Title

The metabolic interplay between dietary carbohydrate and exercise and its role in acute appetite-regulation in males: a randomised controlled study

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KEY POINTS

- Carbohydrate and exercise independently influence key appetite-regulating hormones
- Temporal changes in post-exercise appetite are linked to acetate, lactate, and PYY
- Post-exercise energy intake is associated with GLP-1 and succinate levels

ABSTRACT

Understanding the metabolic determinants of post-exercise appetite-regulation would facilitate the development of adjunctive-therapeutics to supress compensatory eating behaviours and improve the efficacy of exercise as a weight loss treatment. Metabolic responses to acute exercise are however dependent on pre-exercise nutritional practices, including carbohydrate intake. We therefore aimed to determine the interactive effects of dietary carbohydrate and exercise on plasma hormonal and metabolite responses and explore mediators of exercise-induced changes in appetite-regulation across nutritional states. In this randomised crossover study, participants completed four 120 min visits: (i) control (water) followed by rest; (ii) control followed by exercise (30 min at ~75% $\dot{V}O_2$ max); (iii) carbohydrate (75 g maltodextrin) followed by rest; and (iv) carbohydrate followed by exercise. An ad libitum meal was provided at the end of each 120 min visit, with blood sample collection and appetite assessment performed at pre-defined intervals. We found that dietary carbohydrate and exercise exerted independent effects on the hormones GLP-1 (Carbohydrate: 16.8 pmol/L, Exercise: 7.4 pmol/L), ghrelin (Carbohydrate: -48.8 pmol/L, Exercise: -22.7 pmol/L) and glucagon (Carbohydrate: 9.8 ng/L, Exercise: 8.2 ng/L) that were linked to the generation of distinct plasma 1H-NMR metabolic phenotypes. These metabolic responses were associated with changes in appetite and energy intake, and plasma acetate and succinate were subsequently identified as potential novel mediators of exercise-induced appetite and energy intake responses. In summary, dietary carbohydrate and exercise independently influence gastrointestinal hormones associated with appetite regulation. Future work is warranted to probe the mechanistic importance of plasma acetate and succinate in post-exercise appetite-regulation.

Keywords: exercise; appetite; carbohydrate; metabolism.

INTRODUCTION

Chronic exercise interventions typically produce only modest weight loss, especially when compared to dietary energy restriction (Miller *et al.*, 1997). This relative lack of efficacy is primarily attributed to compensatory eating behaviours, in which the energy expended through exercise is compensated for by an increase in energy intake (Martin *et al.*, 2019). Subjective appetite responses and compensatory energy intake following acute exercise however display marked inter-individual variability (Hopkins *et al.*, 2014; Goltz *et al.*, 2018). Understanding the mechanisms that regulate exercise-induced changes in appetite-regulation (appetite and energy intake responses) would facilitate the development of targeted therapeutics for obesity.

Exercise-induced appetite and energy intake responses have largely been attributed to changes in the systemic concentrations of gastrointestinal hormones implicated in appetite regulation, including glucagon-like peptide 1 (GLP-1), peptide YY (PYY), and (active) ghrelin (Martins *et al.*, 2007). However, acute exercise exerts changes beyond gastrointestinal hormone release, producing large-scale shifts in metabolism that may also drive exercise-induced changes in appetite and energy intake (Contrepois *et al.*, 2020).

Evidence is growing that the metabolic effects of acute exercise are dependent on pre-exercise nutritional practices, including carbohydrate intake (Hargreaves *et al.*, 2004; Vieira *et al.*, 2016; Frampton *et al.*, 2021; Edinburgh *et al.*, 2022). Acute carbohydrate ingestion also modulates GLP-1, PYY, and ghrelin concentrations (Karhunen *et al.*, 2008), as well as producing extensive shifts in the plasma metabolite profile (Ho *et al.*, 2013). We therefore hypothesised that the concurrent provision of carbohydrate and exercise would produce interactive hormonal and metabolite responses that would possess important downstream implications for appetite-regulation.

Our work therefore aimed to determine the interactive effects of carbohydrate and exercise on the plasma hormonal and metabolite responses and explore potential mediators of exercise-induced changes in appetite and energy intake across nutritional states.

RESULTS

Study design

In a randomised-crossover fashion, 12 male participants completed four study visits that involved the consumption of a control (water) or carbohydrate beverage (75 g maltodextrin, 300 kcal), followed by a 30-minute rest or exercise session (75% $\dot{V}O_2$ max on a cycle ergometer). Maltodextrin was selected over other carbohydrate sources because of its low sweetness (BeMiller, 2019) and thus facilitate blinding. This created four study interventions: (i) Control and Rest (Con-Rest), (ii) Control and Exercise (Con-Ex), (iii) Carbohydrate and Rest (Carb-Rest), and (iv) Carbohydrate and Exercise (Carb-Ex). Serial blood samples, visual analogue scale (VAS) assessments, and pulmonary gas measurements were taken periodically during each 120-minute visit, with an *ad libitum* meal presented following visit completion (Figure 1A).

Carbohydrate and exercise independently and interactively modulate the hormonal milieu

Initially, we performed robust linear mixed model analyses to identify the independent and interactive effects of carbohydrate and exercise on the hormonal milieu (Figure 1B and 2). Our results revealed that carbohydrate and exercise independently increase the anorexigenic hormone GLP-1 (Carb: 16.8 pmol/L [95% CI, 8.5 to 25.0 pmol/L], Ex: 7.4 pmol [95% CI, 3.0 to 11.7 pmol/L]; Figure 1B), while acting independently to decrease circulating levels of orexigenic hormone (active) ghrelin (Carb: -48.8 pmol/L [95% CI, -67.8 to -29.8 pmol/L], Ex: -22.7 pmol/L [95% CI, -40.6 to -4.8 pmol/L]; Figure 1B). Our results also show that carbohydrate independently increased the anorexigenic hormone PYY (Carb: 3.0 pmol/L

[95% CI, 0.5 to 5.4 pmol/L; Figure 1B). In addition, we investigated the interactive effects of carbohydrate and exercise on insulin, glucagon, and glucose concentrations due to the posited role of glucose homeostasis in appetite regulation (Wyatt *et al.*, 2021). Carbohydrate and exercise exhibited antagonistic and interactive effects on the insulin response, in which carbohydrate increased and exercise decreased circulating levels, and with exercise attenuating the rise in circulating insulin following carbohydrate ingestion (Figure 2). Conversely, carbohydrate and exercise independently increased glucagon concentrations (Carb: 9.8 ng/L [95% CI, 2.1 to 17.6 ng/L], Ex: 8.2 ng/L [95% CI, 3.1 to 13.4 ng/L]; Figure 2). Glucose concentrations were unaffected by exercise, but as expected showed a marked increase following carbohydrate ingestion (Carb: 1.0 mmol/L [95% CI, 0.7 to 1.3 mmol/L]; Figure 2).

Carbohydrate and exercise suppress appetite but exert opposite effects on acute energy balance

The independent and interactive effects of carbohydrate and exercise on appetite, acute energy balance, and substrate oxidation are presented in Figure 1B and 2. Carbohydrate and exercise showed independent appetite-suppressive effects (Carb: -8 mm [95% CI, -14 to -2 mm], Ex: -8 mm [95% CI, -14 to -2 mm]; Figure 1B). However, there were no independent effects of carbohydrate or exercise changes on *ad libitum* meal energy intake. (Figure 1B). As *ad libitum* meal energy intake was the sole component of acute energy balance that was allowed to vary in our study design (pre-exercise energy intake and exercise energy expenditure were fixed), *ad libitum* meal energy intake had the potential to exert a large influence over acute energy balance. Carbohydrate independently increased whilst exercise independently decreased acute energy balance (total energy intake minus total energy expenditure in each study intervention), thus exercise without carbohydrate ingestion resulted in the lowest acute energy balance (Figure 1B).

Carbohydrate and exercise generate distinct metabolic phenotypes

Data obtained from ^{1}H-NMR metabolic profiling analysis were modelled using repeated measures partial least squares discriminant analysis (RM-PLS-DA) to distinguish metabolic phenotypes between study interventions at pre-defined time points (T = 0, T = 30, T = 120). The metabolic phenotype was highly distinguishable between all study interventions immediately post exercise (T = 30), characterised by high a degree of explained variance ($R^2Y \geq 0.99$) and capability of prediction ($Q^2Y > 0.7$ with the exception of Con-Ex vs Carb-Ex; Figure 3A) that showed a lower capability of prediction ($Q^2Y = 0.48$). Differences between study interventions were present until the end of the study visit (T = 120) but were less pronounced at later time points (Figure 3B). No differences between study interventions were detected at baseline (T = 0).

