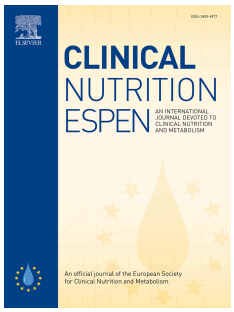


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# High dietary nucleotide consumption for one week increases circulating uric acid concentrations but does not compromise metabolic health: a randomised controlled trial

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**Abstract**

**Background & Aims:** Elevated circulating uric acid concentrations have been linked to various cardio-metabolic diseases. Bolus consumption of a nucleotide-rich dietary protein source increases postprandial serum uric acid concentrations. We assessed the impact of twice-daily nucleotide-rich mixed-meal consumption for one week on postabsorptive serum uric acid concentrations, insulin sensitivity (IS), glycaemic control and the plasma lipidome. **Methods:** Twenty healthy adults participated in a randomised, controlled, parallel-group trial in which they consumed a 7 d fully-controlled eucaloric diet where lunch and dinner contained either nucleotide-depleted (LOW) or high-nucleotide (HIGH) mycoprotein. Postabsorptive blood samples were obtained pre, throughout and post-intervention, and oral glucose tolerance tests were performed pre- and post- intervention. Daily waking urine samples and 24h continuous blood glucose measurements were collected throughout. **Results:** Postabsorptive serum uric acid concentrations remained unchanged in LOW but increased throughout the intervention week in HIGH (from 295±17 to 472±29 $\mu mol \cdot L^{-1}$ by day 6; $P<0.05$). Urinary uric acid did not change throughout the intervention in either group. The intervention did not affect indices of IS, 24h glycaemic control, nor had a meaningful impact on the plasma lipidome. **Conclusions:** One week of twice-daily consumption of nucleotide-rich mixed-meals increases postabsorptive serum uric acid concentrations above clinically acceptable thresholds but these changes are not associated with deleterious effects on IS, daily glycaemic control or plasma lipid composition.

**Clinical trial registry:** NCT02984358 (https://clinicaltrials.gov/ct2/show/NCT02984358)

**Keywords:** Nucleotides, nucleic acids, serum uric acid, metabolic health, mycoprotein

**Abbreviations**

CGMS, continuous glucose monitoring system; CHO, carbohydrate; iAUC, incremental area under the curve; HIGH, high-nucleotide group; LOW, low-nucleotide group; OGTT, oral glucose tolerance test; NRM, nuclear magnetic resonance; RM, repeated measures; RMR, resting metabolic rate; SEM, standard error of the mean

## Introduction

Nucleotides are organic molecules comprising a nitrogenous base (purine or pyrimidine), a five-carbon sugar and one or more phosphate groups, which are widely involved in human metabolism (e.g. as the constituent molecules of nucleic acids) (1). Nucleotides can be obtained endogenously by *de novo* synthesis or from salvage pathways (~10 and ~90% of total endogenous production, respectively), or exogenously, via dietary intake. The dietary intake of nucleotides (primarily in the form of RNA and DNA) can vary widely between individuals but is typically <1 g/day (2, 3). Evidence suggests that nucleotides are conditionally-essential nutrients, with an increased dietary requirement during periods of rapid growth (e.g. most infant feed formulae are enriched with nucleotides; (4, 5)), in certain disease states due to the requirement to support repair of rapidly turning-over cell types (e.g. those contained within the gastrointestinal and immune systems) (6, 7), as well as in recovery from extensive trauma and in intensive care, such as in burns patients (8). Additionally, emerging data imply beneficial effects of nucleotide supplementation on exercise performance and recovery (9).

Despite this conditional requirement for dietary nucleotides, concerns have been raised regarding high intakes in humans (10). Dietary nucleotides are digested and absorbed as pyrimidines and purines (1), with a major metabolic end-product of purine metabolism being uric acid. Epidemiological and observational studies have reported that serum uric acid concentrations positively correlate with the development of gout (11), hypertension (12) and metabolic syndrome (13), and is a predictor of type 2 diabetes (14), though causal mechanistic links remain to be established. Although uric acid homeostasis is tightly regulated at the level of hepatic (formation) and renal (clearance) function (15), short term ingestion of high-dose (> 2 g/day) isolated (or yeast-derived) nucleotides results in an acute elevation of circulating uric acid concentrations above clinically acceptable levels (i.e., >420 $\mu mol \cdot L^{-1}$ in men and >360 $\mu mol \cdot L^{-1}$ in pre-menopausal women (16-19). Despite an absence of data translating these

findings to more nutritionally relevant conditions, the FAO/WHO/UNICEF Protein Advisory Group recommended the limit of additional dietary nucleic acid load from single-cell protein-rich novel foods to be set at 2 g/day (20). This has implications for potential benefits of increasing/supplementing with dietary nucleotides, as well as providing industrial challenges for the production of novel and sustainable, nucleotide-containing dietary protein sources (21). Mycoprotein, a sustainable fungal-derived dietary protein source (22), is naturally rich in RNA-derived nucleotides (~ 10 g per 100 g dry weight), but heat-treated during production to reduce RNA content (to under 2 g per 100 g dry weight) (23) for commercial products to comply with FAO/WHO/UNICEF recommendations (20, 21). We recently reported that the ingestion of a nucleotide rich (1.7 g) mycoprotein-containing mixed-meal transiently and modestly increased (by 12%) postprandial serum uric acid concentrations in healthy adults for 12 hours compared with ingesting nucleotide-depleted mycoprotein (24). However, it is not clear whether repeatedly ingesting such nucleotide-rich meals would have a chronic and cumulative effect of elevating circulating uric acid concentrations above clinically acceptable levels, or whether urinary clearance would also adaptively increase. Moreover, a detailed investigation of the relationship between circulating uric acid levels and markers of metabolic health (and therefore the potential relevance to disease progression) is absent.

In the present study, we applied a one-week fully controlled dietary intervention in healthy young adults where the major source of dietary protein at lunch and dinner was either nucleotide-depleted or nucleotide rich mycoprotein, with energy and macronutrient contents of the diets matched. We hypothesised that one week of high-nucleotide mycoprotein consumption would increase postabsorptive serum uric acid concentrations and impair markers of metabolic health (i.e. oral glucose tolerance, indices of insulin sensitivity, glycaemic control and the plasma metabolome).

## Subjects and methods

*Participants and medical screening*

Twenty healthy, recreationally active, young adults (age: 24±1 y; BMI: 24±1 kg/m$^2$; male = 8 and female = 12) participated in the present study. Subjects' characteristics are presented in **Table 1**. Prior to participating, each subject attended a screening visit to ensure eligibility. Blood pressure, body mass, height and body composition (determined by air displacement plethysmography; Bodpod; Life Measurement, Inc., Concord, CA, USA) were measured at screening. The participants also completed a general health questionnaire and the International Physical Activity Questionnaire (IPAQ) (25). Vegetarians, vegans, smokers, and participants taking regular medication or suffering from chronic diseases were excluded, to maintain a relatively homogenous participant cohort and/or to avoid any alterations in metabolic health status. Participants included were recreationally active (partook in regular exercise or sport at a non-competitive level, two to five days a week), normotensive (≤140/90 mmHg), and had a BMI between 18.5 and 30 kg/m$^2$, in order to minimise the likelihood of existing metabolic dysfunction. All participants were informed of the study's purposes, procedures and risks, and provided written informed consent. This study is part of a wider project investigating the effects of mycoprotein consumption on metabolic health, part of which has been published previously (26). The study was conducted at the Nutritional Physiology Research Unit, Department of Sport and Health Sciences, St. Luke's Campus, University of Exeter, between January and December of 2017, and was approved by the University of Exeter's Sport and Health Sciences Ethics Committee (Ref No: 161026/B/07) in accordance with the Declaration of Helsinki. This project was registered at ClinicalTrials.gov (NCT02984358).

*Experimental Protocol*

The present study was a randomised, controlled, parallel design trial, with participants being randomly allocated into one of two dietary interventions which differed with respect to the inclusion of either a nucleotide-depleted (as is available commercially) mycoprotein (LOW; *n*=10) or a specifically produced experimental high nucleotide, mycoprotein (HIGH; *n*=10 (24)) as the main protein source at lunch and dinner. Participants were allocated sequential numbers at the time of screening which were then used as the only identifiable characteristic for all documents containing participant information, and were randomised into groups using an online randomiser (http://www.randomization.com/), with stratification by sex. **Figure 1** shows an overview of the study design. All subjects underwent a period of habitual data collection as well as data collection during their allotted intervention.

*Habitual data collection*

Habitual data collection took place either during the 2 weeks before (LOW; *n*=7, HIGH; *n*=7) or between 2 and 4 weeks following (LOW; *n*=3, HIGH; *n*=3) the experimental period. Subjects were asked to complete a 3-day food diary to assess their habitual dietary intake, following consultation with a qualified nutritionist concerning how to complete this in as much detail as possible. All food and drink consumed were recorded for three consecutive days, including two weekdays and one weekend day. The diaries were analysed for energy and macronutrient content using Nutritics (Nutritics Professional Nutritional Analysis Software, Swords, Dublin, Ireland). Participants wore a GENEActiv Original accelerometer (ActivInsights, Kimbolton, UK), a wrist-worn device to measure daily physical activity by intensity, on their non-dominant wrist, for 5 consecutive days (including both week and weekend days). Physical activity data from the GENEActiv monitors were processed using GENEActiv excel macros. The 5 days of habitual physical activity data were compiled into an individual average for each participant and the same was done for the 7 days of the intervention.

Glucose sensors were placed subcutaneously at the side of the abdomen and connected to a continuous glucose monitoring system (CGMS; Dexcom G4 Platinum, San Diego, California, USA) to measure interstitial glucose concentrations (calibrated to blood glucose concentrations measured via finger prick 4 times per day) every 5 minutes for the same 5 days as those where accelerometry data were collected. During all habitual data collections, participants were instructed not to change their normal routines.

