The impact of mycoprotein and dietary nucleotide intake on metabolic health, exercise metabolism and endurance performance in humans

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Abstract

Mycoprotein is a sustainably-produced, fungal-derived dietary protein source and its consumption has been shown to improve acute postprandial glycaemic responses and decrease circulating cholesterol concentrations. However, whether these findings translate to habitual mycoprotein consumption improving physiologically relevant, longer-term changes in insulin sensitivity (IS) and/or glycaemic control has not yet been investigated. Mycoprotein 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), for commercial products to comply with FAO/WHO/UNICEF recommendations limiting the additional dietary nucleic acid load from single-cell protein-rich novel foods to 2 g/day. These recommendations are based on data showing that short term ingestion of high-dose (> 2 g/day) isolated (or yeast-derived) nucleotides results in elevations in circulating uric acid concentrations above clinically acceptable levels (i.e. of > 420 $\mu mol \cdot L^{-1}$). Epidemiological and observational studies have reported that serum uric acid concentrations positively correlate with the development of gout, hypertension and metabolic syndrome, and are a predictor of type 2 diabetes, though causal links remain to be established.

Despite these concerns regarding dietary nucleotides, serum uric acid concentrations and metabolic health, evidence supports an increased dietary requirement for nucleotides during periods of rapid growth (e.g. infancy) and stress, such as in certain disease states. Muscular exercise is one of the most common states of physiological stress and emerging data imply beneficial effects of dietary nucleotide supplementation on exercise performance and recovery. Dietary nucleotide supplementation has been shown to reduce post-exercise

stress hormone response, improve markers of immune health, and result in improvements to muscular strength, force production and time to exhaustion. It has been suggested that dietary nucleotides might positively affect energy production but studies have not yet examined this. Increased production of ATP modulated by dietary nucleotide intake prior to submaximal moderate-intensity exercise might lead to improved exercise performance, potentially by sparing muscle glycogen. Additionally, these potential benefits to energy production might also lead to improved recovery following glycogen depleting exercise. Ribose, a pentose monosaccharide that makes up the structure of nucleotides, has also been proposed as a limiting factor in the production of ATP. As such, dietary ribose (like dietary nucleotide) intake might modulate fuel utilisation and improve energy production and exercise performance in a depleted state, as well as assist skeletal muscle cells in recovery from fatigue; but further research to establish these effects is necessary.

The studies described in this thesis, performed in healthy human volunteers, have focused on two main topics. Firstly, the impact of both mycoprotein and dietary nucleotide intake on markers of metabolic health was assessed. Secondly, the potential for dietary nucleotides and ribose to impact muscle ATP and glycogen concentrations and endurance exercise muscle metabolism, performance and recovery.

The major findings were that substituting meat/fish for mycoprotein twice daily for 1 week did not modulate whole-body IS or glycaemic control but resulted in changes to plasma lipid composition, the latter primarily consisting of a coordinated reduction in circulating cholesterol-containing lipoproteins. The ingestion of a nucleotide-rich mixed meal was found to increase serum uric acid concentrations for ~12 h but did not influence postprandial blood glucose or

serum insulin concentrations. Accordingly, twice-daily consumption of high-nucleotide mycoprotein for one week led to sustained increases in serum uric acid concentrations (above clinically relevant thresholds), but not to any associated deleterious effects in IS, glycaemic control or plasma lipid composition. Finally, the twice-daily ingestion, for 2 weeks, of a nucleotide-rich mycoprotein drink, with or without added ribose, did not influence skeletal muscle ATP content, muscle fuel utilisation during exercise, exercise performance or the metabolic or performance-related recovery from exercise.

The present thesis documents the first investigations into the potential impact of mycoprotein on IS and extends on previous observations of the beneficial effects of mycoprotein intake on blood lipid profile by demonstrating how rapidly these benefits ensue. Further novel findings were that high nucleotide intake, even when incorporated into mixed meals, leads to a cumulative increase in serum uric acid concentrations, which after three to five days become clinically significant. Despite this, our results clearly show measures of IS and glycaemic control remained unaffected. Furthermore, it is described in this thesis the first attempt to increase resting skeletal muscle ATP concentrations in humans using dietary nucleotide and ribose supplementation and to assess their impact in muscle fuel utilisation during fatiguing exercise and after acute recovery. The findings of this thesis have theoretical and practical applications regarding the use of sustainable alternative protein sources on the prevention and treatment of cardiometabolic risk factors and add valuable knowledge to our understanding of the role of dietary nucleotides and ribose in muscle fuel utilisation and exercise performance.

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Declaration

All of the procedures described in this thesis, including venepunctures and cannulations, oral glucose tolerance tests, placement of continuous glucose monitoring systems, diet preparation, exercise tests, blood, urine and skeletal muscle sample preparation, expired gas analysis and biochemical tissue analysis have been performed by myself, with the following exceptions.

The serum and urine uric acid analyses described in Chapters 3, 4, 5 and 6 were carried out at the Clinical Chemistry department of the Royal Devon & Exeter NHS Foundation Trust. Nuclear magnetic resonance (NMR) metabolomics analysis, in Chapters 3 and 4, was performed at the MRC Integrative Epidemiology Unit at the University of Bristol. The quantification of muscle nucleotides (ATP and IMP), phosphocreatine (PCr) and creatine by ion-pair reversed-phase chromatography, in Chapter 6, was carried out by Jamie Blackwell.

Dr Benjamin Wall, Professor Francis Stephens and Dr Marlou Dirks performed the muscle biopsies described in Chapter 6. Fellow PhD students in the Nutritional Physiology research group occasionally performed venepunctures and cannulations, prepared supplements and assisted in the exercise biopsies in Chapter 6.

I hereby declare that the present thesis has been composed by myself and that it is a record of my work, except when help has been acknowledged. No part of this thesis has been submitted in any other application for a higher degree and all sources of information have been referenced.

Mariana de Oliveira Cardoso Coelho, December 2020

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List of publications

Abstracts

Coelho, M., Monteyne, A. J., Dirks, M. L., Finnigan, T. J. A., Stephens, F. B., & Wall, B. T. (2018). Substituting meat/fish for mycoprotein for one week does not affect indices of metabolic health irrespective of dietary nucleotide load or serum uric acid concentrations in healthy young adults. *Proceedings of the Nutrition Society*, *77*(OCE4).

First author papers

Published

Coelho, M. O., Monteyne, A. J., Dunlop, M. V., Harris, H. C., Morrison, D. J., Stephens, F. B., & Wall, B. T. (2019). Mycoprotein as a possible alternative source of dietary protein to support muscle and metabolic health. *Nutrition Reviews*, *78*(6), 486-497.

Coelho, M. O., Monteyne, A. J., Kamalanathan, I. D., Najdanovic-Visak, V., Finnigan, T. J., Stephens, F. B., & Wall, B. T. (2020). Short-Communication: Ingestion of a Nucleotide-Rich Mixed Meal Increases Serum Uric Acid Concentrations but Does Not Affect Postprandial Blood Glucose or Serum Insulin Responses in Young Adults. *Nutrients*, *12*(4), 1115.

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Monteyne, A. J., Coelho, M. O., Porter, C., Abdelrahman, D. R., Jameson, T. S., Jackman, S. R., Blackwell, J. R., Finnigan, T. J., Stephens, F. B., Dirks, M. L., & Wall, B. T. (2020). Mycoprotein ingestion stimulates protein synthesis rates to a greater extent than milk protein in rested and exercised skeletal muscle of healthy young men: a randomized controlled trial. *The American Journal of Clinical Nutrition*.

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Monteyne, A. J., Dunlop, M. V., Machin, D. J., Coelho, M. O., Pavis, G. F., Porter, C., Murton, A. J., Abdelrahman, D. R., Dirks, M. L., Stephens, F. B., & Wall, B.T. (2020) A mycoprotein based high-protein vegan diet supports equivalent daily myofibrillar protein synthesis rates compared with an isonitrogenous omnivorous diet in older adults: a randomized controlled trial. *British Journal of Nutrition*

List of abbreviations

6-PGDH	6-phosphogluconate dehydrogenase
AA	amino acid
ADP	adenosine diphosphate
AGDase	Amyloglucosidase
AMP	adenosine monophosphate
ANOVA	analysis of variance
APO	Apolipoprotein
ATP	adenosine triphosphate
AUC	area under the curve
BMI	body mass index
BMR	basal metabolic rate
BP	blood pressure
CDP	cytidine diphosphate
CON	control group (Chapter 3), control condition (Chapter 6)
CGMS	continuous glucose monitoring system
CHD	coronary heart disease
CHO	carbohydrate
CI	confidence interval
CMP	cytidine monophosphate
CONGA	continuous overlapping net glycaemic action
CONSORT	Consolidated Standards of Reporting Trials
CTP	cytidine triphosphate
CV	coefficient of variance

CVD	cardiovascular disease
DALY	disability-adjusted life-years
DHA	docosahexaenoic acid
DNA	deoxyribonucleic acid
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
EF	extraction factor
ELISA	enzyme-linked immunosorbent assay
EXH	glycogen depleting cycling exercise protocol (Chapter 6)
FAO	Food and Agriculture Organization
FDR	false discovery rate
GLP-1	glucagon-like peptide 1
Glu-6-P	glucose-6-phosphate
Glu-6-PDH	glucose-6-phosphate dehydrogenase
GMP	guanosine monophosphate
H-NU	high nucleotide condition (Chapter 4)
HBA1c	glycated haemoglobin
HCl	hydrochloric acid
HDL	high-density lipoprotein
HEC	hyperinsulinaemic-euglycaemic clamp
HIGH	high nucleotide mycoprotein group (Chapter 5)
HK	hexokinase
HOMA-IR	homeostatic model assessment of insulin resistance
HPLC	high-performance liquid chromatography
iAUC	incremental area under the curve

IDL	intermediate-density lipoprotein
IMP	inosine monophosphate
IPAQ	International Physical Activity Questionnaire
IS	insulin sensitivity
ISI	insulin sensitivity index
ITP	inosine triphosphate
$KHCO_3$	potassium bicarbonate
KH_2PO_4	monopotassium phosphate
L-NU	low nucleotide condition (Chapter 4)
LDL	low-density lipoprotein
LH	lithium heparin
LOW	nucleotide-depleted mycoprotein group (Chapter 5)
MRI	magnetic resonance imaging
MRS	magnetic resonance spectroscopy
MS	mass spectrometry
MS	multiple sclerosis
MSU	monosodium urate
MYC	mycoprotein group (Chapter 3)
NAD^+	nicotinamide adenine dinucleotide (oxidised)
NADH	nicotinamide adenine dinucleotide (reduced)
Na_2HPO_4	disodium phosphate
NaOH	sodium hydroxide
NCD	noncommunicable disease
NCEP: ATP III	National Cholesterol Education Program's Adult Treatment Panel III
NMR	nuclear magnetic resonance

NUC	high nucleotide condition (Chapter 6)
OGIS	oral glucose insulin sensitivity
OGTT	oral glucose tolerance test
PAL	physical activity level
PC	plasma cholesterol
PCA	perchloric acid
PCr	phosphocreatine
PDA	photodiode-array
PERF	work output performance test immediately after glycogen depleting exercise (Chapter 6)
PPP	pentose phosphate pathway
PRPP	5-phosphoribosyl-1-pyrophosphate
PYY	peptide YY / peptide tyrosine tyrosine
R-5-P	ribose-5-phosphate
RDA	recommended dietary allowance
REC	work output performance test after 3-hour acute recovery from glycogen depleting exercise (Chapter 6)
RIB	high nucleotide + ribose condition (Chapter 6)
RM	repeated measures
RMR	resting metabolic rate
RNA	ribonucleic acid
RT	retention time
SAM	significance analysis of microarrays
SCFA	short-chain fatty acid
SD	standard deviation
SEM	standard error of the mean

SGOT	serum glutamic oxaloacetic transaminase
SIgA	salivary immunoglobulin A
TBA	tetrabutylammonium dihydrogen phosphate
TC	total cholesterol
TEA	triethanolamine
TMP	thymidine monophosphate
TTP	thymidine triphosphate
UDP	uridine diphosphate
UMP	uridine monophosphate
UNICEF	United Nations Children's Fund
VLDL	very-low-density lipoprotein
$\dot{V}CO_2$	carbon dioxide production
$\dot{V}O_2$	oxygen consumption
$\dot{V}O_2$ max	maximal oxygen consumption
WC	waist circumference
WHO	World Health Organization

Chapter 1:

General introduction

1.1 Sustainable nutrition and metabolic health

1.1.1 Sustainable nutrition

Developing a nutritionally sustainable future for the world's population, projected to grow from approximately 7.3 billion to more than 9 billion by 2050, is an urgent contemporary issue (1). This projected increase is coupled with global trends of rises in urbanization, social mobility, and wealth creation, all factors expected to exacerbate the global food demand (1). As a result, current and future generations must view developments in the understanding of human nutrition through the lens of mounting challenges associated with the sustainability of increased food production.

The demand to produce sufficient dietary protein for the world's population is driven by demographics and compounded by accumulating scientific data that support protein consumption at levels greater than the currently accepted Recommended Dietary Allowances (RDAs; i.e. 0·75–0·8 $g \cdot kg^{-1}$ per day in the UK and the USA (2, 3)) in various populations. For instance, evidence suggests that muscle mass maintenance in older adults (4-7), the promotion or retention of muscular training adaptations in athletes (8, 9), and successful weight management (10) are all supported by modest increases in dietary protein intake above the current RDAs. It is clear, therefore, that the global need for dietary protein production is a pressing societal issue that is gathering momentum.

The majority of data supporting the refinement of dietary protein requirements has been obtained from studies examining the *in vivo* metabolic handling or adaptive responses to animal-derived protein ingestion (11-13). The carbon, water, and land-use footprints of animal-derived protein production are 8 to 80 times, 50 to 150 times, and 30 to 220 times greater, respectively, than those of

many plant-based proteins (with variations dependent on the protein source and the method of quantification) (14). The mounting data concerning the environmental cost of intensive animal-derived dietary protein production is resulting in shifting social attitudes and government initiatives towards more sustainable sources, with vegan, vegetarian, and flexitarian diets becoming increasingly popular (15). As a result, research that investigates non–animal-derived protein sources (including their nutritional value and metabolic handling), is applicable to a progressively larger demographic, and the impact of such evidence will increase accordingly.

1.1.2 Metabolic health

Noncommunicable diseases (NCDs) are responsible for 41 million deaths yearly, corresponding to 71% of all deaths worldwide (16). Cardiovascular disease (CVD) became the leading cause of death globally in the 20th century. It is estimated that 17.9 million people die from CVD each year, an estimated 31% of all global deaths (16). Along with CVD, cancers, respiratory diseases and diabetes account for most NCD deaths, with these 4 groups of pathologies being implicated in over 80% of all premature NCD deaths (16). Aside from being a pressing health issue, NCDs are also a significant financial burden and disproportionally affect vulnerable and socially disadvantaged people. Global health financing has steadily increased over the past two decades and is projected to continue to increase in the future and to display persistent discrepancies between countries (17).

Cardiometabolic risk factors predict the development of cardiovascular and metabolic disease. According to the World Health Organization (WHO), there are four key cardiometabolic risk factors which contribute to the development of

NCDs (18): elevated blood pressure, overweight and obesity, hyperglycaemia and hyperlipidaemia. These four factors are the essential components of what is called the metabolic syndrome, as agreed upon by the WHO, the National Cholesterol Education Program's Adult Treatment Panel III (NCEP: ATP III) and the European Group for the Study of Insulin Resistance (however all have different specific definitions) (19, 20). This concept of metabolic syndrome has been evolving for nearly 100 years, having first been described by Kylin, a Swedish physician, in 1923 (21). These metabolic impairments tend to cluster and interact in an individual to determine the level of risk of developing multifactorial NCDs, such as CVD, cancer and diabetes. Due to the rising prevalence of NCDs, it has become increasingly necessary to quantify cardiometabolic risk factors and to develop high-resolution methods which allow for accurate and detailed measures.

Guidelines for the diagnosis and treatment of blood pressure are frequently revised (22) and there has been a shift in focus from measuring total body fat to measuring intra-abdominal, or visceral, adipose tissue, as this type of obesity has been associated with a substantially higher risk for diabetes and CVD (23). Additionally, defining hyperglycaemia and hyperlipidaemia is a complex challenge. The hyperinsulinemic-euglycemic clamp (HEC) is considered the *gold standard* for the measurement of insulin sensitivity/resistance, but it is a costly, time consuming and invasive method (24). As such, a variety of surrogate measures have been utilised for this purpose, such as insulin sensitivity indices, derived from fasting glucose and insulin or from oral glucose tolerance tests (OGTT), postprandial glucose handling and 24-hour glucose excursions measured using continuous glucose monitoring systems (CGMS). Finally, hyperlipidaemia generally refers to the measurement of blood triglycerides and

total cholesterol, as well as of HDL and LDL cholesterol, and of the ratio between these two types of cholesterol (colloquially referred to as "good" and "bad" cholesterol, respectively). However, it is now possible to obtain more detailed analyses of the complete blood lipid profile. Nuclear magnetic resonance (NMR) spectrometry is a novel methodology which has emerged in the past two decades as one of the principal analytical techniques used in metabolomics (25). NMR spectrometry has the ability to quantify metabolite levels, is highly reproducible and, most importantly, has been identified as a promising method for lipidomics study (25, 26). Methodological advances will no doubt continue to improve our current ability to identify and modulate cardiometabolic risk factors in order to achieve more beneficial health outcomes.

The relationship between dietary habits, cardiometabolic risk and chronic NCDs is well established and improving global dietary habits could potentially prevent one in every five deaths (27). In 2017, 11 million deaths and 255 million disability-adjusted life-years (DALYs) were attributable to dietary risk factors (27). This data, derived from epidemiological studies, does not clarify the mechanisms by which dietary factors can have deleterious effects on metabolic health and intervention studies are required to disentangle the impact of high energy consumption and the individual health effects of a variety of foods and nutrients.

Energy-dense diets, often combined with a sedentary lifestyle, have contributed to a drastic increase in the prevalence of obesity, insulin resistance and metabolic syndrome, especially in developed countries. The growing obesity epidemic, along with its associated health complications (28), requires nutritional approaches to induce and sustain weight loss. Although weight loss via caloric restriction under laboratory conditions is relatively straightforward to achieve (29), it tends to be more difficult under free-living conditions. Furthermore, subsequent

weight regain appears to be the major barrier to longer-term weight management (30). The primary reasons for these difficulties include a lack of satiety while maintaining an energy deficit (31) and a decline in the basal metabolic rate as a result of loss of muscle mass (32, 33). Diets relatively high in protein (generally those diets in which absolute protein intake is simply maintained while an energy deficit is created by restricting carbohydrates or fats) have been suggested as a potential solution to these issues (10, 30). For instance, when volunteers are subjected to ad libitum weight loss diets (i.e. more representative of free-living attempts at weight loss), those consuming diets higher in protein generally lose body mass and maintain this loss more effectively than those on lower-protein diets (34, 35). This seems primarily attributable to the satiating effects of protein ingestion, which results in overall lower energy intake (34) since isoenergetically controlled weight-loss interventions show equivalent weight loss irrespective of protein content (34, 35). It is also true that higher-protein diets increase overall daily energy expenditure as a result of enhanced diet-induced thermogenesis and energy expenditure during sleep (36, 37). Furthermore, during isoenergetically controlled weight-loss studies, it has typically been shown that higher-protein diets increase the ratio of fat mass loss to lean mass loss, which constitutes overall body weight loss (38). The effects of dietary protein intake during weight loss on satiety, daily energy expenditure, and lean mass retention, underline the importance of ensuring the consumption of enough dietary protein during periods of caloric restriction, as well as when maintaining energy balance, to optimise metabolic health (10, 30, 34).

Animal products are high in protein, however, they are also relatively energy-dense, higher in salt and saturated fat and low in fibre and, as such, might be expected to contribute to metabolic disease. High intakes of meat, particularly

processed meat (and, to a lesser degree, unprocessed red meat) are associated with an increased risk of CVD and type 2 diabetes, potentially due to higher consumption of saturated fat, dietary cholesterol, sodium and nitrates/nitrites (39). In 2017, the global intake of red and processed meats was 18 and 90 % greater, respectively, than the "optimal" intake (27). As such, a potential detrimental effect of excessive intake of red and, especially, processed meats, to metabolic health is now a major public health concern (40), but mechanistic evidence supporting deleterious effects in humans is still lacking.

Sustainable and health-promoting alternatives to animal-based protein sources could be instrumental in maintaining energy balance and metabolic health, not at the expense of adequate protein intakes. It is worth noting that plant-based diets are associated with reduced incidence of obesity and, consequently, with a decreased risk of developing type 2 diabetes and metabolic syndrome. Plant-based diets are also associated with an increased intake of fibre and a decreased intake of saturated, trans, arachidonic, and docosahexaenoic fatty acids (41). These differences are interesting from the point of view of the aetiology of NCDs and explain a potential protective role of plant-based diets in metabolic health, which is independent of differences in energy intake. As such, alternative non-animal protein sources might play a crucial role not only in ensuring the sustainability of our food systems, but also in supporting energy balanced diets, whilst ensuring appropriate dietary protein intake levels. Dietary interventions assessing the impact of alternative protein sources on cardiometabolic risk factors are needed to provide scientific evidence of the potential beneficial effects of these alternative protein sources.

