The brain is subject to significant energetic stress of its own during exercise [217-220], with neural tissue having one of the highest rates of ATP turnover of any organ. Brain fuel selection is of paramount importance to maintain optimal neural function. The hierarchical position of the brain demands a constant supply of glucose to power executive tasks, which may be disrupted or inadequate during physiological stress [221, 222]. Ketone bodies are the primary alternative substrate to glucose that can be metabolised by neural tissue [223, 224], evolved to prolong survival during starvation[225]. For this reason ketone bodies may be an ideal supplemental fuel source for the CNS during physical stress, limiting central fatigue by maintaining the delicate energetic balance of the brain. It is possible therefore that CNS conditions could influence the performance of peripheral muscular work, and indirectly fuel selection during highly demanding physical exertion [226]. This may explain the divergent profiles in power output over the 30 min test, where any subjective sense of fatigue may have been buffered resulting in a higher fatigue threshold, and greater muscular work.

Duration and intensity

In this study, no evidence was found to support a positive influence of supplemental ketones on short maximal intensity bouts with a significant anaerobic emphasis (2 km trial). In contrast, during more prolonged lower intensity efforts, performances were improved (30 min trial). The metabolic explanation for this may be surmised from the low fractional contribution to energy production available from circulating substrates over short durations of exercise. Additionally during intense efforts, anaerobic production of ATP may outweigh any small contribution to energy production provided by ketosis. However during repeated intense efforts, the cumulative contribution to oxidative energy production provided by ketone bodies may no longer be negligible, and could result in fatigue resistance during multiple efforts. Further investigation is needed to explore this hypothesis. Importantly, these data indicate no deleterious impairment to performance during single intense efforts.

Limitations

It was not feasible to collect muscle tissue or blood during exercise in this study, the detailed metabolic effects of ketone oxidation during exercise requires further characterisation. In particular, possible alterations in mitochondrial energy transduction and interactions between substrates during ketosis may provide insight into the mechanisms governing cellular energetic efficiency. From an evolutionary perspective, ketones have evolved to conserve carbohydrate stores. However the 30 min duration of exercise used in this trial may not have caused exhaustive 'fuel depletion', and the relative effect of ketosis in sparing intramuscular fuel reserves is unknown. Further investigation of the role of supplemental ketones in prolonged exhaustive exercise may reveal additional performance improvements, possibly via the attenuation of muscle glycogen use. Likewise it would be of great value to examine the tissue specific effects of increased ketone availability on muscle heart and brain function during exercise and the integration of these systems in determining exercise capacity.

Applied interpretation

Presently, the relationship between a 30 min ergometer trial and a directly interpretable competition test for water rowing is unknown. This limitation is true of any laboratory-based inference on real

world performance. In an attempt to maximise the internal validity of an exercise test, laboratory conditions enable the tightest control of repeat test variance, but may sacrifice external validity in the process. The demonstrable effect that ketosis had on 30 min performance in this study should therefore be subject to cautious interpretation if applied to a real world competition scenario. However, the translation of these findings towards a potential benefit for competitive sport would be worthy of further investigation.

4.6 Conclusion

The addition of a circulating supply of ketone bodies to supplement the fuel requirements of energetically demanding exercise may have significant performance-improving benefit for the trained athlete. Ketone bodies may provide an additional fuel supply to power endurance exercise, providing a metabolic alternative to conventional substrates, carbohydrate and fat.

5 The effects of substrate on aerobic exercise metabolism

5.1 Thesis Context

An exponential decay in intramuscular glycogen occurs with sustained high intensity exercise [227, 228]. Once intramuscular glycogen becomes depleted, a marked decline in exercise performance is inevitable. Nutritional fuelling strategies designed to increase the exogenous contribution to energy demand during exercise have been the mainstay of sports nutrition for decades [229, 230]. While the common objective may be to attenuate the decline in muscular carbohydrate supply to preserve physical endurance, the best methods to achieve this remain contentious. Furthermore the intracellular mechanisms regulating fuel selection during exercise are poorly understood, with complex interaction between substrates and energy demand [202, 231-233].

5.2 Introduction

Previously, a nutritional ketosis was shown to have a beneficial effect on endurance performance lasting 30 min. One possible explanation for these findings is a profound alteration in fuel selection, which may supplement the supply of carbon units to the TCA cycle, and decreased rates of glycogenolysis during prolonged exercise. Providing ketone bodies to spare intramuscular reserves mimics the physiology of starvation, where ketone bodies provide fuel for oxidation and act as signals limiting glucose and glycogen metabolism [91, 234]. Ketone metabolism in starvation is a natural evolutionary protection mechanism, providing an alternative source of fuel to the brain, heart and skeletal muscle[52]. The supplementation of ketone bodies in physiologic states other than starvation may make use of our body's hardwired metabolic response to elevated blood ketones. Ketones can be readily oxidised by working muscle, and exert a strong influence over glycolytic flux in vivo. Elevated concentrations of ketones in a perfused working rat heart resulted in the suppression of glycolytic flux, even reporting a promotion of glycogen synthesis during continuous hydraulic work [45, 46].

However, the 'glucose- FFA cycle' of preferential mitochondrial substrate oxidation described by Randle [20] did not consider the influence of ketone bodies as a potential energy source in the

dynamic balance of fuel selection. In fact, until now, the concept of altering nutrition to reduce rates of glycogenolysis has been confined to the study of carbohydrate or fat [235-240].

Numerous studies have investigated dietary and pharmacological strategies to increase the exogenous provision of carbon units to the TCA cycle during exercise. Conflicting reports over the benefits of raising circulating fatty acids to spare glucose metabolism [241-245], and many studies failing to show convincing benefits of carbohydrate feeding [246-251] make a unifying hypothesis regarding optimal dietary strategy difficult. In addition, variations in exercise intensity, duration and study population further complicate the interpretation of fuel selection during exercise.

The cellular mechanisms that control mitochondrial preference for substrates during exercise are still to be fully understood. As previously mentioned the 'glucose-FFA' cycle is inadequate as a model of fuel selection during heavy exercise, as working muscle becomes increasingly reliant on glycolysis to provide acetyl-CoA to the TCA cycle, regardless of free fatty acid (FFA) availability. In addition, the presence of ketone bodies in these conditions makes clear inferences on mitochondrial substrate preference nearly impossible. Ketone bodies could provide a logical alternative for the delivery of carbon units to the TCA cycle, free of the limitations in mitochondrial and cell transport that restrict fat and carbohydrate utilisation. Further to acting as an alternative carbon supply, the greater enthalpy of ketone combustion over pyruvate could provide greater potential energy for conservation in the bonds of ATP. Therefore mimicking the physiology of starvation during exercise (by raising circulating ketone concentrations) may alter the hierarchical preference of mitochondrial substrate selection, effecting an improvement in energetic performance.

In order to test this hypothesis, a population of high performance endurance athletes performed repeated fatiguing exercise following dietary administration of carbohydrate, fat, or ketone ester. The goal of each dietary strategy was to provide optimal nutritional conditions to promote the respective oxidation of each substrate. Muscle biopsy was performed to quantify subcellular changes in muscle glycolytic intermediates, TCA cycle intermediates, carnitine derivatives and ketone metabolites. The

main objective of this study was to provide a greater understanding of the alterations in substrate selection during ketosis, and the potential mechanisms responsible for these changes.

5.3 Methods

Subjects

Table 9. Physical characteristics of study athletes

Athlete	Mean (+/- SEM)
Age (yr)	27.6 (+/- 1.6)
Height (m)	1.91 (+/- 3.5)
Weight (kg)	91.3 (+/- 5.2)
Fat %	11.8 (+/- 0.6)
LBM (kg)	79.6 (+/- 5.5)
H_20 %	62.7 (+/- 0.8)
$VO_{2\ Max}$ (L/min)	5.35 (+/- 0.2)
W_{max} (W)	392 (+/- 12)
BMI (kg/m^2)	24.8 (+/- 0.7)

Ten male high performance endurance athletes were recruited to undertake this trial. All had a minimum of 6 years endurance training in rowing, road cycling, or swimming. Seven of the athletes were former national champions or world top 8 performers. Physical characteristics are presented in Table 9.

All athletes provided written informed consent after having the invasive procedures, and methodological risks explained by the study investigator. Athletes volunteered for muscle biopsy procedures, and were free to withdraw their consent for this procedure without affecting their participation in the trial.

General design

Each athlete completed three experimental trials consisting of 60 min of constant load cycling at 75% of W_{Max} following the ingestion of isocaloric drinks of carbohydrate, fat or ketone ester. Trials were performed in a randomised single blinded cross over design, with a minimum of 1 week between trials. A further visit for the determination of individual resting ketone kinetic profiles using an identical administration protocol was performed on a separate non-exercising study day.

Preliminary testing

All participants undertook an incremental exercise test to exhaustion (see General Methods 2.5) on an electronically braked cycle ergometer (Monark 928e, Sweden) for the determination of $VO_{2\ Max}$ and W_{Max} at least 1 week prior to the first trial. The same cycling ergometer was used for the steady state exercise tests.

Experimental trials

Athletes reported to the testing facility in the John Radcliffe Hospital at 0800 hours following an overnight fast of 10 hours. Participants rested whilst catheters were inserted and blood samples obtained, before ingesting a drink containing carbohydrate, emulsified fat, or ketone monoester (Figure 6). Drinks were ingested 15 min prior to the onset of exercise, over a 5 min time interval. Respiratory gas collections were performed continuously for 2 min prior to the onset of exercise and for the initial 10 min of each exercise bout. Thereafter respired gases were collected for 2 min intervals at 25 min, 35 min, 45 min, 50 min, and 60 min. At 45 min elapsed time, athletes were stopped for 2.5 min and asked to ingest a second smaller volume beverage as a 'top up' for the remaining 15 min of exercise.

All exercise was performed under controlled conditions at 20-22^{o} C, with additional cooling provided by floor mounted fans. Every effort was made not to disturb athletes during blood sampling or gas exchange measurements, and athletes were not permitted musical distraction.

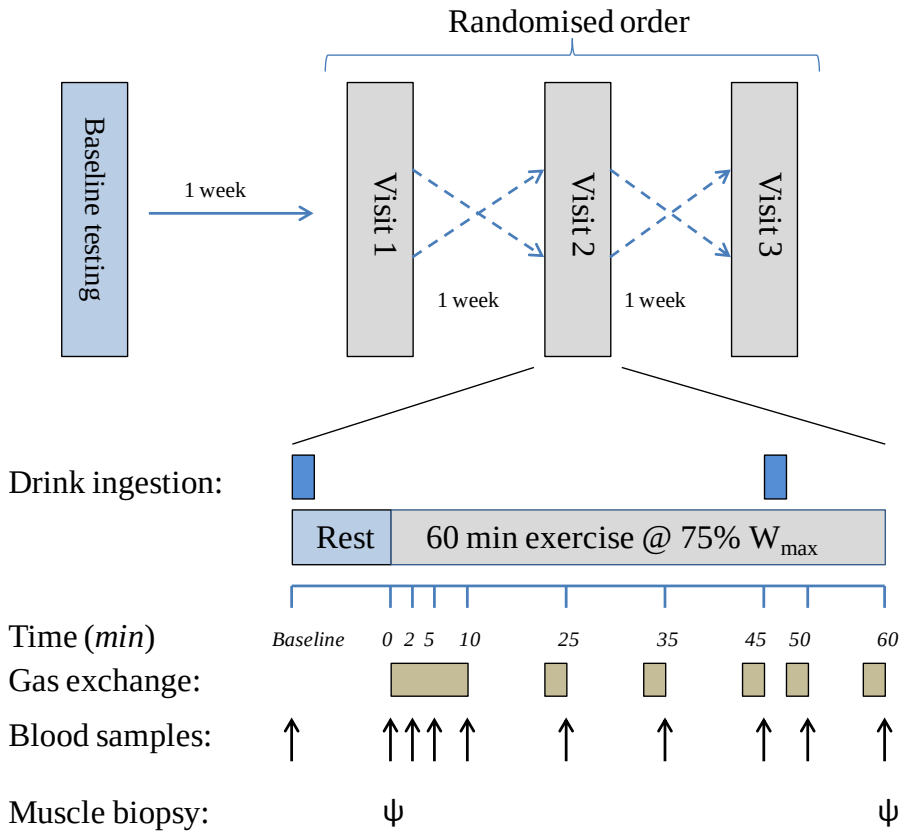


Figure 21 Study design schematic showing preliminary testing, randomised cross over design and exercise protocol with sampling points on each occasion.

Diet and activity prior to testing

Athletes undertook a diet diary of 3 representative days during a single week, to provide a standardised diet plan for pre-testing calorie intake. Prior to each testing visit, athletes were asked to consume identical calorie intakes in the 24 hour period, consisting of either a normal diet, or a high fat very low carbohydrate diet. To assist athletes in execution of high fat, low carbohydrate diets, meal instructions and plans were given to each athlete before the dietary period, and asked to record all intakes to ensure compliance.

Athletes were asked to refrain from hard physical exertion 48 – 24 hours before each visit, and no physical exertion beyond activities of daily life within 24 hours of each test.

Substrate preparation and delivery

Drinks were isocaloric in energy composition, and taste matched using sweeteners (Neotame™, NutraSweet, USA) or bitter additive (Symrise, product number 648352, UK) to ensure blinding (Chapter 3 preparation 2). Calories of substrate were bodyweight adjusted, and dosed to ensure a minimum carbohydrate delivery of 1.2 g/min of exercise [154, 252, 253] 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 a long chain triglyceride (Calogen™, Zoetermeer, Netherlands), Carbohydrate (Maltodextrin, fructose ratio 5:1, Gu Gels, Berkeley, USA), or ketone monoester.

All subjects drank a 75% dose of beverage 10 min prior to the start of exercise, and the final 25 % at 45 min into each trial. During the trial subjects were allowed water *ad libitum.*

Blood sampling and analysis

As described in General Methods section 2.6.

Blood samples (1-2 ml) were obtained at the start of exercise, 2 min, 5 min, 10 min, 25 min, 35 min; 45 min, 50 min, and 60 min elapsed time (Figure 21). Once removed from the catheter, blood was immediately stored in cooled blood tubes containing EDTA, and stored in an ice bath. Samples were analysed according to methods previously described (General Methods 2.7).

Indirect calorimetry

Gas exchange measurements were performed according to methods previously described (General Methods 2.4).

Muscle biopsy and analysis

Muscle tissue was obtained from the Vastus Lateralis muscle before and immediately after each exercise bout (General Methods 2.8). Samples were immediately frozen in liquid nitrogen, and stored at -80º C until further analysis. Muscle samples were homogenised to extract aqueous and organic metabolites, and analysed using a Quattro triple Quadrupolar mass spectrometer (see Appendix 9.2 for elaborated methods).

Statistics

Statistical analysis was performed for significant effects using 3-way ANOVA for repeated measures. Post hoc testing using Tukey correction was used to determine significant effects between arms. One way repeated measures ANOVA with post hoc Tukey correction was used to determine differences in resting vs. exercise ketone kinetics. Statistical significance was determined as $p < 0.05$. All data is presented as the mean +/- SEM unless otherwise stated. Muscle metabolite concentrations are presented in arbitrary units (AU), or as a comparative ratio to carbohydrate (Figure 30). One athlete withdrew consent for biopsy procedures during the trial and this data was not included in the analysis. Two athletes were unable to complete the full 60 min of exercise due to exhaustion (one single trial each on carbohydrate and fat feeding arms) and all data collected up to this point were included in the analysis.

5.4 Results

5.4.1 Gas exchange

No differences in resting or exercising oxygen uptake were observed between trial arms (Table 10), with oxygen uptake increasing ~8 fold from resting conditions on all arms during exercise. Respiratory exchange ratios (RER) were not different between arms at rest, although considerable variation in average RER values were observed between subjects. During the initial 5 minutes of exercise RER values were significantly higher on the carbohydrate arm ($p < 0.05$ vs. Ketone and Fat) and tended to remain higher throughout exercise. No differences were observed between fat and ketone trials.

Respiratory exchange ratios were all close to unity (~0.9 or above) during exercise, indicative of exercise intensities being primarily reliant on carbohydrates for fuel (see later discussion).

Table 10 Pulmonary gas exchange at rest and exercise following the administration of dietary substrate drinks.

Time	**VO_2 (L/min)**			**RER**		
	Ketone	*CHO*	*Fat*	*Ketone*	*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 expressed as mean +/- SEM. ‡ $p < 0.05$ between CHO and Fat.* $p < 0.05$ between CHO and ketone.

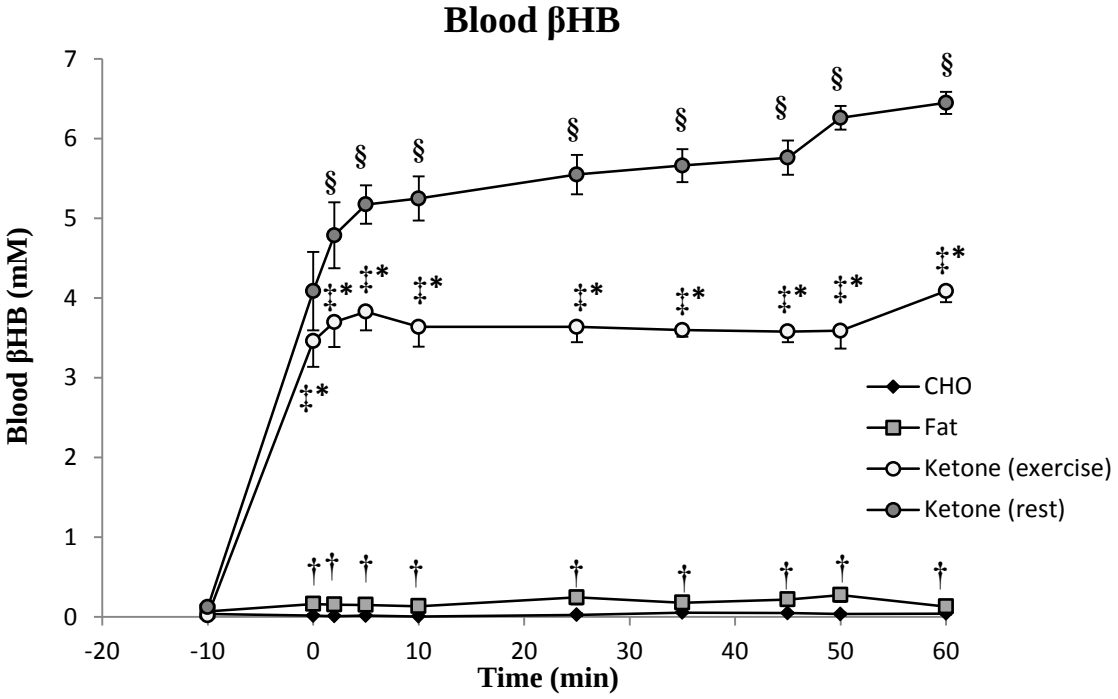


Figure 22 βHB concentrations were significantly lower at all time points after the onset of exercise compared to resting conditions. § $p < 0.05$ resting vs. exercise. ‡ $p < 0.05$ Ketone vs. Fat. * $p < 0.05$ Ketone vs. Carbohydrate. † $p < 0.05$ Fat vs. Carbohydrate.