*Experimental test days*

Participants reported to the laboratory at ~08.00 h on day 0 (prior to starting the dietary intervention) and on day 8 (the morning following the intervention) after an overnight fast and refraining from intense exercise and alcohol consumption for at least 24 h, to undertake two identical experimental test days. A cannula was placed retrogradely in a dorsal hand vein and the hand was then placed in a heated box (55ºC) for arterialised-venous blood sampling before a fasted arterialised-venous blood sample was collected (27). Fasted measurements of oxygen consumption ($\dot{V}O_2$) and carbon dioxide production ($\dot{V}CO_2$) were collected using a facemask and the Metamax 3B (MM3B) portable indirect calorimetry system (Cortex, Leipzig, Germany) for 30 minutes. Carbohydrate and fat oxidation rates, as well as resting metabolic rates (RMR), were calculated using the Frayn equations (28). Subsequently, an oral glucose tolerance test (OGTT) was performed. Briefly, participants ingested 75 g glucose (dextrose, BulkPowders, Colchester, United Kingdom) dissolved in 350 mL water in 5 minutes or less (with the exact time being recorded for each participant in the first visit and replicated on the last test day). Arterialised-venous blood samples were then collected for 2 h, at 15 min intervals, for the measurement of glucose and insulin concentrations and the subsequent calculation of glucose tolerance and IS. Indirect calorimetry was performed throughout the OGTT period, with the exception of the first 15 min following glucose ingestion.

*Dietary intervention*

Basal metabolic rate (BMR) was estimated using the Henry equations based on age, gender, and weight (29). Though all indirect estimates of BMR have inherent limitations, the Henry (Oxford) equations have been reported to be the most accurate and rigorously tested in modern healthy populations (Ramirez-Zea, 2005; SACN, 2011) and are recognised by the British Dietetic Association. The IPAQ was used to calculate a physical activity level (PAL) factor (30). Individual energy requirements were then calculated by multiplying the participant's BMR and PAL. Thereafter, an individual 7-day meal plan was designed for each participant with all food prepared, weighed and packaged in-house in the Nutritional Physiology Unit's research kitchen facility. Nutritional information for the two diets is provided in **Table 2**. Subjects consumed a diet clamped at 1.2 g of protein per kg of body weight per day, with 30% of their energy being provided by fat and the remainder from carbohydrates (~50–55%; variation due to different energy requirements and the clamping of protein intake). The foods and meals consumed were identical between the two groups, except for the type of mycoprotein included, in the form of (also identical) Quorn Foods products (Quorn chicken pieces, Quorn mince, Quorn fillets and Quorn roast chicken slices). A document and diary detailing the plan were provided to the subjects to track compliance with the dietary intervention, log meal times and provide recipe instructions. Thereafter, participants were required to visit the laboratory at ~08.00 h in the overnight fasted state on days 2, 4 and 6, where a venous blood sample was collected via venepuncture, body mass was measured wearing light clothing (seca 703 column scale, seca GmbH & Co. KG, Hamburg, Germany), and the next two days of food were provided. In these interim visits, the researchers discussed with the participants any questions or issues that may have arisen and, in the event of any substantial weight change (>0.5 kg, with the same upward or downward trend on two consecutive visits), the energy content of the next

two days was adjusted (altering CHO-rich foods only). The GENEActiv accelerometer was worn for the duration of the one-week intervention and on day 2 a glucose sensor was placed and the CGMS device connected to collect continuous glucose data for the last 5 days of the intervention. Additionally, participants collected urine samples every morning for the duration of the intervention (9 samples; from day 0 to day 8). Small sterile screw-top containers were used to collect a mid-stream sample of the first urination of the day, which the participants kept sealed inside a plastic bag, refrigerated, until the next time they visited the laboratory (either on the same day or the day after). Following the one week intervention (i.e. day 8), participants were required to repeat the experimental test day where a further OGTT was performed as described above.

*Mycoprotein nucleotide content and composition analyses*

Nucleotide content and composition analysis were performed using a Shimadzu Nexera X2 UHPLC-PDA system (Shimadzu Scientific Instruments, Columbia, Maryland, USA) on a Supelcosil LC-18-T column, 15 cm x 4.6 mm ID (3 µM particles) at 24ºC and an injection volume of 10 µL. The mobile phase was composed of 0.03 M potassium dihydrogen phosphate and 10 mM tetrabutylammonium hydrogen sulphate, adjusted to pH 7.5 with sodium hydroxide (mobile phase A) and 0.03 M potassium dihydrogen phosphate and 10 mM tetrabutylammonium hydrogen sulphate, pH 7.5 containing 20% (v/v) methanol (mobile phase B). Nucleotides were separated using a gradient elution method; the mobile phase started with 40% of mobile phase B for 2.5 min, the composition of mobile phase B in the mobile phase was gradually increased to 100% in 32.5 min and remained at this composition for 2 min. The mobile phase B composition was dropped to 40% in 0.5 min and remained at 40% for a further 2.5 min. The flow rate was 0.7 mL/min, and the nucleotides were detected by UV at a wavelength of 254 nm with a total run time of 40 min.

*Plasma and serum collection and analyses*

One mL of each blood sample was collected into FX blood collection tubes (Becton Dickinson, Franklin Lakes, New Jersey, USA) containing powdered sodium fluoride and potassium oxalate, and glucose was immediately analysed using the YSI 2300 STAT PLUS Biochemistry Analyser (YSI, Yellow Springs, Ohio, USA). Four mL of blood were collected into LH (lithium heparin) plasma tubes (Becton Dickinson) and immediately centrifuged. The remaining 4 mL of each blood sample were collected into SST tubes (containing spray-coated silica and a polymer gel for serum separation; Becton Dickinson) and left at room temperature for at least 30 min. All tubes were centrifuged at 4ºC and 4000 RPM, and aliquoted (one aliquot designated for each of the below analyses) plasma and serum were stored at -80ºC.

One aliquot of each serum sample was transported to the Clinical Chemistry department of the Royal Devon & Exeter NHS Foundation Trust and analysed for uric acid concentrations using the Roche Cobas 702 module of the Cobas 8000 analyser (Roche, Basel, Switzerland) and Roche Uric Acid Kits (Cobas; UA2). Insulin concentrations were analysed in serum samples using DRG ELISA kits (DRG International, Springfield, New Jersey, USA). Plasma samples were sent to the MRC Integrative Epidemiology Unit at the University of Bristol for metabonomics analysis by nuclear magnetic resonance (NMR) spectroscopy. NMR spectroscopy and mass spectrometry (MS) are the key technologies in the metabolomics field, however, MS cannot analyse lipoproteins, making NMR currently the only high-throughput methodology capable of quantifying these metabolites cost-effectively (31). Biomarker concentrations quantified by this NMR approach have been shown to be highly consistent with concentrations obtained from standardised clinical chemistry analyses (32). For a detailed description of the experimental protocol, including sample preparation and NMR spectroscopy please see previously published work (31-33). The data were processed using the Nightingale

Health's NMR-based blood biomarker analysis platform, which provides 224 quantified metabolomic measures per sample (142 primary concentrations plus 82 selected ratios and molecule diameters), including the lipid concentrations and composition of 14 lipoprotein subclasses, fatty acids, amino acids, glycolysis-related measures and ketone bodies.

*Insulin sensitivity*

Five different insulin sensitivity indices (34-38), all validated against the hyperinsulinaemic-euglycaemic clamp technique, were calculated pre- and post- intervention using the blood glucose and serum insulin concentrations measured in the fasting state and during the OGTTs. The homeostatic model assessment (HOMA-IR) is calculated solely from fasting concentrations of glucose and insulin and has been shown to provide a reasonable estimate of hepatic insulin sensitivity (34). The Matsuda index uses OGTT glucose and insulin concentrations, as well as their corresponding fasting values, and represents a combined estimate of both hepatic and peripheral tissue insulin sensitivity (36). The Cederholm, OGIS and Gutt indices mainly focus on peripheral insulin sensitivity and muscular glucose uptake by measuring OGTT glucose clearance (35, 37, 38).

*Continuous glucose monitoring system (CGMS)*

The Dexcom G4 Platinum CGMS sensor was placed in the participants' abdominal subcutaneous fat, using a dedicated applicator. A transmitter was then attached to the sensor and glucose data, collected every 5 minutes, were automatically sent to a receiver. The participants were instructed to carry the receiver at all times and to calibrate the monitor 4 times a day at regular intervals (in the morning and before main meals) by pricking their fingers with disposable lancets and using Contour Next blood glucose meters (Bayer, Leverkusen, Germany). Data from the days when the sensor was inserted and removed were excluded (i.e.

days 2 and 8) to ensure the final dataset comprised of stable measurements only. Days with data for fewer than 70% of the total time-points were also excluded on the principle that these would not be representative. The remaining data were analysed for glycaemic control (24 h average glucose, glucose area under the curve (AUC) and two-hour postprandial glucose concentrations) and glycaemic variability (SD, CONGA1 and CONGA2). To calculate the CONGA1 and CONGA2 indices, the SD of the differences between each glucose concentration reading and the reading obtained 1 (CONGA1) or 2 (CONGA2) hours prior was determined (39).

*Statistical analyses*

A sample size calculation was performed, based on the assumption of a 30% increase in serum uric acid concentrations with repeated high-nucleotide mycoprotein consumption for a week (calculated from an extrapolation of the results from a previous study conducted in our laboratory (40)) and determined that 9 participants were needed in each group to provide a power of 80% and a CI of 95%. Ten participants per group were recruited to account for a potential 10% dropout rate (of which there were none). Recruitment and testing were ended once the trial was fully recruited according to this *a priori* power calculation.

All data are expressed as means ± standard errors (SEM). Participant baseline characteristics, dietary intake and physical activity data were analysed using multiple unpaired *t*-tests. The two groups were compared, for most parameters, using a two-way ANOVA with repeated measures [RM] (with condition and time [RM] as factors). Bonferroni *post hoc* tests were performed in the event of significant main effects to locate individual differences. Blood glucose and serum insulin concentrations during the pre- and post- intervention OGTTs were analysed with three-way ANOVAs (condition, time and test day as factors). Additionally, for the aforementioned parameters, incremental Area Under the Curves (iAUC) were calculated and a one-way

ANOVA was performed to detect any significant effect of treatment. Carbohydrate and fat oxidation data were averaged as fasting and fed responses and analysed with three-way ANOVAs (condition, fasted or fed state, and test day as factors).