1.2 Mycoprotein, dietary nucleotides and metabolic health

1.2.1 Mycoprotein

Following a United Nations prediction of increased population growth and world famine by the 1980s, a search for microorganisms which could be developed as a primary high-protein food source began in 1967, led by the Rank Hovis McDougall (RHM) group (42). Over 3000 soil samples were collected around the world. Mycoprotein was first found in one of these samples, collected from a field in Marlow, Buckinghamshire. Initially denominated *Fusarium graminearum*, it was later reclassified as *Fusarium venenatum* (43).

Mycoprotein is a whole food source produced by continuous flow fermentation of the filamentous fungus *Fusarium venenatum* (a detailed description of the production processes is provided by Finnigan (42)). Strictly defined temperature, pH, nutrient concentration, dissolved oxygen and growth rate conditions need to be maintained constant for the production of mycoprotein (44). The resultant product is a high-protein, high-fibre, and relatively low-energy complete food ingredient (**Table 1.1**) that is textured (via freezing) and flavoured into a variety of products under the trade name Quorn (Marlow Foods; Stokesley, North Yorkshire, UK). The manufacturing of these products utilises 10 times less water and one eighth the amount of land when compared with the production of beef, and their carbon footprint is up to thirteen times lower than beef and up to four times lower than chicken (45, 46). Thus, the sustainability credentials of mycoprotein production position this protein as an attractive alternative protein source to temper environmental concerns associated with increased production of dietary protein (46, 47).

Following the development of mycoprotein in the 1960s, initial experiments in humans during the 1970s established the basic feasibility, tolerability, and metabolic impact of mycoprotein consumption, after which mycoprotein became commercially available in 1985. By the end of the 1990s, once the basic understanding that mycoprotein was safe and did not lead to unfavourable health outcomes had been established, this research in humans began to wane. A complete list and summary of published human mycoprotein studies performed to date is shown in **Table 1.2**. In recent years, however, demographical changes, increased incidence of NCDs and environmental challenges, coupled with the sustainability credentials and potential applications of mycoprotein for metabolic health have reignited interest in this novel food source.

Table 1.1 **Nutritional content of mycoprotein, commercially available protein isolates, Quorn vegan pieces, and a selection of commonly consumed protein sources***

Protein source	Nutrient composition per 100 g						
	Protein (g)	Fat (g)	Carbohydrate (g)	Fibre (g)	Energy (kcal)	Energy (kJ)	Leucine (g)
Mycoprotein (dry weight)	45	13	10	25	340	1423	3.9
Whey protein	80	7	5	< 0.1	402	1682	8.6
Milk protein	80	1	6	< 0.1	350	1464	7.0
Quorn pieces	15	3	4	5	113	473	1.2
Whole egg, raw	13	10	< 1	<1	143	598	1.1
Beef mince (5%), raw	21	5	0	0	137	573	1.7
Chicken meat, raw	21	3	0	0	119	498	1.6
Cod meat, raw	18	1	0	0	82	343	1.4

* Data adapted from the internal analyses of Dunlop et al (2017) (48), from Gorissen et al (2018) (49), and from Food Data Central (50)

Table 1.2 **Human studies investigating the metabolic effects of mycoprotein**

Reference	No. of participants	Participants	Type of study	Type of intervention	Duration of intervention	Study findings
Udall et al (1984) (51)	100	Healthy adults	Double-blind crossover trial	Mycoprotein-based cookie supplementation (20 g dw per day) vs control cookies	30 d	6.9% ↓ in PC No changes in BW or other blood markers (glucose, urea, nitrogen, sodium, potassium, calcium, phosphorus, uric acid, creatinine, lactic acid dehydrogenase, alkaline phosphatase, amylase, SGOT, total protein, albumin, TGs, CBC) No changes in urine markers (pH, glucose, protein, ketones, white and red blood cells)
Turnbull et al (1990) (52)	17 (9 mycoprotein, 8 control)	Healthy adults with total cholesterol between 5.2 and 6.2 $mmol{\cdot}L^{-1}$	Randomised controlled parallel-group trial	Mycoprotein (≈ 191 g Quorn per day) vs meat during a fully controlled diet	3 wk	13% ↓ in PC 9% ↓ in plasma LDL-C (12% ↑ in control group) 12% ↑ in plasma HDL-C (11% ↓ in control group) 53% ↓ in TGs (in both groups) No differences in BW or BP No changes in fasting insulin and glucose No changes in Apo A-I or Apo B

Turnbull et al (1992) (53)	21 (11 mycoprotein, 10 control)	Healthy adults with total cholesterol > 5.2 mmol·L^{-1}	Blinded randomised controlled parallel-group trial	Mycoprotein-based cookie supplementation (26.9 g dw per day) vs control cookies	8 wk	7.9% ↓ in PC 12.6% ↓ in plasma LDL-C No changes in plasma HDL-C or TGs No changes in Apo A-I or Apo B No differences in BW
Turnbull et al (1993) (54)	13	Healthy females (non-restrained eaters)	Randomised controlled crossover trial	Energy-matched mycoprotein-based meal vs chicken-based meal	2 d	24% ↓ in 24-h energy intake on day of meal 16.5% ↓ in 24-h energy intake on day after meal ↓ in prospective food consumption and desire to eat 3 h after meal
Burley et al (1993) (55)	18	Healthy adults	Randomised controlled crossover trial	Energy-matched mycoprotein-based meal vs chicken-based meal	2 d	18% ↓ in energy intake in subsequent meal ↓ in 24-h energy intake on day of meal (resulting from no compensation after reduction in subsequent meal) No differences in 24-h energy intake on day after meal No overall differences in eating rate or motivation to eat. Significant ↓ in hunger 4 h after meal

Nakamura et al (1994) (56)	15	Healthy males	Randomised parallel-group trial	Mycoprotein-based cookies/crisps supplementation (18 g or 24 g dw per day)	8 wk	4.3% ↓ in PC in 24-g mycoprotein group
Ishikawa (1995) (57)	37	Hypercholesteremic patients, with total cholesterol > 220 mg·dL^{-1}	Double-blind randomised controlled parallel-group trial	Mycoprotein-based cookie supplementation (12 g or 24 g dw per day) vs control cookies	4 wk	↓ in PC
Homma et al (1995) (58)	52	Healthy males	Randomised crossover trial	Mycoprotein-based crisps supplementation (18 g or 24 g dw per day)	4 wk	6.7% ↓ in PC in 24-g mycoprotein group
Turnbull & Ward (1995) (59)	19	Healthy adults	Double-blind randomised controlled crossover trial	Mycoprotein-based milkshake (20 g dw) vs control milkshake	120 min	↓ in glycemia (13% at 60 min) ↓ in insulinemia (19% at 30 min and 36% at 60 min)
Williamson et al (2006) (60)	42	Overweight premenopausal women	Randomised controlled crossover trial	Mycoprotein-based preload meal vs tofu or	1 d	12.3% ↓ in energy intake at lunch 20 min after mycoprotein preload

				chicken-based preload meals before lunch		when compared with chicken preload No difference in intake at dinner (no compensation) No differences in subjective ratings of hunger or satiety
Ruxton & McMillan (2010) (61)	31 (21 mycoprotein, 10 control)	Healthy adults	Controlled parallel-group trial	Mycoprotein-based diet (≥ 88 g ww per day; 21 g dw per day) vs animal-based diet	6 wk	↓ in PC in individuals with baseline cholesterol ≥ 4.19 $mmol{\cdot}L^{-1}$ No changes in TC, LDL-C, HDL-C, TGs, glucose, BP, BMI, or WC for sample as a whole
Bottin et al (2016) (62)	Part A: 36 Part B: 14	Overweight and obese adults	Single-blind randomised controlled crossover trial	Part A: Energy-matched mycoprotein-based preload meal (44 g, 88 g, or 132 g ww) vs chicken-based meal (equivalent amount of chicken and macronutrient matched at each protein content)	180 min	10% ↓ in energy intake at lunch after high mycoprotein preload when compared with high chicken preload 9% ↓ in 24-h energy intake following mycoprotein ingestion 8%, 12%, and 21% ↓ in insulin iAUC after low, medium, and high mycoprotein preload, respectively 21% and 16% ↓ in insulinogenic and disposition indices, respectively, following mycoprotein ingestion 9% ↑ in Matsuda Index following mycoprotein ingestion

				Part B: Macronutrient-matched mycoprotein-based meal (132 g ww) vs chicken-based meal		No differences in appetite ratings No differences in postprandial glucose concentrations No differences in plasma GLP-1 or PYY No differences in gastric emptying No differences in resting energy expenditure and substrate utilization
Dunlop et al (2017) (48)	12	Healthy men	Single-blind randomised controlled crossover trial	Mycoprotein-based drinks (20 g, 40 g, 60 g, and 80 g dw) vs milk protein drink	240 min	Equivalent postprandial AA bioavailability between protein-matched amounts of mycoprotein and milk protein. Slower but more sustained hyperinsulinemia and hyperaminoacidaemia compared with milk when protein matched. Dose-response effects on all parameters until 60–80 g of mycoprotein consumed

Abbreviations and symbols: AA, amino acid; APO, apolipoprotein; BMI, body mass index; BP, blood pressure; GLP-1, glucagon-like peptide 1; HDL-C, high-density lipoprotein cholesterol; iAUC, incremental area under the curve; LDL-C, low-density lipoprotein cholesterol; PC, plasma cholesterol; PYY, peptide YY/peptide tyrosine tyrosine; SGOT, serum glutamic oxaloacetic transaminase; TC, total cholesterol; WC, waist circumference; ↑, increase; ↓, decrease.

1.2.2 Mycoprotein and metabolic health

Myself and colleagues have conducted and published a review on mycoprotein as a possible alternative source of dietary protein to support muscle and metabolic health (63). Research into the effects of mycoprotein in health started in the 1980s when, during an initial human investigation establishing the tolerability of mycoprotein, an interesting ancillary observation was made (51). Human volunteers who consumed 20 g of mycoprotein (dry weight) per day for 30 days, in the form of supplemental cookies, showed an approximately 7% decrease in blood cholesterol concentrations (from 4.86 to 4.53 $mmol{\cdot}L^{-1}$) (51), which replicated earlier findings in animals (64-66). Follow-up studies focusing on mycoprotein consumption and cardiometabolic health further investigated the impact of its intake in blood lipid profile and were able to confirm and add to this initial observation (52, 53, 56-58). Turnbull et al (52) performed a 3-week dietary intervention study in which participants consumed 191 g of mycoprotein-containing products (~ 40 g of mycoprotein dry weight) per day as part of a fully controlled and laboratory-supervised diet aimed at maintaining energy balance in individuals with mildly elevated blood cholesterol concentrations. The mycoprotein intervention resulted in reduced total cholesterol (from 5.54 to 4.81 $mmol{\cdot}L^{-1}$; 13% decrease) and low-density lipoprotein cholesterol (LDL-C) (from 4.16 to 3.78 $mmol{\cdot}L^{-1}$; 9% decrease) and increased high-density lipoprotein cholesterol (HDL-C) (from 0.58 to 0.65 $mmol{\cdot}L^{-1}$; 12% increase). These results were especially striking, considering the control group generally showed opposite responses (instead of simply no change). In their follow-up work, Turnbull et al (53) reported the effects of mycoprotein consumption in free-living individuals (a 0.95 $mmol{\cdot}L^{-1}$, or a 16% reduction, and a 0.34 $mmol{\cdot}L^{-1}$, or 21% reduction, in total cholesterol and LDL-C, respectively) to be similar to those observed previously,

even though overall energy, macronutrients, and fibre (around 6 g) intake remained the same across groups. Three additional studies, all conducted in Japan, confirmed this cholesterol-lowering effect of supplemental mycoprotein (12 to 24 g of mycoprotein dry weight per day in free-living conditions), although to a smaller degree (4 – 7 % reduction in total cholesterol) (56-58).

The beneficial metabolic effects of mycoprotein consumption have also been shown to extend to acute postprandial glycaemic control (59, 62). It was reported that 20 g of mycoprotein (dry weight) consumed during an OGTT resulted in reduced postprandial glycaemia and insulinaemia compared with an isonitrogenous, isoenergetic control condition (soy and skimmed milk) in healthy, young adults (59). A recent study in overweight adults reported that mycoprotein consumption (~ 40 g, dry weight), compared with an energy- and macronutrient-matched chicken meal, reduced postprandial insulinaemia, but not glycaemia (62).

Finally, mycoprotein has been shown to induce an acute thermogenic response (similar to that seen following the ingestion of other (animal) protein sources) (48), as well as to increase satiety (54, 55, 60), effects which, coupled with its low energy density, highlight mycoprotein's potential in supporting weight management. Turnbull et al (54) demonstrated that consumption of a mycoprotein meal resulted in acute appetite suppression and a subsequent reduction in ad libitum food consumption for the remainder of the day (by 24%) and for the following day (by 17%) when compared with an isoenergetic and isonitrogenous chicken meal. Similar findings were reproduced by Burley et al (55) and Williamson et al (60) when study participants consumed approximately 30 g and 10 g (dry weight) of mycoprotein, respectively. The effects of mycoprotein on satiety and thermogenesis, along with the high-protein/low-

energy content of mycoprotein, position this food source as an intriguing approach to support a diet (under ad libitum conditions) aimed at weight loss or maintenance. Well-controlled, longer-term laboratory weight loss studies comparing mycoprotein with other protein sources are warranted. It is important to note, however, that the aforementioned effects of mycoprotein in blood lipid profile and postprandial glycaemic control were observed in energy balanced conditions, which raises the question of which mechanisms led to these beneficial metabolic effects. My colleagues and I have argued that the cholesterol-lowering effect of mycoprotein consumption, as well as its impact on postprandial glycaemic control, is probably related to the fibre content (or type) it contains (63).

1.2.3 Fibre in mycoprotein and metabolic health

Following the findings by Udall et al (51), early mycoprotein research was focused on the potential health effects of the dietary fibre in mycoprotein. This stemmed from a body of epidemiological studies showing that higher fibre intakes (typically from fruit, vegetables, and cereals) are associated with reduced blood cholesterol concentrations, improved blood lipid profiles, and reduced incidence of myocardial infarction and coronary heart disease (67-70). Such findings have been confirmed by intervention studies in which increased consumption of dietary fibre has been reported to improve peripheral insulin sensitivity and to lower blood cholesterol concentrations and glycated haemoglobin (HBA1c) in both healthy individuals and patients with type 2 diabetes (71, 72). Whilst the design of the first study by Turnbull et al (52) did not explore the mechanisms underpinning the observed cholesterol-reducing effects of mycoprotein, given that the lipid composition and the energy, macronutrient, and cholesterol contents of the diets

were similar across groups, it was assumed that the fibre content was responsible for the changes in blood lipids.

The increased dietary fibre content could have conceivably exerted its cholesterol-lowering effect by altering LDL-C synthesis/degradation or cholesterol clearance in peripheral tissues or by increasing the binding of fibre to neutral sterols, cholesterol, or bile acids in the intestine, resulting in a decreased amount of cholesterol entering the circulating pool. However, it is noteworthy that the beneficial effects of higher-fibre diets on circulating cholesterol concentrations do not always extend to improvements in the specific lipid sub-fractions of LDL-C and HDL-C (73). It is thus interesting to ponder whether the *type*, rather than simply the amount, of dietary fibre contained within mycoprotein may, at least in part, explain the beneficial effects of mycoprotein consumption on circulating cholesterol concentrations.

Dietary fibre in mycoprotein is composed of two-thirds β-glucan and one-third chitin, which together form a fibrous insoluble matrix that is relatively rare in more traditional food sources. Recent *in vitro* investigations have dug deeper mechanistically and are beginning to shed light on potential mechanisms by which the specific fibre profile of mycoprotein may affect the gut microbiota to bring about these cholesterol-lowering effects in humans. Upon entering the large intestine, dietary fibres become available for fermentation by the gut microbiota (74). Fermentation of dietary fibres leads to the production of short-chain fatty acids (SCFA), primarily acetate, propionate, and butyrate, in a molar ratio of approximately 60:20:20 (75). Production of SCFA, and propionate, in particular, has been shown to reduce hepatic cholesterol synthesis via inhibition of b-hydroxy-b-methylglutaryl coenzyme A reductase (the rate-limiting enzyme in cholesterol synthesis) (76) and suppress adipose tissue lipolysis (77). In human

studies using inulin propionate ester, which delivers propionate directly to the large intestine, propionate has been demonstrated to reduce LDL-C and improve liver function and insulin sensitivity (78, 79). However, current evidence shows the effect of propionate to be inconsistent, with another study suggesting that propionate consumption leads to insulin resistance and compensatory hyperinsulinemia (80). *In vitro* colonic models have shown that mycoprotein and its purified dietary fibre are fermentable, capable of producing SCFA (81). Both mycoprotein and purified mycoprotein dietary fibre produce increased propionate and butyrate at the expense of acetate, and increasing colonic propionate production inhibits the incorporation of plasma acetate into cholesterol (82). Consequently, available data now suggest that the digestive and metabolic properties of the unique fibre profile present in mycoprotein warrant future (*in vivo*) research.

Regarding the impact of mycoprotein intake on postprandial glycaemic handling, the causative mechanism is likely linked to the amount (4 g and 7 g) and type of fibre contained in mycoprotein in these 2 studies (59, 62), as viscous polysaccharides can reduce postprandial glycaemia and insulinaemia (83), and 5 g of β-glucan has previously been shown to modulate glycaemia and insulinaemia when consumed with a high carbohydrate load (84). Though the chitin-glucan matrix is insoluble and not viscous, chitin is likely to undergo alkaline deacetylation to produce the viscous polysaccharide chitosan at some point in the gastrointestinal tract. In turn, this may confer resistance to the flow induced by gastrointestinal motility, reducing contact time in the small intestine and resulting in slower gastric emptying and, consequently, nutrient absorption (85). Irrespective of the mechanism, no data are yet available to confirm whether these acute effects on postprandial glycaemia extend to robust changes in insulin

sensitivity or habitual glycaemic control when mycoprotein is incorporated into the daily diet.

1.2.4 Mycoprotein and dietary nucleotides

Nucleotides are a group of molecules comprising a nitrogenous base (either purine or pyrimidine), a five-carbon sugar and one or more phosphate groups, and used widely within human metabolism (e.g. as the constituent molecules of nucleic acids and in the structure of energy-rich molecules, such as ATP) (86). Nucleotides are a non-essential nutrient, as they can be obtained endogenously by *de novo* synthesis or from salvage pathways (less costly, hence derive ~ 90 % of free nucleotides; production and catabolism of purines are relatively constant between 300 and 400 mg per day) (87), but are also obtained exogenously from dietary intake (widely variable, but generally 0.1 - 2 g/day) (88). Nucleotides are present in moderately high concentrations in meat, fish and shellfish, but especially in animal internal organs, such as in liver, brain and kidneys (89). Nucleotides are also present in yeast, such as baker's yeast and brewer's yeast, and, consequently, in fermented drinks, such as beer.

When mycoprotein is produced, its fungal biomass is naturally rich in RNA (and thus, RNA-derived nucleotides), which must be reduced to meet required safety standards (90). This reduction is achieved by subjecting the culture broth to a short heat treatment (68 ºC, 20 minutes), which allows for RNA breakdown by endogenous enzymes, whilst minimising the loss of biomass (30-33 % loss) and retaining proteins (91). The total nucleotide content of the final mycoprotein paste – which is then used as a raw material for the production of a variety of food products – is reduced to approximately a fifth of what it was initially (from ~ 10%

to ~ 2% of mycoprotein dry weight), a load similar to what is provided by meat products.

1.2.5 Dietary nucleotides, uric acid and metabolic health

When consumed in the diet, nucleic acids (RNA and DNA) are broken down and absorbed, in the form of the aforementioned nitrogenous bases, pentose sugars and phosphate ions, by the action of the enzymes ribonuclease/deoxyribonuclease, phosphatase, and nucleosidase (**Figure 1.1**)

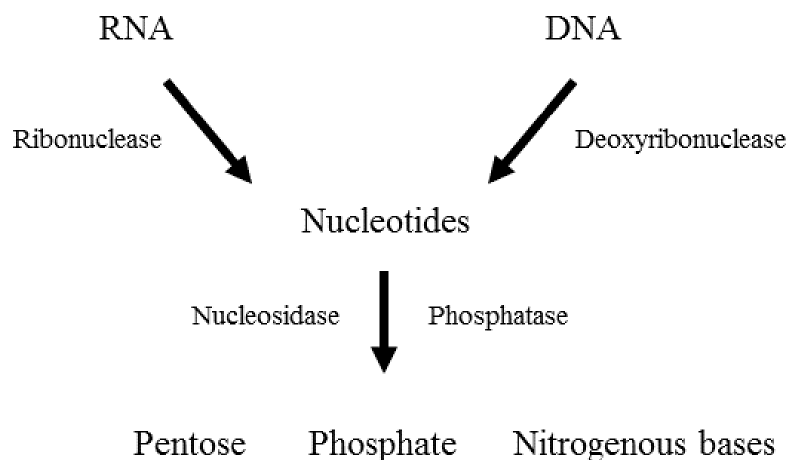


Figure 1.1 **RNA and DNA digestive pathways**

In humans, the end products of pyrimidine catabolism are β-amino acids, ammonia and carbon dioxide, and the main metabolic end product of dietary purine nucleotides is uric acid. Uric acid is formed in the liver, intestines and other tissues, such as the muscle (92). In addition to being formed from dietary purines, nucleic acids from live and dying cells are also degraded into uric acid. Uric acid is transported in the blood to the kidneys and excreted in urine (93). Unlike other mammals, humans and apes lack the liver enzyme uricase, which catalyses the

oxidation of uric acid to allantoin. Allantoin is a diureide of glyoxylic acid and it is more soluble than uric acid. In most animals, allantoin is the final product of purine nucleotide metabolism, being excreted in urine. In humans, the lack of uricase coupled with other factors, such as high dietary purine nucleotide intake, renal under-excretion of uric acid, conditions of excessive cell and purine turnover, and/or genetic factors that result in uric acid overproduction might lead to elevated blood uric acid concentrations, or hyperuricaemia (94). Based on reference ranges (i.e. the predicted interval including 95% of values of a reference group), most countries define normal serum uric acid concentrations as 3.5 to 7.2 $mg{\cdot}dL^{-1}$ (~ 210 to 430 $\mu mol{\cdot}L^{-1}$) in adult males and postmenopausal women and 2.6 to 6.0 $mg{\cdot}dL^{-1}$ (~ 160 to 360 $\mu mol{\cdot}L^{-1}$) in premenopausal women (95).