^{1}H-NMR metabolic profiling analysis identified 23 unique small metabolites that showed significant differences over time between study interventions (Figure 3A and 3B). Quantitative measurements were available for 14 of these metabolites (Jiménez *et al.*, 2018), which were used for subsequent analysis (Figure 4). Carbohydrate ingestion produced differential amino acid and amino acid derivative responses characterised by an increase in creatine concentrations (Carb: 0.005 mmol/L [95% CI, 0.002 to 0.009 mmol/L) and a simultaneous decrease in branched chain amino acid (isoleucine [-0.01 mmol/L], leucine [-0.02 mmol/L], and valine [-0.01 mmol/L]) concentrations (Figure 4). Exercise increased concentrations of creatine (Ex: 0.008 mmol/L [95% CI, 0.003 to 0.013 mmol/L]) and the amino acids alanine (Ex: 0.10 mmol/L [95% CI, 0.07 to 0.13 mmol/L]) and glutamate (Ex: 0.03 mmol/L [95% CI, 0.00 to 0.06 mmol/L]; Figure 4). Concentrations of the TCA cycle intermediates citrate (Ex: 0.04 mmol/L [95% CI, 0.02 to 0.06 mmol/L]) and succinate (Ex: 0.011 mmol/L [95% CI, 0.009 to 0.013 mmol/L]), as well as acetate (Ex: 0.08 mmol/L [95% CI, 0.06 to 0.09 mmol/L]) and lactate (Ex: 2.9 mmol/L [95% CI, 2.1 to 3.8 mmol/L]) also increased in response to exercise, with only lactate increasing in response to carbohydrate ingestion (Carb: 0.5 mmol/L [95% CI, 0.2 to 0.7 mmol/L]; Figure 4). Exercise resulted in increased concentrations of 3-hydroxybutyrate (BHB; Ex: 0.037 mmol/L [95% CI, 0.007 to

0.067 mmol/L]) and pyruvate (Ex: 0.015 mmol/L [95% CI, 0.008 to 0.022 mmol/L]; Figure 4). Ketogenesis was however suppressed by carbohydrate ingestion as demonstrated by decreased BHB (Carb: -0.068 mmol/L [95% CI, -0.010 to -0.036 mmol/L]) and acetone (Carb: -0.04 mmol/L [95 % CI, -0.06 to -0.02 mmol/L]) concentrations (Figure 4). Carbohydrate and exercise exerted antagonistic and interactive effects on glycerol concentrations, with carbohydrate ingestion suppressing and exercise elevating concentrations, and with exercise generating higher concentrations when carbohydrate was not ingested (Figure 4).

^{1}H-NMR metabolic profiling analysis also revealed differences between interventions in ^{1}H-NMR peaks assigned to HDL and LDL/VLDL (Figures 3A and 3B). Consequently, quantitative lipoprotein measurements were analysed (Jiménez *et al.*, 2018), with data presented for main lipoprotein fractions and parameters (Figure 5; sub-fraction data are available via online repository). Carbohydrate ingestion resulted in an increase in high-density lipoprotein cholesterol (HDL-C; Carb: 2.0 mg/dL [95% CI, 0.2 to 3.8 mg/DL]; Figure 5), whereas exercise resulted in an increase in intermediate- density lipoprotein particle number (IDL-P; Ex: 9.7 nmol/L [95% CI, 2.9 to 16.5 nmol/L mg/dL]) and cholesterol concentrations (IDL-C; Ex: 1.7 mg/dL [95% CI, 0.6 to 2.8 mg/dL]), as well as an increase in apolipoprotein A2 (Apo-A2; Ex: 1.3 mg/dL [95% CI, 0.3 to 2.3 mg/dL]; Figure 5). No other main effects of carbohydrate and exercise, or any interaction between carbohydrate and exercise, was detected.

Changes in circulating acetate, lactate, and PYY are associated with exercise-induced appetite suppression

To identify temporal relationships between outcomes, we used repeated measure correlation analyses to create within-intervention correlation networks (Figure 6). These analyses highlighted distinct relationships between outcomes that were dependent on carbohydrate intake and exercise (Figure 6A). Acetate, alanine, Apo-A2, carbohydrate oxidation, total

energy expenditure, lactate, and valine showed a high degree of connectivity (≥15 total number of connections) across multiple study interventions (Figure 6B). Irrespective of carbohydrate intake, higher concentrations of acetate, lactate, and PYY were associated with decreased appetite during exercise study interventions (Figures 6A). Furthermore, of these outcomes, only higher concentrations of acetate were associated with decreased appetite in the resting state (Figure 6A). GLP-1 and PYY showed positive correlations across most study interventions (Figure 6A). GLP-1 also exhibited strong positive correlations with insulin following carbohydrate ingestion (Figure 6A), and with total energy expenditure and carbohydrate oxidation during exercise study interventions (Figure 6A). The only identified hormone-metabolite relationships were positive correlations between GLP-1 and acetate as well as between GLP-1 and lactate during exercise without carbohydrate ingestion (Figure 6A).

Identification of metabolic predictors of *ad libitum* energy intake

Congruent with prior research (Holt *et al.*, 2017), subjective appetite did not correlate with subsequent *ad libitum* meal energy intake within study interventions (see online repository). While subjective appetite may reflect eating latency (King *et al.*, 2013), it does not appear to be predictive of energy intake, and therefore any observed relationship with subjective appetite may not necessarily be present for energy intake.

We therefore investigated the capacity of the preceding metabolic environment during the 120-minute study visit period (represented as time-averaged AUC) to predict subsequent *ad libitum* meal energy intake within each study intervention using partial least squares regression (Figure 7). For all study interventions, one latent variable was created, with variable importance in projection (VIP) scores >1 (Kuhn & Johnson, 2013) used to identify outcomes important in the prediction of subsequent *ad libitum* energy intake (Figure 7A). VIP scores identified GLP-1 and creatine to be important predictors of subsequent energy intake, exhibiting VIP scores >1 in three of the study interventions. Similarly, glucose, alanine,

citrate, succinate, acetone, pyruvate, glycerol, and Apo-A2 also appeared to be important predictors, possessing a VIP score >1 in two of the study interventions. Of these metabolites, only succinate was an important predictor of meal energy intake in both exercise interventions irrespective of carbohydrate intake. Follow-up within-intervention simple univariate regression analyses were also performed to explore the direction of relationship between important predictors (VIP >1) and meal energy intake (Figure 7B) and highlight the consistent negative association between GLP-1 and succinate release with subsequent *ad libitum* meal energy intake in both exercise conditions.

DISCUSSION

Dietary carbohydrate and exercise both exert profound changes on human metabolism that have important implications for appetite-regulation and energy intake. Here, we show that dietary carbohydrate and exercise generate independent or interactive effects on gastrointestinal and pancreatic hormones associated with appetite regulation. Utilising ^{1}H-NMR metabolic profiling analysis, we also demonstrate that dietary carbohydrate and exercise generate distinct plasma metabolic phenotypes, leading to the identification of novel putative mediators of exercise-induced changes in appetite and energy intake.

GLP-1, PYY, and ghrelin have well-recognised roles as key regulators of appetite and energy intake (Steinert *et al.*, 2017), thus the primary outcome of the study was to establish the independent or interactive response of these hormones to carbohydrate intake and exercise. We show that dietary carbohydrate and exercise independently increase GLP-1 levels and decrease ghrelin concentrations (carbohydrate also independently increased PYY). Consequently, both carbohydrate and exercise create a hormonal milieu conducive to appetite and energy intake suppression. Despite the pattern of response for gastrointestinal hormones across study conditions being reflected in subjective appetite responses, this was not observed for meal energy intake, suggesting other factors may contribute to the observed between-condition differences in energy intake.

In accord with previous research (Frampton *et al.*, 2022), exercise without carbohydrate ingestion (which can also be regarded as 'fasted exercise') resulted in the lowest acute energy balance. The long-term implications of this finding are nevertheless unclear due to the inherent trade-off between internal and ecological validity. For example, the time at which participants ate was fixed and therefore any effect of eating latency (possibly as the result of gastrointestinal hormone and metabolite modulation) on subsequent energy intake was unknown. Further work is thus needed to investigate if the lower acute energy balance when exercise is performed in the fasted state translates into greater weight loss with fasted exercise training.

We also provide, for the first time, extensive characterisation of the acute lipoprotein response to exercise, showing a transient increase in IDL-P, IDL-C, and Apo-A2 concentrations following acute exercise. Although chronic changes in fasting levels of these lipoprotein parameters are associated with modified cardiovascular disease risk (Hodis *et al.*, 1997; Birjmohun *et al.*, 2007), the increase observed with acute exercise is likely a physiological response to the increased energy demands imposed by exercise, and thus represents an increase in energy mobilisation. Furthermore, the characterisation of the acute lipoprotein response to exercise may also facilitate the understanding of how chronic exercise modifies lipoprotein profiles and associated cardiovascular disease risk.