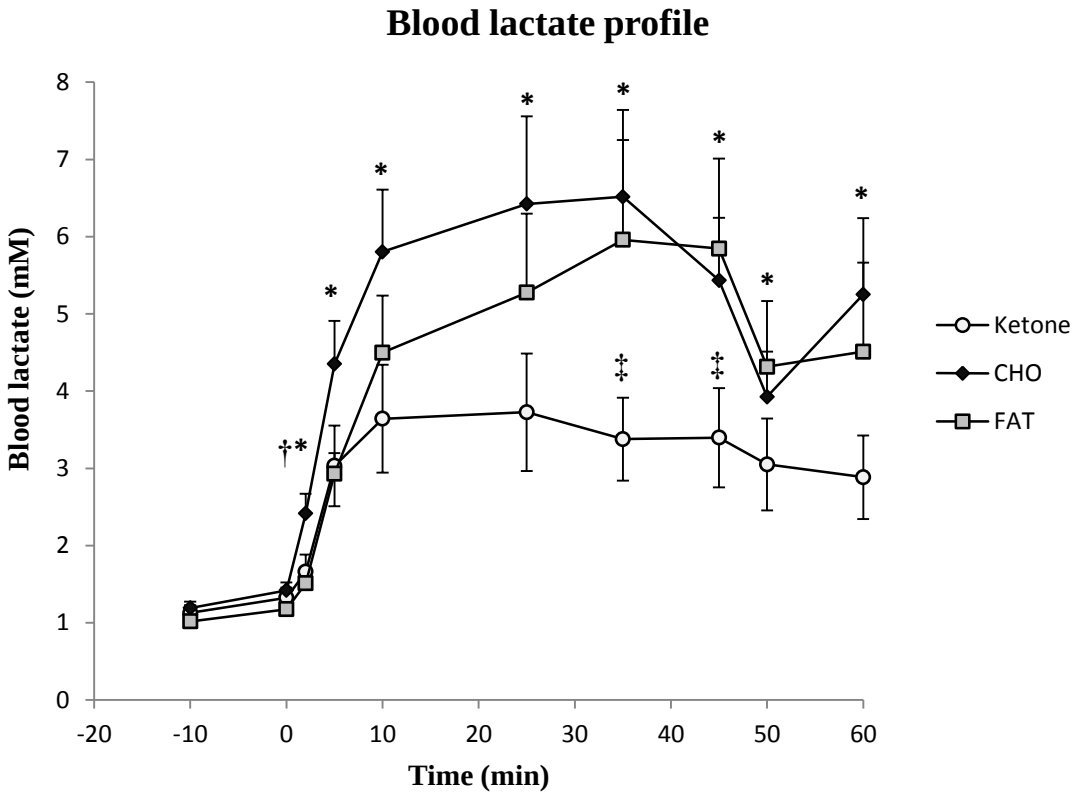


Figure 23 Blood lactate was significantly reduced compared to fat and carbohydrate during exercise with a nutritional ketosis. Carbohydrate ingestion resulted in elevated lactate concentration during the early part of exercise, with concentrations on the fat arm taking longer to reach peak concentration at 45 min. † indicates a significant difference ($p < 0.05$ 3 way repeated measures ANOVA with Tukey post hoc correction) between Fat and Ketone trials, * $p < 0.05$ between CHO and Ketone trials, and ‡ $p < 0.05$ between Fat and Carbohydrate trials. Data expressed as mean +/- SEM.

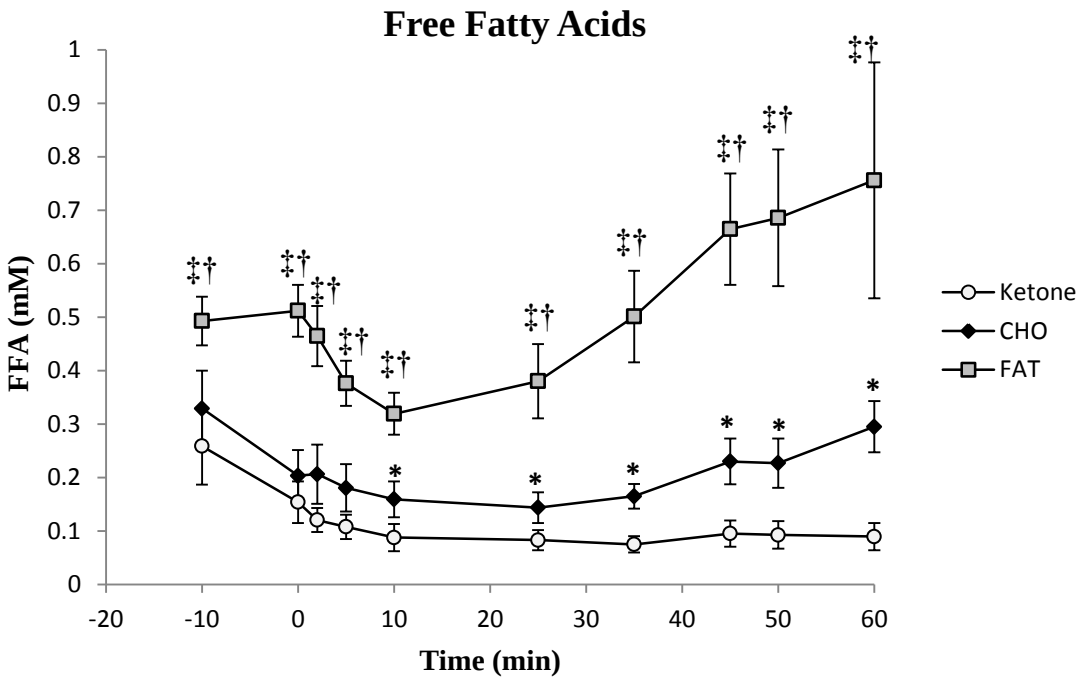


Figure 24 Plasma fatty acid levels at baseline, following drink ingestion and subsequent exercise for 60 min at 75 % of W_{max}. † indicates significant difference ($p < 0.05$ 3 way repeated measures ANOVA with Tukey post hoc correction) between Fat and Ketone trials, * $p < 0.05$ between CHO and Ketone trials, and ‡ $p < 0.05$ between Fat and Carbohydrate trials.

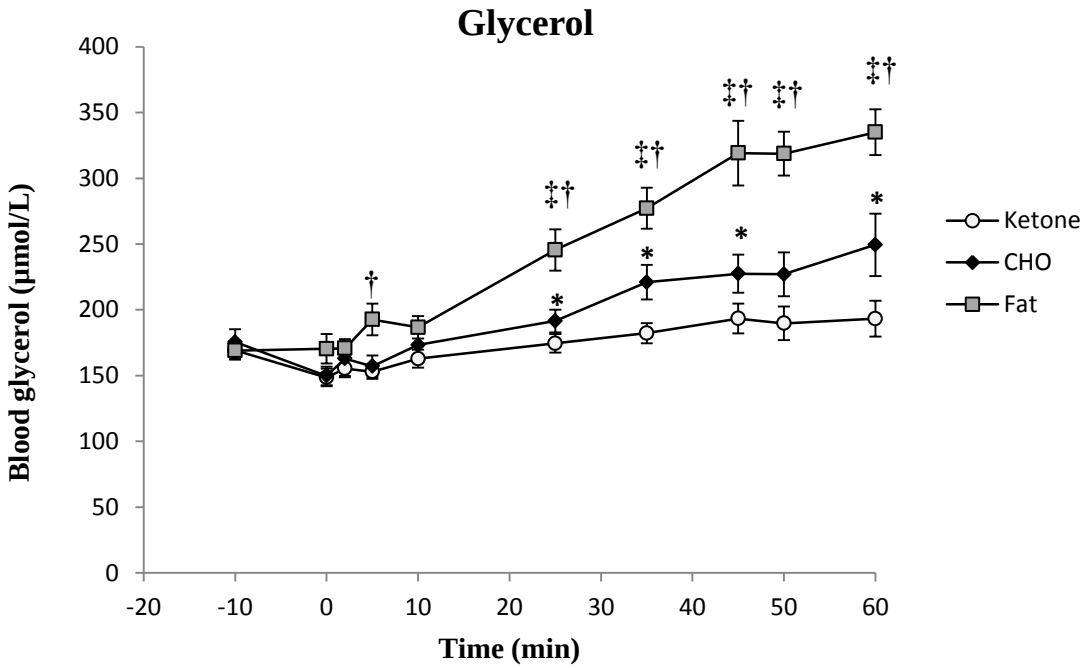


Figure 25 Plasma glycerol concentrations. † indicates significant difference ($p < 0.05$ 3 way repeated measures ANOVA with Tukey post hoc correction) between Fat and Ketone trials, * $p < 0.05$ between CHO and Ketone trials, and ‡ $p < 0.05$ between Fat and Carbohydrate trials.

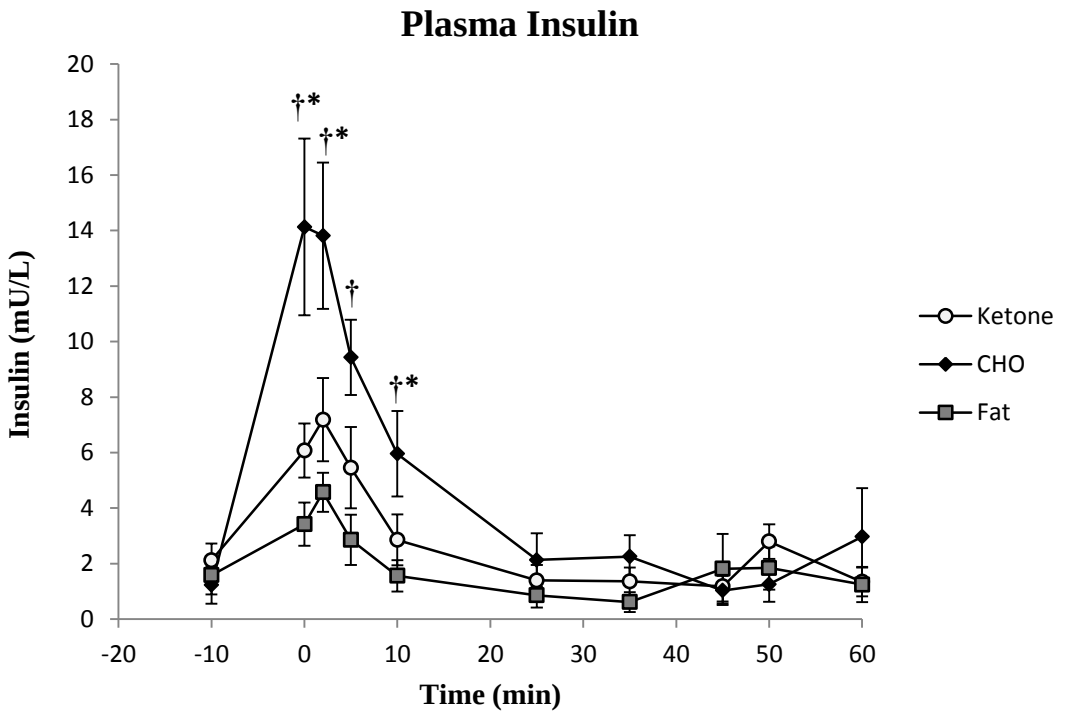


Figure 26 Plasma insulin levels. † indicates significant difference ($p < 0.05$ 3 way repeated measures ANOVA with Tukey post hoc correction) between Fat and Ketone trials, * $p < 0.05$ between CHO and Ketone trials, and ‡ $p < 0.05$ between Fat and Carbohydrate trials.

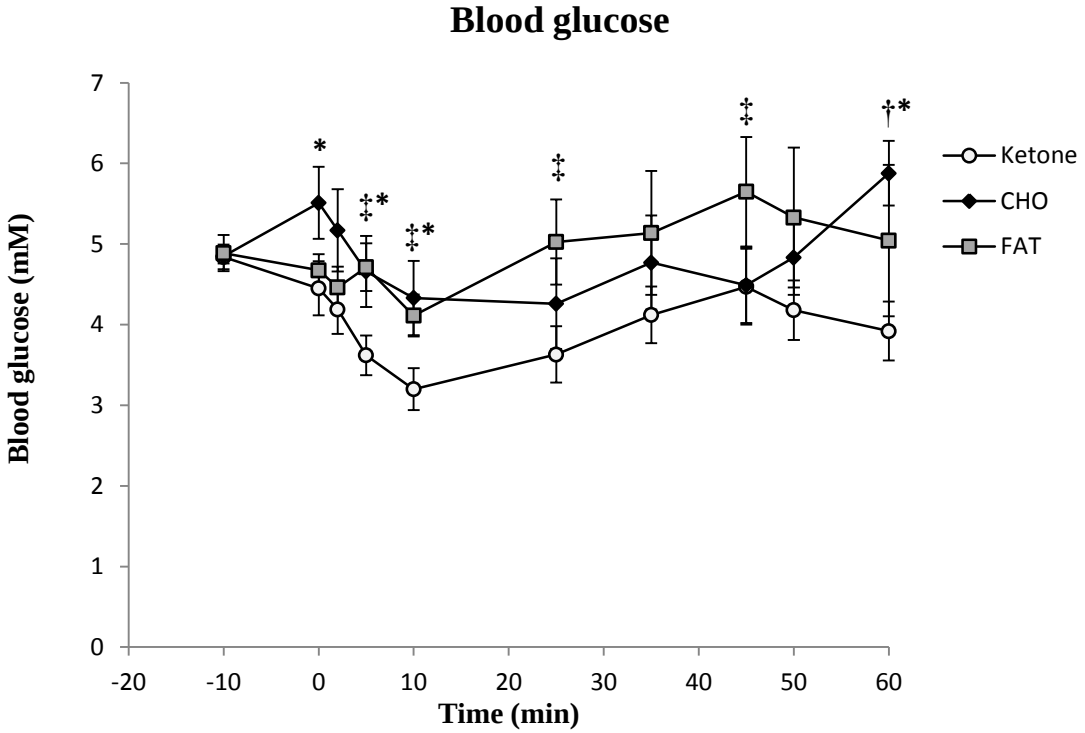


Figure 27 Blood glucose levels. † indicates a significant difference ($p < 0.05$ 3-way repeated measures ANOVA with Tukey post hoc correction) between Fat and Ketone trials, * $p < 0.05$ between CHO and Ketone trials, and ‡ $p < 0.05$ between Fat and Carbohydrate trials.

5.4.2 Blood metabolites

Oral ingestion of an initial drink containing 573 mg/kg BW of ketone monoester resulted in a rapid 28 fold rise in circulating βHB above overnight fasted levels (0.13+/-0.03 mM), reaching 3.46+/- 0.32 mM after ten min of rest (Figure 22). Blood ketones remained above 3.5 mM throughout the 60 min duration of exercise; however blood concentrations were significantly lower at all time-points during exercise than during the non-exercising control arm. βHB concentrations after the ketone drink were significantly (~3.4 mM $p < 0.001$) higher than concentrations observed following drinks containing long chain fats or carbohydrate. As would be expected, a small (~0.07 mM), but significant increase in βHB was observed on fat compared with the carbohydrate arm ($p < 0.05$).

Lactate concentrations were not significantly different at baseline, or at rest between arms (Figure 23). However after the onset of exercise blood lactate concentrations remain significantly lower in the presence of elevated ketone bodies. In particular, identical exercise performed during ketosis resulted in average lactate concentrations ~2-3 mM lower than after carbohydrate drinks ($p < 0.05$ 2 min-60 min), and significantly lower than after fat feeding 30 min, and 45 min into the exercise trial.

Free fatty acid concentrations were significantly higher at baseline after 24 hours of high fat low carbohydrate intake on the fat arm (Figure 24). FFA remained significantly elevated throughout the trial compared with either carbohydrate or ketone feeding ($p < 0.05$) reaching 0.76 mM at the end of exercise. Baseline FFA concentrations were not different between carbohydrate and ketone trials, however after ingestion of substrate drinks, FFA concentrations fell on both arms. Ketosis significantly suppressed the rise in FFA seen after 25 min of exercise in both fat, and to a lesser extent, carbohydrate feeding trials ($p < 0.05$).

Divergent patterns in the exercise induced rise in blood glycerol were observed between respective trials (Figure 25). Concentrations of blood glycerol were highest following fat feeding, % higher at than carbohydrate and ketone trials respectively after 60 min of cycling ($p < 0.01$). Blood glycerol concentrations were lowest during ketosis, significantly less than carbohydrate trials after 25 min of exercise ($p < 0.05$).