Serum uric acid is filtered in the kidneys' glomeruli, and approximately 90% of filtered uric acid is reabsorbed, implying a considerable physiological role (87). Uric acid is a strong reactive oxygen species (ROS), peroxynitrite scavanger and antioxidant, which contributes to about half of the antioxidant capacity of human plasma (92, 96, 97). There are high concentrations of uric acid in the cytosol of human cells, namely in the liver, as well as in vascular endothelial cells and in nasal secretions, where it performs antioxidant functions (98). It has been hypothesised that these antioxidant properties of serum uric acid might be associated with protective effects in immune and neurological functions.

A near null prevalence of multiple sclerosis (MS) has been noted in individuals with hyperuricaemia (99). This is likely due to higher uric acid levels blocking the production of peroxynitrite, believed to be responsible for myelin degradation in MS. Conversely, there is a strong association of low serum uric acid concentrations with increased incidence of MS and raising uric acid levels has

been suggested as a potential treatment for MS (100, 101). Low serum uric acid concentrations have also been associated with Parkinson's disease. A recent systematic review reports that patients with Parkinson's disease display significantly lower serum uric acid concentrations and suggests that manipulating serum uric acid might be a viable strategy to adopt in the treatment and prevention of Parkinson's disease (102). It is important to note, however, that both MS and Parkinson's disease are complex disorders and that simply manipulating serum uric acid concentrations might be insufficient by itself, yet might still contribute to slow deterioration and prevent relapses and exacerbations. Uric acid might have a further therapeutic role in ischemic stroke. Increased serum uric acid concentrations have been found to be associated with good clinical outcomes following acute ischemic stroke (103). Building on this finding, several preclinical and clinical studies support a promising beneficial effect of uric acid therapy in ischemic stroke (104-106). This might be particularly true in patients treated with mechanical thrombectomy (107, 108). In humans, uric acid therapy has been shown to prevent early ischemic worsening after acute stroke in thrombolysed patients (106) and to reduce infarct growth in women with acute ischemic stroke treated with alteplase (105). Finally, studies have also investigated the effects of serum uric acid concentrations in later life cognition, however here the results have been mixed, with studies reporting higher uric acid concentrations being associated with both better and worse cognition outcomes, as well as both faster and no changes in cognitive decline (109-112).

In addition to these protective effects in neurological and autoimmune disorders, serum uric acid might also be associated with benefits in immunity from blood-borne pathogens and in endothelial function. Uric acid is involved in the development of type 2 immune responses, triggered by macroscopic parasites

(113), and stimulates the synthesis of arachidonic acid, which has been shown to be an effective schistosomicide in *in vitro*, animal and human studies (114-117). Furthermore, there are suggestions that uric acid might play a critical role in tissue healing as a scavenger of oxygen free radicals, a mobiliser of progenitor endothelial cells and a supporter of the adaptive immune system (118). Interestingly, a recent study involving patients with hypouricemia due to SLC22A12/URAT1 loss-of-function mutations (which encodes blood vessels and kidney proximal tubular cells transporter URAT1) has reported that extremely low levels of serum uric acid lead to endothelial dysfunction *in vivo* (119). This stands in opposition to a theory that uric acid causes endothelial and/or kidney disease by impairing endothelial function.

Despite all of these potential antioxidant, and thus anti-inflammatory, benefits, uric acid can be itself an agent of inflammation. When the plasma concentration of uric acid exceeds its solubility (around 7 $mg \cdot dL^{-1}$, or 420 $\mu mol \cdot L^{-1}$), monosodium urate (MSU) crystallises (120). MSU crystals deposit in the body, namely in the joints and soft tissues, and elicit an inflammatory response. Gout is the complex form of arthritis resulting from the inflammatory reaction of joint tissues when MSU crystals form within the joint (121). In addition to this role in the aetiology of gout (122), epidemiological and observational data have reported that circulating uric acid concentrations positively correlate with the development of hypertension (123) and metabolic syndrome (124), and are a predictor of type 2 diabetes (125), though causation (particularly by diet) has not been established. It is theorised that some of uric acid's properties, which might have favoured survival in periods of famine or stress, play a role in the pathophysiology of cardiometabolic diseases. Uric acid can help to preserve sodium retention and blood pressure (126), and might contribute to the development of insulin resistance and mild

obesity, through direct effects on the adipocytes, as well as through interfering with insulin's actions by reducing endothelial nitric oxide (127-129). Despite having antioxidant properties in the extracellular environment, *in vitro*, *ex vivo* and animal studies have shown that uric acid is pro-oxidant in the intracellular environment. Hence, uric acid might contribute to metabolic dysfunction by stimulating intracellular oxidative stress and reducing nitric oxide (130). Clearly, uric acid is a molecule involved in a variety of metabolic pathways with complex biological roles, which cannot be simply described as beneficial or harmful and are intricately shaped by their environment, physiological systems and concentrations.

It was recognised at the beginning of the 20th century that feeding (purine nucleotide rich) yeast resulted in increased urinary uric acid excretion. Later, in the late 60s, a few studies demonstrated that human consumption of large boluses (>2 g/day) of isolated or (single-cell) yeast-derived nucleotides would result in acute increases in circulating uric acid concentrations (131-133). However, the impact of dietary nucleotide induced elevated uric acid concentrations in other markers of metabolic health remains to be investigated. Nevertheless, these findings informed the FAO/WHO/UNICEF (Food and Agriculture Organization/ World Health Organization/United Nations Children's Fund). Protein Advisory Group recommendation to limit the additional dietary nucleic acid load from single cell protein novel foods to no more than 2 g/day (134, 135). How this translates to practical nutrition is unclear, since the ingestion of high nucleotide mixed-meals upon circulating uric acid concentrations has not been investigated. Moreover, whether elevations of serum uric acid influence, and therefore may be causatively linked to, markers of metabolic health, such as, acutely, postprandial glucose and insulin responses, or more chronic effects in

insulin sensitivity and lipid profile, is also unknown. Considering the protective potential of elevated serum uric acid concentrations in infectious, autoimmune and neurological disorders, it becomes particularly important to clarify its role in the development of metabolic disease.

The previously mentioned RNA/nucleotide depleting heat treatment which mycoprotein is subjected to during its production ensures compliance with the FAO/WHO/UNICEF Protein Advisory Group dietary nucleotides recommendations. Dunlop et al (48) demonstrated the effectiveness of this process. The ingestion of nucleotide-depleted mycoprotein up to quantities of 40 g did not influence postprandial circulating uric acid concentrations (48), with even 60 g causing only a transient (~2 h) and modest (from ~350 to ~380 $\mu mol \cdot L^{-1}$; well below clinically relevant thresholds of >420 $\mu mol \cdot L^{-1}$ (95, 136)) rise, and in line with the consumption of other protein sources (137). However, it is not known what impact the ingestion of non-nucleotide depleted mycoprotein has upon the postprandial serum uric acid response, insulin sensitivity and other markers of metabolic health.

1.3 Dietary nucleotides, ribose and muscle fuel utilisation

1.3.1 ATP and skeletal muscle fuel utilisation

Energy for all types of biological functions, including exercise, is provided chemically by adenosine triphosphate (ATP). In skeletal muscle, contraction is sustained by the hydrolysis of ATP by myosin ATPase (138). The energy released during ATP hydrolysis allows myosin to enter its activated "cocked" state and induces the formation of the cross-bridge with actin. Concomitantly, ATP is also required by the sarcoplasmic reticulum for the active reuptake of calcium ions, which restores the tropomyosin inhibition of cross-bridge formation, permitting subsequent cross-bridge formation and the continuation of muscle contraction (138). Resting intramuscular stores of ATP, used as an immediate source of energy, are small and can be depleted by maximal exercise in approximately 2 seconds, but a plethora of metabolic pathways drive the regeneration of ATP, promoting the restoration of intramuscular levels (139).

Human skeletal muscle can generate energy anaerobically (i.e. contract without using oxygen) using the phosphagen system or the glycolytic system (138). The phosphagen system relies on the limited intramuscular stores of phosphocreatine (PCr), which can be broken down enzymatically to creatine and phosphate, then transferred to ADP to re-form ATP, generating energy rapidly and at a high rate. The glycolytic system involves the anaerobic breakdown of glucose to pyruvate and pyruvate's conversion to lactate, and has a larger total capacity for producing energy than the phosphagen system (although at a lower rate, resulting in lower power output). Other pathways to regenerate ATP require the use of oxygen and rely on the provision and catabolism of carbohydrate or lipid substrates from intramuscular stores and the bloodstream (138). The human body is a dynamic

environment where each cell is continually switching the proportions of each type of substrate that is oxidised and/or produced. This adaptation is crucial and is achieved only through the several regulatory mechanisms involved in controlling energy transformation and utilisation. Skeletal muscle has the capacity to rapidly modulate the rate of energy production and changes in fuel utilisation in response to exercise.

1.3.2 Muscle fatigue, muscle nucleotide pool and glycogen depletion

Muscle fatigue has been defined as the failure to maintain an expected force, leading to reduced performance of a certain task (140). Voluntary contraction and fatigue are complex mechanisms, where energy supply and deficiency play a major role. Metabolic changes at fatigue can depend on the type of exercise performed but a common denominator is the decreased capacity to generate ATP paired with high ATP turnover, leading to increased catabolism of the adenine nucleotide pool in the muscle cell (141). The breakdown of ATP to ADP and the rephosphorylation of ADP back to ATP constitute the ATP-ADP cycle, whereby energy is utilised and produced (142). When the rate of utilisation of ATP surpasses the rate of ADP phosphorylation, the muscle content of ADP and AMP increases. This, coupled with a decrease in muscle pH due to lactate accumulation, favours the activation of the enzyme AMP deaminase. Consequently, the adenine nucleotide pool in contracting skeletal muscle is decomposed through deamination of AMP to inosine monophosphate (IMP) and ammonia (NH_3) (143). Muscle IMP is typically very low at rest and after low-intensity exercise, but increases after moderate and high-intensity exercise. An enhanced rate of AMP deamination has been shown to coincide with muscle fatigue during both short-term strenuous exercise and prolonged exercise at a

submaximal intensity (144). The decrease of the total adenine nucleotide pool in the muscle at fatigue is generally higher than the increase in muscle IMP (144). This reduction in the muscle total nucleotide pool is small and is rapidly recovered after exercise (145).

During very-high-intensity exercise, fatigue will typically develop within 5 mins of the onset of exercise and might be associated with disruption of the energy supply (i.e. PCr depletion), product inhibition (by the accumulation of lactate, hydrogen ions and inorganic phosphate) and/or due to factors preceding cross-bridge formation, such as a disruption of calcium handling (138). During prolonged exercise (i.e. an exercise intensity which can be sustained for 30 to 180 mins, corresponding to 60 to 85 % of maximal oxygen consumption), the rate of ATP production is lower and, therefore, PCr, CHO and fat can all contribute to ATP resynthesis. At the onset of prolonged exercise, PCr and anaerobic glycolysis make significant contributions to muscle ATP production, potentially to offset delays in muscle blood flow and oxygen extraction, as well as in substrate oxidation. Following the first few minutes of exercise, a steady state of energy demand and delivery is reached, and the oxidation of CHO and fat become the principal source of fuel. CHO provide the greatest amount of ATP per unit of oxygen utilised than any other substrate and is the most important fuel source during this type of activity. Skeletal muscle glycogen is broken down into glucose via the process of glycogenolysis. The resulting glucose contributes to ATP regeneration, both with or without oxygen, via anaerobic or aerobic glycolysis (**Figure 1.2**). When glycogen is used as a substrate, three molecules of ATP are generated for each glucose undergoing glycolysis. Due to the relatively large glycogen store available and the rapid rate at which glycolysis can proceed, the energy supplied in this manner is crucial for the performance of intense exercise

(138). Extensive research has established the link between sufficient CHO availability/replete glycogen stores and improved endurance exercise performance (146, 147). Accordingly, fatigue after prolonged exercise (1-2 h of hard exercise) is primarily brought about by CHO depletion, but the exact biochemical mechanisms underpinning this are still unclear (138).

Carbohydrates are stored in the human body in the form of glycogen, a multibranched polymer of glucose residues. The two major sites of glycogen storage are the liver and skeletal muscle (138). The glycogen content of skeletal muscle at rest is approximately 14 – 18 g per kg wet mass (80-100 mmol glucosyl units kg^{-1}). The leading theory for the association between glycogen depletion and muscle fatigue pertains to glycogen's role as an essential substrate for the muscle contractile function. It has been observed that, following prolonged glycogen-depleting exercise, PCr decreases along with an increase in free ADP and IMP (148, 149). As such, glycogen depletion results in a reduction in the rate of ATP regeneration and consequent inadequate energy supply (due to ATP depletion) to sustain excitation and contraction. However, there is also a strong association between low glycogen and decreased muscle function even after recovery periods, where ATP levels would be normal (150, 151). Therefore, glycogen content might be intimately related to other metabolic changes which modulate the excitation-contraction coupling, such as the sarcoplasmic reticulum calcium handling.

A variety of studies have manipulated endogenous (glycogen stores) and exogenous (CHO intake) availability of CHO before and/or after training sessions to alter performance and exercise-induced adaptations (152). Optimal glycogen stores allow maintenance of exercise performance for longer periods of time and high carbohydrate availability before, during and after exercise bouts has

generally been promoted (153). Guidelines have been published to ensure glycogen resynthesis post-exercise in both the short term (0-6 hours) and longer-term (25-48 hours), between exercise events or training sessions to allow for optimal repeated performances (147). However, deliberately training in conditions of reduced CHO availability has been suggested to contribute to training-induced adaptations of human skeletal muscle, such as increasing maximal mitochondrial enzyme activities and/or mitochondrial content, increasing rates of lipid oxidation and even improving exercise capacity (154). Skeletal muscle glycogen and ATP concentrations are intimately associated and manipulating skeletal muscle ATP could be as useful in exercise training, performance and in recovery between sessions as carbohydrate/glycogen manipulation, however, effective strategies to its implementation and measurement have to not been yet been developed.

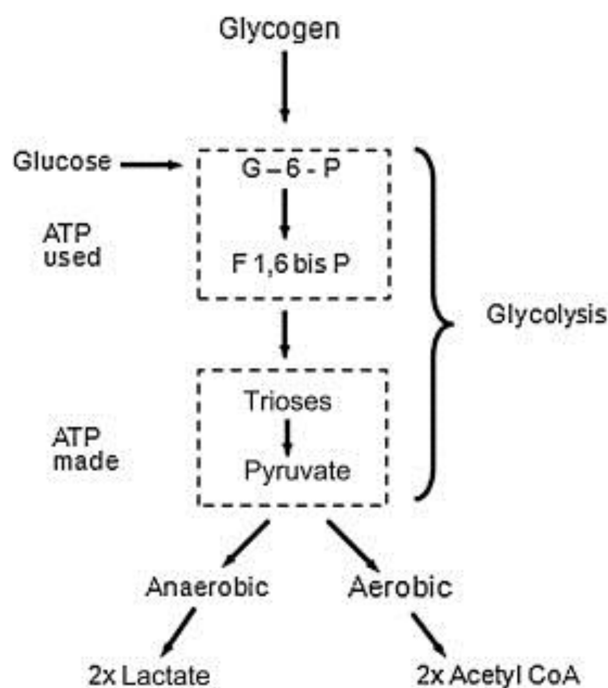


Figure 1.2 **Simplified glycolysis pathways.** Figure from Opie (2014) (155)

1.3.3 Ribose

Ribose is a pentose monosaccharide, part of the structure of certain nucleic acids. Ribose is produced in the body through the pentose phosphate pathway (PPP), a metabolic pathway parallel to glycolysis that generates NADPH, pentoses, and ribose 5-phosphate (R-5-P) (156). The PPP is key to both the *de novo* and salvage pathways in nucleic acid regeneration. Glucose-6-phosphate, an intermediate in the PPP is converted through several intermediate steps to R-5-P and then to 5-phosphoribosyl-1-pyrophosphate (PRPP) (157). Once formed, PRPP is indirectly converted to IMP that, in turn, is ultimately converted to AMP. Finally, AMP is rephosphorylated to replenish decreased ATP pools. Additionally, PRPP plays a role in the salvage pathway by combining with products of adenine nucleotide catabolism (adenine, hypoxanthine, or inosine) to form AMP (157). **Figure 1.3** summarises the role of ribose in adenine nucleic acid synthesis.

The PPP is a slow process that requires the enzymes glucose-6-phosphate dehydrogenase (Glu-6-PDH) and 6-phosphogluconate dehydrogenase (6-PGDH), which have low-level activity in the skeletal muscle. Exogenous ribose is able to bypass these rate-limiting steps of Glu-6-PDH and 6-PGDH in the formation of PRPP by entering the PPP and being converted to R-5-P by ribokinase, thereby enhancing adenine nucleotide salvage and synthesis (158, 159). Ribose is crucial for both the *de novo* and salvage pathways and, as such, R-5-P has been proposed as a limiting factor in the production of ATP. Dietary ribose might offer a potential benefit in energy metabolism by attenuating the loss and/or enhancing the recovery of adenine nucleic acids (157).

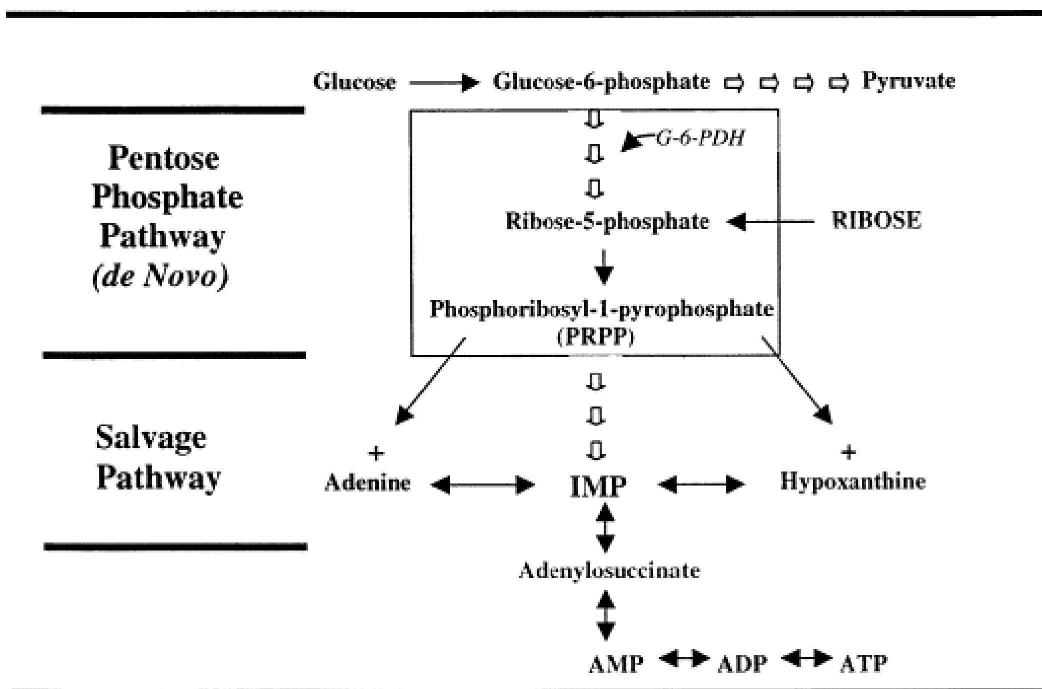


Figure 1.3 **The role of ribose in adenine nucleic acid synthesis.** Figure from Dodd et al (2004) (157)

1.3.4 Nucleotides, ribose and muscle fuel utilisation

Despite worries of the potentially deleterious effects of excessive nucleotide consumption, evidence supports an increased dietary requirement for nucleotides during periods of rapid growth (e.g. most infant feed formulae are enriched with nucleotides (160, 161)), 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 (162, 163)) as well as in recovery from extensive trauma and intensive care, such as in burns patients (164). Muscular exercise is one of the most common states of physiological stress and emerging data imply beneficial effects of dietary nucleotide supplementation on exercise performance, recovery and exercise modulated immune response (165-171). A list and summary of published human studies investigating the

effects of dietary nucleotide supplementation on exercise performance and recovery is shown in **Table 1.3**.