Exercise is often accompanied by a temporary suppression of appetite commonly referred to as exercise-induced anorexia (King *et al.*, 1994). Correlation network analyses resulted in the identification of key relationships between the plasma metabolome and exercise-induced appetite suppression. PYY, lactate, and acetate all displayed strong negative correlations with appetite in both exercise interventions irrespective of carbohydrate intake. The relationships between PYY and lactate and exercise-induced appetite suppression are expected and have been reported previously (Martins *et al.*, 2007; Vanderheyden *et al.*, 2020). Indeed, a recent study highlights that exercise-induced lactate is used as a precursor

for N-lactoyl-phenylalanine (Lac-Phe) synthesis, a metabolite shown to possess anorexic properties in rodents following pharmacological administration (Li *et al.*, 2022). Whilst exercise was shown to raise circulating Lac-Phe in humans, its association with the marked inter-individual exercise-induced changes in appetite and energy intake were not reported (Hopkins *et al.*, 2014; Goltz *et al.*, 2018).

In contrast, a relationship between appetite and acetate during exercise has not been previously identified. The intestine is the primary site of acetate production in the fasting and postprandial state (Kirschner *et al.*, 2021), with skeletal muscle becoming a major contributor to acetate production during exercise (Van Hall *et al.*, 2002). Despite limited human evidence, exogenous administration of acetate has been shown to suppress appetite in rodent models, acting via a central homeostatic mechanism (Frost *et al.*, 2014). Indeed, increasing circulating acetate concentrations through intravenous infusions to levels similar to that achieved during exercise has been shown to increase GLP-1 concentrations (Freeland & Wolever, 2010), suggesting that acetate may regulate exercise-induced appetite suppression by direct and indirect mechanisms.

Appetite and energy intake are related but distinct constructs. Therefore, components of the plasma metabolome that were implicated in appetite suppression could not be assumed to be implicated in energy intake suppression. Consequently, we used partial least squares regression analyses to identify potential metabolic predictors of energy intake. GLP-1 and succinate were the only two components of the plasma metabolome to be identified as important predictors of energy intake in both exercise study interventions. Again, the identification of GLP-1 is unsurprising, and highlights the importance of this hormone in the relationship between dietary carbohydrate, exercise, appetite and energy intake. Recent studies investigating GLP-1 receptor agonists and exercise training have reported greater weight-loss when using both interventions concurrently versus either intervention alone (Lundgren *et al.*, 2021). This suggests that therapeutics based on mediators of exercise-

induced changes in appetite and energy intake may be highly efficacious with respect to weight-loss, particularly when employed as a co-intervention alongside exercise training. A possible role for circulating succinate has, however, not been previously reported. Pre-clinical evidence indicates that succinate administration can decrease energy intake via an upregulation of intestinal gluconeogenesis (Wang *et al.*, 2019), and may also play a key role in skeletal muscle remodelling following exercise (Reddy *et al.*, 2020).

Like acetate, lactate and succinate are produced in substantial amounts by contracting skeletal muscle during exercise (Juel & Halestrap, 1999; Reddy *et al.*, 2020), suggesting that muscle-derived metabolites may be key regulators of energy intake in the acute period following exercise. Despite participants exercising at the same relative intensity, our data also highlight notable inter-individual differences in acetate, lactate, and succinate responses across exercise interventions (Figure 8A). Furthermore, acetate and lactate responses exhibited strong positive correlations between exercise interventions (Figure 8B), suggesting that participants truly vary in their metabolic profile with exercise. It has been recently demonstrated that appetite and energy intake responses to exercise also show substantial inter-individual variation (Hopkins *et al.*, 2014; Goltz *et al.*, 2018). Differences in acetate, lactate, and succinate concentrations may therefore contribute to the variation in post-exercise appetite and energy intake responses, and consequently be a key therapeutic target for interventions aiming to augment exercise-induced weight loss. However, acetate, lactate, and succinate concentrations during exercise interventions were not related to bodyweight, fat-free mass, or $\dot{V}O_2$ max (Figure 8C), suggesting that increasing skeletal muscle mass or aerobic fitness is unlikely to alter the concentration of these metabolites. As well as being muscle-derived, acetate, lactate and succinate are generated from gut microbial metabolic activity (Macfarlane & Macfarlane, 2003). Consequently, the observed variation in circulating levels of these metabolites could partly be explained by individual differences in gut bacterial composition and metabolism.

The findings of our study must, however, be interpreted in the context of its limitations. This study only measured energy intake, and thus energy balance, in the immediate period following exercise completion. Therefore, any compensatory responses that may occur beyond this period would not be observed, and thus our findings cannot necessarily be applied to exercise training studies. Quantitative measurements were only available for select metabolites and thus the response of some metabolites (e.g. isobutyrate) that showed differential responses across study interventions in the ^{1}H-NMR metabolic profiles were not entered into subsequent correlation and regression models. Our analysis may have therefore missed key metabolites involved in exercise induced changes in appetite and energy intake. The cohort used in the present study only included young lean males; the translation of our findings to other populations (such as individuals with obesity or females) cannot be assumed. For example, we observed increases in glucagon concentrations following carbohydrate ingestion, a response that may be only present in this demographic, and not in individuals with obesity and associated metabolic diseases (Wagner *et al.*, 2017). Furthermore, the identification of plasma metabolites involved in exercise-induced appetite and energy intake must be considered exploratory rather than confirmatory. Future investigations should attempt to manipulate succinate and acetate concentrations in the context of exercise, via the ingestion of oral agents that raise or lower their concentrations, to further elucidate their role in energy intake around exercise. Lastly, the carbohydrate and exercise dose selected for this study were chosen in order to maximise gastrointestinal hormone response, and may therefore not reveal synergisms between carbohydrate and exercise that can only be observed at sub-maximal doses.

In conclusion, we show that dietary carbohydrate and exercise generate independent and interactive effects on various components of the plasma metabolome, resulting in the generation of distinct metabolic phenotypes. We also provide, for the first time, extensive characterisation of the lipoprotein response to exercise and identify plasma acetate and succinate as novel putative regulators of exercise-induced appetite and energy intake

suppression. Future investigations are needed to establish if treatments to augment plasma acetate and succinate provide therapeutic opportunities to suppress post-exercise appetite and energy intake responses.

METHODS

Study approval

This study was granted ethical approval (South West - Frenchay Research Ethics Committee; 19/SW/007) and conducted in line with the Declaration of Helsinki. All participants provided written informed consent prior to enrolment. This study is registered at clinicaltrials.gov (NCT04019418).

Participants

This study recruited 12 healthy male participants aged 18 to 40 years old with a body mass index (BMI) of 18 to 30 kg/m^2 (inclusive) between February 2019 and February 2020 (Figure S5). Females were excluded due to the influence of the menstrual cycle on appetite control, gastrointestinal hormone release, and exercise metabolism (Dye & Blundell, 1997; Brennan *et al.*, 2009; Oosthuyse & Bosch, 2010). Full eligibility criteria are provided at ClinicalTrials.gov.

Participants had an age of 24 ± 5 years (mean ± SD) with a BMI of 21.9 ± 2.1 kg/m^2 and a $\dot{V}O_2$ max of 40.2 ± 8.7 ml/kg/min. Detailed participant characteristics are provided via the online repository.

Study design

This study was a semi-double blind, randomised, four-period crossover, placebo-controlled design.

All participants first attended a screening visit to assess eligibility. Eligible participants returned to undertake a maximal oxygen uptake ($\dot{V}O_2$ max) test to determine absolute exercise intensity for study visits. Participants then completed four study visits in a randomised order: (i) control beverage and rest session; (ii) control beverage and exercise session; (iii) carbohydrate beverage and rest session; and (iv) carbohydrate beverage and exercise session. Study visits were separated by a minimum of three days.

The study design is described as semi-double blind as both participants and researchers were blinded to the nature of the beverage, but not to the rest or exercise session.

All study visits and assessments were performed at the NIHR Imperial Clinical Research Facility at Hammersmith Hospital (London, UK).

Screening visit

Participants attended a screening visit following a ≥ 4 hour fast. A blood test (glucose and full blood count), height and weight measurements, and an electrocardiogram (ECG) were performed to determine participant eligibility. Participants were excluded if they had had an abnormal ECG, blood values outside the reference range, a BMI of <18 or >30 kg/m^2, a history of metabolic disease, and/or started a new medication within the last 3 months likely to interfere with energy metabolism, appetite regulation and hormonal balance.

$\dot{V}O_2$ max assessment

All assessments were performed on an ergometrics 900 bicycle ergometer (ergoline, Germany). Participants began the assessment by cycling at 50 watts, after which the intensity was increased by 25 watts every three minutes until volitional exhaustion. Pulmonary gas measurements (oxygen consumption and carbon dioxide production) were taken throughout the assessment via a Quark CPET metabolic cart (COSMED, Italy). If participants did not achieve a respiratory quotient ≥1.1 at the point of volitional exhaustion, the assessment was repeated at a separate study visit. After completion of the assessment,

participants were presented with an *ad libitum* meal identical to that received during study visits to facilitate familiarisation.