For the NMR metabonomics measures, a % Δ change from pre- to post- intervention was calculated for each of the 224 metabolites for each participant. The measures were divided into three groups (concentrations, ratios and dimensions) and analysed using multiple *t*-tests for the dimension measures ($n$ = 3) and using Significance Analysis of Microarrays (SAM) for the concentration and ratio measures due to the large number of tests ($n$ = 142 and n = 79, respectively) and stringency of conventional post-hoc corrections. A heat-map was designed for the significant metabolites and these were organised into clusters. Missing data were handled using imputation in a linear interpolation manner. Statistical significance was set at $P<0.05$. For the SAM analysis, the delta (tuning parameter which determined the False Discovery Rate (FDR) threshold) was set at 0.7 for the analysis of metabolomics ratios, resulting in an FDR of 0 and at 0.8 for metabolite concentrations, resulting in an FDR of 0.125. An FDR of 0.1 was set for metabolite dimensions analysis. NMR metabolomics analyses were carried out in MetaboAnalyst 4.0 (Wishart Research Group, University of Alberta, Edmonton, Alberta, Canada). All other calculations were performed using GraphPad Prism version 7.0 (GraphPad Software, San Diego, California, USA).

## Results

*Nutritional intervention*

Body mass was not different between habitual testing and at the outset of the intervention in either group (from 69±6 to 70±6 kg in LOW and from 72±4 to 72±3 kg in HIGH; $P>0.05$), nor did body mass change during the intervention in either group (70±6 and 71±3 kg post-intervention in LOW and HIGH, respectively; $P>0.05$) indicating participants remained in energy balance throughout the entirety of the study period in both groups.

The nutritional content of the prescribed diets, the actual food consumed during the intervention according to food logs, and participants' habitual diets are presented in **Table 2**. Prescribed diets and actual food consumed did not differ in any parameter, and so all other comparisons were made using the habitual and actual diets only (i.e. omitting the prescribed diet data). There were no significant differences in the energy and fat intakes between the groups' habitual diets (both $P>0.05$), nor did these parameters change between habitual intake and during the intervention in either group (all $P>0.05$). Additionally, there were no significant differences in the carbohydrate and protein intakes between the groups' habitual diets nor between the groups' intervention diets (all $P>0.05$), but there was a reduction in protein intake and an increase in carbohydrate intake from habitual to intervention diets, which were equivalent in both groups (time effects; $P<0.05$, intervention effects; $P>0.05$). Habitual fibre intake was not different between groups ($P>0.05$), but increased equivalently (interaction effect; $P>0.05$) in both groups from the habitual diet to the intervention (time effect; $P<0.05$) by 31 and 27 % in the LOW and HIGH groups, respectively. Quorn products provided 38±2 and 41±1 % of total dietary protein intake in LOW and HIGH, respectively, which did not differ between groups ($P>0.05$). The LOW and the HIGH groups consumed comparable amounts (215±16 and 239±15 g, respectively) of Quorn products daily, corresponding to

181±13 and 202±13 g wet weight (45±3 and 51±3 g dry weight) of mycoprotein per day, respectively (all $P>0.05$).

The nucleotide content of LOW and HIGH mycoprotein and the consequent daily mycoprotein-derived dietary nucleotide intake in both groups are shown in **Table 3**. The daily nucleotide load of the mycoprotein consumed in the intervention diet was ~5 fold greater in the HIGH compared with LOW diet (4.46±0.29 and 0.89±0.06 g, respectively; $P<0.0001$). This translated into an average per meal mycoprotein nucleotide load of 0.29±0.03 g and 1.51±0.10 g at lunch and 0.59±0.04 g and 2.95±0.23 g at dinner, in LOW and HIGH, respectively ($P<0.0001$). The remaining foods present in the diets, such as dairy products, cereals and vegetables, were all low in purine nucleotides (<50 mg/100 g) (41, 42). Based on reference values, these foods amounted to an estimated additional purine load of 137±7 mg in LOW and 127±5 mg in HIGH ($P>0.05$ across groups), which was therefore negligible as a proportion of daily intake compared with that contained within the mycoprotein. Nucleotide content data for some of the products consumed in the diets were not available, but we estimated this as precisely as possible based on existing data (41-43) and ensured there were no differences in the diets between groups.

*Physical activity*

Physical activity data are shown in **Table 4**. Habitual physical activity was not different between LOW and HIGH groups when expressed as average daily total activity time, light activity, moderate activity, vigorous activity, or sedentary time (all $P>0.05$). None of the physical activity parameters changed during the intervention when compared with habitual levels in either group (all $P>0.05$).

*Serum and urine uric acid concentrations*

**Figure 2** shows serum (A) and urine (B) uric acid concentrations throughout the intervention. There were no differences in fasting serum uric acid concentrations between groups at baseline (260±13 μmol·L$^{-1}$ in LOW and 295±17 μmol·L$^{-1}$ in HIGH; $P$>0.05). Serum uric acid concentrations remained constant in LOW but increased above baseline in HIGH (time, condition and time x condition interaction effects; all $P$<0.0001) on days 2 (402±19 μmol·L$^{-1}$; $P$<0.05), 4 (455±25 μmol·L$^{-1}$; $P$<0.05) and 6 (472±29 μmol·L$^{-1}$; $P$<0.05), before decreasing (when compared with day 6, but remaining elevated compared with baseline; both $P$<0.05) on day 8 (409±23 μmol·L$^{-1}$). Urine uric acid concentrations were not different between groups at baseline and did not change throughout the intervention in either group (all main effects; $P$>0.05).

*Insulin sensitivity*

Fasting blood glucose and serum insulin concentrations did not differ between groups at baseline (both $P$>0.05), and fasting serum insulin concentrations did not change throughout the intervention in either group (from 12.3±2.4 to 12.7±1.7 and from 14.0±2.1 to 11.7±1.1 mU·L$^{-1}$ in LOW and HIGH, respectively; $P$>0.05). Fasting blood glucose concentrations decreased throughout the intervention (time effect, $P$<0.05), equivalently so in both groups (interaction effect; $P$>0.05) (4.6±0.1, 4.4±0.1, 4.3±0.1, 4.4±0.1 and 4.5±0.1 mmol·L$^{-1}$ in LOW and 4.5±0.1, 4.2±0.1, 4.1±0.1, 4.2±0.1 and 4.4±0.1 mmol·L$^{-1}$ in HIGH, for days 0, 2, 4, 6 and 8 of the intervention, respectively). Despite this, baseline IS reflected by the HOMA-IR was not different between groups (2.5±0.5 and 2.8±0.5 in LOW and HIGH, respectively; $P$>0.05) and did not change during the intervention in either group ($P$>0.05; **Figure 4A**). Blood glucose and serum insulin concentrations during the two OGTTs performed pre- and post- intervention in the LOW and HIGH groups are shown in **Figure 3**. Both parameters increased with CHO ingestion ($P$<0.0001) and peaked between 30 and 45 minutes of the OGTT, at around 8 mmol·L$^{-}$

$^{1}$ and 100 mU·L$^{-1}$ for blood glucose and serum insulin concentrations, respectively, with no differences detected over time or between groups (all $P$>0.05). Blood glucose iAUC and serum insulin iAUC during the OGTT (displayed in Figure 3C,F) also did not differ between groups, or over time (both $P$>0.05). Consequently, there were no differences between groups at baseline or over the intervention for any of the OGTT derived calculations of IS ($P$>0.05 for Cederholm, Matsuda, Gutt and OGIS) (**Figure 4)**.

*Continuous glucose monitoring system (CGMS)*

Average daily blood glucose values derived from interstitial continuous glucose monitoring were aggregated for the habitual data collection period (5.4±0.1 mmol·L$^{-1}$ in LOW and 5.5±0.1 mmol·L$^{-1}$ in HIGH) and each of the intervention days, in the two groups (5.7±0.2, 5.5±0.1, 5.4±0.2, 5.3±0.2 and 5.6±0.1 mmol·L$^{-1}$ in LOW and 5.7±0.1, 5.4±0.1, 5.3±0.1, 5.3±0.1 and 5.3±0.1 mmol·L$^{-1}$ in HIGH, for days 3 to 7 of the intervention, respectively). Habitual data demonstrated no differences between groups ($P$>0.05), and there was an effect of the intervention in average daily glucose concentrations (time effect; $P$<0.005), but this was not different between groups (condition and interaction effects; both $P$>0.05). No differences were found between groups during the intervention in the average glucose concentrations during the two-hour postprandial periods after the participants' evening meals (6.0±0.3, 5.9±0.2, 5.6±0.2, 5.9±0.2 and 6.1±0.2 mmol·L$^{-1}$ in LOW and 6.2±0.3, 5.5±0.3, 5.9±0.2, 5.5±0.3 and 5.7±0.3 mmol·L$^{-1}$ in HIGH, for days 3 to 7 of the intervention, respectively; $P$>0.05 for time and interaction effects). An effect of time ($P$<0.05) was also found for measures of glycaemic variability between groups, expressed as standard deviation (SD), CONGA1, or CONGA2, with variability increasing from the habitual data to the intervention for all indices, but no differences between groups detected (main effects of condition and interaction effects, all $P$>0.05).

*Indirect calorimetry*

There were no differences in RMR between groups before the intervention (1692±119 Kcal in LOW and 1667±113 Kcal in HIGH; $P>0.05$), and there were no main effects of time, condition or any interactions (all $P>0.05$). An effect of CHO ingestion was detected for both carbohydrate (increasing) and fat (decreasing) oxidation rates ($P<0.0001$). No interaction or condition effects were found (all $P>0.05$), such that neither CHO nor fat oxidation rates changed during the intervention or across groups. The relative contributions of fat and carbohydrate oxidation to total energy expenditure in both the fasted and fed state are displayed in **Figure 5**. A numerical increase in the relative contribution of fat to energy expenditure after the intervention was observed for the HIGH group; however, this did not reach statistical significance ($P>0.05$).