In 2004, Jordan et al (165) first examined whether supplying healthy male participants with an enterically-coated oral ATP supplement would increase circulating ATP concentrations and improve anaerobic performance and strength measures. After the 14 day supplementation period with either a low-dose nucleotide supplement (150 mg per day), a high-dose nucleotide supplement (250 mg per day) or a placebo, they found no differences between groups for any of the study parameters. However, several small treatment effects on muscular strength for the participants consuming the high dose supplement were identified. Further studies have observed no changes in blood ATP concentrations following its intake, as well as no acute effects in exercise performance, calling into question the efficacy of administrating oral ATP (172-174). Physiologically, ATP might be poorly absorbed or inadequately delivered to the skeletal muscle, potentially due to its high molecular weight (165). As such, it is possible that providing precursors to ATP production might be more beneficial than ATP supplementation *per se*. These precursors are potentially rate-limiting to the *de novo* or salvage pathways of ATP and other nucleic acids.

Evidence supports a beneficial role of dietary nucleotides on immune function in recovery from infection, and malnutrition and starvation, but nucleotide supplementation has also been shown to attenuate exercise-induced immunosuppression. McNaughton et al (166, 167) found beneficial effects of nucleotide supplementation in preventing immunosuppression after both moderate-intensity and short-term high-intensity exercise, by reducing blood levels of cortisol and increasing concentrations of salivary immunoglobulin A (SIgA). Ostojic et al (168, 169) later confirmed and expanded on these results by

using a sublingual nucleotide supplementation approach, which aside from having beneficial effects in immune function, also improved participants running time to exhaustion, suggesting endurance performance was sensitive to nucleotide supplementation. It is conceivable that this performance benefit was a result of the manipulation of the muscle ATP content by the nucleotide supplement, however, no measures of muscle metabolism were collected during this study. More recently, Sterczala and colleagues (171) identified a potential benefit of nucleotide supplementation in improving isometric force after heavy resistance exercise, which again, could potentially be a result of increases in muscle ATP content. Despite some heterogeneity in the nucleotide formulations used in these studies and, consequently, in their findings, the existing data seem to point toward dietary nucleotides having a role in exercise performance by attenuating the stress response, reducing immunosuppression and muscle damage, and perhaps modulating muscle energy production.

It has been suggested that dietary nucleotide associated improvements in exercise performance might be due to a positive effect on energy production and oxidative stress in the mitochondria (175). Animal studies have also demonstrated anti-fatigue effects of dietary nucleotides, again potentially by improving mitochondrial function and inhibiting oxidative stress (176). Increased production of ATP, modulated by dietary nucleotide intake, might lead to increased resting skeletal muscle stores of ATP (ATP ‘loading’) and consequently to improved exercise performance, potentially by sparing muscle glycogen. No available studies have investigated the feasibility of this approach. ATP ‘loading’, unlike CHO loading, might not be a viable strategy due to cellular energy balance mechanisms and, as such, the timing of intake of dietary nucleotides might require some consideration. Providing dietary nucleotides at a time of ATP

depletion might potentiate acute ATP replenishment and lead to improved recovery when dietary nucleotides are consumed following ATP/glycogen depleting exercise. This potential for dietary nucleotides to modulate muscle fuel utilisation in humans warrants further investigations.

Ribose has also been proposed as a limiting factor in the production of ATP. Ribose is necessary for the synthesis of PRPP – critical for both *de novo* and salvage pathways of nucleic acid synthesis. Hellsten and colleagues observed that one week of frequent intense training was sufficient to lower resting muscle adenine nucleotide concentrations and, importantly, that oral intake of ribose after training enhanced the rate of adenine nucleotide resynthesis, probably by increasing the rate of PRPP synthesis (177). As such, dietary ribose intake (like dietary nucleotide intake) might modulate fuel utilisation and increase energy production, leading to improvements in exercise performance in a depleted state, as well as assisting muscle cells in recovery from fatigue; but further research to establish these effects is necessary. Providing dietary nucleotides and ribose concomitantly is a potential strategy to optimise the rate of adenine nucleic acid production (i.e. ATP), and consequently improve exercise performance, which deserves further study.

Table 1.3 **Human studies investigating the effects of dietary nucleotide supplementation on exercise performance and recovery**

Reference	No. of participants	Participants	Type of study	Interventio n	Type of exercise	Duration of interventio n	Main study findings
Jordan et al (2004) (165)	27 (9 per group)	Healthy males	Double-blind randomised controlled parallel-group trial	Low dose (150 mg/day) ATP, high dose ATP (225 mg/day) ATP, or matched placebo	Wingate test Bench press	14 d	No changes in blood/plasma ATP No changes in Wingate testing parameters or lactate accumulation 6.6% ↑ 1RM, 18.5% ↑ repetitions to fatigue during set 1 of post-testing and 22% ↑ total lifting volume (within-group differences in the high dose ATP group)
McNaughton et al (2006) (166)	14 (7 per group)	Moderately trained healthy males	Double-blind randomised controlled parallel-group trial	Nucell nucleotide-rich dietary supplement (1.8 g/day) or placebo	Moderate intensity endurance exercise	60 d	↑ in post-exercise SIgA concentrations in the nucleotide group ↓ in post-exercise cortisol concentrations in the nucleotide group No differences in amount of work completed, or blood lactate
McNaughton et al (2007) (167)	30 (10 per group)	Moderately trained healthy males	Double-blind randomised controlled	Nucell nucleotide-rich dietary supplement (1.8 g/day),	Short-term high-intensity exercise	60 d	↑ in pre-exercise SIgA concentrations in the nucleotide group

			parallel-group trial	placebo or control			↓ in post-exercise cortisol concentrations in the nucleotide group No differences in amount of work completed, blood lactate, LDH, or CK concentrations
Herda et al (2008) (174)	24	Healthy males	Double-blind randomised controlled crossover trial	AdenylPyro-G (625 mg) + Cordy-cAMP (350.8 mg) nucleotide-rich dietary supplements or placebo	Vertical jumps, isometric and isokinetic leg extensions, and dynamic constant external resistance forearm flexion	1 h (acute effect)	No differences in any performance parameter
Ostojic et al (2012) (168)	38 (19 per group)	Healthy young males	Double-blind randomised controlled parallel-group trial	Sublingual nucleotides (50 mg daily over 3 portions) or placebo	Incremental treadmill exercise to exhaustion	14 d	↑ in pre-exercise SIgA concentrations in the nucleotide group ↑ in serum IgA concentrations in the nucleotide group ↑ in NKC count in the nucleotide group ↑ in NKC cytotoxic activity in the nucleotide group ↑ in offset of post-exercise drop of salivary immunoglobulins and lactoferrin in the nucleotide group

							No differences in fasting salivary antimicrobial proteins
Ostojic et al (2013) (169)	30 (15 per group)	Physically active healthy males	Double-blind randomised controlled parallel-group trial	Sublingual nucleotides (50 mg daily over 3 portions) or placebo	Incremental treadmill exercise to exhaustion	14 d	4.8 % ↑ in time to exhaustion in the nucleotide group 3.5% ↑ in pre-exercise SIgA concentrations in the nucleotide group 36.1% ↑ in NKC cytotoxic activity concentrations in the nucleotide group No differences in white blood cell count, NKC number, peak rate of perceived exertion, peak heart rate, and peak running speed during the exercise test
Riera et al (2013) (170)	20 (10 per group)	Elite male taekwondo athletes	Double-blind randomised controlled parallel-group trial	Inmunactive ® nucleotide formulation (480 mg/day) or placebo	Cycloergometer exercise test to exhaustion under cold conditions	30 d	↑ recovery from exercise-induced lymphopenic response in the nucleotide group ↑ lymphoproliferative response in the nucleotide group No differences in performance parameters, skin and core temperature, heart rate, RPE, blood lactate or SIgA
Sterczala et al (2016) (171)	20	Healthy males and females	Double-blind randomised controlled	nuBound dietary supplement (278 mg/day dietary	Acute heavy resistance exercise protocol (Smith	14 d	↓ in post-exercise cortisol and MPO concentrations in the nucleotide condition

			crossover trial	nucleotides) or placebo	machine back squats)		↓ CK concentrations 24 h after exercise in the nucleotide condition ↑ isometric force immediately, 24 h and 48 h after exercise in the nucleotide condition No differences in blood lactate, uric acid, neutrophil, lymphocyte and monocyte counts, or countermovement jump peak power.

Abbreviations and symbols: 1RM, 1 rep max; ATP, adenosine triphosphate; CK, creatine kinase; IgA, immunoglobulin A; LDH, lactate dehydrogenase; MPO, myeloperoxidase; NKC, natural killer cell; RPE, rate of perceived exertion; SIgA, saliva immunoglobulin A; ↑, increase; ↓, decrease.

1.4 Summary and research questions

Developing sustainable dietary protein sources is a pressing socioeconomic and environmental concern, and there is an obvious need to develop a robust evidence base to inform the use of such alternative sources. Mounting evidence shows that the incorporation of modest amounts of mycoprotein into the diet positively influences blood lipid profile, and acute mycoprotein ingestion attenuates postprandial glycaemia and insulinaemia. These responses may be mediated by the amount of fibre (chitin and β-glucan) in mycoprotein, as well as by their unique digestive and metabolic properties, though a comprehensive and *in vivo* mechanistic understanding is still lacking. It is not known how rapidly circulating cholesterol is affected when mycoprotein is incorporated into the diet, and a full characterization of the lipid subfraction responses is not yet available. Furthermore, whether alterations of acute postprandial glycaemic control translate into improved insulin sensitivity or habitual glycaemic control when mycoprotein is incorporated into the daily diet is also unclear.

Mycoprotein is naturally rich in RNA-derived nucleotides. It is not known what impact the ingestion of non-nucleotide depleted mycoprotein has upon the postprandial serum uric acid response, insulin sensitivity and other markers of metabolic health (and therefore the potential relevance to disease progression).

Lastly, emerging data imply beneficial effects of nucleotide supplementation on exercise performance and recovery, potentially by modulating ATP and glycogen metabolism. Ribose, a pentose monosaccharide that makes up the structure of nucleotides, has been proposed as a limiting factor in the production of ATP. Increased production of ATP modulated by dietary nucleotide and ribose intake, prior to submaximal moderate-intensity exercise, might lead to improved exercise performance by sparing muscle glycogen; however, this has not yet been

investigated. Additionally, these potential benefits to energy production might also lead to improved recovery following glycogen depletion.

Based on the review of the existing literature, several research questions emerged, which are addressed in the present thesis:

1. What are the effects of repeatedly consuming mycoprotein as the main source of dietary protein on markers of metabolic health?
2. What is the effect of ingesting a single nucleotide-rich mixed-meal on the concentrations of circulating uric acid, glucose and insulin?
3. What is the effect of repeatedly consuming nucleotide-rich mixed-meals on markers of metabolic health?
4. What is the effect of consuming a high-nucleotide supplement, with or without added ribose, on resting skeletal muscle ATP and glycogen concentrations, on depleted endurance exercise muscle metabolism and performance, and on the acute recovery from a depleted state?

Finally, after identifying these research questions, several key hypothesis, which are tested in the present work, were defined:

1. One week of mycoprotein consumption as the main source of dietary protein at lunch and dinner improves whole-body insulin sensitivity, 24 h free-living glycaemic control and the plasma lipidome in healthy young adults.
2. The ingestion of a single nucleotide-rich mixed-meal acutely increases circulating uric acid concentrations and impairs postprandial glucose handling in healthy young adults.
3. One week of high-nucleotide mycoprotein consumption as the main source of dietary protein at lunch and dinner leads to a sustained increase in serum uric acid concentrations and deleterious effects to whole-body

insulin sensitivity, 24 h free-living glycaemic control and the plasma lipidome in healthy young adults

4. Twice-daily ingestion, for 2 weeks, of a nucleotide-rich mycoprotein drink leads to improvements in resting skeletal muscle ATP and glycogen concentrations and in depleted endurance exercise muscle metabolism and performance, in young, healthy, untrained adults. The addition of ribose to the drink leads to further improvements in these parameters.
5. Consumption of a high-nucleotide, high-carbohydrate mycoprotein drink improves the acute recovery of muscle ATP and glycogen concentrations, as well as endurance exercise performance, in young, healthy, untrained adults. The addition of ribose to the drink leads to further improvements in these parameters.

Chapter 2:

General methods

2.1 Overview

Within the current thesis, the work in Chapters 3 and 4 have been published in the *British Journal of Nutrition* and *Nutrients*, respectively. Chapters 5 and 6 are currently under preparation/review. Data collection for Chapters 3 and 5 was conducted simultaneously, with the participants recruited and data presented for the mycoprotein group (MYC) in Chapter 3 being the same as the low-nucleotide mycoprotein group (LOW) in Chapter 5. The full details of the methods used in Chapters 3, 4, 5 and 6 are detailed within these chapters. Additional methods and validations not detailed within those chapters are discussed below.

2.2 Serum uric acid in venous and arterialised-venous blood samples

In chapters 3, 4 and 5, participants had a cannula placed retrogradely in a dorsal hand vein and the hand was then placed in a heated box (55ºC) for arterialised-venous blood sampling (178). Other blood samples were collected via venepuncture from an elbow vein. As such, serum uric acid concentrations were measured in both venous and arterialised-venous blood samples. In order to ensure that observed changes were due to the interventions and not to differences between sampling approach, six volunteers were asked to report to the laboratory after an overnight fast; a venous blood sample from an elbow vein and an arterialised blood sample from a dorsal hand vein were simultaneously collected into SST tubes (containing spray-coated silica and a polymer gel for serum separation; Becton Dickinson). Aliquoted serum samples were 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). A Bland-Altman plot and a Pearson

correlation were used to analyse the agreement between the venous and arterialised-venous fasting uric acid concentrations (**Figure 2.1**). There was a very strong positive correlation between the two measurements (r = 0.999; P<0.0001; 95% limits of agreement: from -3.568 to 8.235).

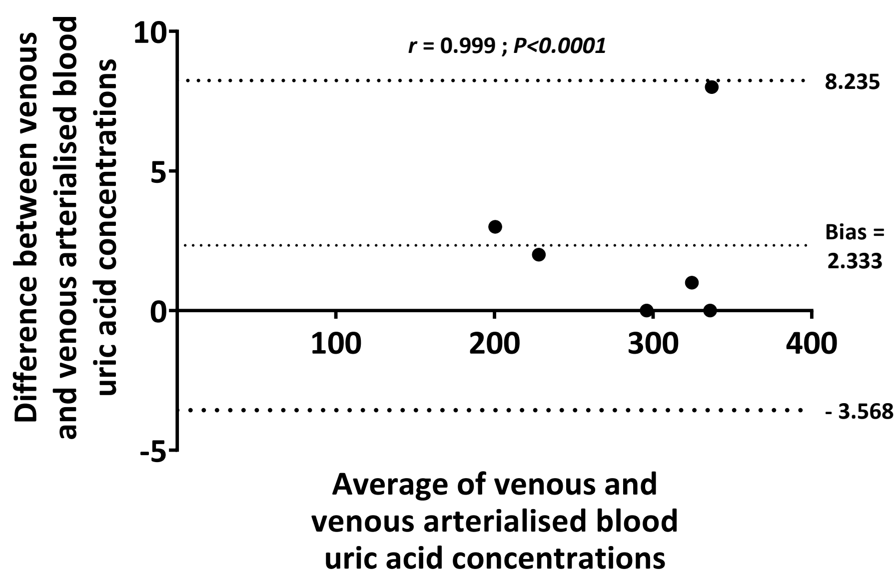


Figure 2.1 **Bland-Altman analysis plot for uric acid concentrations measured in venous and arterialised-venous blood, representing the consistency of the variant results between the two sample types.** There was a very strong positive correlation between the two samples (r = 0.999; *P*<0.0001).

2.3 Nuclear magnetic resonance (NMR) based metabolomics

In chapters 3 and 5, plasma samples from research participants were sent to the MRC Integrative Epidemiology Unit at the University of Bristol for metabolomics analysis by NMR spectroscopy. The samples were collected into lithium heparin plasma tubes (Becton Dickinson), immediately centrifuged, stored at –80°C and transported to the facility in dry ice.

A Janus Varispan liquid handling robot (PerkinElmer, Waltham, MA, USA) was used to mix the plasma with a buffer and to move the material to 96-format racks

of NMR tubes. The racks were subsequently inserted into the robotic sample changer, cooled to refrigerator temperature. NMR spectrometers, such as the one used for these analyses (Bruker Avance III HD 500MHz spectrometer) assure high throughput by possessing automated shimming, accurate temperature control, and stable electronics.

The data were then processed using the Nightingale Health's NMR-based blood biomarker analysis platform which, by using advanced proprietary software with integrated quality control, converts the spectral information to absolute concentrations of 224 metabolomic measures (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. This quantification of metabolic measures in absolute units, rather than relative to another measure, can currently be achieved without external standards added to the blood samples, utilising Bayesian modelling. The Nightingale Health's NMR metabolomics platform employs a targeted approach, with a defined set of metabolites being quantified and, as such, it is not designed for novel biomarker discovery.

2.4 $\dot{V}O_2$ max and glycogen-depleting exercise protocol

In chapter 6, participants performed a continuous incremental $\dot{V}O_2$ max test on an electronically braked cycle ergometer (Lode Excalibur Sport, Lode, Netherlands). Participants began cycling at a power output approximate to their body weight (generally 60 – 80 W) and the resistance was then increased by 20 – 40 W every 3 mins until volitional exhaustion. Measurements of oxygen consumption ($\dot{V}O_2$) were collected using a facemask and the Metamax 3B portable indirect calorimetry system (Cortex, Leipzig, Germany) throughout the

test and were used to determine each participant's maximal oxygen output ($\dot{V}O_2$ max). During the experimental test days, participants completed an exercise protocol based on their $\dot{V}O_2$ max test results and designed to induce glycogen depletion. Participants began cycling at a power output corresponding to 40-50% of their $\dot{V}O_2$ max. They cycled at this intensity for 10 mins and measurements of oxygen and carbon dioxide consumption were collected for the final 3 mins. At the end of the 10 mins, the power output automatically increased to correspond to 70-75% of the participant's $\dot{V}O_2$ max. The participants were instructed to keep their revolutions at a minimum of 70 and to cycle until volitional fatigue and for at least 1 hour continuously. When participants reached fatigue and could not keep cycling above the indicated revolutions, they were permitted a 5 min break, after which they got back on the ergometer until achieving fatigue again. This was repeated until participants were not able to keep cycling for over 2-3 mins (usually after 3 or 4 bouts). This method has been previously shown to induce almost complete muscle glycogen depletion and a substantial depletion of muscle ATP content (179). **Table 2.1** displays a summary of the exercise performed by the participants.