Study visits

Participants were asked to refrain from strenuous exercise, caffeine, and alcohol intake, and to standardise their evening meal the day prior to each study visit. Participants were also asked to fast overnight from 20:00 the evening prior to each study visit (drinking water was permitted).

Participants arrived at the research facility at 09:00 ± 1 hour upon which an intravenous cannula was inserted into the antecubital vein to permit serial blood sampling. Participants were also asked to void their bladder, with all urine thereafter collected for the remainder of the study visit. Following the collection of baseline measurements, participants either consumed a control or carbohydrate beverage. Participants were given five minutes to consume the beverage, after which participants either rested or exercised for 30 minutes. Following completion of the rest or exercise session, participants remained rested for a further 90 minutes at which point an *ad libitum* meal was provided.

Venous blood samples and 100 mm visual analogue scales (VAS) were collected at baseline and at 15-minute intervals following beverage ingestion (T = 15, 30, 45, 60, 75, 90, 105, 120). Pulmonary gas measurements were also performed for 15-minute intervals throughout the visit (baseline, T = 15-30, 45-60, 105-120).

Interventions

Beverages

The control beverage consisted of 300ml of water only. The carbohydrate beverage constituted 300ml of water with 75g of maltodextrin (equating to 75g of carbohydrate [300 kcal]; MyProtein, UK). This amount of carbohydrate has previously been shown to modulate

gastrointestinal hormone release (Meek *et al.*, 2021). Carbohydrate was provided in liquid form as non-sweet maltodextrin and served in opaque bottles at 4°C to facilitate blinding.

Rest and exercise sessions

Participants laid semi-recumbently on a bed for 30 minutes for the rest session. For the exercise session, participants exercised on an ergometrics 900 bicycle ergometer (ergoline, Germany) for 30 minutes at 75% $\dot{V}O_2$ max at a cadence ≥ 80 pedal revolutions per minute. This exercise intensity has previously been shown to modulate gastrointestinal hormone release (Ueda *et al.*, 2009).

Beverage-exercise interval

Most studies investigating the effect of carbohydrate provision prior to exercise on energy intake have involved participants undertaking exercise several hours following carbohydrate ingestion (Gonzalez *et al.*, 2013; Bachman *et al.*, 2016; Edinburgh *et al.*, 2019). The influence of carbohydrate ingestion on physiological responses such as subjective appetite, GLP-1 and PYY release is transient (Steinert *et al.*, 2011), and thus largely attenuated by the time exercise is performed. As a result, any potential interaction between acute carbohydrate ingestion and exercise on these responses is lost. We therefore used a beverage-exercise interval of five minutes to ensure that changes in appetite-related hormones due to both carbohydrate ingestion and exercise coincided.

Ad libitum meal

The *ad libitum* meal consisted of dried durum wheat semolina pasta (Tesco, UK), tomato and herb pasta sauce (Tesco, UK), and olive oil (Tesco, UK). This meal possessed an energy density of 5.3 kJ/g (1.3 kcal/g) and contained 21.2 g of carbohydrate, 3.0 g of fat, and 3.3 g of protein per 100 g.

Participants were given 20 minutes to consume the meal and were asked to eat until 'comfortably full'. Participants were also asked to refrain from using mobile telephones, laptops or other distractions during this period.

Measurements

Primary outcome measures

Blood samples were analysed for GLP-1 and PYY using previously established in-house radioimmunoassays (Adrian *et al.*, 1985; Kreymann *et al.*, 1987) at all time points. Active ghrelin was measured using a commercial ELISA (EZGRA-88K, Merck, Germany) at T = 0, 30, 60, and 120 only.

Secondary outcome measures

Blood samples were analysed for glucose using a colorimetric enzymatic activity assay (GL364, Randox, UK) at all time points. Insulin (HI-14K, Merck, Germany) and glucagon (GL-32K, Merck, Germany) were measured using commercial radioimmunoassays at T = 0, 15, 30, 45, 60, 90, and 120.

Commercial assays were performed as specified by the manufacturer's instructions. Intra- and inter-assay coefficients of variability are available via the online repository.

Pulmonary gas measurements (oxygen consumption and carbon dioxide production) and urea content of urine were used to calculate substrate oxidation (carbohydrate and fat oxidation) (Frayn, 1983) and total energy expenditure (Weir, 1949).

Ad libitum meal energy intake was calculated by measuring the difference in mass of the *ad libitum* meal prior to and following consumption. Total energy intake was calculated by adding the energy content of beverages to *ad libitum* meal energy intake. Acute energy balance was calculated by subtracting total energy expenditure from total energy intake.

Subjective appetite and nausea were measured by VAS. Subjective appetite was assessed using four questions relating to hunger, pleasantness, prospective consumption, and fullness. These scales were then converted into a single composite appetite score (CAS) using the formula: CAS = [hunger + pleasantness + prospective consumption + (100 – fullness)]/4

^{1}H NMR metabolic profiling analysis of blood plasma

Water-suppressed ^{1}H NMR spectroscopy was performed at 310 K using a 600 MHz Bruker Avance III NMR spectrometer (*in vitro* diagnostics research (IVDr)) equipped with a 5-mm BBI Z-gradient probe, high-order shims, and automated tuning and matching (Bruker Biospin GmBH, Rheinstetten, Germany). Previous to the analysis, a quantitative calibration was performed using the standard high-throughput protocol (Dona *et al.*, 2014). Bruker IVDr (B.I.) methods were used to extract lipoprotein data (B.I. LISA^{TM}) and to quantify small molecule metabolites (B.I. Quant-PS 2.0^{TM}) (Jiménez *et al.*, 2018). For each sample, three experiments were acquired in automation: two 1D ^{1}H NMR spectra were acquired using standard one-dimensional pulse sequences, the first one with saturation of the water resonance only (noesygppr1d, standard Bruker pulse program), and the second one with the same feature and with a block to filter out macromolecular/protein signals from the spectrum (Carr–Purcell–Meiboom–Gill, cpmgpr1d); ^{1}H−^{1}H 2D *J*-resolved (*J*-Res) experiment with water suppression was also acquired (jresgpprqf). The parameter sets used for acquisition and processing were according to the literature (Dona *et al.*, 2014).

Randomisation and blinding

Randomisation sequences were generated using an online randomisation tool (randomizer.org). Sequences were then placed into opaque envelopes, sealed, and allocated to participants prior to their first study visit by a member of the study team.

Participants were informed of the type of study visit (exercise or rest session) upon arrival of each study visit.

Beverages were created and labelled (A or B) by an external researcher prior to study visits. Beverage contents was revealed after VAS scoring, assays, and statistical analysis had been performed.

Statistics

All statistical analyses included data from the 12 participants that competed all study visits.

Robust linear mixed model analysis

Time-averaged area under the curve (AUC) was calculated for all longitudinal outcomes and used for subsequent robust linear mixed effect model analysis. Unadjusted values were used for *ad libitum* meal energy intake, total energy intake, total energy expenditure, and acute energy balance.

Robust linear mixed effects models were fitted using the R package 'robustlmm' (Koller, 2016) to evaluate the independent and interactive effects of carbohydrate and exercise on all outcomes. Fixed effects included in the model were carbohydrate and exercise, and an interaction between carbohydrate and exercise. Random effects included in the model were participant, an interaction between participant and carbohydrate, and an interaction between participant and exercise. Random effects with zero variance were removed from the model. The Satterthwaite's degrees of freedom method implemented in the R package 'lmerTest' (Kuznetsova *et al.*, 2017) was used to derive P values for fixed effects. If a significant interaction effect between carbohydrate and exercise was detected, multiple comparisons of estimated marginal means were performed using the R package 'emmeans' (Lenth, 2021) with the Tukey adjustment being applied. Independent effects were defined as a significant main effect of carbohydrate and/or exercise in the absence of a significant interaction effect. Interactive effects were defined as a significant interaction effect (Slinker, 1998). Estimated

marginal means of carbohydrate and exercise were only calculated when no significant interaction effect was detected. Statistical significance was set at $P < 0.05$.

NMR data processing and statistical analysis

The multivariate data analysis was performed on the 1D ^{1}H CPMG spectra. Each spectrum was automatically phased and baseline corrected using Topspin 3.5 pl 7 (Bruker BioSpin GmbH, Rheinstetten, Germany), and then digitized over the range δ -0.5 to 10 and imported into MATLAB (2014a, MathWorks, Natick, U.S.A.). The spectra were referenced to the doublet of the anomeric proton signal of a-glucose at δ 5.23. The spectral regions corresponding to the internal standard (δ -0.5 to 0.6), water (δ 4.3 to 5.1) and noise (δ 5.4 to 5.7, 5.8 to 6.7, and 8.5 to 10.00) were excluded to give full resolution spectra (~11.7 K spectral data points or variables per spectrum). Prior to multivariate data analysis, the spectra were normalized using the probabilistic quotient method (PQN) (Dieterle *et al.*, 2006).