*Nuclear magnetic resonance (NMR) based metabonomics*

**Table 5** lists the 224 metabolites measured by NMR metabonomics. One participant in the HIGH group was excluded from the metabonomics analysis due to being an extreme outlier (presented non-plausible changes of over 300% in most metabolites between measurements, likely due to a methodological error during sample preparation; leaving LOW, $n=10$; HIGH, $n=9$). No differences between groups were found for 208 (127 concentrations, 79 ratios and 2 dimensions) of the quantified targets. **Figure 6** and **Table 6** summarise the significant changes found in 16 of the targets (15 concentrations and 1 dimension). Twelve lipid concentrations of HDL cholesterol fractions changed to a greater degree ($P<0.05$) in LOW (12-26 %) compared with HIGH (0-5 %). Specifically, total cholesterol in HDL (HDL-C) decreased by 12.6±0.00 % in the LOW group (from 1.14±0.07 to 1.00±0.06 $mmol \cdot L^{-1}$) but only by 2.6±0.00 % in the HIGH group (from 1.15±0.07 to 1.12±0.07 $mmol \cdot L^{-1}$; $P<0.05$) and total cholesterol in HDL2 (HDL2-C) decreased by 18.6±0.00 % in the LOW group (from 0.72±0.07 to 0.58±0.05 $mmol \cdot L^{-}$

$^{1}$) and by 3.0±0.00 % in HIGH (from 0.72±0.07 to 0.69±0.06 mmol·L$^{-1}$; $P$<0.05). Similarly, apolipoprotein A1 decreased by 7.5 % in LOW (from 1.24±0.05 to 1.14±0.04 mmol·L$^{-1}$) but only by 2.7 % in HIGH (from 1.26±0.04 to 1.22±0.04 mmol·L$^{-1}$; $P$<0.05) and the mean diameter for HDL particles had a slight decrease of 1.26 % in LOW, whilst remaining constant in HIGH ($P$<0.05).

**Discussion**

The present study investigated the impact of consuming nucleotide-rich meals at lunch and dinner for one week on serum uric acid concentrations, insulin sensitivity, glycaemic control and plasma lipid composition in healthy young adults. We report that high dietary nucleotide load led to a gradual and sustained increase in serum uric acid concentrations, to a magnitude above clinically acceptable thresholds. However, we did not observe any associated changes in indices of whole-body insulin sensitivity or 24 h free-living glycaemic control, and only modest effects on the plasma lipidome. The present findings should be considered in the design and implementation of various clinical nutrition formulations, and in the design and production of novel foods aimed at the wider population, particularly protein-rich single celled sources. Both of these nutritional applications may result in novel products with high RNA contents for a variety of reasons, which clearly have knock on public health implications.

Studies carried out in the 1960s and 70s established a causal link between dietary nucleotide intake and increased serum uric acid concentrations (17-19). Daily, short-term (5-9 days), ingestion of large quantities (> 2 g) of nucleotides isolated from yeast resulted in elevated serum uric acid concentrations above clinically acceptable thresholds (i.e. >420 $\mu mol \cdot L^{-1}$ in men and >360 $\mu mol \cdot L^{-1}$ in pre-menopausal women (16)); that is, to levels associated with the development of a variety of cardio-metabolic diseases (12-14, 16). Recently, we reported that the consumption of a single high-nucleotide (1.7 g) mixed meal increased serum uric acid concentrations for 12 hours, but below these clinical thresholds (24). Therefore, in the present work, we hypothesised that repeatedly ingesting such nucleotide-rich meals for 1 week would have a chronic and cumulative effect on serum uric acid concentrations resulting in a persistent rise beyond clinical thresholds. We applied a tightly controlled one-week nutritional intervention providing two groups of well-matched (see Table 1) healthy young adults with identical diets with the exception of the daily nucleotide load, with the intervention itself having

minimal (and equivalent across groups) effects on habitual dietary intake and physical activity levels (see Tables 2 and 4). The control group consumed dietary nucleotides at a level representative of a typical western diet (0.89 g/day; (2, 3)) while the intervention group received a high daily nucleotide load (4.46 g/day) delivered via the consumption of non-nucleotide depleted mycoprotein at lunch and dinner within normal mixed meals (2, 3)) (see Table 2). While serum uric acid concentrations remained unchanged in the control group, the ~5 fold increase in daily nucleotide intake in the intervention group led to a gradual and steady increase in serum uric acid concentrations with values peaking at ~60 % above baseline after 5 days (see Figure 2). Importantly, this rise resulted in the clinically accepted values (16-19) to indicate hyperuricaemia for men (420 $\mu mol \cdot L^{-1}$) and pre-menopausal women (360 $\mu mol \cdot L^{-1}$) being surpassed by day 6 of the intervention (group mean of 472 $\mu mol \cdot L^{-1}$, with men and women reaching 490 $\mu mol \cdot L^{-1}$ and 460 $\mu mol \cdot L^{-1}$, respectively, and 4/4 male and 5/6 female volunteers surpassing the thresholds). As a result, we provide convincing support for our hypothesis that innocuous and transient increases in circulating uric acid concentrations as a result of ingesting a single nucleotide-rich mixed meal (24) accumulate over repeated meals resulting in persistent hyperuricaemia. Accordingly, our data are in line with previously established guidelines (20, 21) to limit daily dietary nucleotide load to avoid hyperuricaemia. Further, our data also confirm the requirement for industrial practices to incorporate RNA lowering procedures (e.g. (23)) for the production of novel alternative dietary protein sources to bring nucleotide load of meals in line with other dietary protein sources.

The primary motivation for establishing clinical thresholds to define hyperuricaemia has been the association between higher circulating uric acid concentrations and a variety of inflammatory and/or cardiometabolic conditions, such as gout (11), hypertension (12), (pre) type 2 diabetes (14) (44, 45), and metabolic syndrome (13), leading to speculations of a causative role on disease progression (46). We have previously shown that the acute increase

in circulating uric acid concentrations following a single nucleotide-rich meal was not associated with impaired postprandial glucose handling (24). However, while clear mechanistic links have not been established, *in vitro* experimentation has shown that uric acid is a pro-oxidant in the intracellular environment implying metabolic dysfunction could be promoted by increased but *sustained* cellular oxidative stress (47-52). Accordingly, a second aim of the present work was to test the hypothesis that nutritionally elevating serum uric acid concentrations for several days in otherwise healthy individuals would impair indices of cardiometabolic health, lending support for a causative relationship. Given the sustained and remarkable hyperuricaemia achieved throughout the intervention in the intervention group only, we had a clear framework within which to test this mechanistic hypothesis. Nevertheless, we report no differences in daily glycaemic control, calculated liver or peripheral insulin sensitivity (see Figures 3 and 4), or postprandial glucose tolerance either within or between groups. Collectively, therefore, the dramatically elevated circulating uric acid concentrations paired with no impact on a wide array of indices of metabolic health, within the context of a tightly controlled intervention, provide convincing evidence for the lack of a causative effect of elevated serum uric acid on metabolic health. It is plausible that hyperuricaemia is more a *consequence* of disease progression and therefore an accurate predictor, which would be supported by studies that have shown hyperinsulinaemia can induce hyperuricaemia by reducing renal excretion of uric acid (53). A clear limitation in this interpretation of our data, however, is the tightly controlled nature of our design necessitated a relatively short intervention period. To allow more definitive conclusions, future work will be required to assess whether chronic (nutritionally induced) hyperuricaemia would modulate cardiometabolic health over multiple weeks, months or even years.. In addition, an interesting auxiliary finding was a lowering of fasting blood glucose concentrations that was observed in *both* groups (see Figure 3) throughout the intervention. This is in line with our previous

observations (26) that daily mycoprotein consumption subtly reduced fasting blood glucose concentrations compared with a control group consuming meat and fish, despite an absence of changes in insulin sensitivity or daily glycaemic control. While we do not have a satisfactory explanation for this somewhat paradoxical observation (i.e. in the face of no changes in insulin sensitivity or glycaemic control), it is of interest that we have reproduced the finding and the effect appears uninfluenced by the nucleotide content of the mycoprotein. Future work is clearly warranted to establish whether this represents early indications of improvements in insulin sensitivity that are not detectable after only one week and/or without the use of hyperinsulinaemic euglycaemic clamps.

In the present work, the method by which we delivered low or high dietary nucleotide loads within the context of nutritionally relevant mixed meals was the daily consumption of mycoprotein at lunch and dinner. We have previously reported (26), using a targeted and quantitative NMR based metabonomics approach, that substituting meat and fish for mycoprotein at main meals considerably impacts the plasma lipidome. Specifically, cholesterol and various cholesterol lipoprotein moieties are rapidly (after 7 days) and substantially (by 7-27 %) decreased with daily mycoprotein compared with meat/fish consumption. This is likely due to the increased fibre intake (and perhaps type of fibre) that consuming mycoprotein typically induces, potentially mediated by a consequent increase in the gut production of short-chain fatty acids (22, 26). Indeed, here we observed a considerable (approx. 30%) increase in dietary fibre intake compared with habitual intakes, and identical across both groups (Table 2). Given the relevance of plasma lipid signatures for the longer-term prediction of metabolic health (54, 55), we applied the same metabonomics platform in the current study (see Table 6 and Figure 6). In line with our whole body and dynamic assessments of metabolic health, we observed minimal differences (in 208 of the 224 targets) between groups. Indeed, the previously reported (26) effects of mycoprotein consumption *per se* were largely observable in

both groups (e.g. 14 and 8%, 14 and 11% and 19 and 13% reductions in total plasma cholesterol, plasma free cholesterol and plasma LDL cholesterol in LOW and HIGH nucleotide groups, respectively), and therefore a result of mycoprotein consumption *per se* and not the nucleotide content. However, we did note differences in the concentrations of twelve HDL cholesterol fractions, as well as in the total cholesterol concentrations of HDL and HDL2, in apolipoprotein A1 and on the mean diameter of the HDL particles which decreased (to a greater extent) in the control compared with high nucleotide group. We, therefore, confirm the previously reported (26) favourable effects of daily mycoprotein consumption on the plasma lipidome. To our knowledge, this work represents the first attempt to investigate any effects of dietary nucleotide consumption on any plasma metabolic profile.

A final consideration of our work is the physiological regulation of dietary nucleotide metabolism. After peaking at 5 days into the intervention, serum uric acid concentrations declined back towards baseline levels, and below the cut-off for clinical hyperuricaemia (Figure 2). This perhaps implies the onset of an adaptive response to restore homeostasis in the face of a persistent increase in dietary nucleotide load. As a purine metabolic end-product, uric acid is primarily excreted in urine (the kidneys eliminate two-thirds while the gastrointestinal tract eliminates the remainder) (56). However, we observed no changes in daily waking urinary uric acid concentrations despite rising serum levels during the intervention in the high nucleotide group (see Figure 2). This finding could simply represent a lack of sensitivity of our approach given we collected only waking samples (rather than 24 h collections which would have encompassed total excretion, as well as postprandially when it would have potentially been acutely elevated), and the concentration of uric acid in urine is orders of magnitude greater than in serum (i.e. millimolar vs micromolar range, respectively). However, we cannot rule out a metabolic adaptation within specific tissues (e.g. skeletal muscle), whereby excess dietary nucleotide provision resulted in alternative metabolic fates (e.g. increased production of DNA,

RNA, ATP or other nucleotide containing organic molecules). Future work should pursue this possibility given the potential ergogenic effects of nucleotide supplementation on exercise performance (9, 57-59).