Table 2.1 **$\dot{V}O_2$ max test and glycogen depleting exercise**

$\dot{V}O_2$ max ($ml \cdot kg^{-1} \cdot min^{-1}$)	37 ± 1
$\dot{V}O_2$ max load (W)	200 ± 15
40% $\dot{V}O_2$ max load (W)	56 ± 3
70% $\dot{V}O_2$ max load (W)	118 ± 8
Duration of continuous exercise (mins)	67 ± 3
Number of extra exercise bouts	4 ± 0
Total exercise duration (mins)	87 ± 4

2.5 Muscle glycogen extraction and analysis

In chapter 6, muscle glycogen concentrations were determined using the spectrophotometric method of Harris (180). Glycogen was extracted by dissolving 1-6 mg of freeze-dried muscle powder in 100-240 µL of sodium hydroxide (NaOH; 0.1 M), which was then incubated at 80 ºC for 10 min. Subsequently, the samples were neutralised by the addition of 400-960 µL hydrochloric acid (HCl; 0.1 M) in a citric acid buffer (0.2 M; pH 5, containing 0.2 M disodium phosphate, Na_2HPO_4). Amyloglucosidase (AGDase) was then added and the samples were incubated at room temperature for 1 hour, to completely hydrolyse glycogen to glucose. After centrifugation (14000 rpm, 2 mins) the supernatant was extracted and analysed for glucose. An extraction factor was used to calculate the relative amount of glucose in each sample:

$$EF = \frac{Vol_{NaOH} + Vol_{buffer} + Vol_{AGDase}}{Wt_{mp}}$$

EF = extraction factor

Vol_{NaOH} = volume of sodium hydroxide added (µl)

Vol_{buffer} = volume of citrate and hydrochloric acid buffer added (µl)

Vol_{AGDase} = volume of AGDase added (µl)

Wt_{mp} = weight of muscle powder (µg)

Twenty-five µl of the extract or a water blank was added to each well of a 96 well plate and incubated with triethanolamine (TEA) buffer set to pH 8.4, containing 0.75 mM ATP, 1 mM NAD^+ and 1 mM dithiothreitol (DTT), for 5 min. A baseline absorbance was measured with a plate spectrophotometer at a wavelength of

340 nm. The 2-step reaction was begun by adding hexokinase (HK) and glucose-6-phosphate dehydrogenase (Glu-6-PDH) to the assay plate. First, the HK catalysed the phosphorylation of the endogenous glucose by ATP, to form ADP and glucose-6-phosphate (Glu-6-P). And secondly, the Glu-6-P, catalysed by the Glu-6-PDH, reduced NAD^+ to NADH and led to the formation of 6-P-gluconolactone. The assay was incubated for 60 min at room temperature before absorbance being again measured at 340 nm. The change in absorbance due to the reduction of NAD^+ to NADH was used to calculate the muscle glycogen concentration (in glycosyl units mmol·kg^{-1} dm) present in the original sample, as seen below:

$$\text{Glycogen (glycosyl units mmol·kg}^{-1}\text{ dm)} = \frac{(Vol2\times(Abs2-Bla2)-Vol1\times(Abs1-Bla1))\times EF}{(3.4\times SVol)}$$

Vol_1 = initial reaction volume (µl)

Vol_2 = reaction volume with the addition of HK and Glu-6-PDH (µl)

Abs_1 = absorbance of the sample before the addition of HK and Glu-6-PDH

Abs_2 = absorbance of the sample after the addition of HK and Glu-6-PDH

3.4: mM absorption coefficient for NAD^+ at a wavelength of 340 nm (cm^2·µmol^{-1})

SVol = sample volume

EF = perchloric acid extraction factor (l·kg^{-1})

2.6 Muscle metabolites extraction and analysis

In chapter 6, muscle ATP, IMP, PCr and free creatine were extracted and concentrations determined by using an adapted ion-pair reversed-phase chromatography technique described previously (181). Ice cold perchloric acid (PCA; 0.5 M), containing EDTA (1 mM), was added to the freeze-dried muscle

(5-10 mg; 1 ml of PCA per 12.5 mg of muscle powder) and vigorously vortexed intermittently for 10 min whilst being kept on ice. The samples were centrifuged (10000 rpm for 3 mins, 4 ^{o}C) and the supernatant was extracted and neutralised by adding potassium bicarbonate ($KHCO_3$; 2.2 M; 25% of the supernatant's volume). After a second centrifugation (again 10000 rpm for 3 mins, 4 ^{o}C), the supernatant was again removed, and used for the determination of ATP, IMP, PCr and free creatine. The relative amount of these metabolites existing in the extraction was calculated by applying the following extraction factor:

$$EF = \frac{Vol_{PCA} \times (Vol_{sup} + Vol_{KHCO3})}{Vol_{sup} \times Wt_{mp}}$$

EF = extraction factor

Vol_{PCA} = volume of perchloric acid added (μl)

Vol_{sup} = volume of perchloric acid supernatant added (μl)

Vol_{KHCO3e} = volume of $KHCO_3$ added in perchloric acid supernatant (μl)

Wt_{mp} = weight of muscle powder (μg)

ATP, IMP, PCr and free creatine were quantified by ion-pair reversed-phase chromatography with a gradient elution protocol with UV detection (181). Chromatography was performed using a Flexar FL HPLC system (PerkinElmer), consisting of a binary LC pump, autosampler, and photo-diode array detector, simultaneously monitoring absorbance at 210 and 258 nm with 395 nm reference and 5 nm bandwidth. Peaks were integrated and processed using Chromera software (Version 4.1.2.6410; PerkinElmer). Separation was done on a 5-μm Spherisorb ODS2 (Waters, Milford, MA, USA) reversed-phase analytical column (5 μm, 250 mm x 4.6 mm id), with 10 mm guard column. The mobile phase

consisted of buffer A: 25 mM KH_2PO_4 containing 2 $g \cdot L^{-1}$ tetrabutylammonium dihydrogen phosphate (TBA), and buffer B: a 1:3 (v/v) mixture of acetonitrile to 125 mM KH_2PO_4, containing $2g \cdot L^{-1}$ TBA. With a flow of 1.1 $ml \cdot min^{-1}$, and following an equilibrium of 0% B for 3 minutes, 10 µl of sample were injected and mixed by partial flow loop, and then eluted with the following gradient program: 0% B in 2 min, 0 to 70% B in 13 min, maintained at 70% B for 10 min, 70 to 0% B in 2 minutes, then maintained for 7 minutes before the next sample. The eluent was passed into the PDA detector, with peaks determined at 210 nm for creatine (~ 2.6 min RT) and PCr (~14 min RT), and 258 nm for IMP and ATP (~13.5 and 24 min RT, respectively). Peaks were confirmed with spiked samples, and quantified against standard curves of 0, 0.025, 0.05, 0.075, 0.1, 0.2, 0.4, 0.5, 0.6, 0.8 and 1 mM of each analyte.

2.7 Statistical analysis and coefficients of variance

The statistical analysis and the graphs presented in this thesis were completed using GraphPad Prism version 7.0 (GraphPad Software, San Diego, California, USA). Metabolomics analyses were performed using MetaboAnalyst 4.0 (Wishart Research Group, University of Alberta, Edmonton, Alberta, Canada). Statistical significance was set at $P<0.05$ and all data are expressed as means ± standard error of the mean (SEM).

Coefficients of variance (CV) were used to present the relative variability of the analytical procedures used for the determination of the concentrations of blood glucose and lactate, serum insulin and uric acid, and muscle glycogen (**Table 2.2**), and calculated as shown below:

$$\text{CV (\%)} = \frac{Standard\ deviation\ of\ multiple\ measurement\ of\ the\ same\ sample \times 100}{Mean\ of\ multiple\ measurements\ of\ the\ same\ sample}$$

Table 2.2 **Coefficients of variance**

Type of sample	**Measurement**	**n**	**CV (%)**
Blood	Glucose	10	1.9
Blood	Lactate	10	2.2
Serum	Insulin	10	2.7
Serum	Uric Acid	10	0.8
Muscle	Glycogen	10	7.2

Chapter 3:

Daily mycoprotein consumption for 1 week does not affect insulin sensitivity or glycaemic control but modulates the plasma lipidome in healthy adults

The work contained in this chapter has been published as follows:

Coelho, M. O., Monteyne, A. J., Dirks, M. L., Finnigan, T. J., Stephens, F. B., & Wall, B. T. (2020). Daily mycoprotein consumption for 1 week does not affect insulin sensitivity or glycaemic control but modulates the plasma lipidome in healthy adults: a randomised controlled trial. *British Journal of Nutrition*, 1-14.

3.1 Introduction

Growing evidence suggests dietary protein consumption above the current reference daily allowances (i.e. 0.75-0.8 $g \cdot kg^{-1}$ per day in the UK and the USA (2, 3)) may confer metabolic benefits relating to healthy ageing and weight management, such as improved glycaemic control (4-7, 9, 10, 182). In parallel, increasing data are accumulating concerning the environmental cost of intensive animal-derived dietary protein production (1), resulting in shifting social attitudes and government initiatives towards more sustainable sources. As a consequence, the efficacy of non-animal derived, sustainably produced dietary proteins to support glycaemic control and metabolic health is a pressing research focus.

Mycoprotein is a low-energy food source, rich in protein and fibre, derived from the continuous cultivation of the fungus *Fusarium venenatum* (42). For the production of an equivalent amount of edible protein, mycoprotein requires less water and land usage, and has a reduced carbon footprint when compared with meat and dairy (45, 46, 63), positioning it as a sustainable alternative protein source.

Previous work has shown that the ingestion of a single mycoprotein-rich meal in combination with an oral glucose tolerance test (OGTT) results in reduced postprandial glycaemia and insulinaemia compared with isonitrogenous and isoenergetic control meals (59, 62). The careful matching of nutritional conditions in these studies suggests that either mycoprotein was delaying intestinal glucose absorption or improving postprandial (peripheral) glucose uptake, with either effect plausibly linked to the amino acid composition or fibre content (and type) contained within mycoprotein. We have recently shown that protein digestion and amino acid absorption following mycoprotein ingestion are sustained during the

acute postprandial period, highlighting the potential of this alternative protein source to modulate glycaemic control (48). However, whether these findings translate to habitual mycoprotein consumption improving physiologically relevant, longer-term changes in insulin sensitivity (IS) and/or glycaemic control has not been investigated.

Studies that have investigated the incorporation of mycoprotein into the habitual diet (20-60 g dry weight per day for 3-8 weeks using either fully controlled or supplemented free-living nutritional interventions) have reliably shown a 0.4–0.8 $mmol \cdot L^{-1}$ lowering of blood cholesterol concentrations and improvements in LDL:HDL ratios in healthy and hypercholesterolemic individuals (52, 53). These studies designed the nutritional interventions in an energy- and macronutrient-matched manner, and therefore, the higher fibre content of the mycoprotein conditions is probably the causative factor (27-39 g per day in the mycoprotein-based diets *v.* 25-27 g per day in the control diets).

In the present study, we applied a 1-week fully controlled dietary intervention in healthy young adults where the major source of dietary protein at lunch and dinner was obtained from meat and fish (control group; CON) or from mycoprotein (intervention group; MYC) with energy and macronutrient (except fibre) content of the diets matched. We hypothesised that one week of mycoprotein consumption would improve whole-body IS and 24 h free-living glycaemic control. We also applied a novel, targeted NMR-based quantitative metabonomics approach of 224 relevant metabolites that has been epidemiologically validated as a biomarker of IS (183) and would allow further insight as to the impact upon the metabolic profile of mycoprotein consumption.

3.2 Subjects and methods

3.2.1 Participants and medical screening

Twenty healthy, recreationally active, young adults (age: 24±1 y; BMI: 23±1 $kg \cdot m^{-2}$; male = 8 and female = 12) participated in the present study. Subjects' characteristics are presented in **Table 3.1**. 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., CA, USA) were measured at screening. The participants also completed a general health questionnaire and the International Physical Activity Questionnaire (IPAQ) (184). Vegetarians, vegans, smokers, and participants taking regular medication or suffering from chronic diseases were excluded. Participants regularly consuming >2.5 or <0.8 $g \cdot kg^{-1}$ of protein per day were also excluded. Participants included were recreationally active (partook in regular exercise or sport at a non-competitive level, 2-5 days a week), were normotensive, and had a BMI between 18.5 and 30 $kg \cdot m^{-2}$. Half of the female participants (6/12) were taking hormonal contraceptives. When this was not the case, female participants were tested (and their habitual data collected) during the follicular phase of their menstrual cycle, to control for cycle variations in glucose and insulin responses (185). All participants were informed of the study's purposes, procedures and risks, and provided written informed consent. The study was conducted at the Nutritional Physiology Research Unit, Department of Sport and Health Sciences, St. Lukes Campus, University of Exeter, between January and December of 2017, and it was approved by the University of Exeter's Sport and Health Sciences Ethics Committee (reference no: 161026/B/07) in

accordance with the Declaration of Helsinki and registered at ClinicalTrials.gov (NCT02984358).

Table 3.1 **Participants' characteristics**

	CON	**MYC**	***P*-value**
Sex	6 F / 4 M	6 F / 4 M	-
Age (y)	24±1	24±1	0.63
Height (cm)	174±3	171±4	0.64
Body mass (kg)	69±4	69±6	0.93
BMI ($kg·m^{-2}$)	23±1	23±1	0.70
Body fat (% of body mass)	21±4	21±3	0.95
Lean mass (kg)	53±4	55±5	0.82

CON, control group; MYC, mycoprotein group.
Multiple t-tests were used to compare each characteristic in CON and MYC.

3.2.2 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 primary source of dietary protein consumed: meat/fish-derived dietary protein (CON; *n*=10) or mycoprotein (MYC; *n*=10). 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 3.1** 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.

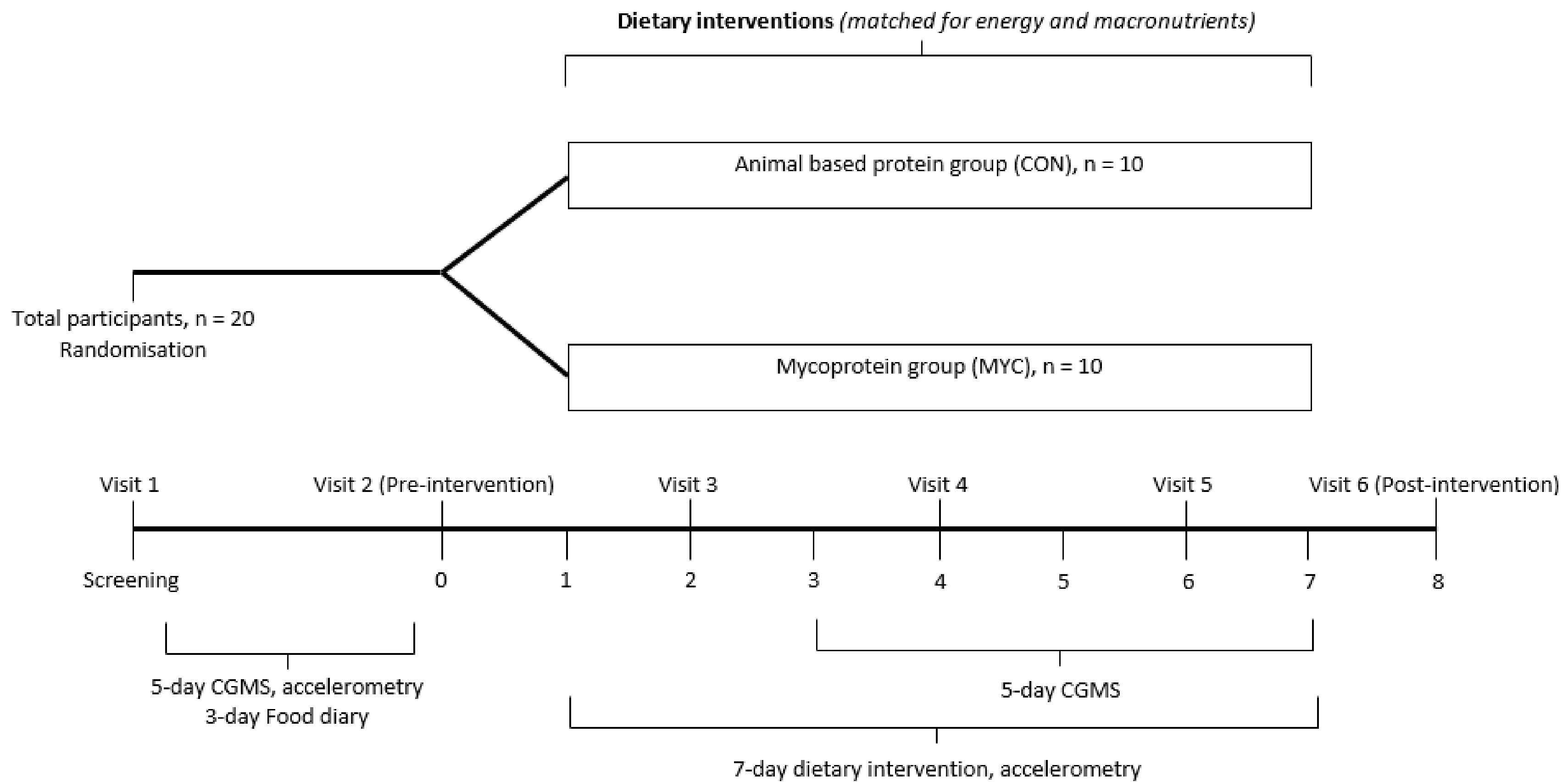


Figure 3.1 **Overview of the experimental protocol.**

3.2.3 Habitual data collection

Habitual data collection took place either during the 2 weeks before (CON; *n*=7, MYC; *n*=7) or between 2 and 8 weeks following (CON; *n*=3, MYC; *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 mins 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.

3.2.4 Experimental test days

Participants reported to the laboratory at about 08.00 hours 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 (178). 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 mins. Carbohydrate (CHO) and fat oxidation rates, as well as resting metabolic rate (RMR), were calculated using the Frayn equations (186). Subsequently, an OGTT was performed. Briefly, participants ingested 75 g glucose (dextrose, BulkPowders, Colchester, United Kingdom) dissolved in 350 mL water in 5 mins 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 a 2 h period 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 mins following glucose ingestion.

3.2.5 Dietary intervention

Basal metabolic rate (BMR) was estimated using the Henry equations based on age, gender, and weight (187). The IPAQ was used to calculate a physical activity level (PAL) factor (188). 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 department's research kitchen facility. Nutritional information for the two diets is provided in **Table 3.2**. Subjects consumed a diet containing 1.2 g of protein per kg of body weight per day (in order to reflect an average UK diet (189)), with 30% of their energy being provided by fat and the remainder from CHO (~50–55%; variation due to different energy requirements and the clamping of protein intake). The meals were identical between the two groups, aside from meat or fish providing the primary protein source in lunches and dinners for the CON group (n=10) and this being replaced by Quorn Foods™ products (to provide the required amount of mycoprotein) in the MYC group (n=10). The CON group consumed meals based on chicken, ham, beef, tuna and salmon. In the MYC group, this was substituted for Quorn chicken pieces, Quorn mince, Quorn fillets and Quorn roast chicken slices. An additional line of interest was the impact of the mycoprotein diet on plasma SCFA concentrations. Acetate, for example, can be produced not only from gut microbial fermentation of dietary fibre (with the mycoprotein diet being high in dietary fibre) but also from hepatic metabolism of alcohol (190). To isolate the impact of the diet, we, therefore, chose not to provide any alcohol during the intervention and required participants to abstain from alcohol for 24 h prior to the start of the intervention. All participants reported adhering to these guidelines. A document and diary detailing the plan were provided to the subjects in order to track compliance with the dietary intervention, log meal times and provide recipe information/instructions. While no formal data concerning tolerability and dietary preferences/liking were collected during the intervention, subjects informally reported no particular disliking of any

foods, nor any adverse events (e.g. gastrointestinal, nausea etc.), and compliance and feedback were similar across groups.

Participants were required to visit the laboratory at about 08.00 hours in the fasted state on days 2, 4 and 6 where body mass was measured wearing light clothing (seca 703 column scale, Seca, 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. The GENEActiv accelerometer was worn for the duration of the 1-week intervention, and on day 2, a glucose sensor was placed and the CGMS connected to collect continuous glucose data for the last 5 days of the intervention. Following the 1-week intervention (i.e. day 8), participants were required to repeat the experimental test day where a further OGTT was performed as described above.

3.2.6 Plasma and serum collection and analyses

From each blood sample, 1 mL 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, OH, USA). A quantity of 4 mL of blood was collected into lithium heparin (LH) 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 mins. All tubes were centrifuged at 4º C and 4000

RPM, and aliquoted (one aliquot designated for each of the below analyses) plasma and serum were stored at -80º C.

One aliquot of each postabsorptive 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, NJ, USA). IL-6 concentrations were measured in plasma samples using Quantikine ELISA kits (R&D Systems, Minneapolis, MN, USA).

Plasma samples were also sent to the MRC Integrative Epidemiology Unit at the University of Bristol for metabolomics 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 in a cost-effective manner (191). Biomarker concentrations quantified by this NMR approach have been shown to be highly consistent with concentrations obtained from standardised clinical chemistry analyses (192). The experimental protocol, including sample preparation and NMR spectroscopy, has been previously described in detail (191-193). The data were then 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. This approach has previously been used to

establish large scale and cross-sectional plasma lipid metabolic profiles of more metabolically compromised populations compared with healthy controls (194, 195) but its use in human nutrition trials is a novel application as, to date, NMR spectroscopy has rarely been applied to investigate changes in response to nutritional interventions (196).

3.2.7 Insulin sensitivity

Five different IS indices (197-201), all validated against the hyperinsulinemic-euglycemic clamp technique, were calculated pre- and post-intervention using the blood glucose and serum insulin concentrations measured in the fasting state and during the OGTT. The homeostatic model assessment of insulin resistance (HOMA-IR) is calculated from solely fasting concentrations of glucose and insulin and has been shown to provide a reasonable estimate of hepatic IS (197). 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 sensitivity (199). The Cederholm, oral glucose insulin sensitivity (OGIS) and Gutt indices focus mainly on peripheral IS and muscular glucose uptake by measuring OGTT glucose clearance (198, 200, 201).

3.2.8 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 mins, was 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 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). Days with data for fewer than 70% of the total time points were also excluded. The remaining data were analysed for glycaemic control (24 h average glucose, glucose area under the curve (AUC) and 2-h postprandial glucose) 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 (202).

3.2.9 Statistical analyses

A power analysis based on the assumption of a 12% increase in the Matsuda Index with mycoprotein consumption (calculated based on previous research (59)) was performed and determined that 8 participants were needed in each group to provide a power of 80% and a 95% CI. Ten participants per group were recruited to account for a potential 20% dropout rate. Recruitment and testing were ended once the trial was fully recruited according to the prior power calculation.

All data are expressed as mean values with their 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 detect individual differences. Blood glucose and serum insulin concentrations during the pre- and post-intervention OGTT were analysed with three-way ANOVAs (condition, time and test day as factors).

Additionally, for the aforementioned parameters, incremental Area Under the Curve (iAUC) was calculated and a one-way ANOVA was performed to detect any significant effect of treatment. CHO 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 metabolomics measures, a % change (Δ) 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 (n = 142 and n = 79, respectively). A heat-map was designed for the significant metabolites and these were organised into clusters. As an internal validation, a Bland-Altman plot and a Pearson correlation were used to analyse the agreement between the YSI and metabolomics fasting glucose data. 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 1 for the analysis of metabolomics ratios, resulting in an FDR of 0.131 and at 0.8 for metabolite concentrations, resulting in an FDR of 0.095. An FDR of 0.1 was set for metabolite dimensions analysis. NMR metabolomics calculations were carried out in MetaboAnalyst 4.0 (Wishart Research Group, University of Alberta, Alberta, Canada). All other calculations were performed using GraphPad Prism version 7.0 (GraphPad Software, San Diego, California, USA).