The data set was auto-scaled and modelled using Partial Least Squares Discriminant Analysis (PLS-DA) in a Monte Carlo Cross-Validation (MCCV) framework and repeated measures (RM) design. Storey-Tibshirani method of correction for multiple testing was used, where variables with $q \leq 0.05$ were considered as significant in the MCCV framework. Goodness of fit (R^2Y) was calculated using the training data, and the goodness of prediction (Q^2Y) from test data (Posma *et al.*, 2018). For discriminant analysis, the number of the intervention was used as dummy variable at each time point (Y = 1, 2, 3 and 4).

Relevant features from the NMR quantitative measurements (e.g. B.I. LISA^{TM}, and B.I. Quant-PS 2.0^{TM}) were also subjected to linear mixed models, correlation networks, and partial least squares regression.

Identification of metabolites for ^{1}H-NMR metabolic profiling analysis

Subset Optimization by Reference Matching (STORM) was used for the identification of significant metabolites using the correlation structure of 1D ^{1}H-NMR data (Posma *et al.*, 2012). *J*-Res spectra were also used for identification purposes. Internal and external databases such as the Human Metabolome Data Base (HMDB; http://hmdb.ca/) and/or the Biological Magnetic Resonance Data Bank (BMRB; http://www.bmrb.wisc.edu) were used for confirmation of assignments.

Correlation network analysis

Correlation networks were created for outcomes measured at T = 0, 30, and 120 using the R package 'rmcorr' (Bakdash & Marusich, 2017). Only outcomes with a significant main effect of carbohydrate and/or exercise, and/or a significant interaction effect were included. Correlations with a coefficient ≥ 0.6 and P value < 0.05 (after correction with Q = 5% (Benjamini *et al.*, 2006)) were displayed graphically using the R package 'circlize' (Gu *et al.*, 2014). A cut-off of ≥ 0.6 was selected as this indicated a strong relationship between the two variables (Campbell & Swinscow, 1997), and therefore more likely to be biologically meaningful.

Partial least squares regression

Partial least squares regression was performed using the R package 'mixOmics' (Rohart *et al.*, 2017) using all outcomes measured in plasma/serum with a significant main effect of carbohydrate and/or exercise, and/or a significant interaction effect. Leave-one-out cross-validation (LOOCV) was employed to select the number of latent variables for each model. Root mean square error of prediction (RMSEP) and goodness of fit (R^2) were calculated via LOOCV using the selected number of latent variables. All variables were scaled and centred prior to analysis.

Sample size determination

A formal sample size calculation was not performed as the interactive effect of carbohydrate and exercise has not been previously investigated. Prior studies have however demonstrated that 12 participants are sufficient to show that carbohydrate ingestion (Steinert *et al.*, 2011) and exercise (Ueda *et al.*, 2009) influence gastrointestinal hormone release. Furthermore, 12 participants have been argued to be sufficient with respect to precision about the mean and variance when no prior information is available (Julious, 2005). The study was stopped once 12 participants had completed all study visits.

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AUTHOR CONTRIBUTIONS

E.S.C and K.G.M conceived and designed the study. J.F, J.P, and A.S.Y.T conducted the study. J.F, J.I.S.C, I.G.P, G.F.B, J.P, A.S.Y.T, A.C.C.O, A.M, and E.S.C performed sample analysis. J.F. and J.I.S.C performed statistical analysis. J.F and E.S.C wrote the first draft of the manuscript, which was critically revised by J.I.S.C, I.G.P, G.F.B, J.P, A.S.Y.T, A.C.C.O, A.M, K.G.M, and G.F. All authors read and approved the final manuscript.

CONFLICTS OF INTEREST

The authors declare no conflict of interest.

AVAILABILITY OF DATA AND MATERIAL

Data and R code used for analysis are available from the Open Science Framework at: https://osf.io/9t2bp/ (DOI: 10.17605/OSF.IO/9T2BP).

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FIGURE CAPTIONS

Abstract: Our work aimed to explore hormonal and metabolite mediators of exercise-induced changes in appetite and energy intake across nutritional states. Twelve-male participants completed four study visits involving intake of water (control) or carbohydrate with a 30 min rest or high-intensity exercise session. Plasma samples were collected throughout the 120 min study periods to quantify gastrointestinal hormone release and 1H-NMR metabolite profiles. Visual analogue scales were used to investigate appetite responses and an ad libitum meal was provided at the end of each study visit to evaluate energy intake. Temporal changes in acetate, lactate and PYY were associated with

supressed appetite responses in both exercise conditions. A consistent negative association between GLP-1 and succinate levels with meal energy intake was found in both exercise conditions.

Figure 1: Study design, the effect of dietary carbohydrate and exercise on gastrointestinal hormones, appetite, ad libitum energy intake, and acute energy balance. (A) Overview of study interventions including serial blood sampling scheme, visual analogue scale assessments, and pulmonary gas measurements. B, baseline. (B) The effect of dietary carbohydrate and exercise on GLP-1, PYY, (active) ghrelin, composite appetite

score (CAS), *ad libitum* meal EI, and acute energy balance. Data are time-averaged AUC for all hormones and CAS. Individual dots represent individual participant values, with yellow dots representing intervention means. P values from robust linear mixed models for main effects of carbohydrate (Carb) and exercise (Ex), and the interaction between carbohydrate and exercise (Carb x Ex) are presented. EI, energy intake.

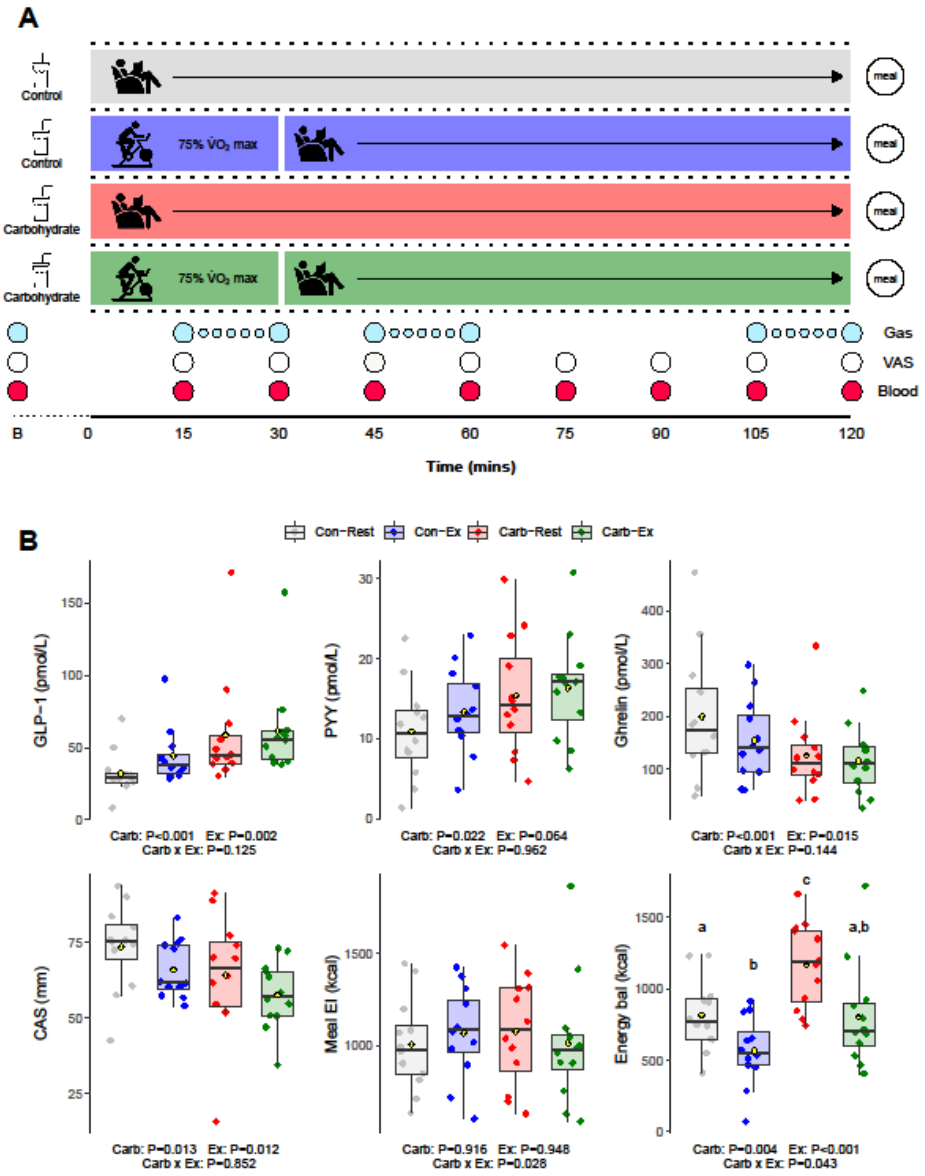


Figure 2: The effect of dietary carbohydrate and exercise on pancreatic hormones, components of acute energy balance, and substrate oxidation. Data are time-averaged AUC for insulin, glucagon, glucose, nausea, carbohydrate oxidation (carb ox), and fat oxidation (fat ox) only. Individual dots represent individual participant values, with yellow dots representing intervention means. P values from robust linear mixed models for main effects of carbohydrate (Carb) and exercise (Ex), and the interaction between carbohydrate and exercise (Carb x Ex) are presented. Interventions with different letters are significantly different from each other (P< 0.05). EE, energy expenditure. EI, energy intake.