Plasma insulin concentrations were significantly elevated following the ingestion of the carbohydrate drink compared with fat and ketone. Insulin concentrations peaked 10 min after the ingestion of carbohydrate, and fell to baseline levels after 25 min of exercise (Figure 26). There were no statistical differences between fat and ketone trials.

No differences in blood glucose were observed between trials at baseline (Figure 27). Blood glucose was initially significantly higher in carbohydrate trials following the ingestion of the substrate drink ($p < 0.01$). Blood glucose fell during the first 10 min of exercise on all arms, but was significantly lower during ketosis than in the fat and carbohydrate arms 5 min and 10 min into exercise.

5.4.3 Muscle metabolites: βHB

NMR methods were used to determine βHB in skeletal muscle tissue before and after exercise on all study arms (Appendix 9.2). At rest, intramuscular concentrations of βHB were increased ~3 fold ($p < 0.01$) compared with carbohydrate and fat arms 10 min after the ingestion of ketone drinks. Ketone bodies remained significantly increased on the ketone arm at the end of exercise, more than double the intramuscular concentrations observed in either fat or CHO arms ($p < 0.05$, Figure 28).

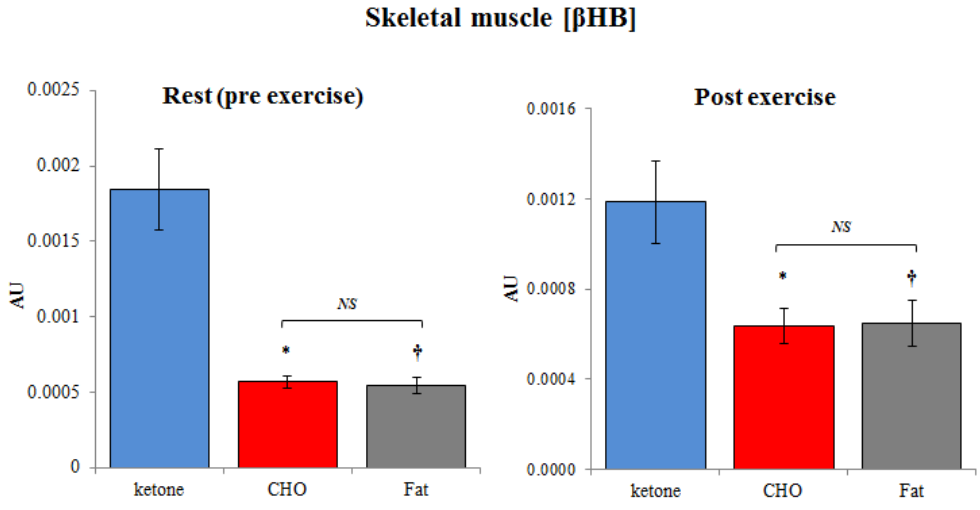


Figure 28 Skeletal muscle concentrations of βHB, determined using NMR methods. Data expressed as the mean +/- SEM. AU = Arbitrary Units. NS = No significant difference between CHO and Fat trials. † indicates a significant difference ($p < 0.05$ 3 way repeated measures ANOVA with Tukey post hoc correction) between Fat and Ketone trials, * $p < 0.05$ between CHO and Ketone trials, and ‡ $p < 0.05$ between Fat and Carbohydrate trials.

5.4.4 Glycolytic intermediates

Intracellular glucose was increased pre exercise in the carbohydrate arm compared with both fat and ketone drinks ($p = 0.02$, $p = 0.03$ respectively). Muscle glycolytic intermediates were significantly reduced at rest during ketosis, with significant reductions in Glyceraldehyde-3-Phosphate ($p < 0.01$), 2&3 Phosphoglycerate ($p < 0.01$), and pyruvate ($P < 0.01$) compared with carbohydrate, and similar changes on the fat feeding condition with the exception of pyruvate ($p = 0.06$). No significant differences were observed between arms in Fructose 1, 6 bisphosphate, Dihydroxyacetone phosphate (DHAP), or 1, 3 Bisphosphoglycerate concentrations at rest.

Following exercise, concentrations of muscle glycolytic intermediates were significantly lower in the ketone arm compared with carbohydrate and fat for all of the assayed intermediates with the exception of DHAP. There were no significant differences observed between fat and carbohydrate arms across the same intermediates (Figure 30).

5.4.5 Muscle metabolites: TCA cycle intermediates

The pool of TCA intermediates remained largely unaffected by changes in substrate provision, both at rest and following exercise. No significant differences were observed in TCA intermediates between arms, with the exception of increased concentrations of oxaloacetate at rest and following exercise on the fat arm ($p < 0.05$ vs. CHO and Ketone). In addition, malate concentrations were reduced on the CHO arm at the end of exercise compared with fat and ketone conditions ($p < 0.05$). The effect of exercise raised the total sum of TCA metabolites ($p < 0.05$) on all arms over resting conditions, but no significant differences in the total sum of metabolites were observed between substrates. Data from TCA intermediates are presented in Table 11.

Table 11 TCA cycle intermediates pre and post exercise.

TCA cycle intermediate	**Study arm**					
Pre exercise	*Ketone*	*+/- SEM*	*CHO*	*+/- SEM*	*Fat*	*+/- SEM*
Citrate/Isocitrate	1397.2	210.7	1439.5	122.3	1823.8	240.0
cis-Aconitate	41.2	6.2	30.1	5.6	57.0	5.8
α-Ketoglutarate	18654.3	3421.5	19193.0	4083.9	16994.9	2241.7
Succinate	1380.6	207.0	1242.4	113.2	1695.1	146.6
Fumarate	527.3	89.8	697.2	95.7	413.5	67.7
Malate	10936.6	2140.0	8902.4	2271.0	11093.9	1316.0
Oxaloacetate	7695.5	1354.5	6446.7	917.2	13440.7†‡	1920.8
Post exercise	*Ketone*	*+/- SEM*	*CHO*	*+/- SEM*	*Fat*	*+/- SEM*
Citrate/Isocitrate	2679.1	388.5	2731.9	213.7	2867.7	240.4
cis-Aconitate	37.4	2.7	44.9	5.9	52.8	5.1
α-Ketoglutarate	24380.5	3365.2	30196.0	5372.4	23736.3	3999.3
Succinate	1701.9	155.0	1617.6	151.2	1725.4	193.2
Fumarate	829.9	70.9	771.6	111.9	656.1	82.9
Malate	25110.1	2685.5	15742.3 *‡	1398.1	29059.9	3793.5
Oxaloacetate	11544.7	1392.4	10635.3	1107.4	19469.4†‡	2438.0

All Data expressed in arbitrary units (AU). Data presented as mean +/- SEM

† $p < 0.05$ Ketone vs. Fat, ‡ $p < 0.05$ CHO vs. Fat, * $p < 0.05$ Ketone vs. CHO

5.4.6 Carnitine derivatives

There was no significant difference in free carnitine concentration between arms in the resting condition. Acyl carnitine concentrations between substrate feeding arms were similar at rest for short, medium and long chain acyl groups (Table 12); however differences in C4, C6 and C16 carnitine concentration were detected between fat and carbohydrate arms, and between ketone and fat in C10:1.

Table 12 Pre exercise muscle Carnitine derivatives

	Carnitine derivatives					
	Ketone	*+/- SEM*	***CHO***	*+/- SEM*	***Fat***	*+/- SEM*
Free carnitine	370178.2	26176.4	375267.6	14039.6	343632.6	24561.4
C2	140376.8	21370.4	121581.1	12604.0	151402.3	14468.7
C3	1362.1	607.3	793.5	136.5	514.3	76.2
C4	283.1	71.4	408.5	71.7	170.3‡	21.6
C5	473.2	225.8	221.3	34.1	141.0	28.2
C6	63.3	22.3	112.5	28.1	35.7‡	6.5
C8	18.6	2.5	37.9	10.6	14.4	2.1
C10	109.2	24.5	178.3	47.6	76.8	10.4
C10:1	18.2	2.4	21.6	4.5	11.2†	1.4
C12	150.1	68.3	302.8	90.3	208.9	47.3
C14	103.6	29.4	146.7	32.4	84.2	10.1
C14:1	158.2	64.9	371.8	113.5	194.9	43.1
C14:2	93.3	44.8	138.9	41.1	108.3	24.3
C16	521.5	235.1	490.6	99.8	244.4‡	43.0
C16:1	599.4	319.1	1576.3	610.0	890.2	205.3
C16:2	403.1	254.0	629.2	287.1	558.0	150.7
C16-OH	86.9	41.1	141.4	49.3	108.7	29.2
C16:1-OH	149.3	79.1	392.8	167.2	273.0	87.1
C18:1	231.6	43.2	529.2	143.8	298.1	34.8
C18:2	581.5	274.8	717.7	175.2	580.4	124.4
C18:1-OH	61.4	32.1	82.1	33.3	84.4	26.8
C18:2-OH	34.4	23.1	59.5	26.6	59.4	26.0

All data expressed in arbitrary units (AU). Data presented as mean +/- SEM.

† $p < 0.05$ Ketone vs. Fat, ‡ $p < 0.05$ CHO vs. Fat.

However following 60 min of high intensity exercise marked differences in free carnitine, acetyl carnitine, and acyl carnitine derivatives were observed between the three substrates. In the ketone arm, significant increases in acetyl carnitine ($p < 0.01$), and short chain C3 carnitine ($p < 0.05$) were observed compared with carbohydrate and fat arms. Short and medium chain carnitine derivatives were significantly increased on the carbohydrate arm, whereas longer chain acyl-carnitines were present in the high fat trials (Table 13). Higher free carnitine was present in the carbohydrate arm at the end of exercise compared with the ketone arm ($p < 0.01$), but not different to fat.

Table 13 Post exercise Carnitine derivatives

	Carnitine derivatives					
	Ketone	*+/- SEM*	***CHO***	*+/- SEM*	***Fat***	*+/- SEM*
Free carnitine	256116.9	20452.0	343011.5*	13474.2	293405.7	24435.2
C2	218502.1	16056.7	166535.6*	16501.1	173060.3†	10941.3
C3	8180.6	1218.8	3275.4*	677.9	3171.0†	576.8
C4	966.1	259.8	3497.4*	981.1	950.8‡	156.5
C5	920.7	278.5	1745.0	313.3	853.3‡	187.9
C6	49.8	13.2	208.4*	53.9	73.5‡	10.3
C8	16.3	3.2	54.7*	10.4	22.8‡	2.1
C10	81.6	9.7	272.7*	45.4	112.1‡	10.3
C10:1	18.0	2.5	50.8*	8.6	23.2‡	2.1
C12	164.7	27.6	429.9	141.1	226.8	33.5
C14	72.5	11.4	159.5*	29.1	90.6	10.4
C14:1	166.7	28.8	552.3	154.1	266.3	35.4
C14:2	99.6	17.1	197.1	48.3	121.2	19.0
C16	296.3	58.7	294.3	38.6	519.7	84.2
C16:1	815.7	178.4	1244.3	318.9	3151.2	1046.8
C16:2	577.9	137.4	621.1	202.2	1326.8	470.7
C16-OH	132.5	32.6	256.5	70.2	669.7†	187.4
C16:1-OH	296.8	82.7	723.1	202.0	2054.0†	742.1
C18:1	237.2	38.4	353.6	60.9	544.7†	99.3
C18:2	776.4	178.1	57.7*	25.3	1320.4‡	337.5
C18:1-OH	105.6	27.7	182.1	56.0	674.5†‡	127.5
C18:2-OH	37.9	13.3	353.6*	60.9	57.7‡	25.3

All data expressed in arbitrary units (AU). Data presented as mean +/- SEM.

† $p < 0.05$ Ketone vs. Fat, ‡ $p < 0.05$ CHO vs. Fat, * $p < 0.05$ Ketone vs. CHO.

5.4.7 Branched chain amino acids

Analysis of branched chain amino acids (BCAA) Leucine/Isoleucine, and Valine revealed significant differences between substrate arms, both before and after exercise. High fat, low carbohydrate diet in the 24 hours prior to the trial elevated resting concentrations of branched chain amino acids ($p < 0.05$) between fat and carbohydrate, and fat and ketone arms (Figure 29). Exercise caused a significant increase in branched chain amino acids in all arms, with the biggest increases in fat and CHO arms ($p < 0.01$ vs. ketone). Carbohydrate and ketone feeding blunted the exercise induced rise in BCAA observed compared to fat feeding for Leucine / Isoleucine and Valine. This effect was most pronounced during ketosis, with significantly lower concentrations of Leucine/Isoleucine compared

with carbohydrate ($p < 0.05$). No difference in Valine concentration was observed between carbohydrate and ketone after exercise ($p = 0.22$).

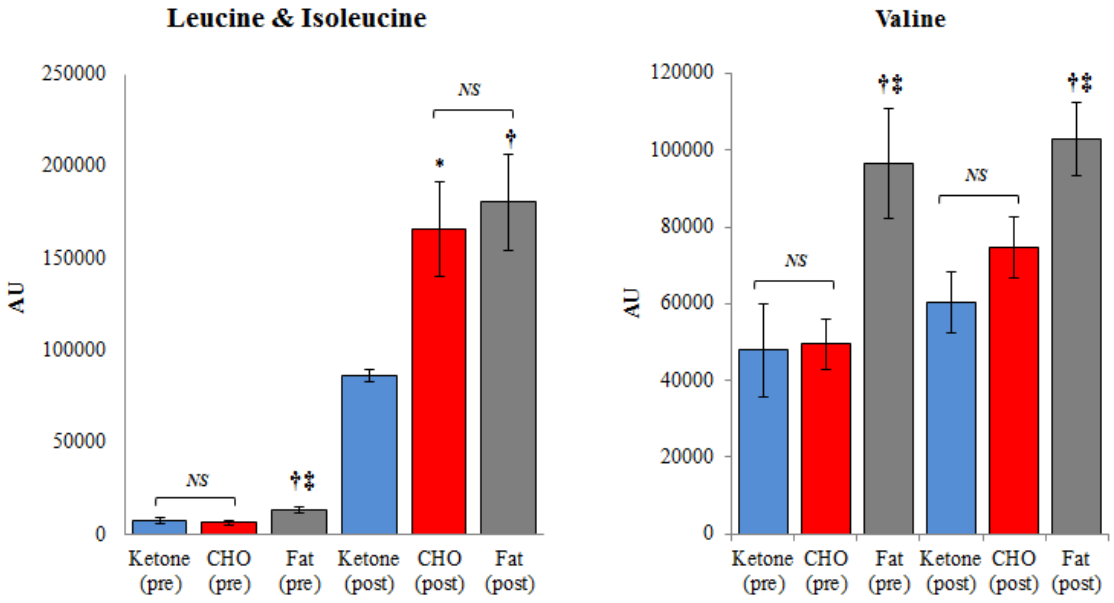


Figure 29 Skeletal muscle concentrations of branched chain amino acids before (pre) and after (post) exercise on the three substrate feeding arms. NS = not significantly different. † indicates a significant difference ($p < 0.05$ 3 way repeated measures ANOVA with Tukey post hoc correction) between Fat and Ketone trials, * $p < 0.05$ between CHO and Ketone trials, and ‡ $p < 0.05$ between Fat and Carbohydrate trials. Data are expressed as mean +/- SEM.