3.3 Results

3.3.1 Nutritional intervention

Body mass was not different between habitual testing and at the outset of the intervention in either group (from 69±4 to 70±4 in CON and 69±6 to 70±6 kg in MYC; $P>0.05$), nor did body mass change during the intervention in either group (70±4 and 70±6 kg post-intervention in CON and MYC, 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 summarised in **Table 3.2**. Prescribed diets and actual food consumed did not differ in any parameter, and so all other comparisons were made using the habitual and actual intervention diets only. 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 CHO intake from their habitual diets to the intervention in both groups (time effect $P<0.05$). Although fibre intake was not different between groups (group effect; $P>0.05$), significant time and interaction effects were detected ($P<0.05$), such that fibre intake increased by 31±2 % in the MYC group only ($P<0.05$). The MYC group consumed 215±16 g of Quorn products daily, corresponding to 181±13 g wet weight (45±3 g dry weight) of mycoprotein per day. In the CON group, 38±1 and 6±1 % of the total protein

consumed was provided by meat and fish, respectively, and in the MYC group, 38±2 % was provided by Quorn products. Dairy provided 13±1 % of the protein in the CON group and 15±2 % in the MYC group (P>0.05), and 32±1 % and 36±2 % of the protein in the CON and MYC groups, respectively, came from non-animal sources (not including mycoprotein; P>0.05). The remaining portion of dietary protein was provided by mixed (plant and animal) sources (e.g. chocolate bars, porridge oat pots, cakes, etc.).

3.3.2 Physical activity

Physical activity data are shown in **Table 3.3**. Habitual physical activity was not different between CON and MYC 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).

3.3.3 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 14.8±1.1 to 14.2±1.7 and from 12.3±2.4 to 12.7±1.7 $mU \cdot L^{-1}$ in CON and MYC, respectively; P>0.05). Pre- and post-intervention fasting blood glucose concentrations displayed a strong trend for an interaction effect (from 4.41±0.08 to 4.58±0.06 $mmol \cdot L^{-1}$, and from 4.55±0.11 to 4.47±0.07 $mmol \cdot L^{-1}$ in CON and MYC, respectively; P=0.05). Despite this, baseline IS reflected by the HOMA-IR was not different between groups (2.9±0.2 and 2.7±0.5 in CON and MYC, respectively; P>0.05) and did not change during the intervention in either group (P>0.05). Blood glucose and serum

insulin concentrations during the two OGTT performed pre- and post-intervention in the CON and MYC groups are shown in **Figure 3.2**. Both parameters increased with CHO ingestion (P<0.0001) and peaked between 30 and 45 mins 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 (P>0.05 for interaction and group effects). Blood glucose iAUC and serum insulin iAUC during the OGTT (displayed in Figure 3.2) also did not differ between groups or over time (both P>0.05). Consequently, there were also 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 3.3** displays these four indices and HOMA-IR for the two time-points in the two groups.

3.3.4 Continuous glucose monitoring system (CGMS)

Average daily glucose values were aggregated for the habitual data (5.5±0.1 mmol·L^{-1} in CON and 5.4±0.1 mmol·L^{-1} in MYC) and each of the intervention days, in the two groups (5.5±0.1, 5.5±0.2, 5.3±0.2, 5.4±0.1 and 5.4±0.1 mmol·L^{-1} in CON and 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 MYC, for days 3 to 7 of the intervention, respectively). Habitual data demonstrated no differences between groups (P>0.05) and this did not change throughout the intervention (P>0.05, for time and interaction effects). No differences were found between groups during the intervention in the average glucose concentrations in the 2-h postprandial period after the participants' evening meal (6.3±0.2, 6.1±0.4, 5.5±0.2, 5.3±0.2 and 5.5±0.2 mmol·L^{-1} in CON, and 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 MYC, for days 3 to 7 of the intervention, respectively; P>0.05 for time and interaction effects). There were also no

differences in glycaemic variability between groups, expressed as SD, CONGA1 or CONGA2 (all $P>0.05$).

3.3.5 Indirect calorimetry

There were no differences in RMR between groups before the intervention (1539±114 kcal in CON and 1692±119 kcal in MYC; $P>0.05$), and there were no main effects of time, condition or an interaction effect (all $P>0.05$). An effect of CHO ingestion was detected for both CHO (increasing) and fat (decreasing) oxidation rates ($P<0.0001$). No interaction or condition effects were found (all $P>0.05$). The relative contribution of fat and CHO oxidation to total energy expenditure in both the fasted and fed state are displayed in **Figure 3.4**.

3.3.6 Plasma IL-6 and serum uric acid concentrations

Fasting plasma IL-6 concentrations did not differ between groups at baseline ($P>0.05$) and did not change throughout the intervention in either group (from 1.7±0.6 to 1.4±0.6 $pg \cdot mL^{-1}$, and from 2.1±0.6 to 1.3±0.4 $pg \cdot mL^{-1}$ in CON and MYC; $P>0.05$ for time and interaction effects). Fasting serum uric acid concentrations were 297±20 $\mu mol \cdot L^{-1}$ in the CON group and 260±13 $\mu mol \cdot L^{-1}$ in the MYC group at baseline ($P>0.05$), and remained constant in both groups throughout the study (main effects of time, condition and interaction; all $P>0.05$).

3.3.7 Nuclear magnetic resonance (NMR) based metabolomics

The 224 metabolites measured by NMR metabolomics are listed in **Table 3.4**. No differences between groups were found for 171 (93 concentrations, 76 ratios and 2 dimensions) of the quantified targets. **Figure 3.5** and **Table 3.5** summarise the significant changes found in 53 of the targets (49 concentrations, 3 ratios and 1

dimension). Forty-five lipid concentrations of different lipoprotein fractions (including VLDL, LDL, IDL and HDL) remained largely unchanged in the CON group (0 – 11 % change) but decreased significantly in the MYC group (7-27 % decreases; all $P<0.05$). Plasma free cholesterol concentrations decreased by 4.00±0.03 % in the CON group (from 0.89±0.06 to 0.86±0.07 $mmol{\cdot}L^{-1}$) but by significantly more (13.99±0.03 %) in the MYC group (from 0.75±0.07 to 0.64±0.06 $mmol{\cdot}L^{-1}$; $P<0.05$) and, similarly, total (including VLDL, LDL, IDL and HDL) plasma cholesterol concentrations decreased by 5.23±0.03 % in the CON group (from 3.00±0.19 to 2.86±0.25 $mmol{\cdot}L^{-1}$) but to a significantly greater degree (by 14.28±0.03 %) in the MYC group (from 2.50±0.26 to 2.12±0.22 $mmol{\cdot}L^{-1}$; $P<0.05$). Plasma LDL cholesterol concentrations decreased by 2.55±0.07 % in the CON group (from 0.88±0.09 to 0.85±0.11 $mmol{\cdot}L^{-1}$) but to a greater degree, 19.33±0.07 %, in the MYC group (from 0.71±0.13 to 0.56±0.11 $mmol{\cdot}L^{-1}$; $P<0.05$) and plasma HDL2 cholesterol decreased by 11.03±0.02 % in the CON group (from 0.91±0.08 to 0.82±0.08 $mmol{\cdot}L^{-1}$) but by 18.58±0.03 % in the MYC group (from 0.72±0.07 to 0.58±0.05 $mmol{\cdot}L^{-1}$; $P<0.05$). DHA and omega 3 fatty acids concentrations decreased by 3.04±0.05 % (from 0.110±0.014 to 0.107±0.015 $mmol{\cdot}L^{-1}$) and 2.78±0.05 % (from 0.30±0.02 to 0.29±0.03 $mmol{\cdot}L^{-1}$) in the CON group and by 17.26±0.03 % (from 0.085±0.009 to 0.070±0.008 $mmol{\cdot}L^{-1}$) and 17.53±0.05 % in the MYC group (from 0.24±0.03 to 0.20±0.02 $mmol{\cdot}L^{-1}$), respectively (both $P<0.05$). HDL dimensions decreased by 1.26±0.00 % in MYC but only by 0.17±0.00 % in CON ($P<0.05$). Interestingly, plasma glucose remained unchanged in the CON group (from 3.8±0.1 to 3.8±0.0 $mmol{\cdot}L^{-1}$) but was reduced by 4.49±0.00 % (from 3.8±0.1 to 3.6±0.1 $mmol{\cdot}L^{-1}$) in MYC, and plasma acetate concentrations increased by 8.5±0.1 % (from 0.055±0.005 to 0.059±0.006 $mmol{\cdot}L^{-1}$) and 43.6±0.1 % (from 0.059±0.005 to 0.083±0.008 $mmol{\cdot}L^{-1}$) in CON

and MYC, respectively. These changes were not significant using the SAM multivariate analyses, but were significant when individually analysed (t-tests, $P<0.05$) which we deemed appropriate given their lack of involvement in the recognised pathways that the remainder of the metabolomics SAM analyses took into account. Changes in plasma total cholesterol, free cholesterol, LDL cholesterol, HDL2 cholesterol, DHA, omega-3 fatty acids, acetate and glucose concentrations are represented in **Figure 3.6**. A Bland-Altman analysis was performed in order to verify the trend for a decrease in blood glucose concentrations determined by YSI against the significant change in NMR-derived analyses of plasma glucose in the MYC group. This also served as a verification of the robustness of the NMR-based metabolomics approach. The Bland-Altman plot to analyse the levels of agreement between the YSI and metabolomics glucose data is represented in **Figure 3.7**. There was a strong positive correlation between the two measurements ($r = 0.60$; $P<0.001$; 95% limits of agreement: from 0.287 to 1.216).

Table 3.2 **Nutritional composition of participants' habitual diets, of the prescribed intervention diet and of their actual intake during the intervention according to the collected logs during the one-week intervention**

	Habitual dietary intake		**Prescribed intervention diet**		**Actual intake during the intervention**	
	CON	**MYC**	**CON**	**MYC**	**CON**	**MYC**
Energy (MJ/d)	8.9±0.7	10.1±0.6	10.1±0.6	11.0±1.0	10.1±0.6	10.9±0.1
Energy (kcal/d)	2120±177	2414±150	2422±155	2624±237	2422±152	2598±247
Protein (g/d)	91±7	107±14	83±5*	84±7*	83±5*	82±7*
Protein (g·kg^{-1} body weight)	1.4±0.1	1.6±0.2	1.2±0.0*	1.2±0.0*	1.2±0.0*	1.2±0.0*
Protein (% total energy)	18.8±1.5	17.6±1.7	13.7±0.4*	13.0±0.5*	13.7±0.4*	12.9±0.6*
Carbohydrate (g/d)	247±29	260±22	331±22*	355±35*	330±22*	350±37*
Carbohydrate (% total energy)	41.6±2.5	43.0±2.1	54.5±0.4*	53.9±0.7*	54.4±0.4*	53.4±1.0*
Fat (g/d)	94±8	99±7	82±5	87±8	82±5	87±8
Fat (% total energy)	36.8±2.0	37.0±1.7	30.3±0.2*	29.8±0.1*	30.5±0.2*	30.2±0.3*
Fibre (g/d)	23±2	26±2	26±2	34±2†	26±2	34±2†

CON, control group; MYC, mycoprotein group.
* Significantly different from habitual diet (time effect; $P<0.05$)
† Significantly different from habitual diet and from CON group (time and interaction effect; $P<0.05$)
Separate two-way repeated-measures ANOVAs were used to compare CON and MYC actual dietary intakes during the intervention with both the habitual diets and the prescribed intervention diets.

Table 3.3 **Daily habitual physical activity and daily physical activity during the intervention**

	Habitual		**Intervention**	
	CON	**MYC**	**CON**	**MYC**
Total activity (mins/day)	241 ± 19	251 ± 34	247 ± 45	295 ± 26
Light activity (mins/day)	83 ± 4	80 ± 7	85 ± 16	94 ± 9
Moderate activity (mins/day)	150 ± 15	158 ± 27	154 ± 27	186 ± 18
Vigorous activity (mins/day)	8 ± 3	12 ± 5	8 ± 3	15 ± 5
Sedentary (mins/day)	656 ± 24	661 ± 26	659 ± 42	654 ± 34

CON, control group; MYC, mycoprotein group.
Multiple two-way ANOVAs were used to compare the different activity levels in CON and MYC habitually and during the intervention.

Table 3.4 **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 lipids 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
XXL-VLDL-TG	Triglycerides in chylomicrons and extremely large 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
XL-HDL-P	Concentration of very large HDL particles	**Lipoprotein Particle 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
L-HDL-L	Total lipids in large HDL	**Fatty Acids and 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
S-HDL-FC	Free cholesterol in small HDL	**Glycolysis Related 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
HDL2-C	Total cholesterol in HDL2	**Branched-chained 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
TG/PG	Ratio of triglycerides to phosphoglycerides	**Fluid Balance and Inflammation**	
PC	Phosphatidylcholine and other cholines	Crea	Creatinine
SM	Sphingomyelins	Alb	Albumin
TotCho	Total cholines	Gp	Glycoprotein acetyls, mainly a1-acid glycoprotein

Table 3.5 **Significant NMR-based metabolomics features identified using either Significant Analysis of Microarrays (concentrations and ratios) or t-tests (dimensions)**

Concentrations	% Δ change CON	SEM	% Δ change MYC	SEM	d.value	stdev	rawp	q.value
XL-HDL-FC	-0.48%	0.05	-25.85%	0.04	-2.4672	0.064471	0.00098592	0.050372
XL-HDL-C	-1.40%	0.04	-23.04%	0.03	-2.3885	0.052223	0.0011972	0.050372
XL-HDL-CE	-1.34%	0.04	-22.11%	0.03	-2.3173	0.051227	0.0016197	0.050372
XL-HDL-L	-1.91%	0.04	-22.18%	0.04	-2.1522	0.055798	0.0033099	0.070958
XL-HDL-P	-1.99%	0.04	-21.94%	0.04	-2.1173	0.055879	0.0038028	0.070958
XL-HDL-PL	-1.84%	0.05	-21.76%	0.04	-1.9285	0.064943	0.006831	0.091044
L-HDL-FC	-9.17%	0.03	-24.54%	0.04	-1.8228	0.045938	0.0088028	0.10266
L-HDL-C	-8.43%	0.02	-21.48%	0.04	-1.5961	0.043399	0.016901	0.12108
IDL-FC	-2.98%	0.04	-18.33%	0.04	-1.585	0.058474	0.017958	0.12108
L-HDL-L	-9.33%	0.02	-20.84%	0.03	-1.5517	0.035824	0.019648	0.12108
L-HDL-P	-9.33%	0.02	-20.62%	0.03	-1.5358	0.035138	0.02007	0.12108
L-HDL-CE	-8.21%	0.02	-20.67%	0.04	-1.5351	0.042784	0.02007	0.12108
DHA	-3.04%	0.05	-17.26%	0.03	-1.4892	0.057088	0.023239	0.12108
M-LDL-TG	-7.08%	0.03	-27.03%	0.09	-1.4817	0.096334	0.024014	0.12108
IDL-C	-2.64%	0.05	-17.21%	0.04	-1.4298	0.063572	0.028732	0.12108
M-LDL-P	-3.61%	0.06	-25.72%	0.10	-1.4276	0.11648	0.028803	0.12108

M-LDL-L	-3.29%	0.06	-25.32%	0.10	-1.4213	0.11663	0.029577	0.12108
XL-HDL-TG	0.33%	0.07	-18.45%	0.07	-1.4058	0.09517	0.031056	0.12108
M-LDL-C	-2.02%	0.08	-27.06%	0.12	-1.3926	0.14145	0.032676	0.12108
L-HDL-PL	-10.54%	0.02	-20.19%	0.03	-1.392	0.030936	0.032746	0.12108
L-LDL-FC	-2.12%	0.04	-14.96%	0.03	-1.3761	0.054964	0.034366	0.12108
L-HDL-TG	-3.43%	0.06	-19.38%	0.05	-1.3743	0.077665	0.034507	0.12108
IDL-CE	-2.46%	0.05	-16.74%	0.05	-1.3649	0.066219	0.035634	0.12108
L-LDL-C	-2.71%	0.06	-19.07%	0.06	-1.3617	0.081812	0.035915	0.12108
IDL-L	-2.71%	0.04	-15.08%	0.04	-1.347	0.053468	0.037535	0.12108
L-LDL-CE	-2.94%	0.07	-21.27%	0.07	-1.3285	0.099596	0.03993	0.12108
FAw3	-2.78%	0.05	-17.53%	0.05	-1.3162	0.073673	0.041338	0.12108
IDL-P	-2.70%	0.04	-14.47%	0.04	-1.309	0.051504	0.04162	0.12108
S-LDL-L	-4.29%	0.06	-24.90%	0.10	-1.3075	0.11925	0.04162	0.12108
IDL-PL	-2.38%	0.03	-13.66%	0.03	-1.306	0.047989	0.041761	0.12108
S-LDL-P	-4.59%	0.06	-25.05%	0.10	-1.3009	0.11887	0.042606	0.12108
L-LDL-L	-3.20%	0.05	-16.65%	0.05	-1.2903	0.065862	0.043592	0.12108
S-LDL-C	-2.61%	0.08	-26.81%	0.13	-1.2792	0.15082	0.045	0.12108
L-LDL-P	-3.44%	0.05	-16.59%	0.05	-1.2753	0.064748	0.045423	0.12108
LDL-C	-2.55%	0.07	-19.33%	0.07	-1.2438	0.096487	0.050775	0.12622

M-LDL-FC	-2.46%	0.04	-20.02%	0.09	-1.2437	0.10285	0.050775	0.12622
FreeC	-4.00%	0.03	-13.99%	0.03	-1.241	0.042177	0.051408	0.12622
S-LDL-FC	-3.64%	0.05	-20.72%	0.10	-1.1778	0.10661	0.060634	0.14192
M-LDL-PL	-3.03%	0.04	-19.60%	0.10	-1.1769	0.1024	0.060845	0.14192
XS-VLDL-CE	3.13%	0.04	-7.16%	0.03	-1.1534	0.050838	0.065352	0.14689
L-LDL-TG	-4.61%	0.03	-14.48%	0.03	-1.1497	0.047456	0.066127	0.14689
LDL-TG	-5.97%	0.03	-15.99%	0.04	-1.1321	0.050121	0.070986	0.1535
S-LDL-TG	-8.70%	0.03	-25.25%	0.10	-1.1253	0.10863	0.072394	0.1535
XS-VLDL-PL	-2.38%	0.03	-12.60%	0.04	-1.1087	0.053763	0.076197	0.15798
XS-VLDL-C	1.26%	0.04	-8.36%	0.03	-1.0857	0.050216	0.081761	0.16583
S-LDL-PL	-4.60%	0.04	-19.57%	0.10	-1.0608	0.10266	0.089507	0.17306
HDL2-C	-11.03%	0.02	-18.58%	0.03	-1.0577	0.03298	0.090563	0.17306
L-LDL-PL	-2.68%	0.04	-11.85%	0.03	-1.0553	0.048557	0.090915	0.17306
Serum-C	-5.23%	0.03	-14.28%	0.03	-1.0515	0.047617	0.092746	0.17306

Ratios	**% Δ change CON**	**SEM**	**% Δ change MYC**	**SEM**	**d.value**	**stdev**	**rawp**	**q.value**
XL-HDL-FC_%	1.11%	0.01	-4.74%	0.01	-3.1601	0.018531	0.0060759	0.18544
L-HDL-FC_%	0.08%	0.01	-5.07%	0.02	-2.9305	0.017575	0.008481	0.18544
IDL-FC_%	-0.38%	0.01	-3.93%	0.01	-2.7984	0.012681	0.010253	0.18544

Dimensions	% Δ change CON	SEM	% Δ change MYC	SEM	t.stat	p.value	- log 10 (p)	FDR
HDL_D	-0.17%	0.00	-1.26%	0.00	3.4955	0.0025822	2.588	0.0077467

CON, control group; MYC, mycoprotein group

Abbreviations: XL-, extremely large; -FC, free cholesterol; -C, total cholesterol; CE, cholesteryl esters; -L, lipid; -P, particles; PL, phospholipids; L- large; IDL-, intermediate-density lipoprotein; M-, medium; FAw3, omega-3 fatty acids; S-, small; XS-, very small; XL-HDL-FC_%, free cholesterol to total lipids ratio in very large HDL; L-HDLFC_%, free cholesterol to total lipids ratio in large LDL; IDL-FC_%, free cholesterol to total lipids ratio in IDL; FDR, false discovery rate; HDL_D, mean diameter for HDL particles.