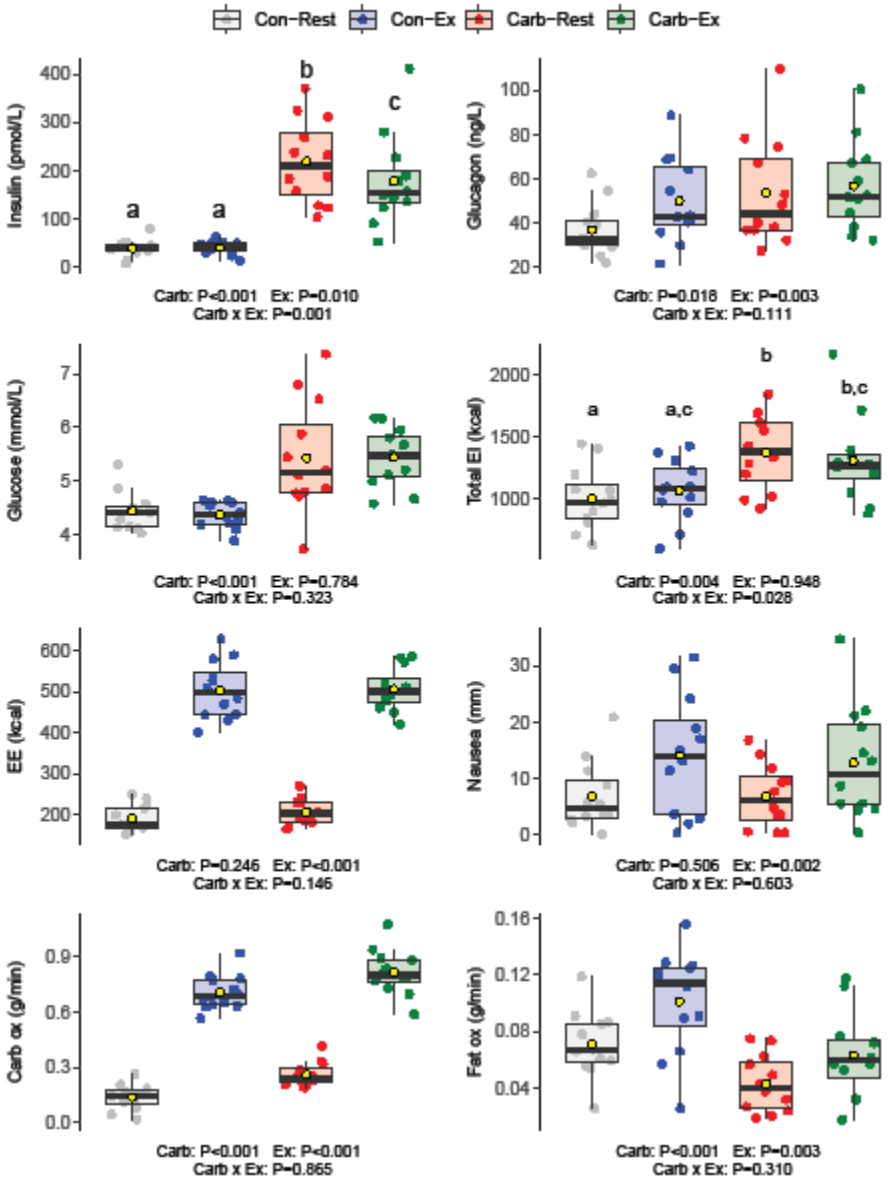


Figure 3: PLS-DA score plot and metabolite matrix. (A) Comparisons between study interventions at T = 30. (B) Comparisons between study interventions at T = 120. Lower matrix displays score plots derived from partial least-squares discriminant analysis demonstrating differentiation in metabolic profiles between study interventions (Con-Rest: grey; Con-Ex: blue; Carb-Rest: Red; Carb-Ex: green). Individual dots represent individual participant metabolic profiles. All models include kernel density estimates (KDE) of the predicted scores for both study interventions under comparison. Upper matrix displays differences in metabolites between study interventions. R^2Y, explained variance. Q^2Y, capability of prediction. NMR peak assignments for significant metabolites are provided in the online repository.

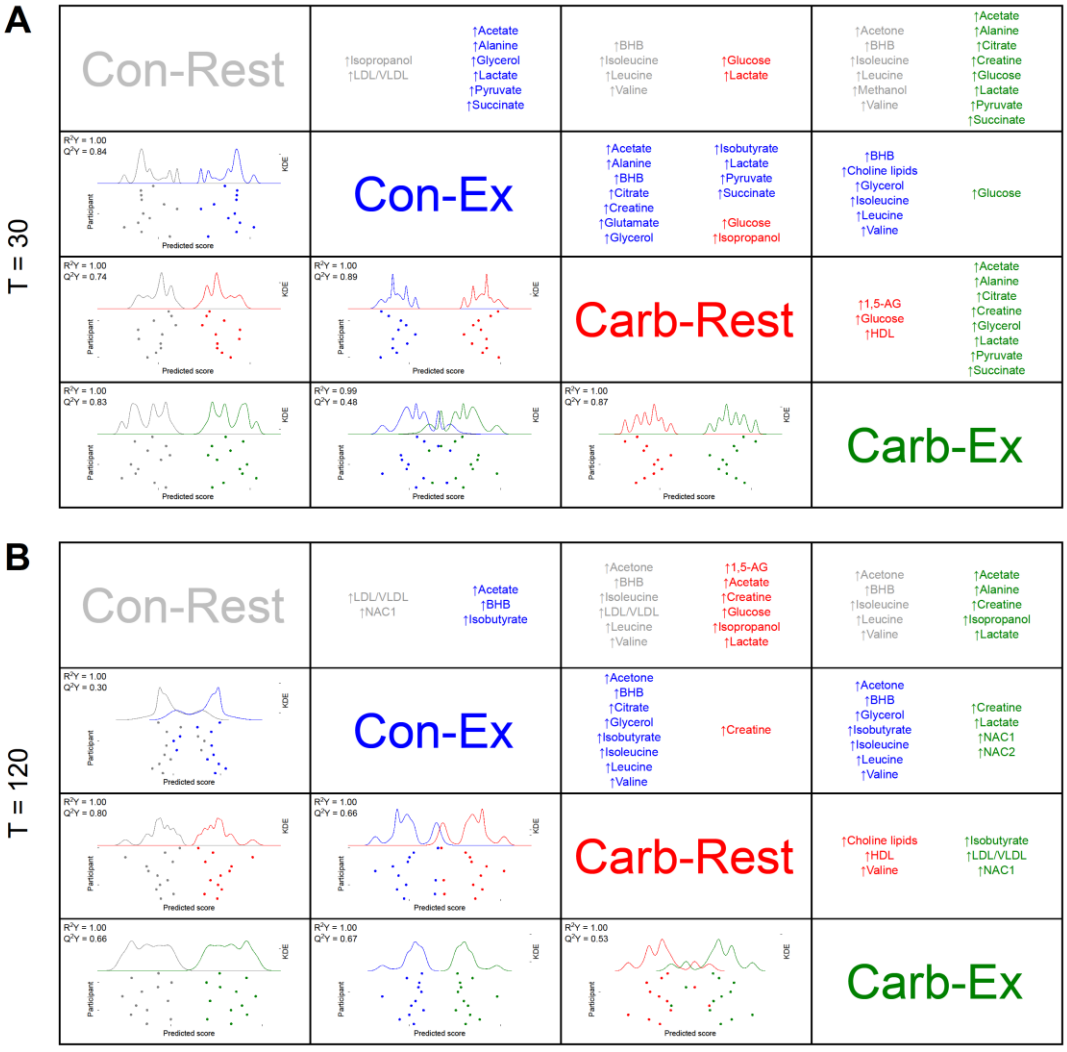


Figure 4: The effect of dietary carbohydrate and exercise on circulating small metabolites. Data are time-averaged AUC for all outcomes. Individual dots represent individual participant values, with yellow dots representing intervention means. P values from robust linear mixed models for main effects of carbohydrate (Carb) and exercise (Ex), and the interaction between carbohydrate and exercise (Carb x Ex) are presented. Interventions with different letters are significantly different from each other (P< 0.05). BHB, 3-hydroxybutyrate.