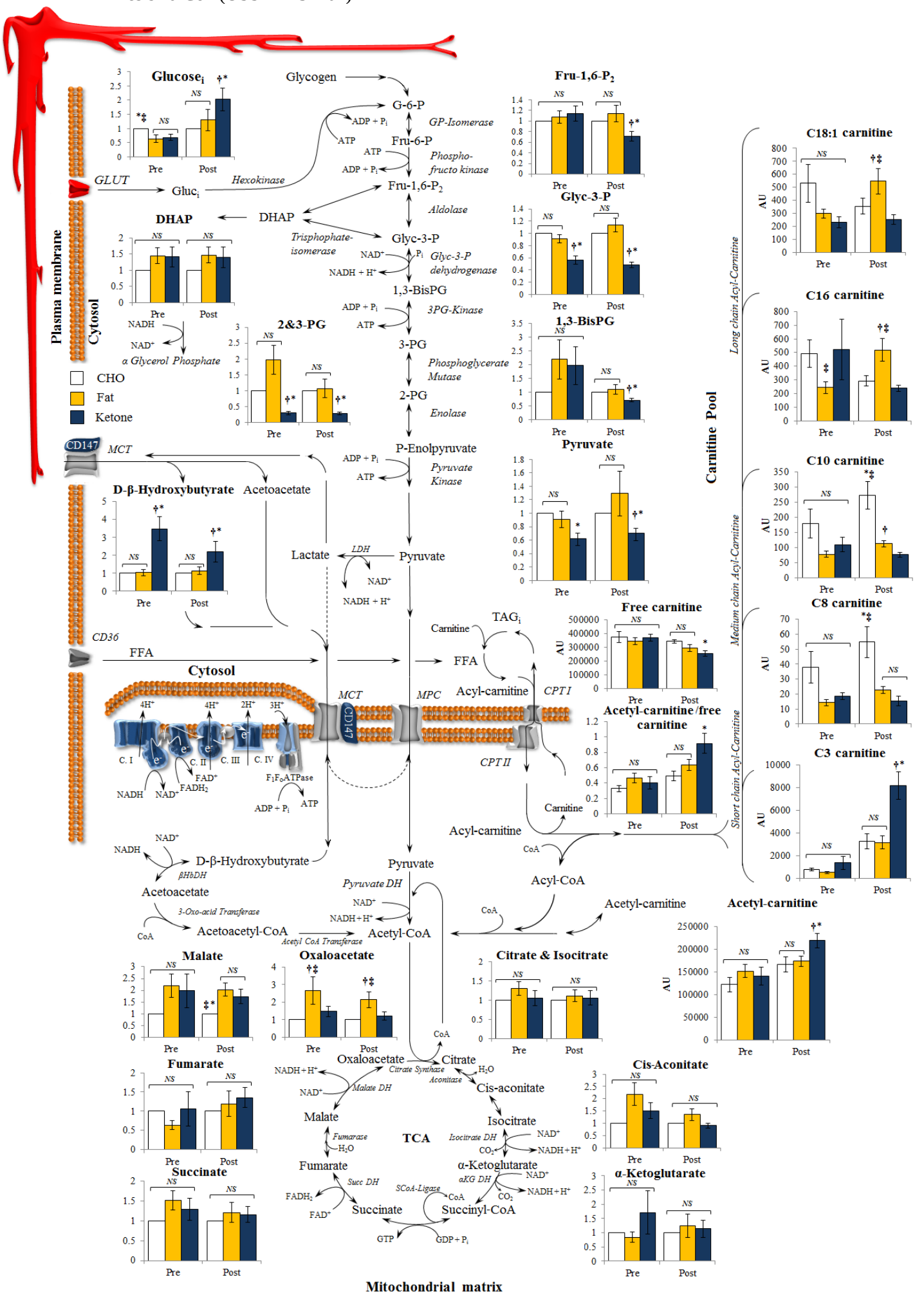

Plasma membrane
Cytosol
CHO
Fat
Ketone
Glucose$_i$
Glycogen
G-6-P
GP-Isomerase
Fru-6-P
Phospho-fructo kinase
Fru-1,6-P$_2$
Aldolase
DHAP
Triosphophate-isomerase
Glyc-3-P
Glyc-3-P dehydrogenase
1,3-BisPG
3PG-Kinase
3-PG
Phosphoglycerate Mutase
2-PG
Enolase
P-Enolpyruvate
Pyruvate Kinase
Pyruvate
LDH
Lactate
GLUT
Gluc$_i$
Hexokinase
ATP
ADP + P$_i$
NAD$^+$
NADH + H$^+$
NADH
α Glycerol Phosphate
2&3-PG
CD147
MCT
D-β-Hydroxybutyrate
Acetoacetate
CD36
FFA
TAG$_i$
Carnitine
Acyl-carnitine
CPT I
CPT II
MPC
C. I
C. II
C. III
C. IV
FAD$^+$
FADH$_2$
F$_1$F$_o$ATPase
βHbDH
3-Oxo-acid Transferase
Acetoacetyl-CoA
CoA
Acetyl CoA Transferase
Acetyl-CoA
Pyruvate DH
Acetyl-carnitine
Malate
Oxaloacetate
Citrate & Isocitrate
Fumarate
Succinate
Malate DH
Citrate Synthase
Aconitase
Cis-aconitate
Isocitrate
Isocitrate DH
α-Ketoglutarate
αKG DH
Succinyl-CoA
SCoA-Ligase
Succ DH
GTP
GDP + P$_i$
TCA
Mitochondrial matrix
Carnitine Pool
Long chain Acyl-Carnitine
Medium chain Acyl-Carnitine
Short chain Acyl-Carnitine
C18:1 carnitine
C16 carnitine
C10 carnitine
C8 carnitine
C3 carnitine
Free carnitine
Acetyl-carnitine/free carnitine
Pre
Post
AU
NS

Figure 30 Schematic representation of the effects of nutritional substrates on working skeletal muscle metabolism pre (Pre) and post (Post) intense exercise. Data for glycolytic and TCA intermediates are expressed as a ratio to carbohydrate (CHO) trials, with Acyl Carnitine values expressed in arbitrary units (AU). All data expressed as mean +/- SEM. † indicates a significant difference ($p < 0.05$, 3 way repeated measures ANOVA with Tukey post hoc correction) between high fat (Fat) and ketone (Ketone) trials. * $p < 0.05$ between CHO and Ketone, ‡ $p < 0.05$ between Fat and CHO trials. *NS* denotes no significant difference between trials. $Gluc_i$ = intracellular glucose, G-6-P = Glucose-6-phosphate, Fru-6-P = Fructose-6-phosphate, Fru-1,6-P_2 = Fructose-1,6-bisphophosphate, DHAP = Dihydroxyacetone phosphate, Glyc-3-P = Glyceraldehyde-3-phosphate, 1,3BisPG = 1,3-bisphosphoglycerate, 2&3PG = 2, and 3 Phosphoglycerate, P-Enolpyruvate = Phosphoenolpyruvate, GP-Isomerase = Glucose phosphate isomerase, LDH = Lactate dehydrogenase, MCT = Monocarboxylate transporter, MPC = Mitochondrial pyruvate transporter, CPT = Carnitine palmitoyltransferase, βHbDH = D-β-hydroxybutyrate dehydrogenase, αKG-DH = α-Ketoglutarate dehydrogenase. The suffix *DH* denotes 'dehydrogenase' to other labelled enzyme complexes

5.5 Discussion

This study sought to determine the effects of dietary substrate feeding on metabolic responses to exercise. In particular this work investigated the comparative effect of a new nutritional source of calories, in the form of a ketone ester, and its physiological effects on fuel selection and signalling during physical stress.

Protocol considerations and workload selection

The intensity of 75 % of W_{max} for 60 min was demanding on participants, with 2 athletes unable to complete the full 60 min on two occasions. The trial workload was deliberately chosen to be close to the limits of maximal sustainable exercise for this duration in trained athletes [254, 255]. The reasons for this are several; firstly, differences in aerobic power arising from subtle shifts in mitochondrial substrate energetics would be exaggerated in a trial of this kind. The heavy sustained nature and longer duration of exercise places massive strain on oxidative ATP production [256, 257], and gives the greatest chance of detecting differences between treatments. Secondly, exercise in this intensity domain (~85% $VO_{2\ Max}$) has been shown to be highly glycolytic, a condition necessary to test whether ketone bodies alter mitochondrial fuel preference during physiological stress. Thirdly, if carbohydrate is acknowledged to be the best nutritional source of energy to power high performance exercise, and previous findings suggest ketone bodies may challenge this notion, then the effects of nutritional ketones would need to be demonstrable during conditions of near maximal effort. Finally, the effects

of acute ketosis on skeletal muscle metabolism have not been described in humans, or during exercise. Therefore it remained to be proven that skeletal muscle metabolism is altered under these conditions.

Glycolysis

This work observed significant decreases in glycolytic intermediate concentration in the presence of elevated ketone bodies. The most pronounced effects can be observed after 60 min of high intensity exercise, where despite elevated intracellular glucose concentrations compared with CHO and Fat arm, all of the assayed glycolytic intermediates during ketosis were reduced. This suggests a significant inhibition of glycolytic flux, despite ample substrate for glycolysis in the form of intracellular glucose [258]. Lower concentrations of glycolytic intermediate were also observed on the ketone arm at rest, suggesting that (when present) ketone bodies rapidly exert hierarchical preference over carbohydrate metabolism. Remarkably, this inhibition of glycolysis remains despite the massive stimulation of exercise, at an intensity which would normally be highly glycolytic [259]. Pyruvate concentrations were considerably lower during ketosis than carbohydrate and fat feeding conditions, which may explain the 2-3mM decrease in blood lactate by simple fact of lower substrate availability for lactate dehydrogenase.

The conventional theory of the ‘Randle Cycle’ suggests that elevated fatty acid availability may inhibit glycolysis by the action of citrate on phosphofructokinase[260]. No significant effects of elevated fatty acids were observed at rest or during exercise on the pool of glycolytic intermediates studied here. In contrast, ketone bodies dramatically reduced glycolytic intermediate concentration under both resting and exercising conditions. This is consistent with work in the perfused rat heart, where metabolic control over glycolysis by ketone bodies was shared over a number of enzymatic steps, and no specific rate limiting step could be identified in the glycolytic pathway [261].

Carnitine pool

Mitochondrial membrane fatty acid translocation is dependent on carnitine availability. Striking differences in acyl carnitine derivatives between substrate arms were present at the end of exercise. On the carbohydrate arm significantly higher free carnitine concentrations were observed, whereas

during ketosis, a reduction in free carnitine, and a greater concentration of acetyl carnitine was observed. This is an interesting finding, as some have proposed that the limitation in fatty acid oxidation observed during exercise is a direct result of an exhaustion of free carnitine supply [21, 262-264]. This implies that ketone bodies are able to generate a greater supply of acetyl units to the TCA cycle than other substrates, and that excess C_2 units were shunted into the carnitine pool, forming acetyl carnitine. Alternatively, elevated acetyl carnitine concentrations, and higher acetyl carnitine / free carnitine ratio during ketosis could be a result of increased intramuscular fatty acid oxidation. Increased activity of β-oxidation could lead to a greater supply of acetyl units to the TCA cycle in conditions of reduced glycolysis, such as those observed here and following ketogenic diets. This could make sound evolutionary sense in conditions of starvation, effectively switching the largest sink of glucose units (skeletal muscle) away from glucose metabolism towards more plentiful fatty acid oxidation. However to achieve this, the action of ketone bodies would have to overcome the inhibition of CPT transport induced by exercise. The mechanism for a possible increase in the efficiency of the carnitine shuttle by the action of ketone bodies is not able to be elucidated in this work. However if ketone bodies were found to increase the intramuscular mobilisation of triacylglycerol stores, and *per se* the efficiency of fatty acid metabolism during exercise, this may breathe new life into the concept of a 'glucose free fatty acid' cycle. Further work is needed to determine if the action of ketone bodies can acutely alter the activity of the CPT/carnitine shuttle, and to determine changes in lipid intermediate flux through the β-oxidation pathway.

TCA pool

Although significant differences between substrates were observed in 'upstream' pathways of glycolysis, and in the acyl carnitine pool, the effect of substrate manipulation on TCA cycle intermediates was negligible. No differences were observed between treatments for the assayed intermediates of the first half of the TCA cycle. However oxaloacetate concentrations were significantly higher at both rest and at the end of exercise on the fat arm compared with either carbohydrate or ketone conditions. This may represent either a lower rate of condensation of acetyl CoA with oxaloacetate to form citrate, or inhibition of citrate synthase activity resulting in

accumulation of oxaloacetate. It is impossible from this current data to delineate between these possibilities, as assays of acetyl CoA concentration were not possible here. This, if lower on the fat arm would support the notion that a shortage of acetyl CoA supply may be responsible for the raised oxaloacetate levels.

Malate concentrations were also significantly decreased on carbohydrate compared with fat or ketone at the end of exercise. No differences in resting malate concentrations were observed between substrates, suggesting that the combined effects of exercise and carbohydrate supply were responsible for the observed reduction in malate concentration.

However these effects on TCA cycle intermediates may be minor in the larger context of the general TCA pool. The sum of the total TCA intermediates was not different between substrate arms either before or after exercise. This suggests that even under the most demanding physiological circumstances the TCA pool is highly regulated to conserve oxidative combustion of carbon fuels [265-268]. This is consistent with evidence during exercise in severely glycogen depleted conditions, where the total pool of TCA intermediates is maintained even at volitional fatigue [269, 270].

Amino acid metabolism

Another interesting finding from this study is that amino acid metabolism was significantly altered by substrate conditions during exercise. It is well known that progressive increases in both muscle and plasma concentrations of amino acids accompany strenuous physical activity [271, 272]. Branched chain amino acids are particularly important as they are able to undergo rapid transamination to sustain the anapleurotic pool during a multitude of physiological stressors [273]. Branched chain amino acids were modestly elevated at rest following only 24 hours of a high fat, low carbohydrate diet, but the effect of 60 min of high intensity exercise was much more dramatic with several fold increases in BCAA within skeletal muscle. Ketone bodies appeared to attenuate the rise in BCAA concentrations, an affect that could be attributed to either a decrease in muscle catabolism during exercise, or a reduced demand for anapleurotic substrates.

Limitations

The metabolomic methods employed in this study to determine concentrations of muscle metabolites were specifically chosen to map a broad variety of pathway intermediates. This allows a snapshot of working metabolism in detail not possible with other methods. However, whilst this method allows accurate comparisons between substrates, and between rest and exercise conditions, it does not provide detail of pathway flux, nor allow the calculation of metabolite ratios. However we can reliably infer from the changes in relative concentration of intermediates that the effects of different substrates significantly altered the major metabolic pathways involved in ATP generation.

It was also not possible with the techniques used here to determine concentrations of the ketone intermediates acetoacetate, and acetoacetyl CoA, as well as the common substrate for TCA cycle oxidation, acetyl CoA. Measurement of glycogen stores were not conducted in the course of this work, due to the finite limits of tissue obtainable by muscle biopsy. It is reasonable to speculate that the dramatic decrease in glycolytic intermediate concentrations below G-6-P, and the preserved intracellular glucose concentrations observed during ketosis, may have decreased the rate of glycogen breakdown [274]. This would appear consistent with the evolutionary objective of ketone bodies to spare carbohydrate stores during starvation. Furthermore, the role of glycogen as a determinant of mitochondrial energetic performance is not clear. Glycogen is metabolised to pyruvate, the end product of glycolysis, in identical fashion to that of exogenous glucose, so its thermodynamic potential would be expected to be similar to that of glucose. The duration of exercise employed in this study was not designed to completely exhaust intramuscular fuel stores; a condition which may influence substrate selection. However the presence of ketone bodies at rest was sufficient to cause a significant decrease in many glycolytic intermediates. This suppression remained after the onset of exercise, suggesting that any differences in glycogen concentration between arms at the end of exercise are unlikely to have accounted for the effects seen here.

In this study the elevation of free fatty acids were modest in comparison to pharmacological methods employed historically to investigate muscle fatty acid oxidation [245, 275-278], however the methods of this study were confined to nutritional interventions. This may have reduced the impact of any

substrate sparing effect contributed by increased fatty acid availability, but does more accurately represent nutritional practice. There were no observed differences in the concentrations of glycolytic intermediates between CHO and Fat trials. This is consistent with previous work suggesting that at this intensity of exercise, elevated fatty acids are not able to override the dominance of carbohydrate as a preferred fuel [279].

Whilst this work details the effects of substrate manipulation towards the upper limits of human aerobic capacity, it is not possible to conclude from this study alone whether these effects are generalisable across all aerobic exercise intensities. Future work should address this question, and aim to quantify the contribution of ketone bodies to energy expenditure during exercise over a range of intensities.

5.6 Conclusions

Alterations in acute substrate provision cause significant shifts in fuel metabolism during exercise. The presence of a novel ketone body provides an alternative carbon source for oxidative phosphorylation during heavy exercise, decreasing glycolysis, and suppressing peripheral lipolysis.

The importance of the carnitine pool as a sink for Acyl groups has received much interest as a possible mechanism for the limitation in fatty acid oxidation. This work shows that marked differences in carnitine chain length are present during exercise, depending on the substrate being metabolised. This suggests a more complex relationship between the carnitine pool and the regulation of fatty acid oxidation.

Finally, these findings also suggest a new hierarchy in substrate preference, which challenges the traditional belief that carbohydrates, when present, exert dominance over other substrates as the preferred fuel for high intensity aerobic exercise.

6 The effects of exercise on ketone handling and elimination

6.1 Thesis context

In previous chapters it has been consistently observed that levels of ketosis are reduced during exercise compared to resting conditions. One reason for this may be the oxidation of circulating ketones cleared from the bloodstream to be used as respiratory fuels. Another potential explanation is elimination of ketone bodies by urinary excretion. This work in this chapter investigated the effects of increasing physical demands on ketone body disposal.

6.2 Introduction

Investigating the effects of exercise intensity on resultant fuel selection has provided valuable insight into the mechanisms controlling substrate energy transformation. It is well accepted that carbohydrate becomes the preferential fuel to power exercise as intensity increases [21, 259]. However in recent chapters it can be seen that ketone bodies may contribute a significant supply of carbon units for oxidation during exercise. Not normally present in such quantities outside of fasting, or specific dietary conditions, βHB may distort the conventionally accepted hierarchy of mitochondrial substrate preference, as discussed in Chapter 5.

However as yet, the effects of exercise intensity on the oxidation of ketone bodies is unclear, and must first start with the examination of the blood disappearance profile of ketone bodies across a range of workloads. Further to this, urinary elimination of circulating ketone bodies has been reported to be proportional to their plasma concentration [280]. However the effects of acute exercise on urinary ketone elimination are unknown.

6.3 Methods

Subjects

Table 14 Physical characteristics of study athletes

Athlete	Mean (+/- SE)
Age	28.6 (+/- 0.7)
Height	1.88 (+/- 4.8)
Weight	87.4 (+/- 6.9)
$VO_{2\ Max}$ (L/min)	5.26 (+/- 0.3)
W_{max} (W)	384 (+/- 18)
BMI	24.4 (+/- 0.8)

Highly trained male endurance athletes (n = 8) from a cycling, running, or rowing background were chosen to participate in this trial (Table 14). All athletes were currently undertaking more than 12 hours per week of endurance exercise, and were routinely used bicycle ergometers for training. All subjects gave their informed consent after having the study risks explained.

Baseline testing and workload prescription

Athletes completed an incremental load maximal test one week prior to study trials to determine W_{max} and $VO_{2\ max}$ allowing calculation of subsequent workloads (General Methods 2.5). Athletes completed three further trials at workloads corresponding to 40% (Low), 60% (Med), and 70% (High) of W_{max} in randomised order.

Blood sampling and analysis

All samples were collected via a 22G iv cannula percutaneously inserted into an antecubital fossa vein. Samples were collected, and analysed according to methods previously described (General Methods 2.6 and 2.7).

Urine collection and analysis

Athletes were asked to void the contents of their bladder immediately before ingesting each ketone containing drink. Upon the completion of each exercise trial, athletes voided into a urine collection container (Simport Plastics Ltd, Beloeil, Canada). This urine volume was recorded and 2ml aliquots immediately frozen at -80ºC until further analysis. Urine was subsequently analysed for βHB using an automated bench top analyser (General Methods 2.7).

Ketosis and dosing considerations

Athletes consumed identical drinks on each visit, consisting of 573mg/kg ketone monoester dissolved in solution. Drinks were consumed 7.5 min before the onset of exercise, following an overnight fast.