In conclusion, twice-daily consumption of high-nucleotide containing mycoprotein for one week increased serum uric acid concentrations above clinically relevant thresholds but did not produce any appreciably deleterious effects on daily glycaemic control, insulin sensitivity or the plasma lipidome.

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**Conflict of Interest**

BTW has received research grants from Marlow Foods. MOCC and AJM receive PhD studentship funding from Marlow Foods and the College of Life and Environmental Sciences, University of Exeter. TJAF is an employee of Marlow Foods. Remaining authors declare no conflicts of interest.

**Authors' contributions**

MOCC and BTW designed research. MOCC recruited, randomised and assigned participants to interventions. MOCC and AJM conducted research. TJAF provided essential materials. I.D.K. and V.N.-V. performed essential analyses. MOCC analysed data. MOCC, FBS and BTW wrote the paper. BTW had primary responsibility for final content. All authors read and approved the final manuscript.

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**Table 1.** Participants' characteristics

| | **LOW** | **HIGH** | ***P* value** |
|---|---|---|---|
| **Sex** | 6 F / 4 M | 6 F / 4 M | - |
| **Age (y)** | 24±1 | 25±2 | 0.47 |
| **Height (cm)** | 171±4 | 173±3 | 0.83 |
| **Body mass (kg)** | 69±6 | 72±4 | 0.66 |
| **BMI (kg/m$^2$)** | 23±1 | 24±1 | 0.53 |
| **Body fat (% of body mass)** | 21±3 | 21±3 | 0.95 |
| **Lean mass (kg)** | 55±5 | 58±4 | 0.65 |

Multiple *t*-tests were used to compare each characteristic in LOW and HIGH.

*Abbreviations:* LOW, low nucleotide mycoprotein group; HIGH, high nucleotide mycoprotein group.

**Table 2.** Nutritional composition of participants' habitual diets, and of the prescribed one-week intervention diet and their actual intake during the intervention according to the collected logs.

| | Habitual dietary intake | | Prescribed intervention diet | | Actual intake during intervention | |
|---|---|---|---|---|---|---|
| | **LOW** | **HIGH** | **LOW** | **HIGH** | **LOW** | **HIGH** |
| **Energy (MJ)** | 10.1±0.6 | 9.5±0.7 | 11.0±1.0 | 10.7±0.6 | 10.9±0.1 | 10.7±0.5 |
| **Energy (kcal)** | 2414±150 | 2263±173 | 2624±237 | 2565±126 | 2598±247 | 2555±122 |
| **Protein (g)** | 107±14 | 98±13 | 84±7 | 88±4 | 82±7 | 87±4 |
| **Protein (g/kg bm)** | 1.6±0.2 | 1.4±0.2 | 1.2±0.0* | 1.2±0.0* | 1.2±0.0* | 1.2±0.0* |
| **Protein (% total energy)** | 17.6±1.7 | 17.8±2.2 | 13.0±0.5* | 13.7±0.3* | 12.9±0.6* | 13.7±0.3* |
| **Carbohydrate (g)** | 260±22 | 267±31 | 355±35* | 342±18* | 350±37* | 342±18* |
| **Carbohydrate (% total energy)** | 43.0±2.1 | 46.1±2.2 | 53.9±0.7 | 53.3±0.5* | 53.4±1.0* | 53.4±0.5* |
| **Fat (g)** | 99±7 | 83±6 | 87±8 | 86±4 | 87±8 | 85±4 |
| **Fat (% total energy)** | 37.0±1.7 | 33.6±1.7 | 29.8±0.1* | 30.0±0.2* | 30.2±0.3* | 29.9±0.2* |
| **Fibre (g)** | 26±2 | 26±4 | 34±2* | 33±2* | 34±2* | 33±2* |

Separate two-way repeated measures ANOVAs were used to compare LOW and HIGH actual dietary intakes during the intervention with both the habitual diets and the prescribed intervention diets.

*Abbreviations:* LOW, low nucleotide mycoprotein group; HIGH, high nucleotide mycoprotein group.

* Significantly different from habitual diet (time effect; $P<0.05$)

**Table 3.** Daily mycoprotein-derived dietary nucleotide intake (in grams) and nucleotide composition of dry mycoprotein (%)

| | LOW | | HIGH | |
|---|---|---|---|---|
| **Nucleotides** | **g/day** | **% mycoprotein dry wt** | **g/day** | **% mycoprotein dry wt** |
| **CMP** | - | - | 0.32 | 0.62 |
| **UMP** | 0.12 | 0.26 | 0.22 | 0.44 |
| **GMP** | 0.10 | 0.21 | 0.14 | 0.28 |
| **TMP** | 0.21 | 0.46 | 1.53 | 3.00 |
| **CDP (** | 0.09 | 0.19 | 0.43 | 0.84 |
| **UDP** | 0.02 | 0.07 | 0.17 | 0.34 |
| **CTP** | 0.21 | 0.47 | 1.62 | 3.18 |
| **ADP** | 0.01 | 0.02 | 0.04 | 0.08 |
| **TTP** | - | - | 0.02 | 0.04 |
| **ITP** | 0.13 | 0.29 | - | - |
| **ATP** | - | - | 0.01 | 0.01 |
| **Total nucleotides** | 0.89 | 1.96 | 4.46 | 8.83 |

*Abbreviations:* LOW, Low nucleotide mycoprotein group; HIGH, High nucleotide mycoprotein group; CMP, Cytidine monophosphate; UMP, Uridine monophosphate; GMP, Guanosine monophosphate; TMP, Thymidine monophosphate; CDP, Cytidine diphosphate; UDP, Uridine diphosphate; CTP, Cytidine triphosphate; ADP, Adenosine diphosphate; TTP, Thymidine triphosphate; ITP, Inosine triphosphate; ATP, Adenosine triphosphate

**Table 4.** Daily habitual physical activity and daily physical activity during the intervention

| | Habitual | | Intervention | |
|---|---|---|---|---|
| | **LOW** | **HIGH** | **LOW** | **HIGH** |
| **Total activity (mins/day)** | 251±34 | 259±14 | 295±26 | 288±27 |
| **Light activity (mins/day)** | 80±7 | 80±9 | 94±9 | 93±11 |
| **Moderate activity (mins/day)** | 158±27 | 168±12 | 186±18 | 182±18 |
| **Vigorous activity (mins/day)** | 12±5 | 11±6 | 15±5 | 14±5 |
| **Sedentary (mins/day)** | 661±26 | 643±17 | 654±34 | 646±32 |

Multiple two-way ANOVAs were used to compare the different activity levels in LOW and HIGH habitually and during the intervention (all $P>0.05$)

*Abbreviations:* LOW, low nucleotide mycoprotein group; HIGH, high nucleotide mycoprotein group.

**Table 5.** List of metabolites measured by NMR-based metabolomics

| **Abbreviation** | **Full Name** | **Abbreviation** | **Full Name** |
|---|---|---|---|
| **Lipoprotein Subclasses** | | **Lipoprotein Subclass Ratios** | |
| XXL-VLDL-P | Concentration of chylomicrons and extremely large VLDL particles | XXL-VLDL-PL_% | Phospholipids to total lipds ratio in chylomicrons and extremely large VLDL |
| XXL-VLDL-L | Total lipids in chylomicrons and extremely large VLDL | XXL-VLDL-C_% | Total cholesterol to total lipids ratio in chylomicrons and extremely large VLDL |
| XXL-VLDL-PL | Phospholipids in chylomicrons and extremely large VLDL | XXL-VLDL-CE_% | Cholesterol esters to total lipids ratio in chylomicrons and extremely large VLDL |
| XXL-VLDL-C | Total cholesterol in chylomicrons and extremely large VLDL | XXL-VLDL-FC_% | Free cholesterol to total lipids ratio in chylomicrons and extremely large VLDL |
| XXL-VLDL-CE | Cholesterol esters in chylomicrons and extremely large VLDL | XXL-VLDL-TG_% | Triglycerides to total lipids ratio in chylomicrons and extremely large VLDL |
| XXL-VLDL-FC | Free cholesterol in chylomicrons and extremely large VLDL | XL-VLDL-PL_% | Phospholipids to total lipids ratio in very large VLDL |

| | | | |
|---|---|---|---|
| | Triglycerides in chylomicrons and extremely large | | |
| XXL-VLDL-TG | VLDL | XL-VLDL-C_% | Total cholesterol to total lipids ratio in very large VLDL |
| XL-VLDL-P | Concentration of very large VLDL particles | XL-VLDL-CE_% | Cholesterol esters to total lipids ratio in very large VLDL |
| XL-VLDL-L | Total lipids in very large VLDL | XL-VLDL-FC_% | Free cholesterol to total lipids ratio in very large VLDL |
| XL-VLDL-PL | Phospholipids in very large VLDL | XL-VLDL-TG_% | Triglycerides to total lipids ratio in very large VLDL |
| XL-VLDL-C | Total cholesterol in very large VLDL | L-VLDL-PL_% | Phospholipids to total lipids ratio in large VLDL |
| XL-VLDL-CE | Cholesterol esters in very large VLDL | L-VLDL-C_% | Total cholesterol to total lipids ratio in large VLDL |
| XL-VLDL-FC | Free cholesterol in very large VLDL | L-VLDL-CE_% | Cholesterol esters to total lipids ratio in large VLDL |
| XL-VLDL-TG | Triglycerides in very large VLDL | L-VLDL-FC_% | Free cholesterol to total lipids ratio in large VLDL |
| L-VLDL-P | Concentration of large VLDL particles | L-VLDL-TG_% | Triglycerides to total lipids ratio in large VLDL |
| L-VLDL-L | Total lipids in large VLDL | M-VLDL-PL_% | Phospholipids to total lipids ratio in medium VLDL |
| L-VLDL-PL | Phospholipids in large VLDL | M-VLDL-C_% | Total cholesterol to total lipids ratio in medium VLDL |
| L-VLDL-C | Total cholesterol in large VLDL | M-VLDL-CE_% | Cholesterol esters to total lipids ratio in medium VLDL |
| L-VLDL-CE | Cholesterol esters in large VLDL | M-VLDL-FC_% | Free cholesterol to total lipids ratio in medium VLDL |
| L-VLDL-FC | Free cholesterol in large VLDL | M-VLDL-TG_% | Triglycerides to total lipids ratio in medium VLDL |
| L-VLDL-TG | Triglycerides in large VLDL | S-VLDL-PL_% | Phospholipids to total lipids ratio in small VLDL |
| M-VLDL-P | Concentration of medium VLDL particles | S-VLDL-C_% | Total cholesterol to total lipids ratio in small VLDL |