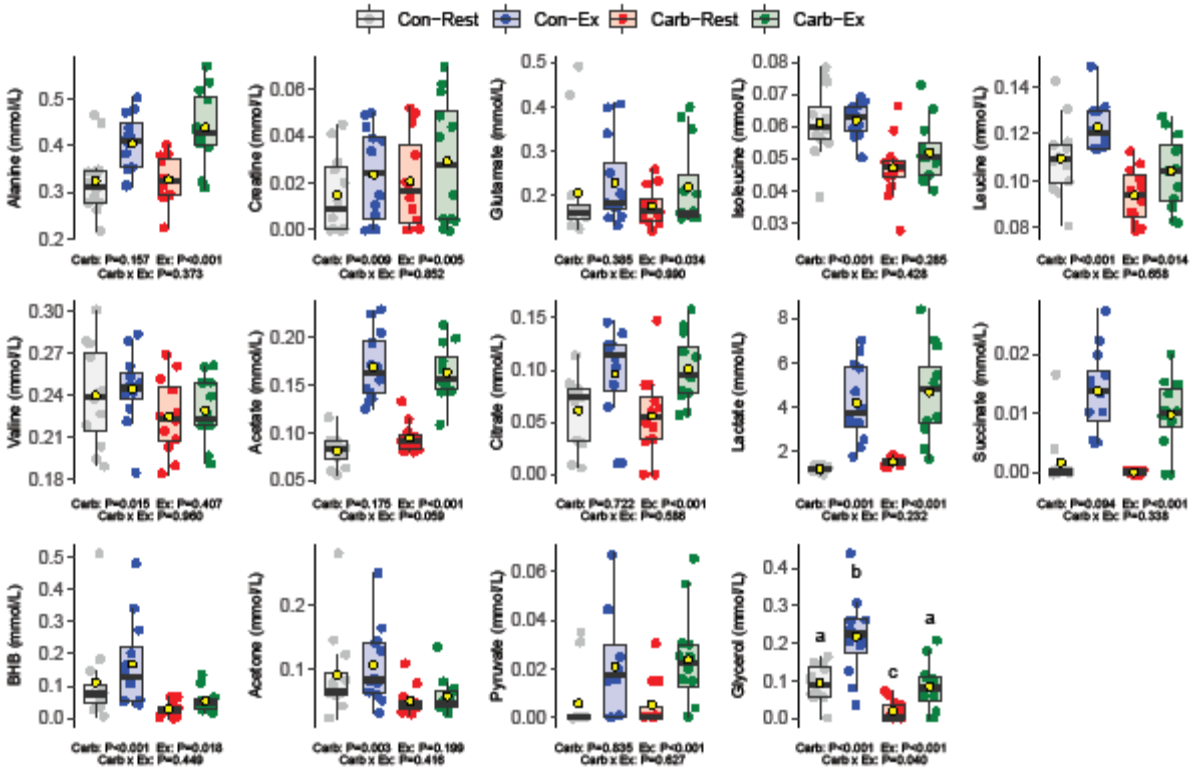


Figure 5: The effect of dietary carbohydrate and exercise on main lipoprotein fractions and parameters. Data are time-averaged AUC for all outcomes. Individual dots represent individual participant values, with yellow dots representing intervention means. P values from robust linear mixed models for main effects of carbohydrate (Carb) and exercise (Ex), and the interaction between carbohydrate and exercise (Carb x Ex) are presented. TC, total cholesterol. TG, total triglycerides. Total-P, total particle number.

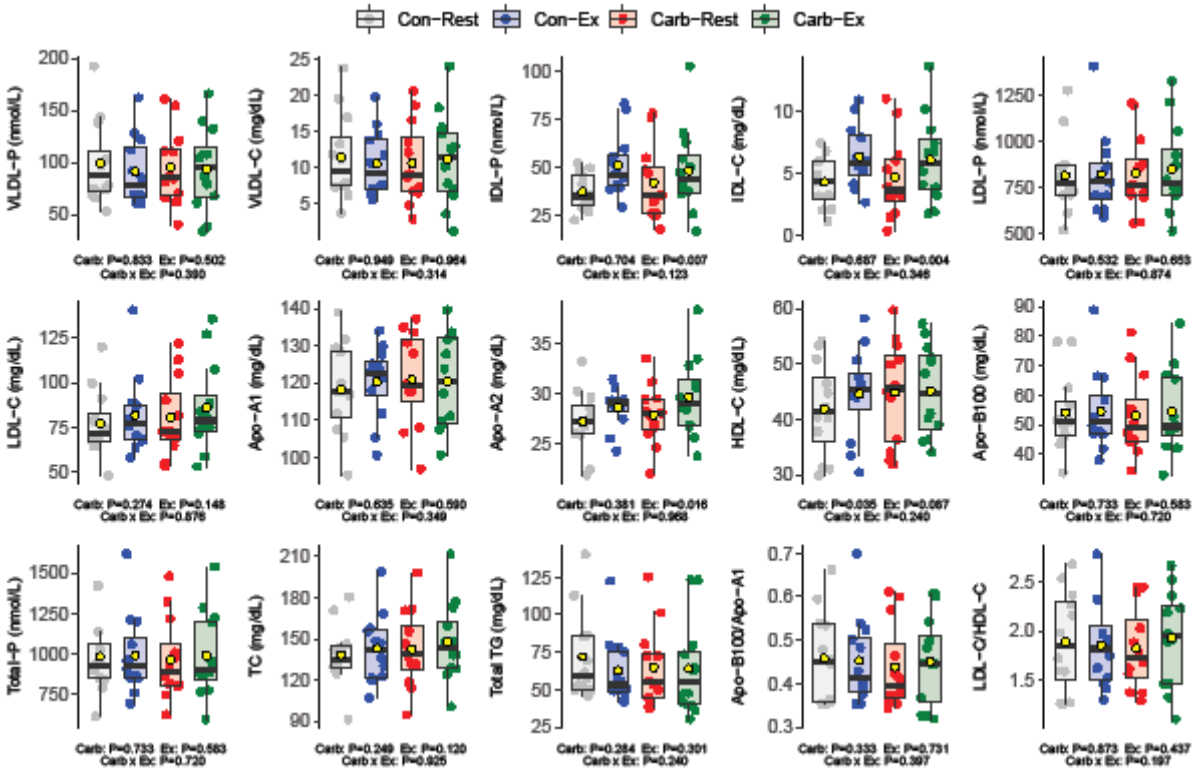


Figure 6: Correlation networks of temporal measurements across study interventions. Repeated measures correlation networks for (A) Con-Rest, Con-Ex, Carb-Rest, and Carb-Ex. (B) The top 10 outcomes with the highest number of connections for each study intervention. Outcomes linked by a green line showed a significant positive correlation, outcomes linked by a red line showed a significant negative correlation. Only outcomes measured at T = 0, 30, and 120 with a significant main effect of carbohydrate and/or exercise, and/or a significant interaction effect were included in the analyses. Correlations with a coefficient ≥0.6 and adjusted P value <0.05 are displayed. r_{rm}, repeated measures correlation coefficient.