Pulmonary gas exchange

Pulmonary gas collections (General Methods 2.4) were made at the same time points to blood sampling, with collections averaged over a 1 min time interval.

6.4 Results

6.4.1 Gas exchange

Oxygen uptake was significantly increased with harder physical work, and a progressive increase in RER during exercise was also observed between exercise intensities (table 15). Resting RER was elevated at baseline, consistent with a 're-feeding' effect following fasting, and the ingestion of study substrate drinks [281].

Table 15

Time	**VO_2 (L/min)**			**RER**		
	40% W_{max}	*60% W_{max}*	*70% W_{max}*	*40% W_{max}*	*60% W_{max}*	*70% W_{max}*
Rest	0.57 +/- 0.03	0.59 +/- 0.05	0.56 +/- 0.05	1.00 +/- 0.04	1.02 +/- 0.06	1.02 +/- 0.06
2	2.24 +/- 0.14	3.10 +/- 0.18*	3.21 +/- 0.25†‡	0.87 +/- 0.02	0.90 +/- 0.02	1.00 +/- 0.04†‡
5	2.27 +/- 0.15	3.04 +/- 0.21*	3.75 +/- 0.23†‡	0.93 +/- 0.03	0.98 +/- 0.01*	1.03 +/- 0.03†‡
10	2.28 +/- 0.10	3.14 +/- 0.25*	3.82 +/- 0.26††	0.94 +/- 0.03	0.97 +/- 0.02	1.02 +/- 0.03†
20	2.36 +/- 0.15	3.25 +/- 0.26*	3.93 +/- 0.23†‡	0.94 +/- 0.03	0.96 +/- 0.02	1.00 +/- 0.02†
30	2.47 +/- 0.14	3.27 +/- 0.25*	4.05 +/- 0.26†‡	0.91 +/- 0.02	0.96 +/- 0.03	0.99 +/- 0.03†
45 (min)	2.35 +/- 0.15	3.24 +/- 0.23*	4.16 +/- 0.27††	0.90 +/- 0.02	0.94 +/- 0.02	0.96 +/- 0.02†

Data are expressed as mean +/- SEM. † $p < 0.05$ Low vs. High, ‡ $p < 0.05$ High vs. Med, * $p < 0.05$ Med vs. Low.

6.4.2 Blood metabolites

Blood ketone kinetics showed consistent patterns in absorption and rates of appearance, following a standardised 573 mg/kg dose of ketone ester. Kinetic profiles were initially very similar between

conditions, but with the onset of different exercise intensity, divergent patterns in steady state ketone concentration were observed (Figure 31).

A moderate correlation was observed between steady state oxygen uptake (average of measurements over the final 25 min of the protocol) and blood βHB (Figure 32).

Blood lactate concentrations were significantly different between trials as would be expected, with new plasma steady state concentrations of lactate achieved after approximately 10 min of constant load exercise (Figure 33).

Blood glucose was not significantly different between bouts of exercise at 40, 60, or 70% of Wmax at any time point during exercise. However blood glucose concentration did follow a consistent pattern on all three trials, falling from resting and baseline conditions with the onset of exercise in all three trials ($p < 0.05$, Figure 34).

Similar findings were observed in plasma FFA concentrations, which again, did not differ in response to the three workloads. As shown previously, plasma FFA fell with the ingestion of ketone drinks, and remained suppressed for the duration of exercise (data not shown).

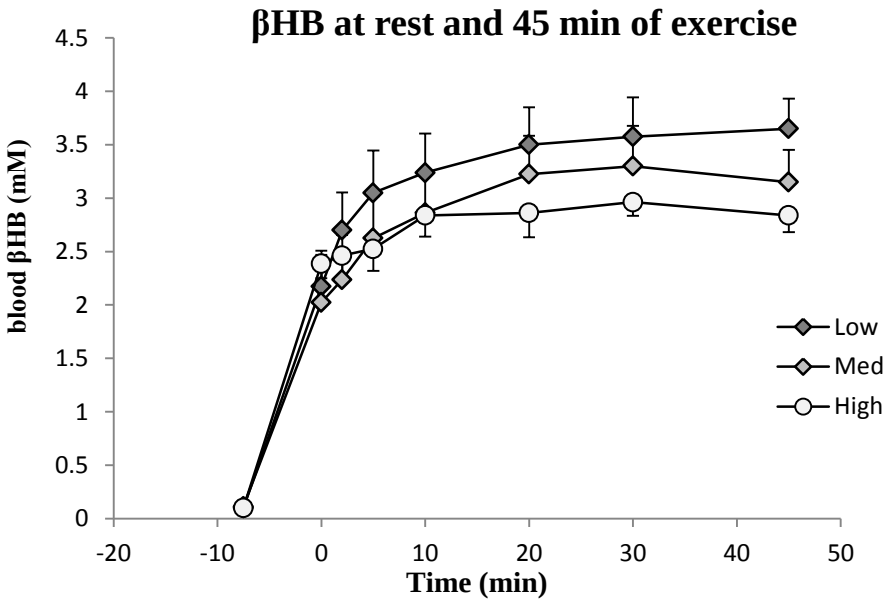


Figure 31 Blood ketone kinetics during 45 min of exercise at low (40%), med (60%) and High (70%) of W_{Max}.

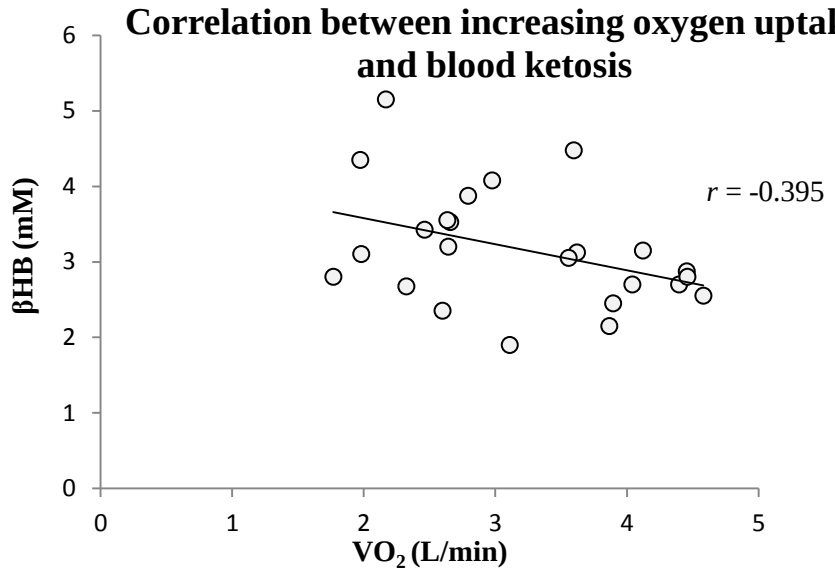


Figure 32 Correlation between steady state oxygen uptake and blood ketosis. Each data point represents the average over the final 25 min of the exercise (p = 0.046, *r* = -0.3952 Two-tailed Pearson's correlation).

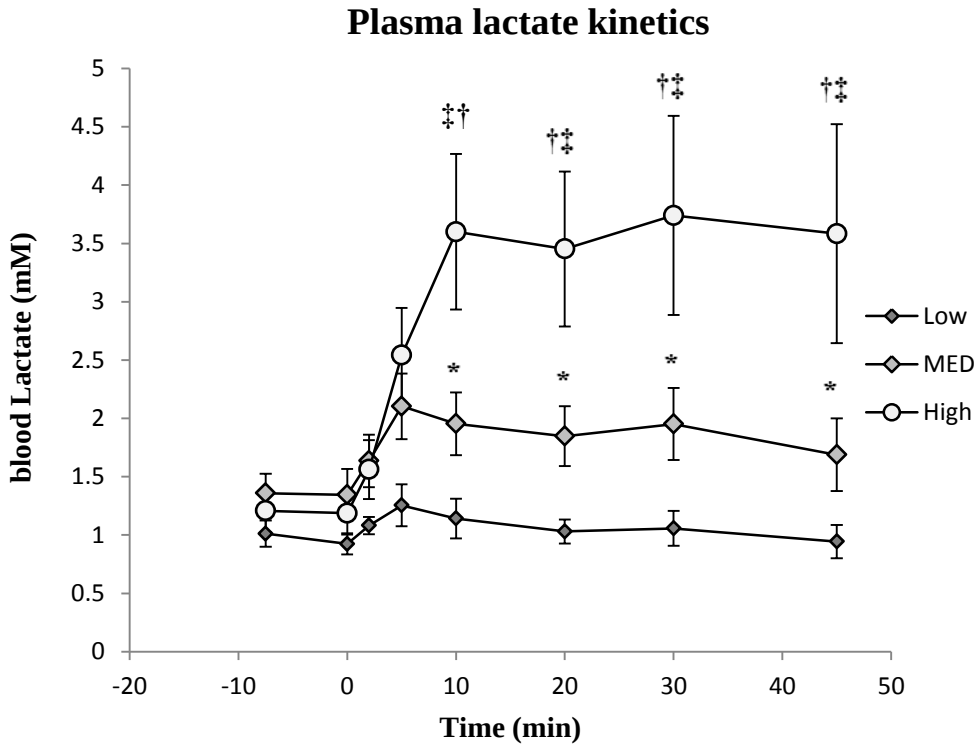


Figure 33 Lactate kinetics during 45 min of exercise at low (40%), med (60%) and High (70%) of $W_{Max.}$ † $p < 0.05$ Low vs. High, ‡ $p < 0.05$ Med vs. High, * $P < 0.05$ Low vs. Med.

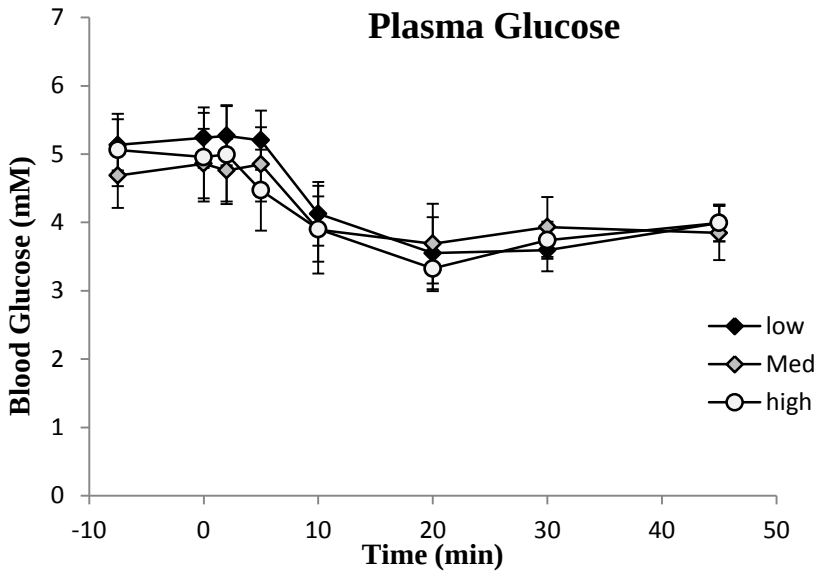


Figure 34 Blood glucose during 45 min of exercise at low (40%), med (60%) and High (70%) of W_{Max}. No significant differences between intensities were detected between arms.

6.4.3 Urinary excretion

Urinary elimination of βHB correlated ($r = 0.595$ $p < 0.05$) with plasma AUC during exercise (Figure 35). Urinary elimination over the course of the study was small in relation to the ingested quantity of ketone ester, and ranged between 0.02 and 0.3 g of βHB (mean 0.1g +/- 0.02).

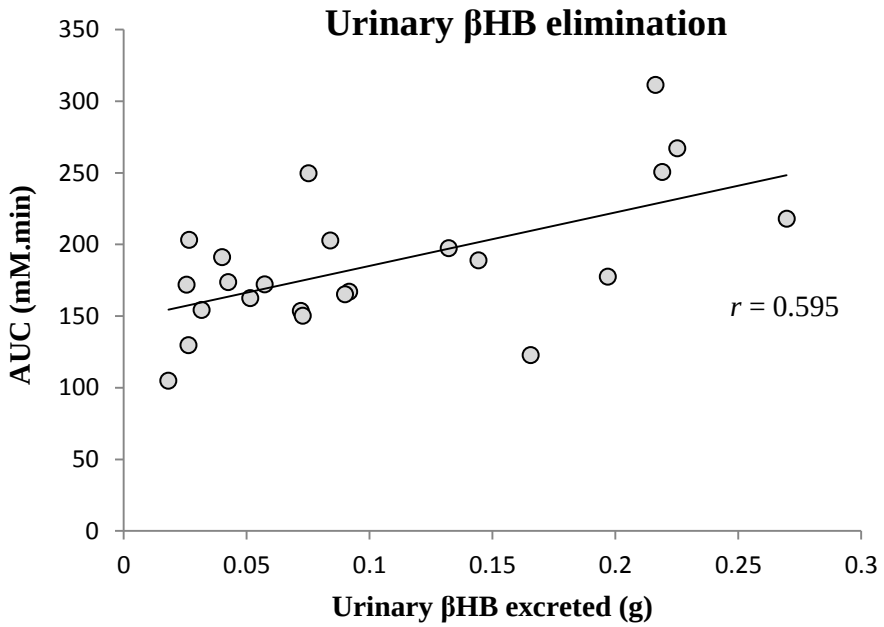


Figure 35 Relationship between urinary excretion of βHB (g) during 45 min of exercise and plasma AUC. ($p = 0.028$, $r = 0.595$ two-tailed Pearson's correlation).

6.5 Discussion

This work determined the relationship between exercise intensity and circulating ketone concentrations during steady state work of various intensities. This study described the relationship between exercise and urinary elimination of ketone bodies, and determined if elimination rather than oxidation could explain the observed differences in plasma ketone kinetics observed in previous chapters.

Effects of exercise on plasma ketone kinetics

An increase in physical workload resulted in lower plasma concentrations of circulating ketone. This alteration in plasma concentration appears to reach a new steady state concentration after 10 min of constant exercise. Increased oxygen demand was inversely proportional to ketone body concentration, indicating that plasma clearance of ketone bodies (via metabolic disposal) was increased at higher workloads. These findings extend the observations of previous chapters, suggesting that not only are ketone bodies readily oxidised during exercise, but the oxidation of ketone bodies is related to physical intensity. This finding has important implications for the current understanding of fuel selection during exercise. Firstly, an increase in ketone oxidation with intensity mirrors that of carbohydrate oxidation, which is in contrast to fat oxidation which reaches a ceiling around 55% of W_{Max} [21, 22, 282]. Secondly, it appears from these data, that during harder aerobic efforts that ketone oxidation becomes a significant source of energy to power exercise, which would cause an error in the presently accepted equations for the calculation of substrate oxidation by indirect calorimetry [283, 284].

Urinary elimination of ketone

It has been well established previously that increased plasma ketone concentrations as a result of fasting or high fat diets cause an increase in the urinary concentrations of ketones [280, 285, 286]. This observation is supported by this work, which showed a clear relationship between plasma AUC and urinary elimination of ketone bodies. However, this relationship has not been investigated in detail during exercise, as performed here. Firstly this work demonstrated that during exercise, renal elimination of ketone bodies is consistent with resting ketosis augmented with ketone infusions [287].

Secondly the amounts of ketone eliminated in the urine cannot account for the changes observed in plasma ketone concentration.

Previous studies have suggested that blood ketosis of 3-5 mM βHB results in urinary elimination rates equivalent to 0.4-2.8 mM/h. [286, 287]. In this work, similar circulating ketone concentrations during exercise resulted in a mean urinary elimination rate (over the period of exercise) of 1.09 mM/h. There were no statistical differences between exercise intensities in the rates of urinary ketone elimination. But like several previous studies, a positive correlation was observed between higher blood ketone concentrations and urinary elimination rates [287, 288]. However, a wide range of urinary ketone elimination rates were observed across the three exercise intensities (~5 fold differences between smallest and largest values), and because renal perfusion during exercise is intensity dependant [289], the possibility of lower rates of ketone excretion at higher intensities cannot be excluded. Further work should aim to determine the relationship between renal perfusion and urinary excretion of ketones during exercise.

6.6 Conclusions

These findings support evidence from previous chapters, and other work, that ketone bodies are oxidised as respiratory fuels during exercise when present in sufficient circulating quantities. Furthermore, there appears to be a relationship between the severity of exercise and the disappearance profile of ketones from the bloodstream. Urinary elimination of circulating ketone during exercise cannot account for the fall in plasma ketone concentrations during exercise, with approximately 0.1-0.3g of ketone eliminated during 45 min of exercise.

7 The effects of ketones and carbohydrates on endurance performance

7.1 Thesis context

In Chapter 4 the performance effects of a mixed substrate formulation containing a ketone ester was compared to a control condition containing isocaloric fat in place of ketone ester. While both drinks contained considerable carbohydrate (45% of calories) the additive effects of the combination of carbohydrate and ketone bodies vs. carbohydrate alone is unknown. Further to this, intestinal absorption of carbohydrates has recently been suggested to limit exogenous oxidation rates[290]. Mixtures of carbohydrate may however enable a higher saturation threshold of intestinal carbohydrate absorption, and provide additional performance benefits during sustained exercise [291, 292].

7.2 Introduction

Fuelling long duration exercise with carbohydrate has been the cornerstone of endurance performance for many decades [230, 293]. Ingestion of soluble carbohydrates at regular intervals before and during exercise increases the supply of oxidisable substrates to all working organs [294]. However, regardless of the strategy employed by the oral route, exogenous carbohydrates cannot provide more than 15-25% of the energy requirements of exercise [295, 296]. Instead, a gradual decline in muscle glycogen is inevitable, leading to a progressive loss of performance [29].