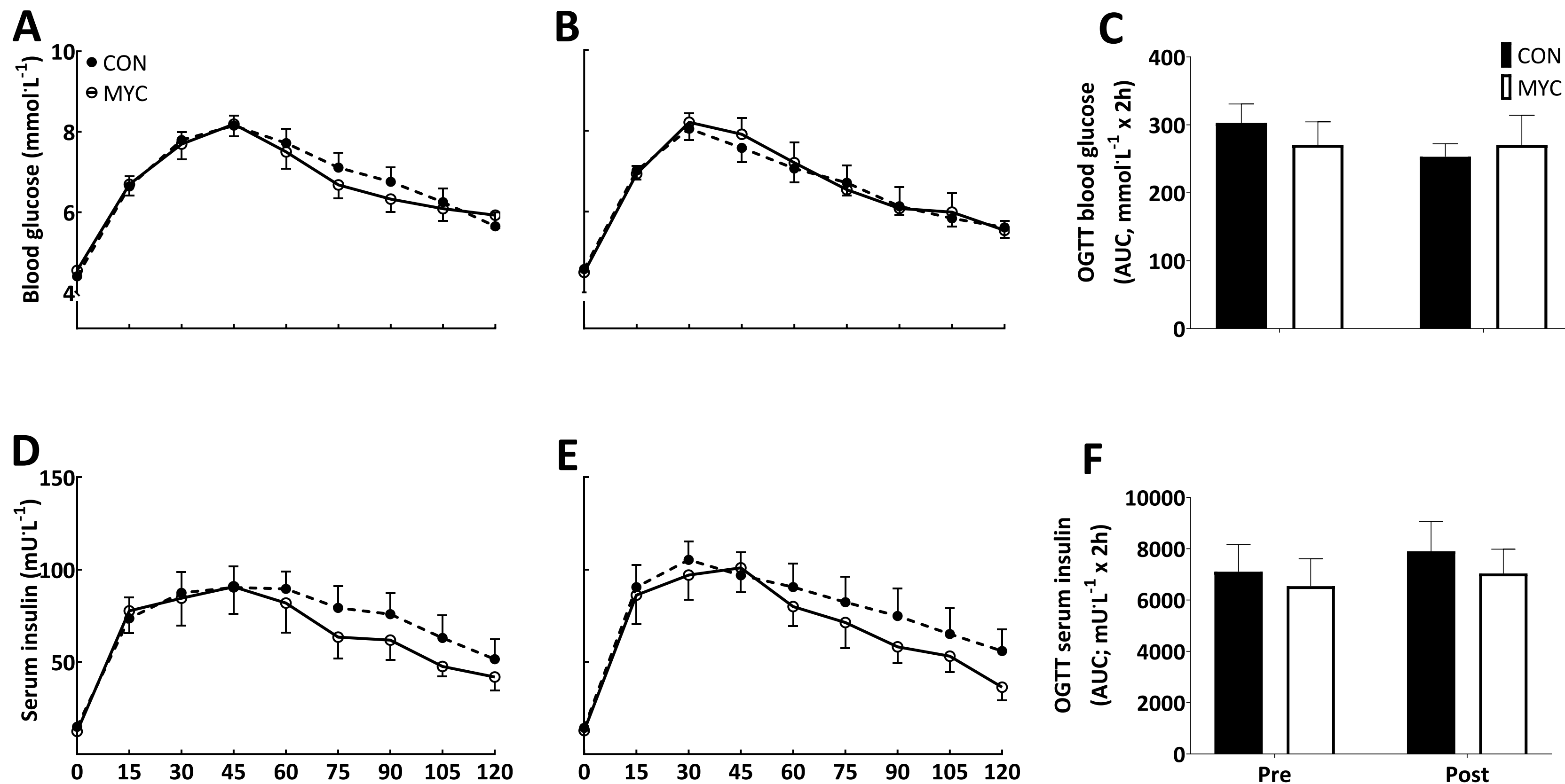


Figure 3.2 **Blood glucose (A, B and C) and serum insulin (D, E and F) concentrations during oral glucose tolerance tests (OGTT) on days 0 (A and D) and 8 (B and E) of a fully controlled dietary intervention with either a meat-based diet (CON) or a mycoprotein-based diet (MYC).** Values are means, with standard errors represented by vertical bars. OGTT data were analysed using three-way ANOVA. Incremental AUC data were analysed using one-way ANOVA. There was a significant effect of carbohydrate 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 incremental AUC and serum insulin incremental AUC, no statistically significant main effects of time or condition (both P>0.05), as well as no interaction effects (P>0·05) were found.

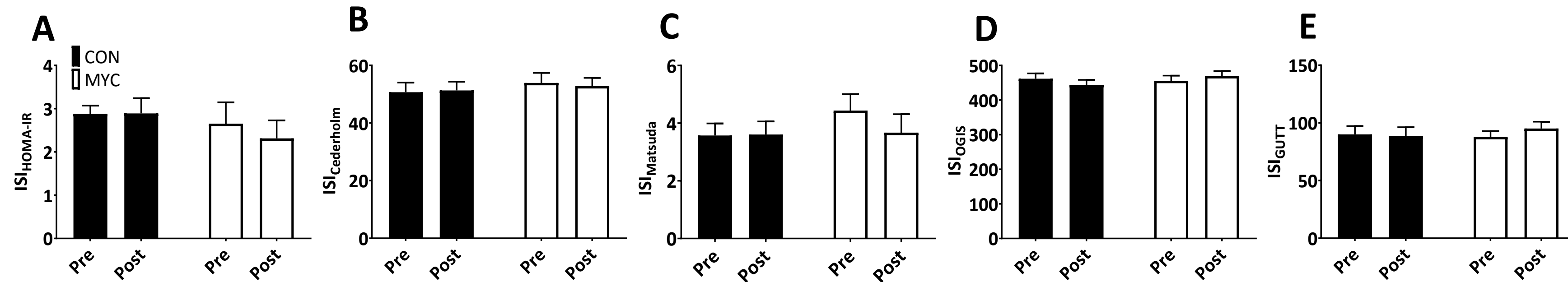


Figure 3.3 **Insulin sensitivity indices (ISI) (A: HOMA-IR, B: Cederholm, C: Matsuda, D: OGIS, E: GUTT) calculated with the blood glucose and serum insulin concentrations measured fasting and during oral glucose tolerance tests (OGTT) pre and post a 1-week fully controlled dietary intervention with either a meat-based diet (CON) or a mycoprotein-based diet (MYC).** Values are means, with standard errors represented by vertical bars. There were no differences between groups at baseline for any of the OGTT calculated insulin sensitivity indices (all P>0.05) and no changes resulted from the intervention (time and interaction effects; all P>0.05)

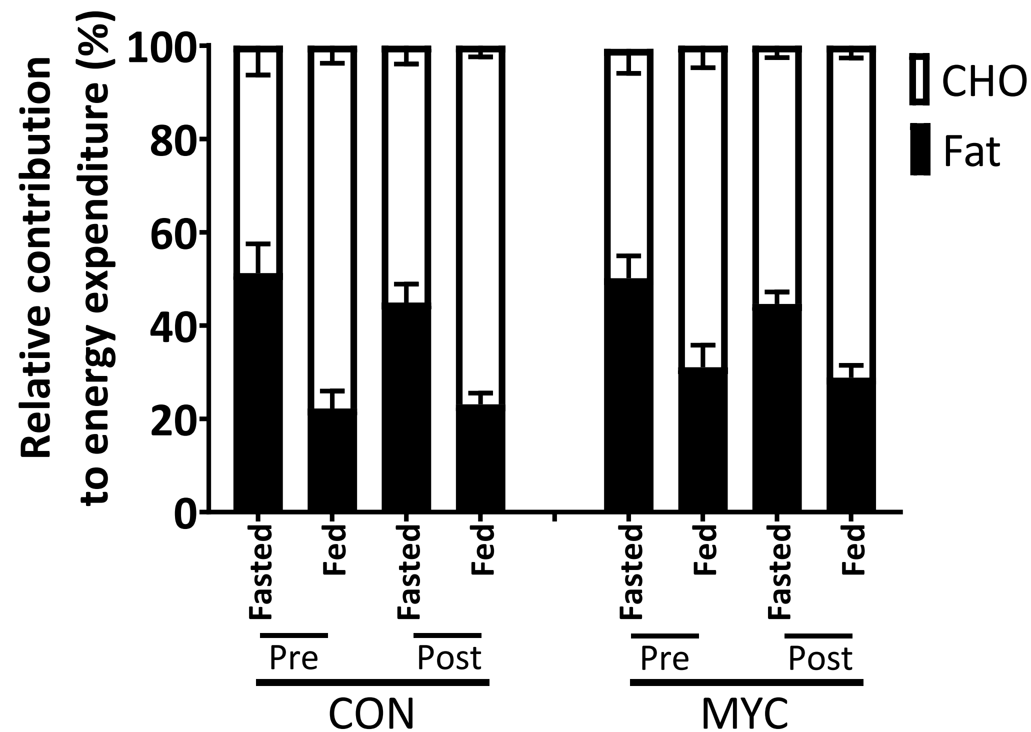


Figure 3.4 **Relative contribution of fat and carbohydrate (CHO) oxidation rates to energy expenditure calculated via indirect calorimetry using the Frayn equations, in the fasted and CHO-fed states, pre- and post- a 1-week fully controlled dietary intervention with either a meat-based diet (CON) or a mycoprotein-based diet (MYC).** Values are means, with standard errors represented by vertical bars. An effect of CHO ingestion was found for both CHO and fat oxidation rates (P<0.0001). No interaction or condition effects were found (all P>0.05).

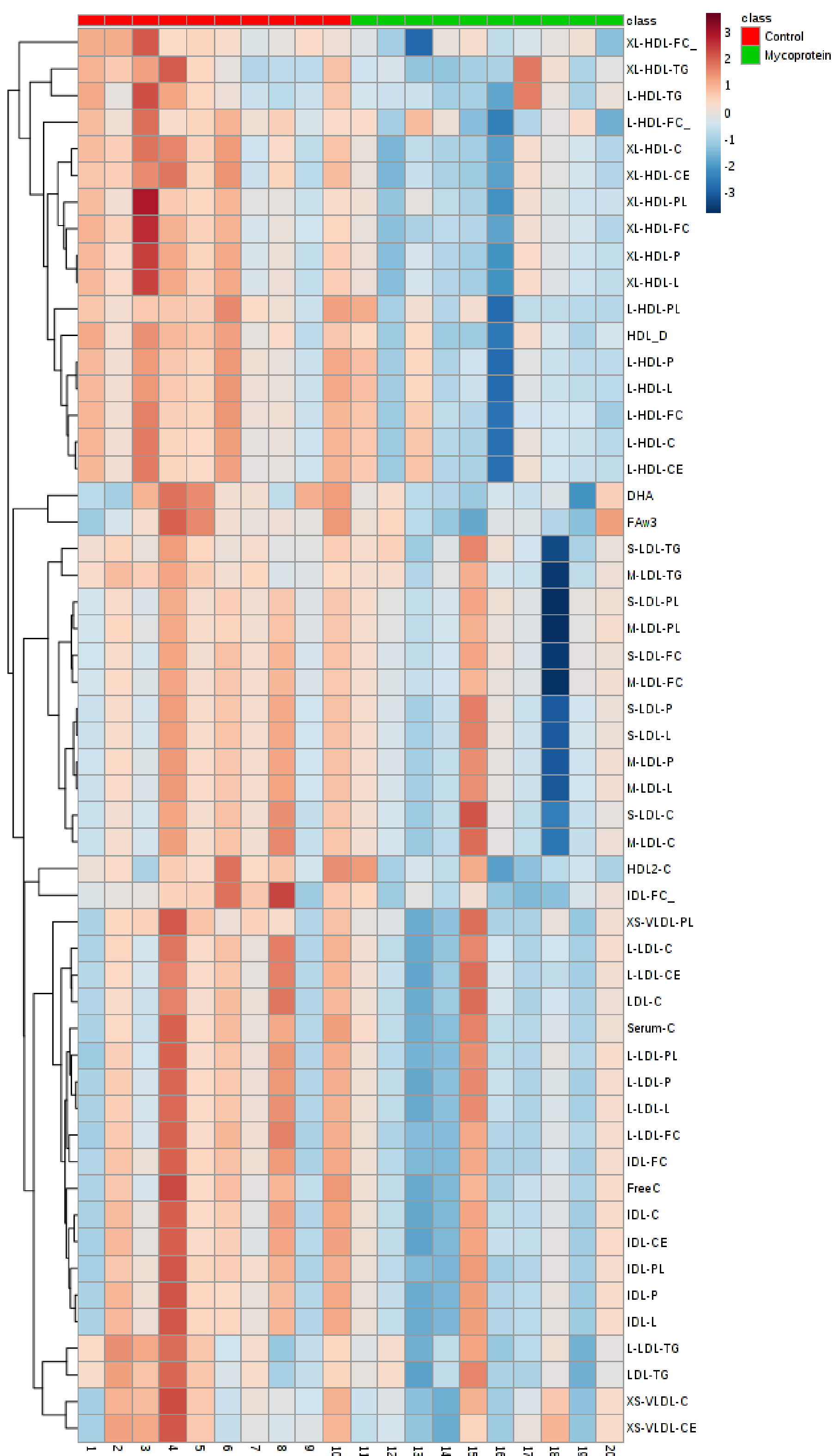


Figure 3.5 **Heat map and cluster representation of NMR-based metabolomics analyses which exhibited significant changes between pre- and post- a 1-week fully controlled dietary intervention with either a meat-based diet (CON) or a mycoprotein-based diet (MYC), calculated by the Δ change for each participant.** Participants in CON are represented in red and participants in MYC are shown in green. For an explanation of abbreviations, see Tables 3.4 and 3.5.

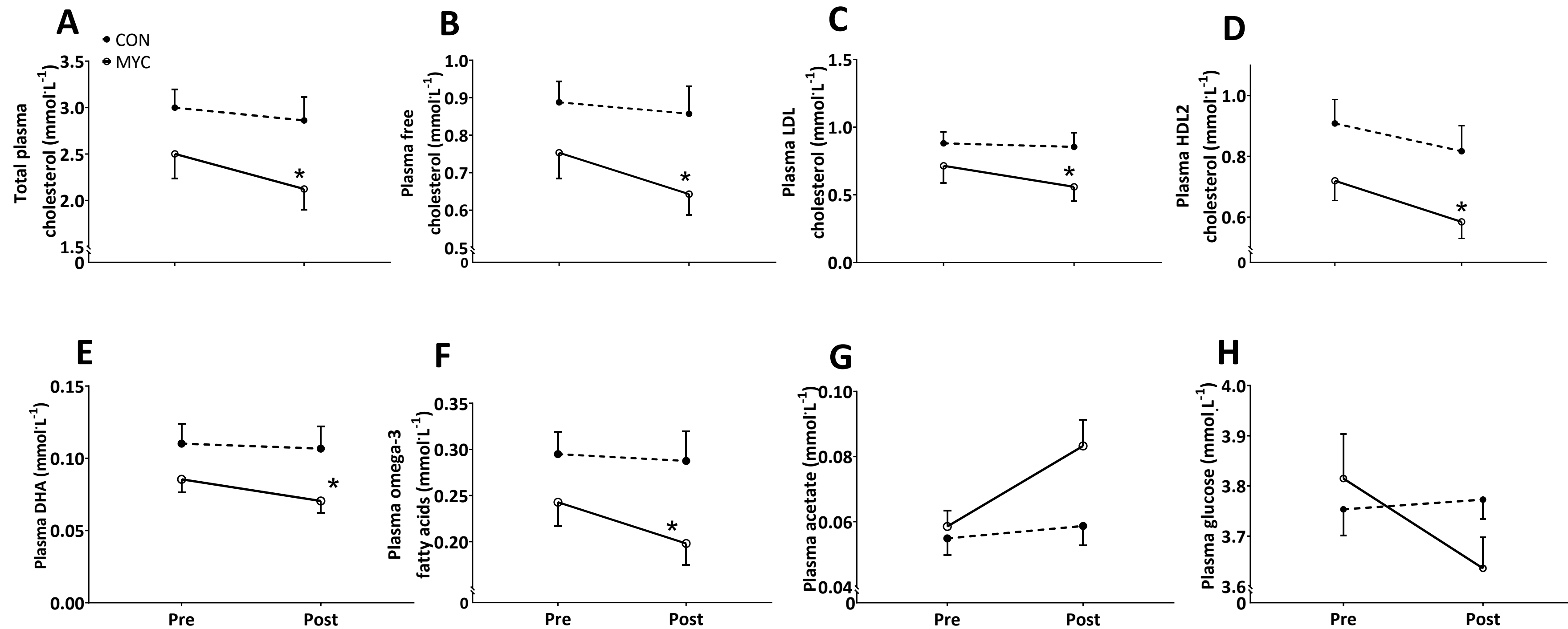


Figure 3.6 **Selected metabolites from the metabolomics analysis considered of particular relevance. Total plasma cholesterol (A), plasma free cholesterol (B), plasma LDL cholesterol (C), plasma HDL2 cholesterol (D), plasma DHA (E), plasma omega-3 fatty acids (F), plasma acetate (G) and plasma glucose (H) as measured by NMR-based targeted metabolomics pre- and post- a 1-week fully controlled dietary intervention with either a meat-based diet (CON) or a mycoprotein-based diet (MYC).** Values are means, with standard errors represented by vertical bars. NMR metabolomics metabolite concentrations were analysed using significance analysis of microarrays (SAM). Total plasma cholesterol, free cholesterol, LDL cholesterol, HDL2 cholesterol, DHA and omega-3 fatty acid were decreased to a larger degree in the MYC group (14-19% decrease) compared with the CON group (3-11 % decrease; $P<0.05$). Plasma glucose remained unchanged in the CON group but was reduced by 4.5±0.1 % in MYC and plasma acetate concentrations increased by 8.5±0.1 % and 43.6±0.1 % in CON and MYC, respectively. The changes in these two variables were significant when individually analysed (t-tests, $P<0.05$) but not when using the SAM multivariate analysis.

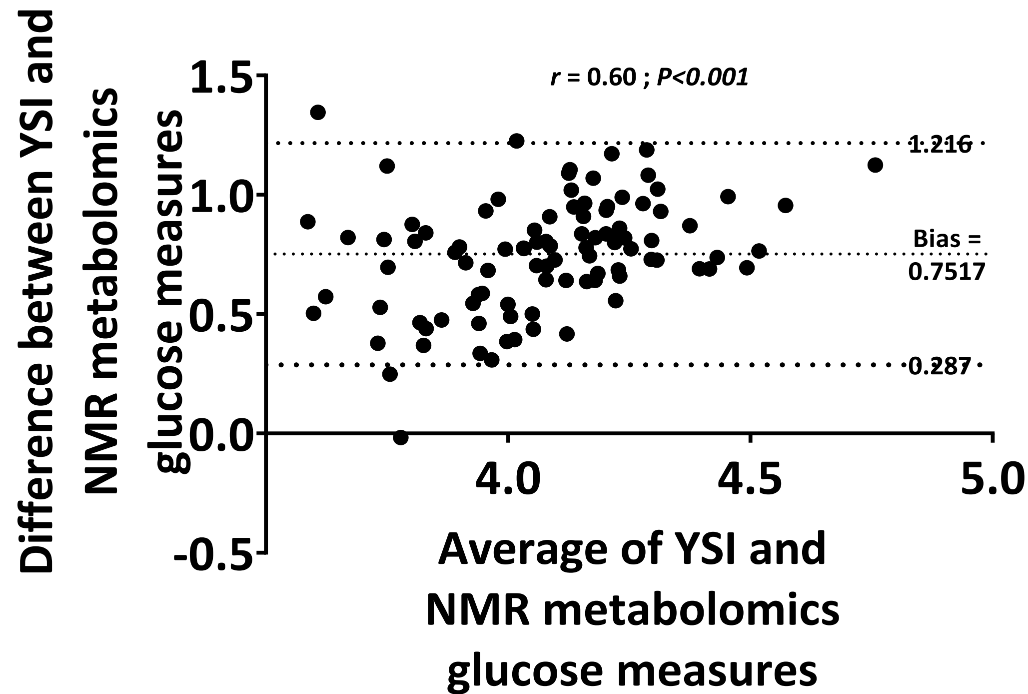


Figure 3.7 **Bland-Altman analysis plot for blood glucose concentrations measured by the benchtop YSI biochemistry analyser and plasma glucose concentrations measured by NMR spectroscopy, representing the consistency of the variant results between the two techniques**. Measurements for every participant's sample, pre- and post- a 1-week fully controlled dietary intervention with either a meat-based diet (CON) or a mycoprotein-based diet (MYC) in CON and MYC were aggregated. There was a strong positive correlation between the two techniques (r = 0.60; P<0.001).

3.4 Discussion

We investigated the impact of substituting meat and fish for mycoprotein as the major source of dietary protein at lunch and dinner during a fully controlled, energy and macronutrient balanced 1-week dietary intervention period on IS, glycaemic control and plasma lipid composition. We report that the mycoprotein intervention did not change indices of whole-body IS or 24-h free-living glycaemic control. However, the mycoprotein intervention had a profound impact on the plasma lipidome, inducing changes generally assumed to be indicative of improvements in long-term cardio-metabolic health.

Earlier studies (59, 62) reported that bolus mycoprotein ingestion improved acute postprandial glucose handling, but no work had investigated whether this translated to longer-term measures of metabolic health. We sought to test the hypothesis that chronic (one week), habitual (twice daily) mycoprotein consumption would improve whole body IS and/or daily habitual glycaemic control under carefully controlled conditions. We applied a nutritional intervention with no differences in energy or macronutrient consumption between groups (except for fibre; see Table 3.2) to young adults (who were well matched across groups; see Table 3.1). As a result, in our control group, despite a shift from habitual to controlled dietary conditions (which can often induce metabolic changes *per se* (52)), we observed no changes in any index of IS or glycaemic control. When substituting meat and fish for mycoprotein as the primary source of dietary protein in lunch and dinner, we also observed no changes in indices of liver or peripheral IS determined during an oral glucose tolerance test (Figures 3.2 and 3.3). Given the per meal mycoprotein consumption (~90 g wet weight) was equivalent/in excess of previous work demonstrating bolus mycoprotein consumption could improve acute glycaemic control (59, 62), this lack of support

for our hypothesis was perhaps surprising. However, those previous studies also indicated the effect was probably mediated by acute postprandial interactions of mycoprotein with dietary CHO, rather than an effect on IS *per se*. Of interest, epidemiological studies have shown total (postprandial) hyperglycaemia and/or the prevalence of (postprandial) hyperglycaemic excursions over the day to be better predictors of longer-term cardio-metabolic health (203). As such, to capture any effects of repeated mycoprotein ingestion on cumulative free-living postprandial glycaemic control (which could feasibly be independent of changes in IS), we applied continuous glucose monitoring throughout the study. However, whether we looked across the entire day or focussed on postprandial periods only, we did not observe any impact of the mycoprotein intervention (compared with either habitual conditions or the control group). We therefore demonstrate that short-term mycoprotein consumption does not impact IS or daily blood glucose control, at least in healthy young adults.