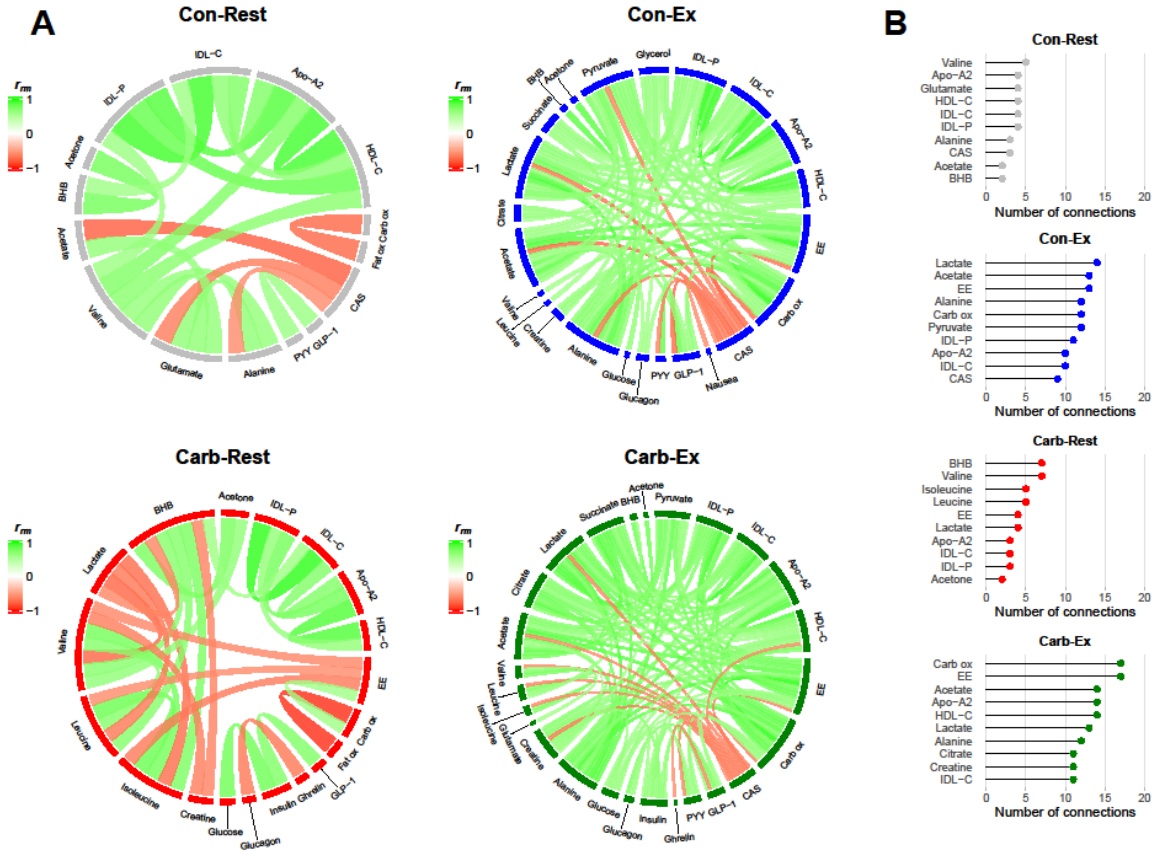


Figure 7: Prediction of *ad libitum* meal energy intake from preceding metabolic environment. (A) Variable importance in projection scores from partial least squares regression models for each study intervention. Only outcomes measured with a significant main effect of carbohydrate and/or exercise, and/or a significant interaction effect were included in the analyses. (B) Simple univariate linear regression plots for outcomes with a VIP score >1. Root mean square error of prediction (RMSEP) and explained variance (R^2) calculated via leave-one-out cross-validation (CV). EI, energy intake.

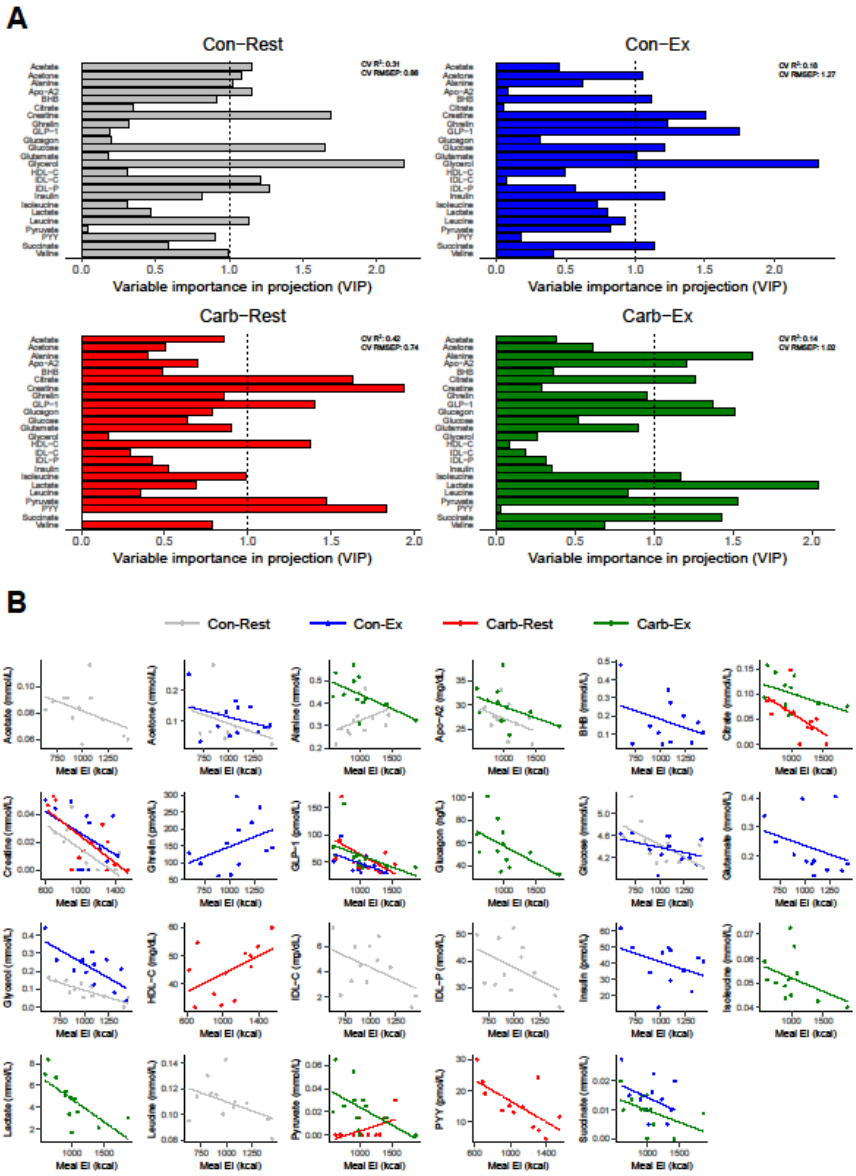
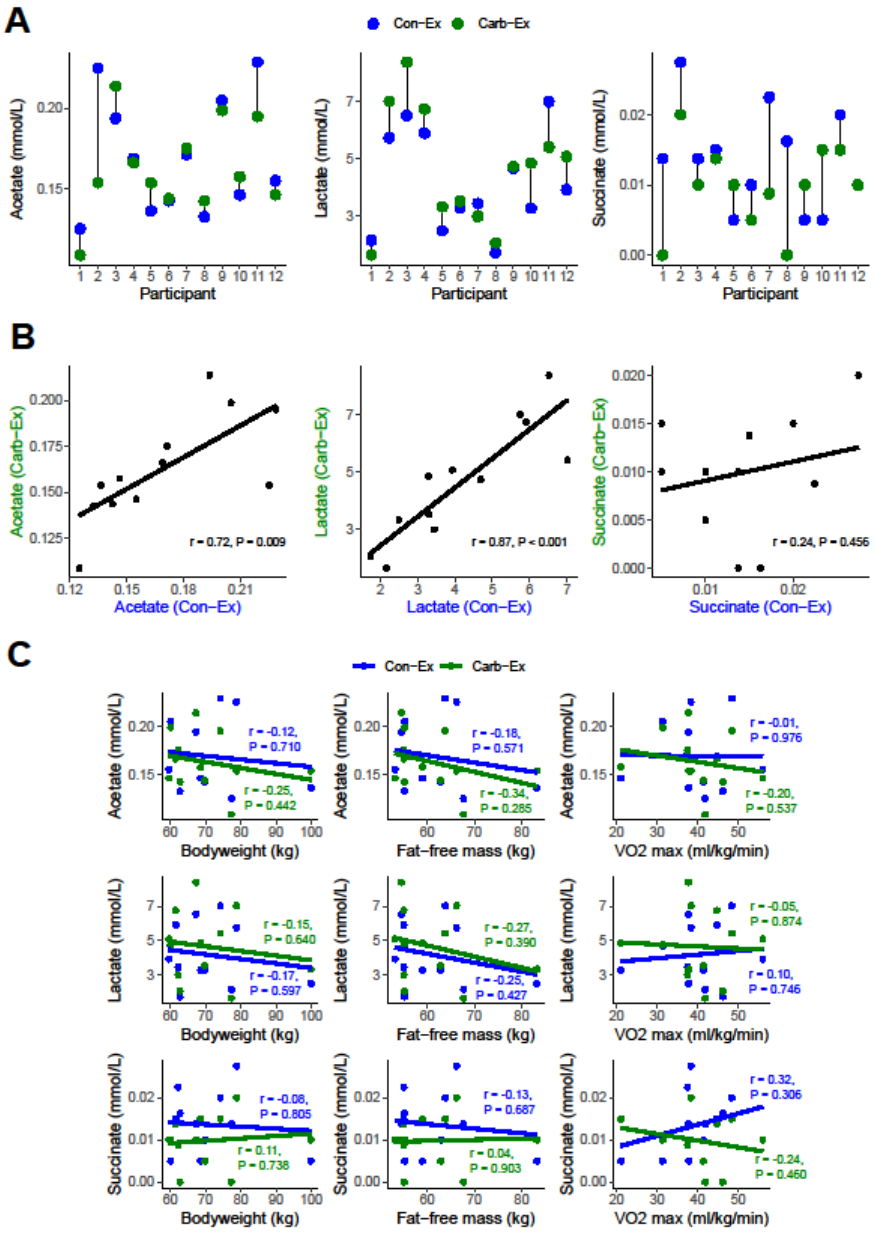


Figure 8: Inter-individual variation in acetate, lactate, and succinate responses during exercise interventions and their relationship with anthropometric and physiological characteristics. (A) Time-averaged AUC data from each participant for both exercise interventions. Length of black line represents difference between exercise interventions. (B) Correlation between Con-Ex and Carb-Ex time-averaged AUC data. (C) Correlation between acetate, lactate, succinate and anthropometric (bodyweight, fat-free mass) and physiological characteristics ($\dot{V}O_2$ max). r, Pearson's correlation coefficient.



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