As reported in multiple studies, gut transport may be the rate limiting step in the effective utilisation of exogenous carbohydrate. Saturation of the sodium dependant glucose transporter (SGLT) limits exogenous dextrose absorption to approximately 1g/min [297]. Multiple transportable carbohydrates such as fructose, are not absorbed via the SGLT1 as is the case for dextrose [298]. GLUT5 mediated transport instead enables an alternative mechanism of absorption, albeit slower than for dextrose [297, 299]. Delivering a mixture of carbohydrates has therefore been shown to increase total exogenous carbohydrate oxidation over plain dextrose by 13-65% [300, 301].

Delivering an alternative substrate such as a ketone body may (as previously discussed in other chapters) significantly alter energetic performance by causing a switch in fuel selection. Furthermore, the oral route of delivery of ketone bodies is able to supply equivalent if not greater total calories than

exogenous carbohydrate via independent pathways of gut transport. Synergistic administration of both carbohydrate and ketones may provide greater total exogenous fuel to power exercise, and may be of additional benefit over carbohydrate alone.

7.3 Methods

Subjects

Table 16 Physical characteristics of study athletes

Athlete	Mean (+/- SE)
Age	29.4 (+/- 1.0)
Height	1.87 (+/- 3.5)
Weight	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	24.3 (+/- 0.9)

Eight endurance trained cyclists and rowers (n=6 male, n=2 female) were recruited to take part in this study (General Methods 2.2). All athletes were training at least 10 hours per week in their respective sports, and were of national or international calibre (Table 16). All subjects provided written informed consent after the study risks were explained.

Baseline testing and workload prescription

All athletes undertook baseline incremental tests to maximum on an SRM® stationary bicycle ergometer (SRM Training Systems, Germany) to allow prescription of subsequent workloads (General Methods 2.5). To familiarise athletes for the time trial section of the performance tests, athletes performed a maximal 30 min effort on the SRM 20 min after the completion of the incremental maximum test.

Blood sampling and analysis

Blood samples were obtained according to methods previously described (General Methods 2.6). Analysis was performed using a bench top analyser (General Methods 2.7).

Pulmonary gas exchange

Gas collections were made at baseline, and at each interval accompanying blood sampling according to methods described earlier (General Methods 2.4).

Substrate drinks

Subjects ingested drinks containing isocaloric quantities of carbohydrate and ketone, or a 1:1:2 mixture of dextrose, fructose, and maltodextrin [302]. In both conditions a minimum of 1.2 g/min of carbohydrate supply was ensured during exercise trials. Additional matched calories from either ketone, or 'multiple transportable carbohydrates' were provided to allow comparisons according to evidence based 'optimal feeding strategy'[294, 303].

Statistics

Paired t-tests (two tailed) were used to compare time trial performances between substrate feeding arms. One way repeated measures ANOVA was used to detect significant effects between blood metabolites. Statistical significance was considered as $p < 0.05$.

General study design and standardisation

Subjects completed two blinded exercise trials following an overnight fast, consisting of 60 min steady state workload at 70% W_{Max} followed by a blinded 30 min time trial for maximum distance. This general study design has been used in several previous literature reports seeking to investigate the cumulative effects of dietary intervention, fatigue, and fuel sparing on endurance performance [241, 304, 305]. 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, with no external stimuli. Blood and pulmonary gas measurements were collected during the first 60 min steady state 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. Consistent encouragement was given at identical intervals to all athletes, and athletes remained blinded to their scores until the end of all testing. Trials were performed at 18ºC in the climate controlled lab in the Clinical Cardiovascular Research Facility (CCRF) at the John Radcliffe Hospital. Power output from each test was recorded automatically every second onto an external study computer.

7.4 Results

7.4.1 Gas exchange

No differences in gas exchange parameters were detected between each arm of this study. Respiratory exchange ratios were consistently close to unity (1.0) during both rest and exercise in both arms, and no differences in oxygen uptake were detected (Table 17).

Table 17

Time	**VO_2 (L/min)**		**RER**	
	Ketone+ CHO	*CHO*	*Ketone+ 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

Data expressed as mean +/- SEM. No significant differences were observed between trials.

7.4.2 Blood metabolites (Prefatigue)

During the 60 min prefatigue, lactate concentrations increased significantly above resting levels, and became divergent between testing conditions after 5 min of steady state work. Lactate concentrations were significantly higher following carbohydrate alone, compared to carbohydrate + ketone; achieving pseudo steady state concentrations ~2mM higher than the ketone containing arm ($p < 0.05$ figure 37). Plasma glucose concentrations increased on both arms following the ingestion of substrate drinks, and fell during the initial 25 min of exercise. Plasma glucose was consistently lower in the ketone containing limb, with the exception of the 25 min sampling point, this did not reach statistical significance (figure 39).

No differences in plasma FFA concentrations were present until the final 15 min of the 60 min prefatigue. However plasma FFA rose steadily in the carbohydrate arm after 25 min of steady

exercise, and was significantly increased compared with the ketone arm after 45 and 60 min ($p < 0.05$, figure 38).

Ketone bodies were increased 30 fold above resting conditions following the administration of oral ketone ester drinks. Blood βHB increased by ~2mM during the 20 min prior to exercise, however with the onset of exercise, blood concentrations initially decreased between 2 min and 10 min ($p < 0.05$, figure 36) before gradually increasing again towards a stable blood concentration of ~2.5 mM. Blood βHB did not change from baseline on the carbohydrate feeding arm, remaining at ~0.05 mM during the course of exercise.

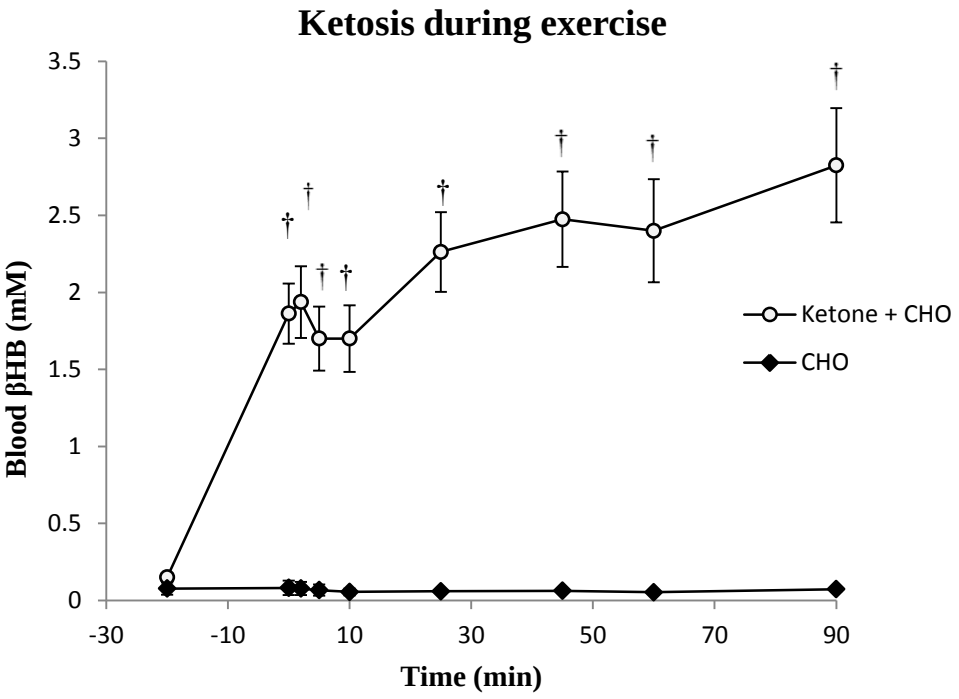


Figure 36 Blood βHB over the 90 min duration of exercise. † indicates a significant difference ($p< 0.05$ one repeated measures ANOVA with post hoc Tukey correction) between ketone and carbohydrate (ketone + CHO) and carbohydrate only trials (CHO). Data are expressed as mean +/- SEM.

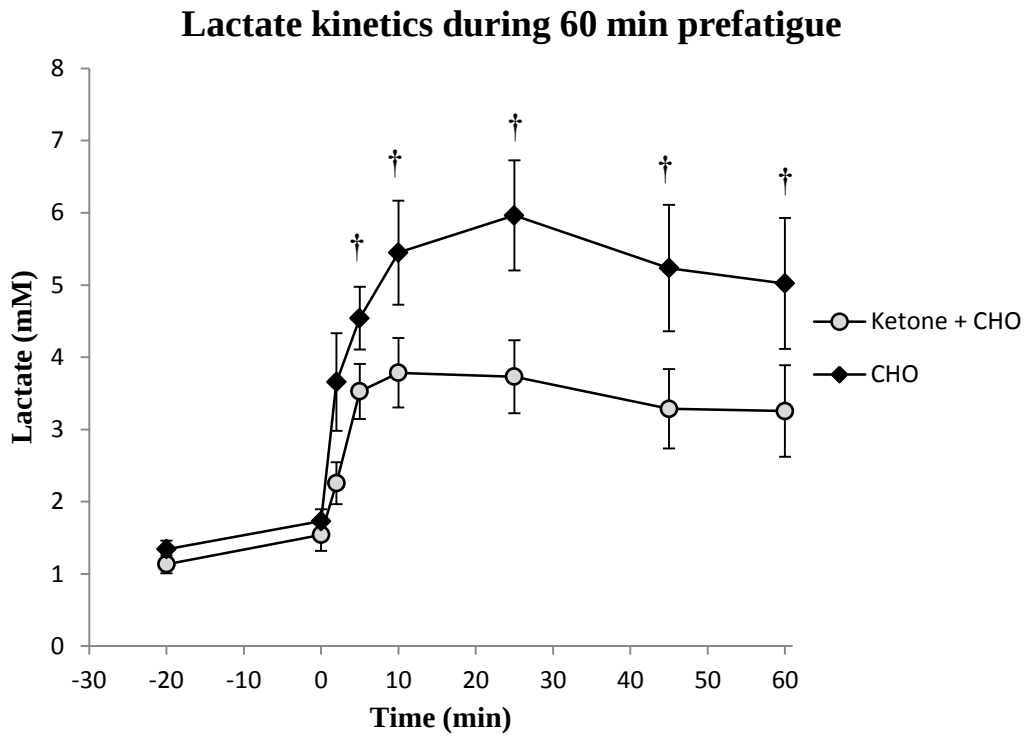


Figure 37 Lactate profiles during 60min of controlled (prefatigue) work at 70% of Wmax prior to the 30 min time trial. † $p < 0.05$ (one way repeated measures ANOVA) between ketone and carbohydrate (Ketone + CHO), and carbohydrate (CHO) alone arms. Data are expressed as mean +/- SEM.

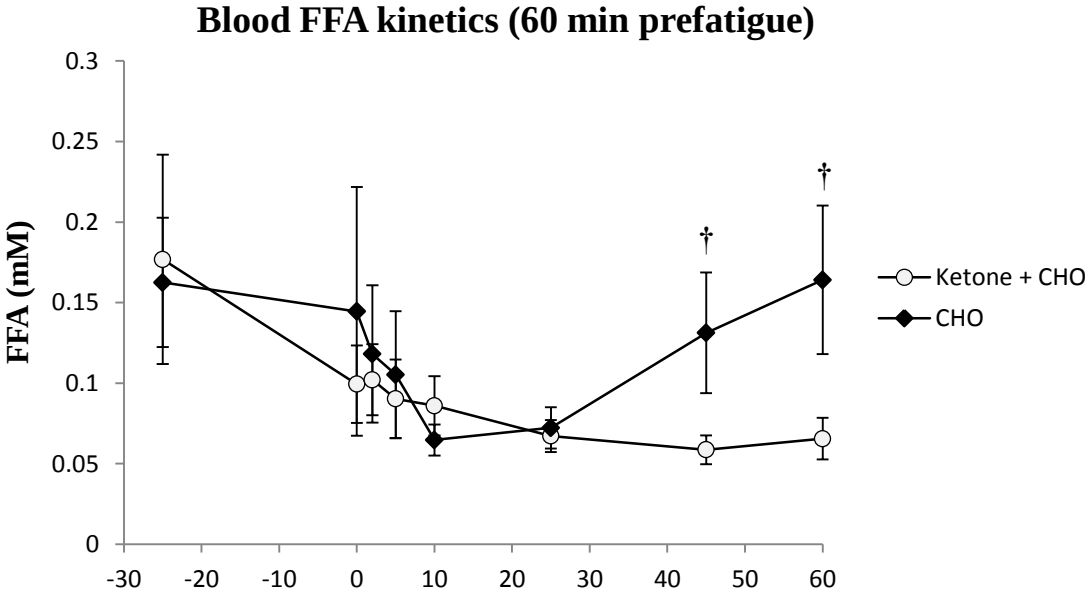


Figure 38 FFA profile during the 60 min of controlled (prefatigue) work at 70% of Wmax prior to the 30 min time trial. † $p < 0.05$ between Ketone and carbohydrate, and carbohydrate (CHO) alone arms. Data are expressed as mean +/- SEM.

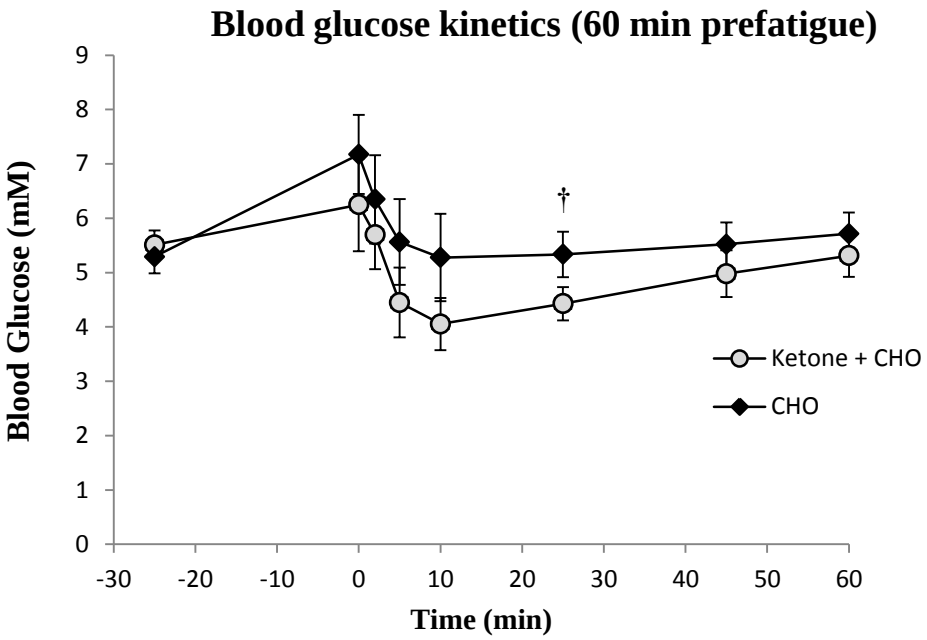


Figure 39 Blood glucose kinetics during 60 min of controlled (prefatigue) work at 70% of Wmax prior to the 30 min time trial. † $p < 0.05$ between Ketone and carbohydrate, and carbohydrate (CHO) alone arms. Data are expressed as mean +/- SEM.

7.4.3 Time trial performance

During the blinded 30 min time trial, athletes cycled an average of 411 m or 2% further ($p < 0.05$, figure 40) on the combined ketone and carbohydrate arm compared to carbohydrate feeding alone. No differences in cadence, heart rate, or pacing strategy were observed over the course of this time trial (figure 41). Of the 8 athletes studied, 7 showed improved performance during ketosis over carbohydrate alone. There was no effect of trial order on subsequent time trial performance (p NS trial 1 vs. trial 2).

7.4.4 Gastrointestinal upset

Subjective measures of gastrointestinal and systemic upset were not recorded during the study, however 2 of the 8 athletes reported significant nausea following the second substrate drink prior to the time trial on the ketone arm. No vomiting occurred during the course of the trial on either arm, and no other adverse effects were reported in the course of the trial.

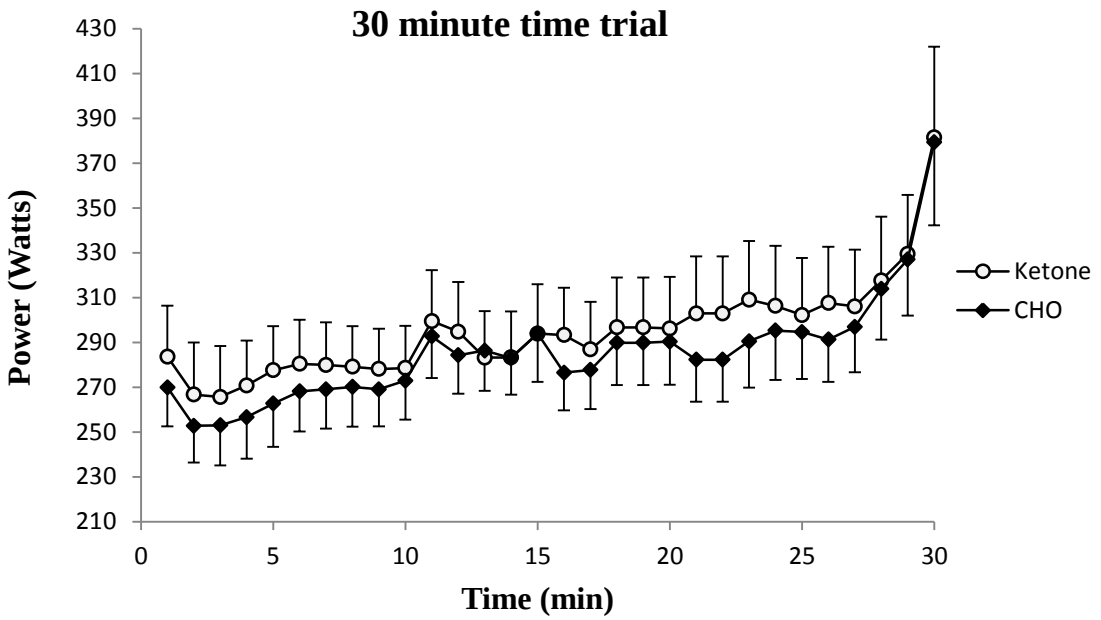


Figure 40 Power output over 30 minute time trial. Data presented as the average power +/- SEM over 1 minute intervals of the time trial. No significant differences in minute-by-minute power output were detected between experimental arms. Data are expressed as mean +/- SEM.