| | | | |
|---|---|---|---|
| M-VLDL-L | Total lipids in medium VLDL | S-VLDL-CE_% | Cholesterol esters to total lipids ratio in small VLDL |
| M-VLDL-PL | Phospholipids in medium VLDL | S-VLDL-FC_% | Free cholesterol to total lipids ratio in small VLDL |
| M-VLDL-C | Total cholesterol in medium VLDL | S-VLDL-TG_% | Triglycerides to total lipids ratio in small VLDL |
| M-VLDL-CE | Cholesterol esters in medium VLDL | XS-VLDL-PL_% | Phospholipids to total lipids ratio in very small VLDL |
| M-VLDL-FC | Free cholesterol in medium VLDL | XS-VLDL-C_% | Total cholesterol to total lipids ratio in very small VLDL |
| M-VLDL-TG | Triglycerides in medium VLDL | XS-VLDL-CE_% | Cholesterol esters to total lipids ratio in very small VLDL |
| S-VLDL-P | Concentration of small VLDL particles | XS-VLDL-FC_% | Free cholesterol to total lipids ratio in very small VLDL |
| S-VLDL-L | Total lipids in small VLDL | XS-VLDL-TG_% | Triglycerides to total lipids ratio in very small VLDL |
| S-VLDL-PL | Phospholipids in small VLDL | IDL-PL_% | Phospholipids to total lipids ratio in IDL |
| S-VLDL-C | Total cholesterol in small VLDL | IDL-C_% | Total cholesterol to total lipids ratio in IDL |
| S-VLDL-CE | Cholesterol esters in small VLDL | IDL-CE_% | Cholesterol esters to total lipids ratio in IDL |
| S-VLDL-FC | Free cholesterol in small VLDL | IDL-FC_% | Free cholesterol to total lipids ratio in IDL |
| S-VLDL-TG | Triglycerides in small VLDL | IDL-TG_% | Triglycerides to total lipids ratio in IDL |
| XS-VLDL-P | Concentration of very small VLDL particles | L-LDL-PL_% | Phospholipids to total lipids ratio in large LDL |
| XS-VLDL-L | Total lipids in very small VLDL | L-LDL-C_% | Total cholesterol to total lipids ratio in large LDL |
| XS-VLDL-PL | Phospholipids in very small VLDL | L-LDL-CE_% | Cholesterol esters to total lipids ratio in large LDL |
| XS-VLDL-C | Total cholesterol in very small VLDL | L-LDL-FC_% | Free cholesterol to total lipids ratio in large LDL |

| | | | |
|---|---|---|---|
| XS-VLDL-CE | Cholesterol esters in very small VLDL | L-LDL-TG_% | Triglycerides to total lipids ratio in large LDL |
| XS-VLDL-FC | Free cholesterol in very small VLDL | M-LDL-PL_% | Phospholipids to total lipids ratio in medium LDL |
| XS-VLDL-TG | Triglycerides in very small VLDL | M-LDL-C_% | Total cholesterol to total lipids ratio in medium LDL |
| IDL-P | Concentration of IDL particles | M-LDL-CE_% | Cholesterol esters to total lipids ratio in medium LDL |
| IDL-L | Total lipids in IDL | M-LDL-FC_% | Free cholesterol to total lipids ratio in medium LDL |
| IDL-PL | Phospholipids in IDL | M-LDL-TG_% | Triglycerides to total lipids ratio in medium LDL |
| IDL-C | Total cholesterol in IDL | S-LDL-PL_% | Phospholipids to total lipids ratio in small LDL |
| IDL-CE | Cholesterol esters in IDL | S-LDL-C_% | Total cholesterol to total lipids ratio in small LDL |
| IDL-FC | Free cholesterol in IDL | S-LDL-CE_% | Cholesterol esters to total lipids ratio in small LDL |
| IDL-TG | Triglycerides in IDL | S-LDL-FC_% | Free cholesterol to total lipids ratio in small LDL |
| L-LDL-P | Concentration of large LDL particles | S-LDL-TG_% | Triglycerides to total lipids ratio in small LDL |
| L-LDL-L | Total lipids in large LDL | XL-HDL-PL_% | Phospholipids to total lipids ratio in very large HDL |
| L-LDL-PL | Phospholipids in large LDL | XL-HDL-C_% | Total cholesterol to total lipids ratio in very large HDL |
| L-LDL-C | Total cholesterol in large LDL | XL-HDL-CE_% | Cholesterol esters to total lipids ratio in very large HDL |
| L-LDL-CE | Cholesterol esters in large LDL | XL-HDL-FC_% | Free cholesterol to total lipids ratio in very large HDL |
| L-LDL-FC | Free cholesterol in large LDL | XL-HDL-TG_% | Triglycerides to total lipids ratio in very large HDL |
| L-LDL-TG | Triglycerides in large LDL | L-HDL-PL_% | Phospholipids to total lipids ratio in large HDL |

| | | | |
|---|---|---|---|
| M-LDL-P | Concentration of medium LDL particles | L-HDL-C_% | Total cholesterol to total lipids ratio in large HDL |
| M-LDL-L | Total lipids in medium LDL | L-HDL-CE_% | Cholesterol esters to total lipids ratio in large HDL |
| M-LDL-PL | Phospholipids in medium LDL | L-HDL-FC_% | Free cholesterol to total lipids ratio in large HDL |
| M-LDL-C | Total cholesterol in medium LDL | L-HDL-TG_% | Triglycerides to total lipids ratio in large HDL |
| M-LDL-CE | Cholesterol esters in medium LDL | M-HDL-PL_% | Phospholipids to total lipids ratio in medium HDL |
| M-LDL-FC | Free cholesterol in medium LDL | M-HDL-C_% | Total cholesterol to total lipids ratio in medium HDL |
| M-LDL-TG | Triglycerides in medium LDL | M-HDL-CE_% | Cholesterol esters to total lipids ratio in medium HDL |
| S-LDL-P | Concentration of small LDL particles | M-HDL-FC_% | Free cholesterol to total lipids ratio in medium HDL |
| S-LDL-L | Total lipids in small LDL | M-HDL-TG_% | Triglycerides to total lipids ratio in medium HDL |
| S-LDL-PL | Phospholipids in small LDL | S-HDL-PL_% | Phospholipids to total lipids ratio in small HDL |
| S-LDL-C | Total cholesterol in small LDL | S-HDL-C_% | Total cholesterol to total lipids ratio in small HDL |
| S-LDL-CE | Cholesterol esters in small LDL | S-HDL-CE_% | Cholesterol esters to total lipids ratio in small HDL |
| S-LDL-FC | Free cholesterol in small LDL | S-HDL-FC_% | Free cholesterol to total lipids ratio in small HDL |
| S-LDL-TG | Triglycerides in small LDL | S-HDL-TG_% | Triglycerides to total lipids ratio in small HDL |
| | | **Lipoprotein Particle** | |
| XL-HDL-P | Concentration of very large HDL particles | **Size** | |
| XL-HDL-L | Total lipids in very large HDL | VLDL_D | Mean diameter for VLDL particles |

| | | | |
|---|---|---|---|
| XL-HDL-PL | Phospholipids in very large HDL | LDL_D | Mean diameter for LDL particles |
| XL-HDL-C | Total cholesterol in very large HDL | HDL_D | Mean diameter for HDL particles |
| XL-HDL-CE | Cholesterol esters in very large HDL | **Apolipoproteins** | |
| XL-HDL-FC | Free cholesterol in very large HDL | ApoA1 | Apolipoprotein A-I |
| XL-HDL-TG | Triglycerides in very large HDL | ApoB | Apolipoprotein B |
| L-HDL-P | Concentration of large HDL particles | ApoB/ApoA1 | Ratio of apolipoprotein B to apolipoprotein A-I |
| | | **Fatty Acids and** | |
| L-HDL-L | Total lipids in large HDL | **Saturation Measures** | |
| L-HDL-PL | Phospholipids in large HDL | TotFA | Total fatty acids |
| L-HDL-C | Total cholesterol in large HDL | UnSat | Estimated degree of unsaturation |
| L-HDL-CE | Cholesterol esters in large HDL | DHA | 22:6, docosahexaenoic acid |
| L-HDL-FC | Free cholesterol in large HDL | LA | 18:2, linoleic acid |
| L-HDL-TG | Triglycerides in large HDL | FAw3 | Omega-3 fatty acids |
| M-HDL-P | Concentration of medium HDL particles | FAw6 | Omega-6 fatty acids |
| M-HDL-L | Total lipids in medium HDL | PUFA | Polyunsaturated fatty acids |
| M-HDL-PL | Phospholipids in medium HDL | MUFA | Monounsaturated fatty acids; 16:1, 18:1 |
| M-HDL-C | Total cholesterol in medium HDL | SFA | Saturated fatty acids |

| | | | |
|---|---|---|---|
| M-HDL-CE | Cholesterol esters in medium HDL | **Fatty Acids (%)** | |
| M-HDL-FC | Free cholesterol in medium HDL | DHA/FA | Ratio of 22:6 docosahexaenoic acid to total fatty acids |
| M-HDL-TG | Triglycerides in medium HDL | LA/FA | Ratio of 18:2 linoleic acid to total fatty acids |
| S-HDL-P | Concentration of small HDL particles | FAw3/FA | Ratio of omega-3 fatty acids to total fatty acids |
| S-HDL-L | Total lipids in small HDL | FAw6/FA | Ratio of omega-6 fatty acids to total fatty acids |
| S-HDL-PL | Phospholipids in small HDL | PUFA/FA | Ratio of polyunsaturated fatty acids to total fatty acids |
| S-HDL-C | Total cholesterol in small HDL | MUFA/FA | Ratio of monounsaturated fatty acids to total fatty acids |
| S-HDL-CE | Cholesterol esters in small HDL | SFA/FA | Ratio of saturated fatty acids to total fatty acids |
| | | **Glycolysis Related** | |
| S-HDL-FC | Free cholesterol in small HDL | **Metabolites** | |
| S-HDL-TG | Triglycerides in small HDL | Glc | Glucose |
| **Cholesterol** | | Lac | Lactate |
| Serum-C | Serum total cholesterol | Cit | Citrate |
| VLDL-C | Total cholesterol in VLDL | **Amino Acids** | |
| Remnant-C | Remnant cholesterol (non-HDL, non-LDL - cholesterol) | Ala | Alanine |
| LDL-C | Total cholesterol in LDL | Gln | Glutamine |