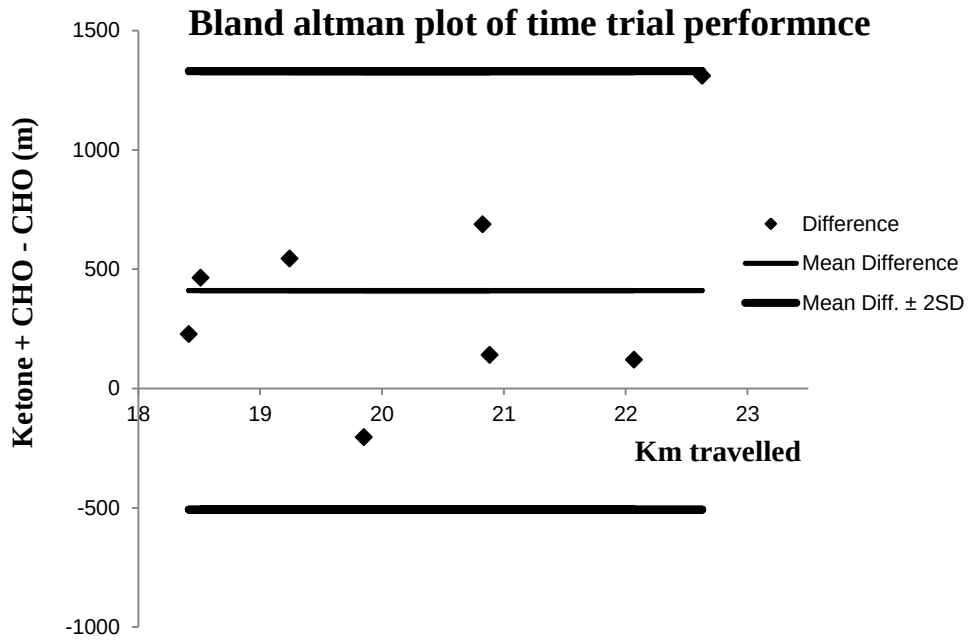


Figure 41 Bland Altman plot of performance during 30 min time trial (distance travelled in m). Average performance difference of 411 m (p = 0.039 two tailed T-test).

7.5 Discussion

This work determined the effects of synergistic administration of carbohydrates and ketones together, in comparison to evidence based strategy to provide maximal carbohydrate provision by multiple

transportable carbohydrates. The combined effects of carbohydrate and ketone feeding improved endurance exercise performance in a highly trained athletic cohort, demonstrating a 2 % improvement in time trial performance.

Study design

This trial was intentionally designed to be strenuous, by employing a relatively high intensity steady workload (prefatigue) prior to an all-out time trial performance. The rationale for this design was to target workloads that under conventional conditions would be primarily glycolytic [259], offering the best chance to detect alterations in carbohydrate metabolism. The prefatigue duration was designed to allow sufficient time so that any subtle metabolic effects of the respective nutritional interventions would be manifest in a change in physical performance. However, in an attempt to minimise the effects of long (boring) exercise on subsequent motivation during time trial performance[306], and to provide standardised time point comparisons in power, a 30 min trial for distance was employed. Blinding athletes to time trial performance also allowed the investigation of the effects of pacing strategy induced by alterations in cerebral substrate supply, or central nervous system governance.

Prefatigue

As previously mentioned, the incorporation of a standardised workload of moderate to long duration before a time trial effort is well established as a sensitive means of assessing the effects of substrate manipulation on exercise performance [292, 307-309]. This prefatigue period also allows investigation of metabolic changes during standardised workloads leading up to the time trial.

In this study, marked differences in metabolic profile were observed during the 60 min of steady state workload at 70% of Wmax. Blood metabolite profiles observed in this study were almost identical to those observed previously in Chapter 6, suggesting that similar alterations in skeletal muscle metabolism were likely to have occurred. Blood lactate was significantly lower during ketosis, compared with the carbohydrate condition, despite the addition of 1.2 g/min of dextrose in the ketone preparation. This finding provides further evidence that ketone bodies may influence carbohydrate metabolism when provided in direct competition. However, a lower lactate production may be better

viewed as an easing of the pressure on glycolytic pathways to provide pyruvate to the anapleurotic pool as a result of ketone oxidation. If the oxidation of ketones exerts feedback control on glycolysis lowering pyruvate production, whilst improving oxidative efficiency, this could reasonably account for the disparate lactate concentrations between arms.

Synergistic action of ketone bodies, glucose, and insulin is already known to improve energetic performance in the isolated perfused rat heart. In this model, hydraulic work increased, oxygen consumption was reduced, and free energy increased following ketone, glucose and insulin administration compared with glucose alone [45]. The findings of this study suggest similar mechanisms may be responsible for the performance changes exhibited here during whole body exercise. However the effects of combined ketone and insulin action on cell energetics during exercise remain unclear.

Blood βHB concentrations were 30 fold higher in the ketone arm, reaching 3 mM by the conclusion of the protocol. This ketosis is somewhat lower than would usually be expected following administration of equivalent amounts in a mixture not containing carbohydrate, as seen in previous chapters (5 and 6). The reasons for this finding are uncertain, and either represents a reduction in the bioavailability of βHB, or an increased disposal of βHB. The former seems conceivable, given the slower rate of appearance of blood βHB at rest than previously observed in preparations not containing carbohydrate. However, the action of insulin on the peripheral disposal of ketone bodies may in fact increase the rate of disappearance from the circulating pool [310]. This increased peripheral disposal of ketone bodies by the action of insulin has been suggested previously [310, 311], and could explain why administration of carbohydrate and ketones together result in a lower circulating ketosis than ‘pure’ ketone solutions. In fact the synergistic delivery of carbohydrates, stimulating insulin release, may have advantageous effects on peripheral utilisation of ketone bodies, and thermodynamic effects on mitochondrial function. In the isolated perfused rat heart, the combination of carbohydrate, ketones, and insulin together increased the concentration of metabolites of the first third of the TCA cycle some 18 fold above glucose alone, increased hydraulic efficiency by 35%, and the ΔG_{ATP}

$_{Hydrolysis}$[45]. These thermodynamic effects of simultaneous carbohydrate and ketone provision may help to explain the improvements in endurance performance observed in this work.

Regardless of the reasons for the lower ketosis, adequate circulating levels were still achieved close to the reported Km for endothelial transport [110, 114], indicating that an adequate supply of ketone bodies were provided to fuel muscular work.

Performance effects

The ingestion of a combined preparation of carbohydrate (dextrose) and ketone resulted in a 2% improvement in performance compared with carbohydrates alone. This finding is remarkable for a number of reasons: firstly, these results challenge decades of traditional sports nutrition which favour carbohydrates as the primary fuel to power exercise. Secondly, these data suggest that ketone bodies may provide an additional source of calories via the oral route in excess of that provided with the most aggressive carbohydrate fuelling strategy. Evidence in support of this can be surmised from the fact that carbohydrate (dextrose) in the ketone arm was fed at significantly lower amounts than in the carbohydrate alone arm. Whilst adequate according to published guidelines to achieve maximal exogenous oxidation rates for dextrose alone, many recent publications have suggested increased exogenous oxidation with a mixture of carbohydrate sources [253, 305, 312, 313]. Despite every effort to provide the maximum possible exogenous energy supply through mixed carbohydrate sources on the carbohydrate arm, this did not result in superior trial performance. In fact, an inferior performance was observed in this study compared to the calorie matched administration of ketone ester. This could mean that gut transport is in fact a major limiting factor to any nutritional delivery of substrate during exercise, and that comparatively, nutritional delivery of ketone bodies is more easily achieved than that of carbohydrate. It remains to be seen if the supply of multiple carbohydrates with ketones could provide further performance improvements, or if larger amounts of oral ketone body are a more effective means of providing exogenous fuel. However limitations to gut transport are almost certainly not the sole explanation for the performance effects observed in this trial. For example, the nature of the exercise conducted here is highly glycolytic, yet the addition of circulating ketones was able to increase exercise performance despite conditions that would usually favour carbohydrate

oxidation. This implies that a switch in fuel selection towards ketone bodies may have an energetically favourable effect on mitochondrial redox state. Therefore, a much deeper understanding of the thermodynamic relationships between fuel selection and mitochondrial function during exercise are needed.

Limitations

Due to the demanding nature of this trial, and the highly specialised cohort tested, study numbers are relatively small. However, they are consistent with the established literature of functional performance testing after dietary intervention [314-317]. As in Chapter 4, obtaining invasive measurements of the physiological processes controlling physical performance were not achievable without providing a confounding distraction. However similar profiles in plasma metabolites to that of previous chapters suggest analogous changes in substrate metabolism underlie the alterations in physical performance observed here.

In this study both preparations contained carbohydrates, so any relative performance effects of ketone bodies with carbohydrate over ketone bodies by themselves is unknown. This would seem a logical next question to investigate, as, should the addition of carbohydrates provide no meaningful enhancement to performance, ketone bodies may become the gold standard substrate to power exercise.

A further question regarding the dose response relationship of circulating ketosis to physical performance is suggested by this work. Exploring the lower and upper limits of performance enhancing efficacy of ketosis is outside the scope of this study, but may be of utility in the treatment of patient populations, or in applied elite sport.

7.6 Conclusion

The addition of ketone bodies to optimal carbohydrate intakes during endurance exercise enhanced physical performance when compared to carbohydrates alone. Ketone bodies may provide additional available fuel to exercising muscle and brain under conditions of energetic stress.

8 General Discussion

8.1 Summary of main findings

1. Administration of an oral ketone monoester resulted in elevated blood ketone levels, without the need for specific dietary manipulation. This oral method of ketosis has predictable dose response, clearance, and kinetic profiles within a normal and athletic population.
2. The onset of exercise during an acute nutritional ketosis led to a fall in plasma ketone concentrations in proportion to the workload undertaken. This fall in blood ketone concentration during exercise cannot be explained by urinary elimination of ketone, and represents increased oxidation of ketone bodies as a respiratory fuel.
3. The addition of circulating ketone bodies to a meal replacement formula resulted in an average improvement in endurance performance of 0.7 % (n=22) during a blinded 30 min rowing trial. No improvement over shorter 'anaerobically' biased 2 km rowing was observed.
4. The synergistic administration of ketone bodies with dextrose, vs. a mixture of carbohydrates resulted in lowered blood lactate levels, and a 2 % improvement in endurance performance during prolonged exercise (n=8).
5. Ketone concentrations increase rapidly in skeletal muscle once plasma levels rise. The increase in intramuscular ketone concentration results in an alternative carbon source for the TCA cycle, suppressing glycolysis. Even the profound stimulation of high intensity exercise for one hour did not overcome this hierarchical shift in substrate preference.
6. An elevated ketone concentration during exercise attenuates the increase in branched chain amino acids released from skeletal muscle.
7. Elevated ketone concentrations mimic the physiology of starvation, suppressing lipolysis, and shifting mitochondrial substrate preference during exercise away from fat and carbohydrate towards ketone bodies. This shift in fuel selection may have advantageous thermodynamic implications for mitochondrial energetics.

8.2 Efficacy of the oral route of delivery for nutritional ketosis

This thesis describes in detail the effects of the acute administration of nutritional ketone bodies from an oral monoester preparation. Two monoester preparations were tested in the course of this thesis, a milkshake based meal replacement [194], and a new preparation developed by the author. The pharmacokinetics of these preparations are described in detail, both at rest and during exercise (chapters 3 and 6).

In the course of this work, a clear dose response relationship was observed between blood ketone levels and dose of ingested ketone monoester. The onset of physiological ketosis is rapid using this method, and reliably results in elevated blood ketone concentrations.

Despite much dedicated effort, it was impossible to make a drink preparation containing this ketone ester which tasted appealing. In fact one current limitation to this method of nutritional ketosis is subject tolerability. While extensive research has already established the safety and toxicology profile, this work and that recently published [193, 194] supports the notion that systemic effects such as nausea, vomiting, dizziness and headache may be problematic in some instances. Notably, these effects were noted more prevalently in the milkshake meal replacements, than the sports water preparations. Furthermore, vomiting occurred in approximately 10% of athletes, whilst the majority reported no ill effects. Importantly even in those who reported feeling unwell during exercise, performance was not seemingly affected, and all except one athlete completed the required exercise protocol.

Important to note is these cohorts are somewhat unusual in their stoicism towards systemic upsets during exercise [318, 319], and are highly motivated to continue exercise that most non-athletes would find difficult. In addition many commercial sports nutrition supplements are well known for their unpleasant taste, so this factor may have also contributed to a greater compliance.

This thesis is the first work to systematically administer this method of nutritional ketone bodies to human subjects, and for these reasons little is currently known of their longer term effects. Only acute administration was therefore investigated in this work.

8.3 Ketone bodies as fuels for muscular work and exercise

Infusion studies and radiolabelled tracers

Previous published evidence on the role of ketone bodies to fuel muscular work have almost always been confounded by the inability to produce elevated ketone concentrations without the effects of starvation, or elevated fatty acids. This lack of facility to induce acute ketosis has meant almost all of the published literature methods have employed infusions of either AcAc or βHB in order to study the role of ketone bodies as oxidative substrates, or signalling molecules in human subjects. Narrowing this search further to those studies conducted during exercise results in only a handful of published reports, derived from at most 26 subjects [88, 320]. None of these studied strenuous exercise, or performed work in athletic cohorts.

These early investigations sought to determine the regulation of ketogenesis, and its role in starvation, obesity and diabetes [321]. However their findings and interpretation of these results leave much to be questioned. Firstly, one of the most significant findings by Fery and Balasse et al was a profound stimulation of exercise on the metabolic clearance of ketone bodies in overnight fasted subjects [88, 322]. In two further studies, subjects performed relatively low intensity exercise (40-50% $VO_{2\ Max}$) for 30 min and 2 hours respectively, during constant infusions of either acetoacetate or βHB [323, 324]. Circulating ketone bodies fell by > 1mM, rates of disappearance of ketones markedly increased, and metabolic clearance of ketone increased ~5-8 fold above resting conditions. Furthermore the % of CO_2 derived from the oxidation of ketones was consistently between 10.1 and 17.6% of total CO_2 suggesting significant oxidation of ketones in these overnight fasted subjects, even at relatively low workloads. Despite these findings the authors concluded '*that the direct effect of exercise on the metabolic clearance of ketones is negligible*' and suggested that during hyperketonaemia '*increased energy requirements are met primarily by fuels other than ketones*'.

Another factor to consider in the interpretation of tracer studies is the well-known problem of incomplete equilibration of ketone bodies in the circulating pool, potentially resulting in significant measurement error when using a single labelled tracer [325-327]. This is almost certainly the case in the recently mentioned infusion studies, and although acknowledged by the authors, makes

calculations of absolute ketone oxidation (reported as 0.4 mM/min) seem uncertain. Another possible source of error in the calculation of ketone oxidation comes from the loss of labelled tracer into the TCA, and bicarbonate pools [328, 329]. Particularly when exercise duration is prolonged, loss of this label may account for significant error in measured specific activity of exhaled CO_2. Compounding this problem, no labelled bicarbonate prime was given to saturate the bicarbonate pool before the onset of exercise [329].

One important aspect of the different responses to hyperketonaemia was also demonstrated by these studies. Relative to the overnight fasted state, infusions of ketone bodies after 3-5 days (or longer) of total fasting was not associated with an increase in ketone oxidation, but rather the reverse [330, 331]. Theories exist as to the cause of this finding of diminished skeletal muscle ketone oxidation during prolonged fasting. Perhaps the most logical explanation being that for evolutionary reasons it makes better sense to conserve fuels that the brain will oxidise during starvation (ketone bodies and glucose), whilst switching skeletal muscle preference to fatty acids, as skeletal muscle represents a large potential sink of carbon units that may exhaust blood derived fuels. However, it is unclear what drives skeletal muscle upregulation of fatty acid oxidation during starvation: either regulation by the ketonaemia itself, or by the raised fatty acid concentrations present during fasting [332, 333]. Administering ketone bodies by means of a dietary ketone ester may allow the examination of this question in further detail, by providing elevated ketone bodies without the associated rise in free fatty acids. This is not possible with traditional infusion methods as subjects would need to remain connected to IV apparatus + infusions of ketone bodies 24 hours a day for at least 5 days. This inconvenience to subjects, not to mention the dangerously large salt load which would accompany the infusion, precludes this as a viable method for testing this concept.

Athletic adaption and changes to skeletal muscle ketone oxidation

Athletic training results in widespread alterations in the capacity of skeletal and cardiac muscle to oxidise substrates [131, 334]. Ketone body production during and after prolonged exercise is a well described feature of exercise metabolism [335-337]. Many have reported decreases in ketone concentration at the end of exercise in trained athletes compared with untrained controls [337-340],

but the potential advantage of a switch to ketone body oxidation under energetically stressful conditions has not been acknowledged. This work suggested a different interpretation for why the body may endogenously produce ketone bodies during times of physical stress; that of an energetically favourable alternative substrate which is 'conserved' under normal circumstances but is preferred by the body in times of need. Ketosis may therefore represent an evolutionary adaption to sustain performance, and why the training adaption's of athletes to harness ketone bodies [341] make them an ideal initial cohort in characterising this novel physiological state.

8.4 Effects of ketosis on endurance performance

In the course of this thesis, exercise has been used as a model of physiological stress, and applied to test the relationship between substrate energetics and human performance. Chapters 4 and 7 explored for the first time in human subjects, the effects of a nutritional supply of ketone bodies on athletic performance.

Endurance exercise performance was consistently improved during nutritional ketosis, provided the exercise is of sufficient duration to be primarily oxidative, rather than anaerobically biased. The magnitude of this effect is significant for the elite athlete, with average improvements in physical performance of between 0.7-2%. It should be noted that for the highly trained athletic cohorts studied in this thesis a performance improvement of this magnitude is enough to break personal, as well as all comers' records. In fact, this nutritional intervention may well provide a novel and superior method of fuelling exercise. As seen in previous chapters, ketosis may improve physical capacity alone, or in combination with other dietary substrates.

The functional performance improvements observed with nutritional ketosis may be due to a number of mechanisms, some of which have been discussed previously in the course of this thesis. Fundamentally the presence of elevated blood ketones appears to positively influence physical performance, and is accompanied by changes in skeletal muscle metabolism. However skeletal muscle is only one aspect of a complex integrated system that ultimately regulates physical performance. For example neuroenergetic considerations are an important factor in the will to

continue exercise [342], particularly in the exertional domains athletes were subjected to in these performance trials. Further to this, cardiac function may well have been positively influenced as a result of altered fuel availability. It seems unlikely however, given the body's evolutionary capacity to utilise ketone bodies, that any single organ system is the sole determinant of this altered physical capacity.

8.5 Fuelling exercise: a new order of substrate selection?

The acute nutritional delivery of ketone bodies creates a physiological state unique to this method. Under normal conditions the body sources virtually no energy from circulating ketone bodies, yet the equivalent ketosis of several weeks of fasting [52] can be delivered in a matter of minutes using this monoester. Providing 3-5mM of circulating ketones to a physiologically ketone- naive state has profound consequences on fuel selection, and substrate metabolism, as described in chapter 6.

Randle described the glucose FFA cycle that suggested an overall substrate hierarchy dominated by fatty acid selection in preference to carbohydrate for oxidative phosphorylation. While generally speaking the Randle cycle approximates substrate hierarchy accurately at rest, the same is not true when cellular conditions change, such as during exercise or other physiological stressors. This thesis sought to explore fuel selection during exercise in greater detail by comparing nutritional supply of ketone bodies with other nutritional substrates. Perhaps surprisingly, this work indicated that during heavy aerobic exercise, ketone bodies may exert control over carbohydrate oxidation, prompting a re-evaluation of accepted substrate hierarchy. Ketone bodies also inhibit peripheral lipolysis, and therefore control plasma FFA oxidation, and are themselves oxidised by skeletal muscle. Whilst ketone bodies almost certainly do not become the sole carbon source for oxidative phosphorylation during exercise, their contribution to, and regulation of mitochondrial energy transduction indicates a new order of substrate preference.

8.6 Thermodynamics of substrate oxidation

Manipulating dietary habits and therefore substrate physiology unquestionably alters human performance. Although poorly acknowledged, the reasons for these effects lie in the thermodynamic relationships at the core of mitochondrial oxidation.

Fundamentally our body is driven by a series of controlled chemical reactions, resulting in the oxidation of carbon substrates to water and CO_2. Thus for a given amount of substance, the maximum amount of non-expansive work that can be obtained from a closed system is denoted by the Gibbs free energy. Described in 1873 [343], this application of the second law of thermodynamics can be readily translated to biological systems, and in its simplest form relates enthalpy and entropy to a conservation of energy. Put formally as:

$$\Delta G = \Delta H - T\Delta S$$

This equation, in part, explains why certain substrates with higher enthalpy yield greater potential energy to power a system. From this equation we can see that the larger the value of Gibbs free energy (denoted as a minus sign) the more energy can be exchanged with the surrounding system. In non-standard chemical conditions such as are present in most biological systems[344], an alternative form of this equation can be used:

$$\Delta G' = \Delta G^\circ + RT\ \ell n\ Q$$

This equation allows the integration of the reaction quotient (Q) into the relationship between free energy and the chemical conditions under which the reaction is taking place. In the case of cellular substrate energetics, the final common endpoint for the complete oxidation of carbon fuels is the conservation of energy in the phosphate bonds of ATP. Therefore applying this concept to the equation above, the inherent energy stored in this bond ($\Delta G_{\text{ATP hydrolysis}}$) can be calculated from the equation:

$$\Delta G' = \Delta G^\circ + RT\ \ell n\ \frac{[ADP][Pi]}{[ATP]}$$

This introduces an important concept for the application of thermodynamics to biological systems; that of near equilibrium relationships between metabolic pathways, each part of a complex interdependent network, with an overall net forward flux [345]. This kinetic linkage between redox couples of the major pathways and the phosphorylation potential of the cell has its origins in the pioneering work of Klingenburg [345], Krebs [346-349], and later Veech [350-354] among others.

Despite the apparent simplicity of oxidising substrates to liberate energy to perform work, the useful free energy of substrate combustion is influenced by the architecture of the metabolic pathway and the enthalpy of that particular substrate. For this reason the available free energy to perform work, the free energy of ATP hydrolysis ($\Delta G'_{ATP}$) is not equivalent for all dietary fuels.

Since the conversion of ADP + Pi to ATP is driven by the electrochemical potential difference across the mitochondrial membranes, the equation for free energy can now be expressed as:

$$\Delta G' = -n\,F\,\Delta E$$

However it should be noted that the donation of electrons to the electron transport chain are from the reducing equivalents NADH and $FADH_2$, both of which can be described as a redox couple with respect to standard membrane potential (that of the Hydrogen electrode E_h) [353]. It becomes apparent that the larger the electrical potential difference between mitochondrial phases created by the pumping of protons into the inter-mitochondrial space [355], the greater the potential free energy. We can express the potential energy of this proton gradient as:

$$\Delta G'\ [H^+]_{Inter}/[H^+]_{Matrix} = RT\,\ell n\,[H^+]_{Inter}/[H^+]_{Matrix} + FE_{\,Matrix/Inter}$$

Where ‘Inter’ and ‘Matrix’ denote the separate mitochondrial phases partitioned by the inner mitochondrial membrane [11]. Therefore the relative supply of reducing equivalents generated by the architecture of each pathway also has a significant influence on mitochondrial potential gradients, and thus the $\Delta G'_{ATP}$.

8.7 Limitations

Substrate oxidation and metabolic control

Quantitative measurements of ketone oxidation in tissues were not possible with the style of study undertaken in this thesis. Conventional substrate oxidation calculations using stable or radioisotopes of carbon may have provided greater insight into the dynamics of fuel selection during exercise. While routinely used in this context, standard isotopic calculations for oxidation rates of fat and carbohydrate do so making several important assumptions. These assumptions may not hold true during ketosis, if attempting to apply the same methodological approaches to ketone oxidation.

Working metabolic control during exercise with and without ketosis is yet to be described in detail. Such a programme of work would require significant quantities of muscle tissue obtained immediately after or during exercise (as in Chapter 5). While needle biopsy techniques do enable muscle tissue to be obtained quickly and relatively simply, many samples may be required at multiple exercise intensities, during several combinations of substrate feeding. This will require multiple subject groups and considerable time and discomfort for participants. Analysis of this tissue is also very time consuming, not to mention challenging with small sample sizes. While extremely interesting, mechanistic metabolic control modelling was regretfully not possible in this thesis for these reasons.

Adequate measures of organ specific function were also not possible during concomitant exercise. These present methodological problems of their own if accurate assessment of LV work, cardiac A-V perfusion, or cerebral A-V substrate supply are desired. The Kety –Schmidt technique [356] has been previously used to determine changes in cerebral blood flow, and substrate oxidation during exercise, but involves retrograde cannulation of the internal jugular vein. These, nor other invasive angiographic techniques to measure cardiac performance, were ethically justifiable for this cohort.

Performance effects and dose response:

Further work to delineate a dose response relationship between plasma ketosis and exercise performance is suggested by this work. In this thesis, performance studies were deliberately aligned with physiological concentrations of ketone bodies, such as would be observed following several days

or weeks of total fasting. However even in this range of circulating concentrations there may exist differences in relative energy contributions from ketosis, which may have performance implications. For example the circulating ketone concentrations in the lower range between 1.5 mM and 3 mM may in fact be less effective that concentrations closer to the reported Km of endothelial transport of 5 mM [11, 110]. Evidence for concentrations much higher than this is poor, and some have suggested that markedly elevated ketone concentrations may saturate transport and result in no further increase in ketone oxidation [323]. It is clear more work needs to be done to determine optimal dosing targets for different experimental conditions, and most likely for different cohorts as well, as previously discussed.

Next steps

Successful grant funding has already been secured using information acquired in the course of this thesis, aiming to further test the effects of nutritional ketone bodies on cerebral and exercise performance in a military cohort (Appendix 9.4). Several small clinical studies in diabetes and Parkinson's disease are already underway. Formal stable isotope studies at rest and during a series of exercise loads are planned to quantitate the contribution of ketone body oxidation to energy expenditure. Further to this, studies in skeletal muscle myopathy (Glycogenoses, and CPT deficiency syndromes) are planned to investigate further the mechanisms of skeletal muscle fuel selection, and therapeutic potential of ketosis.

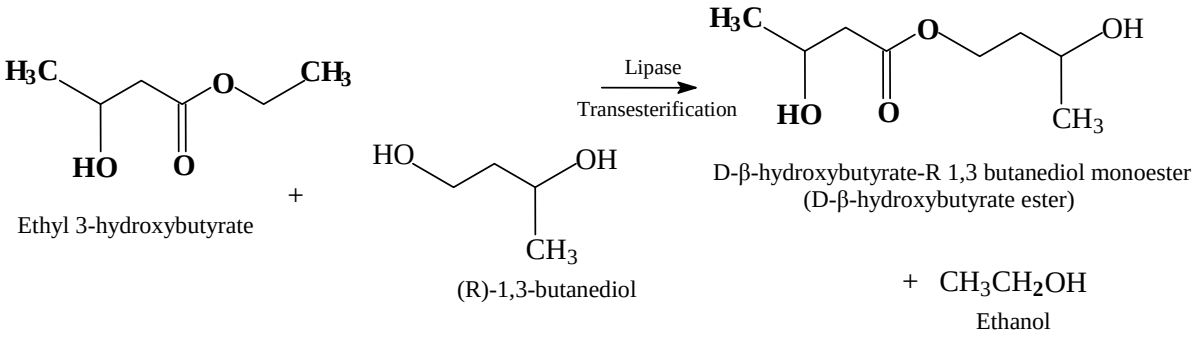
8.8 The future

This thesis is merely a starting point for further work to broaden and deepen our understanding of the roles, and effects of this novel form of ketosis on human physiology. Clinical applications for a metabolic agent capable of optimising cellular energetics are considerable, including cardiac failure, diabetes, and neurological disorders such as epilepsy to name a few. In fact, substrate energetics are such a fundamental part of our very existence it is clear more chapters in this story are yet to be written. However such pathological conditions represent new challenges, in particular the need to supply a chronic ketosis over months or even years. As yet our understanding of the chronic effects of this nutritional ketosis are very limited, but perhaps could hold the key for more profound discoveries yet to come.

9 Appendix

9.1 Manufacture process of ketone monoester

Initially an ethyl ester of D-β-hydroxybutyrate (chemically synthesized) and the > 99% pure R enantiomer of 1,3-butanediol are reacted together in the presence of immobilized lipase from *Candida Antarctica* under mild vacuum to remove the resultant ethanol by-product. Although the D-β-hydroxybutyrate ester could be formed alternatively by direct esterification of (*R*)-1,3-butanediol and D-β-hydroxybutyrate, the transesterification process used here avoids the presence of small amounts of unreacted monomeric D-β-hydroxybutyric acid in the final product.



Each batch of raw monoester was quality tested for impurities by mass spectroscopy, and then shipped to the University of Oxford department of Physiology Anatomy and Genetics (DPAG). Smaller aliquots of raw ester were individually tested for microbial contamination (IFN, Reading, UK). All ketone was stored at or below room temperature in air tight containers to minimize contamination or exposure to environmental esterases.

9.2 Materials and Methods (metabolomic analysis of skeletal muscle)

Metabolite extraction from skeletal muscle

Metabolites were double-extracted from approximately 100mg tissue using a modified Folch method as previously described (Le Belle *et al*., 2002)[357]. The aqueous and organic fractions were separated and further split into 2 identical volumes to allow analysis on multiple platforms.

^{1}H-NMR analysis of aqueous metabolites

Half of the aqueous fraction (~25mg wet weight tissue) was dried down under nitrogen, and resuspended in 600µL D_2O containing 0.09% w/v NaCl (Sigma), 0.01% w/v NaN_3 (Sigma) and 0.25mM 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.).

Carnitine analysis

Half of the aqueous fraction was combined with half of the organic fraction, and 200µL acyl-carnitine standard containing eight deuterated species was added (Cambridge Isotope Laboratories, Inc.). Samples were dried down under nitrogen and butylated with 3M butanolic-HCl (Sigma). Samples were dried down once more before resuspension in 200µL 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 Corporation).

Citric acid cycle and glycolytic intermediates and BCAA analysis

Tissue extracts from the carnitine analysis were recovered, dried down 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. Branched chain amino acids, citric 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)

9.3 World Anti-doping communication (letter)

ukad anti-doping in sport

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www.ukad.org.uk
ukad@ukad.org.uk

Protect

Dr [redacted]
UK Sport
40 Bernard Street
London
WC1N 1ST

13 October 2011

Dear [redacted]

Re: D-β-HYDROXYBUTYRATE-*R*-1,3-BUTANEDIOL MONOESTER

This letter details the processes followed with respect to the research conducted by Oxford University, as headed up by [redacted] regarding the Ketone dietary supplement D-β-HYDROXYBUTYRATE-*R*-1,3-BUTANEDIOL MONOESTER.

The correspondence was initiated with UK Anti-Doping on the prohibited status of Ketones back in April of this year, shortly thereafter (11/04/2011) we met to discuss this topic. At this point only information in the public domain was exchanged (New Scientist article, 25/07/2007) due to the confidentiality agreement (CDA) between UK Sport and Oxford University. Until this initial meeting, and the follow up emails that took place between UK Anti-Doping, the World Anti-Doping Agency (WADA) and UK Sport, it was not known what level of information would be required by UK Anti-Doping and WADA to assess the prohibited status of this Ketone as if the generic category of Ketones could be confirmed as not prohibited, then no further action was required.

The substances categorised as Ketones are diverse. To determine whether the status of β-HYDROXYBUTYRATE-*R*-1,3-BUTANEDIOL MONOESTER was prohibited or not further information regarding the substance needed to be provided to UK Anti-Doping and WADA. A CDA was therefore signed between UK Anti-Doping, Oxford University and UK Sport. [redacted] from Oxford University and UK Anti-Doping met in July to identify the detailed information required. On 26 July, an 18 page dossier was submitted by UK Sport and Oxford University to UK Anti-Doping and, following the signing of a CDA between WADA and Oxford University, it was forwarded to Oliver Rabin, the Director of Science at WADA.

A response received from WADA (08/09/2011) expressed no concern over this substance,

"...Frankly, none of us here in the Science Dept understand why these substances would be of particular concerns from an anti-doping perspective. We would appreciate further clarifications from you..."

UK Anti-Doping has clarified two further points with WADA and received a final response,

UK Anti-Doping is the trading name of United Kingdom Anti-Doping Limited, a company limited by guarantee registered in England and Wales. Registered Number: 6990867

Registered Office: Oceanic House, 1a Cockspur Street, London SW1Y 5BQ.

FS 38189

"At this point in time and based on the confidential information from [redacted] we have reviewed, we have no reason to consider such substances as banned under the 2011 List of Prohibited Substances and Methods.

Should further information or future results justify a review of this judgement, we of course reserve the right to review our current position."

I hope this information is of assistance.

Yours sincerely

Michael Stow
Head of Science and Medicine

9.4 Further studies: Currently in progress

Ministry of Defence
Research Ethics Committee

MOD Research Ethics Committee
(Personnel, Protection and Effectiveness)

From the Chairman
Professor Allister Vale
National Poisons Information Service (Birmingham Unit),
City Hospital, Birmingham B18 7QH

Telephone: 0121 507 4123
e-mail:allistervale@npis.org

Dr Peter Cox
Clinical Researcher
C/-OCMR Department
John Radcliffe Hospital
Oxford OX3 9DU

Our Reference: Protocol 298/PPE/11

Date: 22 April 2012

Dear Dr Cox,

I am delighted to confirm that MODREC has approved Protocol 298/PPE/11.The delay in providing this confirmation was due to the fact that initially I did not receive the MHRA correspondence from you until the MODREC Secretary returned from annual leave. I also needed to confirm that Professor Curran had approved the Questionnaire changes, which she now has I am informed. Finally, could you please remove the "Thanks" on page 32 to the Committee for considering the protocol? It is now approved!

I wish you and your colleagues a successful study and we look forward to receiving in due course a brief summary of the results so that these can be filed in accordance with the arrangements under which MODREC (PPE) operates.

With kindest regards,

Allister Vale MD FRCP FRCPE FRCPG FFOM FAACT FBTS Hon FRCPSG

9.5 Gastrointestinal Symptom questionnaire

Gastrointestinal Symptoms Questionnaire

Please select the number on the scale (for each item) that represents the severity of your gastrointestinal symptoms at this particular moment in time

Upper abdominal problems

	None		Mild		Moderate		Severe		Unbearable
Heartburn	0	1	2	3	4	5	6	7	8
Bloating	0	1	2	3	4	5	6	7	8
Nausea	0	1	2	3	4	5	6	7	8
Vomiting	0	1	2	3	4	5	6	7	8

Lower abdominal problems

	None		Mild		Moderate		Severe		Unbearable
Intestinal cramps	0	1	2	3	4	5	6	7	8
Abdominal pain	0	1	2	3	4	5	6	7	8
Flatulence	0	1	2	3	4	5	6	7	8
Diarrhea	0	1	2	3	4	5	6	7	8

Systemic problems

	None		Mild		Moderate		Severe		Unbearable
Dizziness	0	1	2	3	4	5	6	7	8
Headache	0	1	2	3	4	5	6	7	8
Muscle cramp	0	1	2	3	4	5	6	7	8
Urge to urinate	0	1	2	3	4	5	6	7	8

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