| | | | |
|---|---|---|---|
| HDL-C | Total cholesterol in HDL | His | Histidine |
| | | **Branched-chained** | |
| HDL2-C | Total cholesterol in HDL2 | **amino acids** | |
| HDL3-C | Total cholesterol in HDL3 | Ile | Isoleucine |
| EstC | Esterified cholesterol | Leu | Leucine |
| FreeC | Free cholesterol | Val | Valine |
| **Glycerides and** | | | |
| **Phospholipids** | | **Aromatic amino acids** | |
| Serum-TG | Serum total triglycerides | Phe | Phenylalanine |
| VLDL-TG | Triglycerides in VLDL | Tyr | Tyrosine |
| LDL-TG | Triglycerides in LDL | **Ketone Bodies** | |
| HDL-TG | Triglycerides in HDL | Ace | Acetate |
| TotPG | Total phosphoglycerides | bOHBut | 3-hydroxybutyrate |
| | | **Fluid Balance and** | |
| TG/PG | Ratio of triglycerides to phosphoglycerides | **Inflammation** | |
| PC | Phosphatidylcholine and other cholines | Crea | Creatinine |
| SM | Sphingomyelins | Alb | Albumin |

| | | | |
|---|---|---|---|
| TotCho | Total cholines | Gp | Glycoprotein acetyls, mainly a1-acid glycoprotein |

**Table 6.** Significant NMR-based metabonomics features identified using either Significance Analysis of Microarrays (concentrations) or t-tests (dimensions)

| Concentrations | % Δ change LOW | SEM | % Δ change HIGH | SEM | d.value | stdev | rawp | q.value |
|---|---|---|---|---|---|---|---|---|
| HDL2-C | -18.58% | 0.03 | -3.02% | 0.04 | -2.8015 | 0.046861 | 0.002042 | 0.16908 |
| L-HDL-PL | -20.19% | 0.03 | -3.07% | 0.05 | -2.6278 | 0.056469 | 0.00338 | 0.16908 |
| HDL-C | -12.62% | 0.02 | -2.59% | 0.03 | -2.5069 | 0.031361 | 0.006056 | 0.16908 |
| L-HDL-P | -20.62% | 0.03 | -2.04% | 0.06 | -2.4563 | 0.06701 | 0.007254 | 0.16908 |
| L-HDL-L | -20.84% | 0.03 | -1.79% | 0.06 | -2.4463 | 0.069204 | 0.007394 | 0.16908 |
| L-HDL-FC | -24.54% | 0.04 | 0.32% | 0.09 | -2.343 | 0.097471 | 0.01007 | 0.16908 |
| XL-HDL-FC | -25.85% | 0.04 | 4.95% | 0.12 | -2.3372 | 0.12314 | 0.010352 | 0.16908 |
| L-HDL-C | -21.48% | 0.04 | 0.16% | 0.08 | -2.2583 | 0.087157 | 0.013169 | 0.16908 |
| XL-HDL-C | -23.04% | 0.03 | 2.61% | 0.10 | -2.2543 | 0.10509 | 0.01331 | 0.16908 |
| L-HDL-CE | -20.67% | 0.04 | 0.12% | 0.08 | -2.2313 | 0.084512 | 0.014437 | 0.16908 |
| XL-HDL-CE | -22.11% | 0.03 | 1.89% | 0.10 | -2.2196 | 0.099459 | 0.01493 | 0.16908 |
| XL-HDL-L | -22.18% | 0.04 | 2.91% | 0.11 | -2.1735 | 0.10677 | 0.016408 | 0.16908 |
| XL-HDL-P | -21.94% | 0.04 | 2.75% | 0.10 | -2.1635 | 0.10548 | 0.016831 | 0.16908 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| XL-HDL-PL | -21.76% | 0.04 | 4.01% | 0.12 | -2.0639 | 0.11624 | 0.020915 | 0.1951 |
| ApoA1 | -7.51% | 0.01 | -2.73% | 0.01 | -1.8515 | 0.017173 | 0.036901 | 0.32127 |
| **Dimensions** | **% Δ change LOW** | **SEM** | **% Δ change HIGH** | **SEM** | **t.stat** | **p.value** | **- log 10 (p)** | **FDR** |
| HDL_D | -1.26% | 0.00 | 0.04% | 0.00 | 2.505 | 0.022716 | 1.6437 | 0.068148 |

*Abbreviations:* -C, total cholesterol; L- large; PL, phospholipids; -P, particles; -L, lipid; -FC, free cholesterol; XL-, extremely large; CE, cholesteryl esters; ApoA1, apolipoprotein A1; FDR, false discovery rate; HDL_D, mean diameter for HDL particles; LOW, low nucleotide mycoprotein group; HIGH, high nucleotide mycoprotein group

**Figure Legends**

**Figure 1** Overview of the experimental protocol.

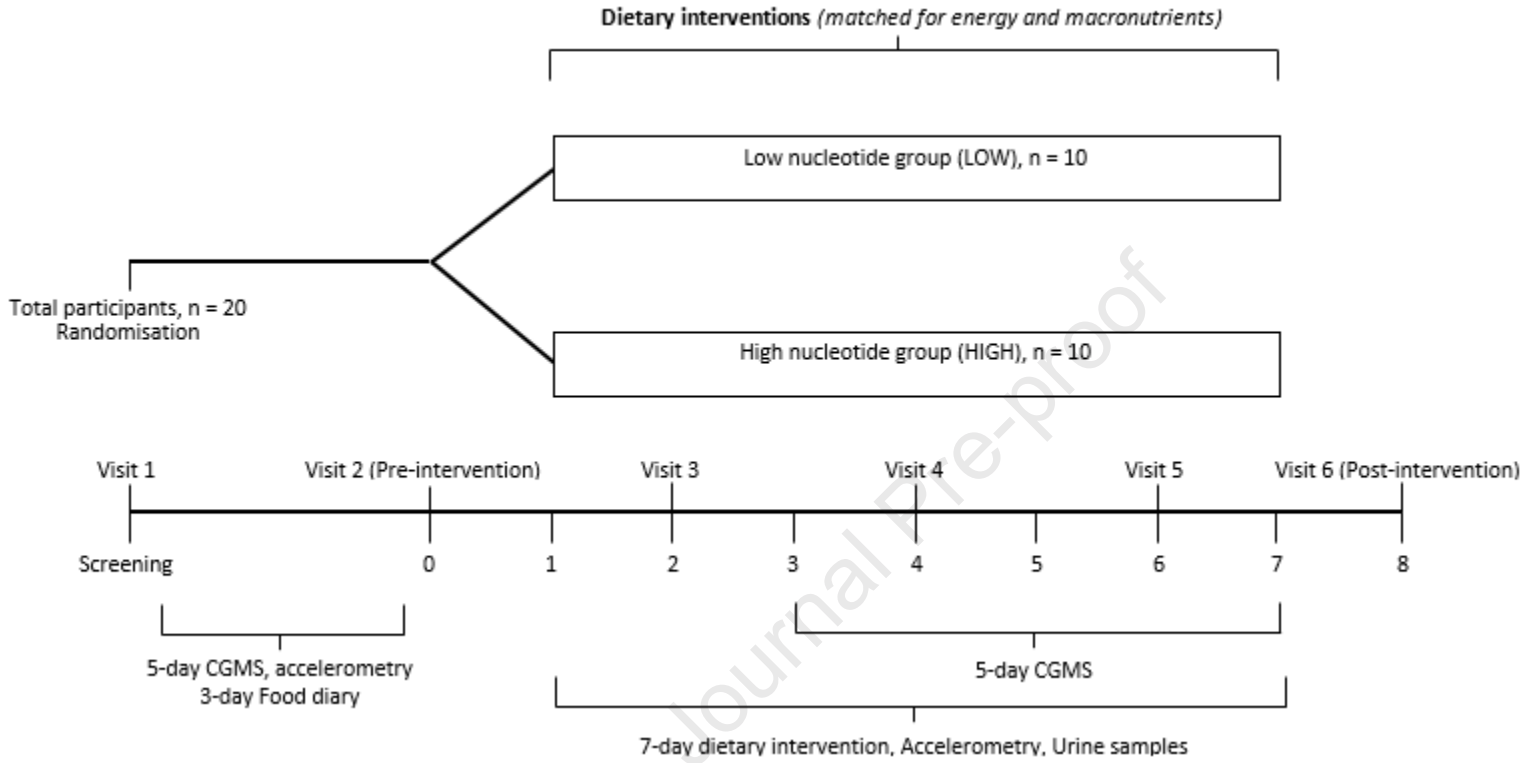
**Figure 2** Serum (A) and urine (B) uric acid concentrations throughout a one week fully controlled dietary intervention with either a low-nucleotide mycoprotein (LOW) or a high-nucleotide mycoprotein (HIGH). Serum samples were collected on days 0, 2, 4, 6 and 8 of the intervention, and urine samples were collected every day. There were no differences in fasting serum uric acid concentrations between groups at baseline ($P$>0.05). Serum uric acid concentrations remained constant in LOW, but steadily increased in HIGH (time, condition and interaction effects; all $P$<0.0001) from baseline to days 2, 4 and 6, before decreasing (but remaining elevated compared with baseline; $P$<0.05) on day 8. There were no differences in urine uric acid between groups at baseline and there were no main effects of time, condition or an interaction effect (all $P$>0.05). * indicates a difference from baseline

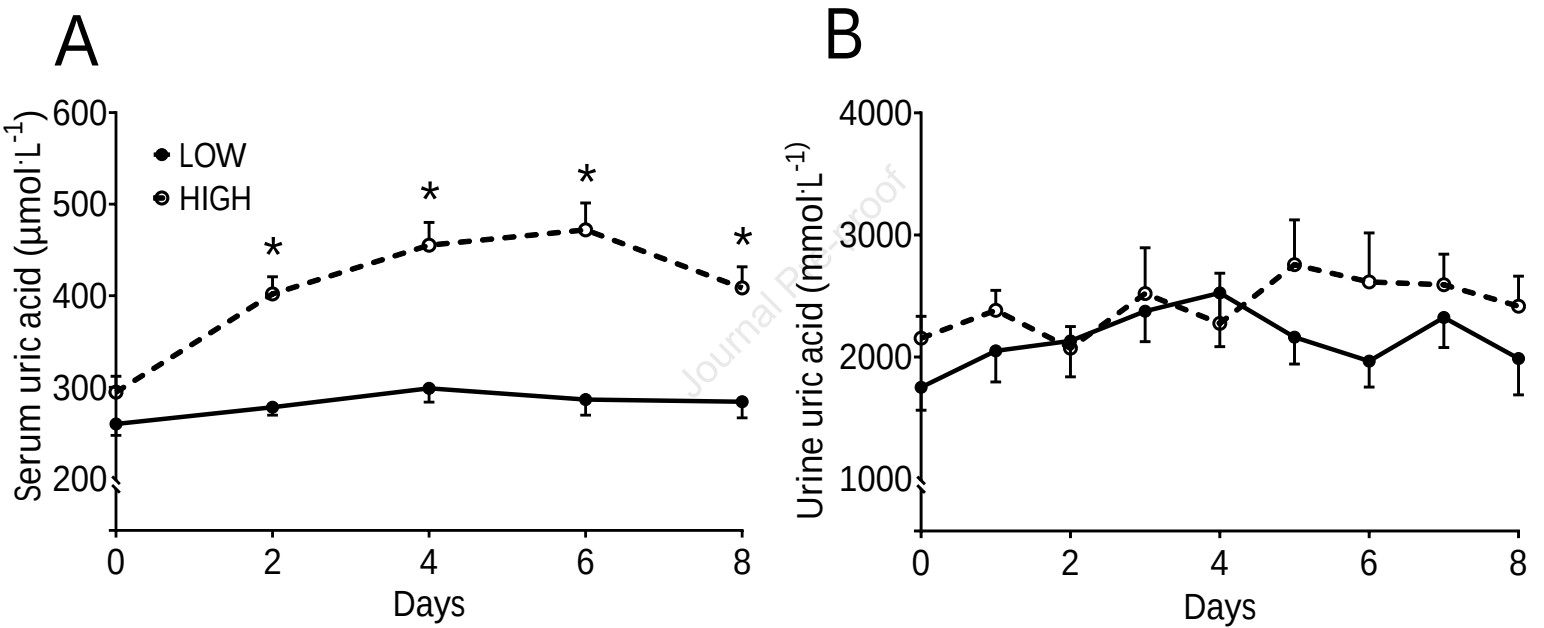
**Figure 3** Blood glucose (A, B and C) and serum insulin (D, E and F) concentrations during oral glucose tolerance test (OGTT) on days 0 (A and D) and 8 (B and E) of a one week fully controlled dietary intervention with either a low-nucleotide mycoprotein (LOW) or a high-nucleotide mycoprotein (HIGH). OGTT data were analysed using three-way ANOVAs. Incremental area under the curve (iAUC) data were analysed using one-way ANOVAs. There was a significant effect of CHO ingestion for blood glucose and serum insulin ($P$<0.0001). No interaction effects or main effects of condition or time were found (all $P$>0.05). For both blood glucose iAUC and serum insulin iAUC, no statistically significant main effects of time or condition (both $P$>0.05), as well as no interaction effects ($P$>0.05) were found.

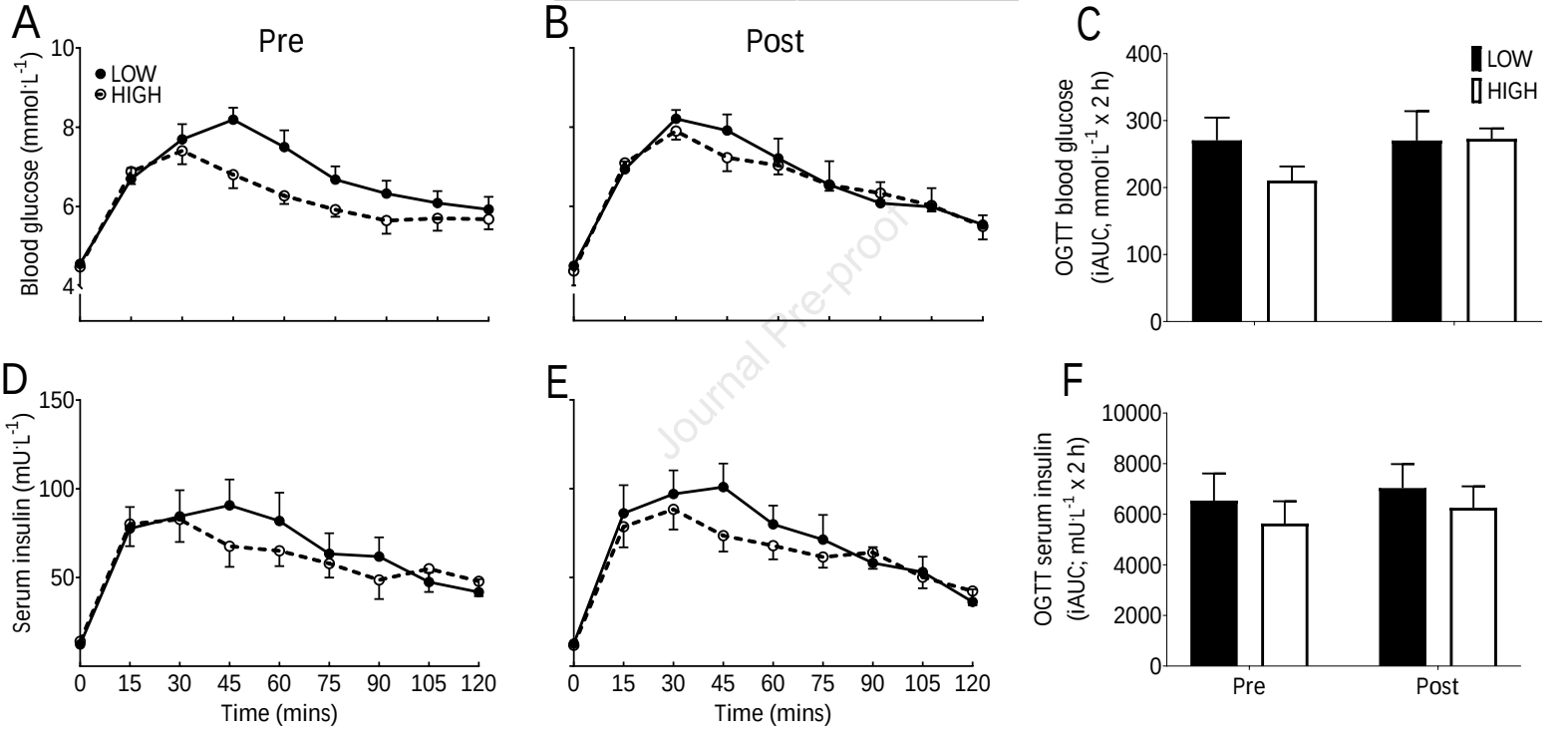
**Figure 4** Insulin sensitivity indices (A: HOMA-IR, B: Cederholm, C: Matsuda, D: OGIS and E: GUTT) calculated with the blood glucose and serum insulin concentrations measured fasting and during oral glucose tolerance tests (OGTT) pre and post a one week fully controlled dietary intervention with either a low-nucleotide mycoprotein (LOW) or a high-nucleotide mycoprotein (HIGH). There were no differences between groups at baseline for any of the OGTT calculated insulin sensitivity (IS) indices (all $P>0.05$) and no changes resulted from the intervention (time and interaction effects; all $P>0.05$)

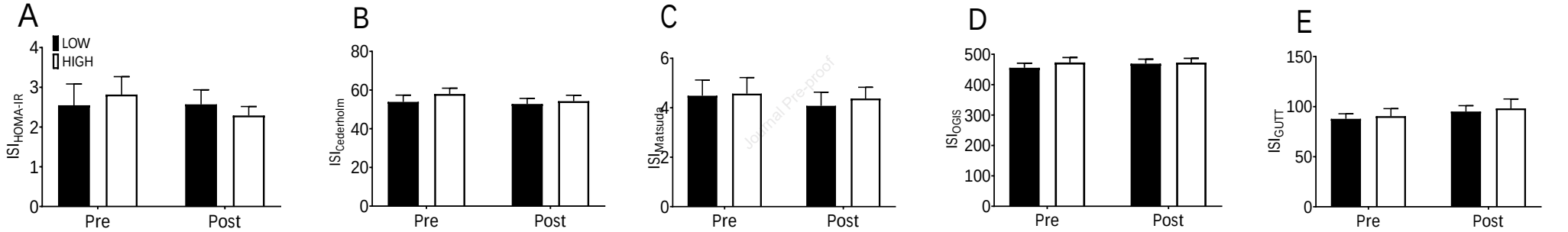
**Figure 5** Relative contribution of fat and carbohydrate oxidation rates to energy expenditure calculated via indirect calorimetry using the Frayn equations, in the fasted and CHO fed states, pre- and post- a one week fully controlled dietary intervention with either a low-nucleotide mycoprotein (LOW) or a high-nucleotide mycoprotein (HIGH). An effect of CHO ingestion was found for both carbohydrate and fat oxidation rates ($P<0.0001$). No interaction or condition effects were found (all $P>0.05$).

**Figure 6** Heat map representing the metabolomics measures which suffered the significant changes between pre- and post- a one week fully controlled dietary intervention with either a low-nucleotide mycoprotein (LOW) or a high-nucleotide mycoprotein (HIGH), as calculated by the $\Delta$ change for each participant. Participants in LOW are represented in green and participants in HIGH are shown in red.








A
LOW
HIGH
ISI HOMA-IR
4
3
2
1
0
Pre
Post
B
ISI Cederholm
80
60
40
20
0
Pre
Post
C
ISI Matsuda
6
4
2
0
Pre
Post
D
ISI OGIS
500
400
300
200
100
0
Pre
Post
E
ISI GUTT
150
100
50
0
Pre
Post