It is worth noting that our participants habitually consumed relatively high protein intakes (i.e. ~1.5 $g \cdot kg^{-1}$/day) and the intervention, therefore, represented an approximately 20% decrease in habitual protein intake. Since high-protein diets have been shown to improve glycaemic control (204) we cannot discount the possibility that the drop in protein intake obscured any potential changes in IS or glycaemic control; though, if so, we would expect these effects across both groups equivalently and our control group also remained unchanged. From a translational perspective, the protein content of the diet plays a large role in determining free-living energy balance, both directly via inducing dietary thermogenesis and indirectly based on the leverage of appetite (10). Given mycoprotein is also a particularly satiating dietary protein source (54, 55, 60), attention should also be paid when considering mycoprotein (and how much) as

a dietary intervention as to whether over- or under-eating is of primary concern for a particular population. It is important that future work extends these findings to more (metabolically) compromised individuals, where such dietary interventions are more likely to induce subtle, but clinically relevant, alterations in indices of metabolic health.

Our findings are in line with previous work that has reported that nutritionally induced acute beneficial effects on postprandial glucose handling do not necessarily translate to longer-term benefits on IS (205). Noteworthy, however, is the lowering effect of the mycoprotein diet on fasting blood glucose concentrations. As a recognised clinical marker of IS, this suggests mycoprotein consumption may support metabolic health, although it is difficult to explain why this was observed in the absence of effects on calculated IA and/or 24 h glucose control. It is possible that mycoprotein consumption specifically altered glucagon sensitivity (potentially due to mycoprotein's high fibre content) (206), or induced early improvements in *β*-cell function (207), but clearly, this warrants further research. It is crucial such research examines the effects of mycoprotein consumption during a longer time period and in various (more metabolically compromised) populations, as these changes may simply have been too subtle to detect in healthy individuals during a relatively short-term intervention. While our data did not largely support our hypothesis, incorporating mycoprotein as a sustainably produced alternative to meat clearly does not *negatively* impact on metabolic health over a 1 week period, an important perspective given the impetus in various populations to reduce animal-derived protein consumption. Indeed no gastrointestinal or other adverse effects were reported during the mycoprotein intervention, and the food substitutes were generally well tolerated/liked. While data concerning food preferences driving eating behaviour

are necessary to evaluate the wider potential/application of such dietary interventions under free-living conditions, our data indicate mycoprotein-containing products are a practical and feasible simple alternative to animal protein sources.

An existing body of work has demonstrated that 3-8 weeks of mycoprotein consumption reduce total plasma cholesterol and plasma LDL concentrations thereby resulting in improved HDL:LDL ratios (a robust marker of beneficial metabolic health outcomes (51-53)). To shed further light on this area we applied a novel NMR-based quantitative and targeted metabonomics approach (208). Strikingly, we found that merely one week of mycoprotein consumption led to coordinated changes in 53 of our 224 targets (see Table 3.5 and Figure 3.5). Specifically, we report decreases in plasma lipoprotein lipid content and, importantly, in plasma total, free, LDL and HDL2 cholesterol. However, unlike in previous studies (52, 53), this reduction in cholesterol was ubiquitous across lipoprotein species and therefore did not impact lipoprotein ratios (e.g. LDL:HDL) (209).

We (63) and others (52, 53) have previously argued that the cholesterol-lowering effect of mycoprotein consumption is probably related to the fibre content (or type) it contains (the most obvious nutritional difference across the diets; 26 vs 34 g daily in CON and MYC, respectively). While our design (not fibre matched across groups) does not allow us to disentangle the interesting potential effects of fibre *quantity* vs *type* (63), the role of higher fibre intake in reducing circulating cholesterol concentrations is also in line with epidemiological (68, 69) and intervention (210) studies (2 to 10 g fibre supplementation per day), with these effects also translating to a reduced risk of coronary heart disease (CHD). We, therefore, provide evidence that an innocuous and feasible dietary intervention

can provide a sufficient increase to dietary fibre intake to place individuals at the top end of this dose-response effect. Furthermore, we extend on previous observations (51-53) by demonstrating how rapidly this effect ensues consequent to mycoprotein consumption.

The mechanism(s) by which increased fibre intake lowers circulating plasma cholesterol may be related to large intestinal fermentation of insoluble fibre fractions via the gut microbiota (63, 211). Short-chain fatty acids (SCFAs), such as acetate, propionate and butyrate, are primary products of fibre fermentation (75). Though SCFA have been shown to have a range of metabolic effects (212-216), of note is the reported effect of (propionate in particular) reducing hepatic cholesterol synthesis (217). *In vitro* colonic models have shown mycoprotein's fibre (which is composed of approximately two-thirds *β*-glucan and one-third chitin (63)) to be fermentable to propionate and butyrate, but at the expense of acetate (81), suggesting such end products could underpin the cholesterol-lowering effect. It is, therefore, somewhat surprising that we observed a considerable (40%) increase in plasma acetate (Figure 3.6) following 1 week of mycoprotein consumption. However, this is in line with previous work showing the acute consumption of other dietary fibres leading to increased postprandial serum acetate concentrations (218), and that fibre (e.g. oat or bran-rich diets) induced reductions in circulating cholesterol are also associated with increases in blood acetate (219). Whether acetate as an *in vivo* end product of mycoprotein bacterial fermentation mediated the plasma cholesterol-lowering effect, either by inhibiting cholesterol synthesis, or by other unknown mechanisms (such as reduced cholesterol absorption or increased peripheral clearance) is not clear, and warrants future (human) research.

The metabolomics approach also revealed non-cholesterol related changes in the plasma lipidome. Of note, mycoprotein consumption decreased plasma DHA and omega-3 fatty acid concentrations (Figure 3.6), presumably due to a lower dietary load. However, given the reported variance in plasma levels of these lipids tends to be related to an individual's last meal, this may be an acute effect rather than reflective of a 'deficiency', particularly over only a one-week period (220). Nevertheless, since omega-3 fatty acids, in particular, have been linked to various desirable health outcomes (221) it would be prudent for future dietary interventions that involve reducing dietary intake of omega-3 (and DHA) lipids to monitor such effects.

In conclusion, the present data show that substituting meat/fish for mycoprotein at lunch and dinner for 1 week does not modulate whole-body IS or 24 h free-living (postprandial) glycaemic control, but considerably impacts upon the plasma lipidome. Mycoprotein represents a sustainable dietary protein source that can be incorporated into the daily diet without compromising short-term metabolic health and facilitating rapid and possibly beneficial changes to the plasma lipidome.

Chapter 4:

Ingestion of a nucleotide-rich mixed meal increases serum uric acid concentrations but does not affect postprandial blood glucose or serum insulin responses in young adults

The work contained in this chapter has been published as follows:

Coelho, M. O., Monteyne, A. J., Kamalanathan, I. D., Najdanovic-Visak, V., Finnigan, T. J., Stephens, F. B., & Wall, B. T. (2020). Short-Communication: Ingestion of a Nucleotide-Rich Mixed Meal Increases Serum Uric Acid Concentrations but Does Not Affect Postprandial Blood Glucose or Serum Insulin Responses in Young Adults. *Nutrients*, *12*(4), 1115.

4.1 Introduction

Free ribonucleic acid (RNA) naturally occurs to varying degrees in many dietary protein sources. Upon ingestion, RNA is digested and absorbed as its constituent nitrogenous nucleotide bases (pyrimidines and purines), pentose sugars and phosphate ions. The primary end-product of purine metabolism is uric acid, which is mainly formed in the liver, transported to the kidneys and excreted in urine (87). Human consumption of large boluses (>2 g/day) of isolated or (single-cell) yeast-derived nucleotides has been shown to result in acute increases in circulating uric acid concentrations (131-133). Epidemiological and observational data have reported that circulating uric acid concentrations positively correlate with the development of gout (122), hypertension (123), and metabolic syndrome (124), and are a predictor of type 2 diabetes (125), though causation (particularly by diet) has not been established. Nevertheless, these findings informed the FAO/WHO/UNICEF Protein Advisory Group recommendation to limit the additional dietary nucleic acid load from single cell protein novel foods to no more than 2 g/day (134, 135). How this translates to practical nutrition is unclear, since the ingestion of a high nucleotide containing mixed meal upon circulating uric acid concentrations has not been investigated. Moreover, whether acute elevations of serum uric acid influence and therefore may be causatively linked to, markers of metabolic health, such as postprandial insulin sensitivity or glucose tolerance, is also unknown.

Mycoprotein is a single-celled fungal-derived protein-rich food source produced by the continuous cultivation of *Fusarium venenatum* and is naturally rich in RNA-derived nucleotides. To comply with the FAO/WHO/UNICEF Protein Advisory Group recommendations, industrial production of mycoprotein for commercial use involves the reduction of RNA content from ~10% to ~2% of its

dry weight. This is achieved by an industrially expensive process of subjecting the culture broth to a short heat treatment (68 °C, 20 minutes) following fermentation, which allows for (endogenous) enzymatic breakdown of nucleotides, without affecting protein content (42). We have previously shown that this process is effective since the ingestion of nucleotide depleted mycoprotein up to quantities of 40 g did not influence postprandial circulating uric acid concentrations (48), with even 60 g causing only a transient (~2 h) and modest (from ~350 to ~380 $\mu mol{\cdot}L^{-1}$; well below clinically relevant thresholds of >420 $\mu mol{\cdot}L^{-1}$ (136)) rise, and in line with the consumption of other protein sources (137). However, it is not known what impact the ingestion of non-nucleotide depleted mycoprotein has upon the postprandial serum uric acid response. By obtaining nucleotide depleted and non-depleted mycoprotein containing food products, we were able to test the hypothesis that the ingestion of a single nucleotide-rich mixed meal would acutely increase circulating uric acid concentrations and impair postprandial glucose handling.

4.2 Subjects and methods

4.2.1 Participants and medical screening

Ten healthy young adults (age: 25 ± 1 years; BMI: 24.4 ± 1.0 $kg \cdot m^{-2}$; male = 4 and female = 6) participated in the present study. Participants' characteristics are presented in **Table 4.1**. 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. Smokers and participants taking regular medication or suffering from chronic diseases were excluded. Participants included were recreationally active (partook in regular exercise or sport at a non-competitive level, two to five days a week), normotensive, and had a BMI between 18.5 and 30 $kg \cdot m^{-2}$. All participants were informed of the study's purposes, procedures and risks, and provided written informed consent. The study was approved by the University of Exeter's Sport and Health Sciences Ethics Committee (Ref No: 170712/A/01) in accordance with the Declaration of Helsinki and was registered at ClinicalTrials.gov (NCT03568864) following the Consolidated Standards of Reporting Trials (CONSORT).

Table 4.1 **Participants' characteristics.**

Sex	6 female / 4 male
Age (years)	25 ± 1
Height (cm)	171 ± 3
Body mass (kg)	72 ± 4
Body mass index ($kg·m^{-2}$)	24.4 ± 1.0
Body fat (% of body mass)	22 ± 4
Lean mass (kg)	57 ± 5
Systolic blood pressure (mmHg)	117 ± 5
Diastolic blood pressure (mmHg)	68 ± 2

4.2.2 Experimental protocol

The present study was a randomised, controlled, double-blind, crossover trial, where participants consumed a meal containing either a low (L-NU) or high (H-NU) amount of dietary nucleotides (1.96% and 8.83% of the mycoprotein (dry weight) in L-NU and H-NU, respectively; **Table 4.2** details the full nutritional composition of the test meals).

Table 4.2 **Nutritional composition of the experimental meals.**

	L-NU	**H-NU**
Energy and macronutrients		
Energy (kJ)	2519	2519
Energy (kcal)	602	602
Protein (g)	28	28
Carbohydrate (g)	58	58
Fat (g)	26	26
Nucleotides (in dry mycoprotein)		
CMP (g/%)	-	0.12/0.62
UMP (g/%)	0.05/0.26	0.08/0.44
GMP (g/%)	0.04/0.21	0.05/0.28
TMP (g/%)	0.09/0.46	0.57/3.00
CDP (g/%)	0.04/0.19	0.16/0.84
UDP (g/%)	0.01/0.07	0.06/0.34
CTP (g/%)	0.09/0.47	0.60/3.18
ADP (g/%)	0.00/0.02	0.02/0.08
TTP (g/%)	-	0.01/0.04
ITP (g/%)	0.06/0.29	-
ATP (g/%)	-	0.00/0.01
Total nucleotides (g/%)	0.38/1.96	1.70/8.83

Abbreviations: L-NU, Low nucleotide condition; H-NU, High nucleotide condition; 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

Participants reported to the laboratory at ~08.00 a.m., after an overnight fast and refraining from intense exercise and alcohol consumption for at least 24 h, to undertake two identical experimental test days, separated by a two-week washout period. A cannula was placed retrogradely in a dorsal hand vein and the hand was then placed in a heated box (55 °C) for arterialised-venous blood sampling (178). A fasted baseline arterialised-venous blood sample was collected and participants ingested the test meal, consisting of a sandwich made of three slices of bread (132 g; Kingsmill 50/50 medium, Allied Bakeries, UK), two packets of mayonnaise (28 g; Hellmann's, Unilever, United States) and eight Quorn vegetarian chicken slices (100 g; Quorn, Marlow Foods, UK), containing either the nucleotide-depleted (L-NU) mycoprotein or the nucleotide-rich (H-NU) mycoprotein (77 g wet weight; corresponding to 19 g dry weight) in 10 mins or less (with the exact time being recorded for each participant in the first visit and replicated on the second experimental test day). Arterialised-venous blood samples were then collected for a 4 h postprandial period, at 15 min intervals for the first hour and at 30 min intervals subsequently. Afterwards, participants were allowed to leave the laboratory and were instructed to return at 8, 12, and 24 h after the ingestion of the sandwich for further (venous) blood sample collection via venepuncture. Participants kept a diary detailing their food intake and physical activity during the 24 h period of the first experimental visit and replicated this for the second experimental visit.

4.2.3 Plasma and serum collection and analyses

One mL of each blood sample was collected into FX blood collection tubes (Becton Dickinson, Franklin Lakes, NJ, USA) and glucose was immediately analysed using the YSI 2300 STAT PLUS Biochemistry Analyser (YSI, Yellow

Springs, OH, USA). Four mL of each blood sample was collected into SST tubes (Becton Dickinson, Franklin Lakes, NJ, USA) and left at room temperature for at least 30 mins to clot, before being centrifuged at 4 °C and 4000 RPM. The aliquoted serum samples were 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) in the Clinical Chemistry department of the Royal Devon and Exeter NHS Foundation Trust. Serum insulin concentrations were analysed using DRG ELISA kits (DRG International, Springfield, NJ, USA). An adapted Cederholm insulin sensitivity index (Cederholm ISI; accounting for the carbohydrate intake from the mixed meal) was calculated using the blood glucose and serum insulin concentrations measured in the fasting state and during the 4 h post-prandial period, to provide an indirect measure of peripheral insulin sensitivity.

4.2.4 Statistical analyses

All data are expressed as means ± standard errors (SEM). Serum uric acid, blood glucose, and serum insulin concentrations were analysed using two-way ANOVAs (with condition and time as factors). Bonferroni post hoc tests were performed in the event of significant main effects. Additionally, incremental Area Under the Curves (iAUCs) were calculated for blood glucose and serum insulin, and paired t-tests were performed to detect any significant effect of condition. The adapted Cederholm ISI data were also analysed using a paired t-test.

4.3 Results

4.3.1 Serum uric acid

Serum uric acid concentrations during the 24 h experimental period are reported in **Figure 4.1**. Baseline fasting serum uric acid concentrations were not different between conditions (274 ± 16 $\mu mol \cdot L^{-1}$ in L-NU and 284 ± 13 $\mu mol \cdot L^{-1}$ in H-NU; $P > 0.05$). Meal ingestion influenced serum uric acid concentrations (effect of time; $P < 0.001$) and it did so divergently between groups (time x condition interaction; $P < 0.001$). In L-NU, meal ingestion modestly (~7 %) and transiently (from 45 mins (279 ± 16 $\mu mol \cdot L^{-1}$) to 4 h (257 ± 14 $\mu mol \cdot L^{-1}$) post-meal consumption) decreased serum uric acid concentrations, with baseline levels restored by 8 h post-meal ingestion. Conversely, in the H-NU condition, following meal ingestion serum uric acid concentrations rose steadily by ~12 %, peaking after 210 mins (319 ± 12 $\mu mol \cdot L^{-1}$; $P < 0.05$), remaining elevated for 12 h and returning to baseline concentrations only after 24 h (302 ± 13 $\mu mol \cdot L^{-1}$; $P > 0.05$).

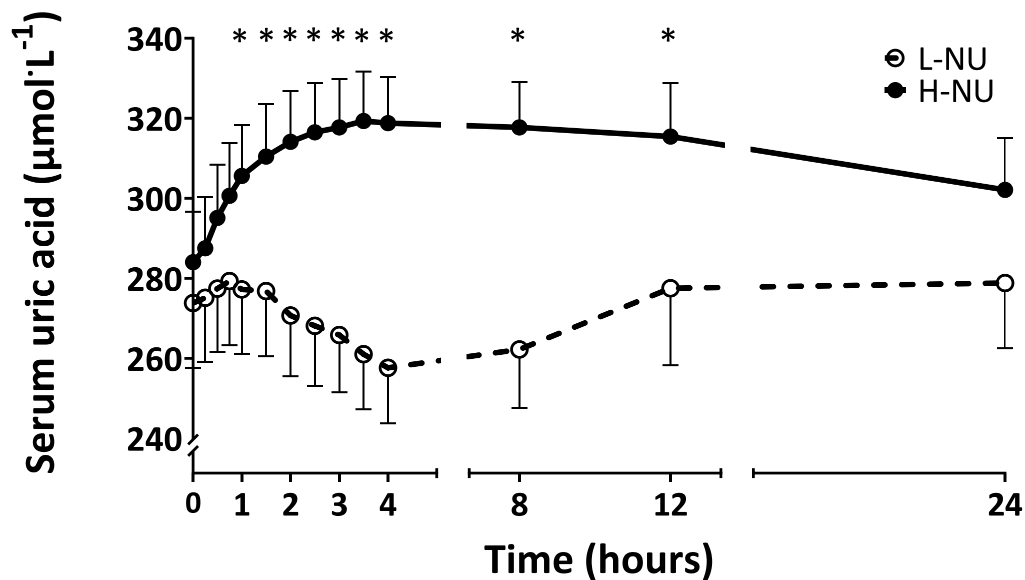


Figure 4.1 **Temporal response of serum uric acid concentrations during a 24 h period following the consumption of a nucleotide-depleted (L-NU) or nucleotide-rich (H-NU) mycoprotein-based mixed meal in healthy young adults (*n* = 10).** Data were analysed using a two-way ANOVA and Bonferroni post-hoc tests. Values are means with SEM represented by vertical bars. Main effect of time ($P < 0.01$), condition ($P > 0.05$) and time x condition interaction ($P < 0.0001$). * denotes significantly different from corresponding baseline value.

When looking at sex-based differences, postabsorptive serum uric acid concentrations were higher in male compared with female participants (314 ± 13 and 264 ± 14 µmol·L^{-1}, respectively; $P < 0.05$). Meal ingestion influenced serum uric acid concentrations similarly across sexes and in line with the cohort as a whole (effect of time, $P < 0.0001$; trend for a time x condition interaction, $P = 0.07$) with similar absolute rises of ~30 to 40 µmol·L^{-1} (peaking after 210 mins at 345 ± 11 µmol·L^{-1} for males and after ~8 h at 305 ± 14 µmol·L^{-1} for females), though this represented seemingly, but not significantly, differing rises expressed as a percentage across sexes (9 ± 2 and 16 ± 4 % for males and females, respectively; $P > 0.05$). In addition, females displayed more persistent elevations of serum uric acid concentrations with 24 h not being sufficient to return to baseline levels (after ~ 24 h: 292 ± 21 µmol·L^{-1}).

4.3.2 Blood glucose and serum insulin

Temporal blood glucose and serum insulin concentrations for the 4 h postprandial period are displayed in **Figure 4.2** (A, C). Fasting blood glucose and serum insulin concentrations did not differ between conditions at baseline (both $P > 0.05$). For both measures, there was a significant effect of time ($P < 0.0001$), such that meal ingestion increased blood glucose and serum insulin concentrations (peaking at 30 mins; glucose: 6.2 ± 0.2 and 6.1 ± 0.2 mmol·L^{-1}, and insulin: 67 ± 10 and 63 ± 8 mU·L^{-1}, for L-NU and H-NU, respectively) and to a similar degree for both conditions (condition and time x condition interaction; $P > 0.05$). Blood glucose iAUC and serum insulin iAUC during the OGTT (displayed in Figure 4.2 (B, D**)**) were also not different between conditions ($P > 0.05$). Consequently, indirect peripheral insulin sensitivity calculated using the adapted Cederholm ISI did not differ between conditions (61 ± 3 and 64 ± 3 mg·L^{2}·mmol^{-1}·mU^{-1}·min^{-1} for L-NU and H-NU, respectively; $P > 0.05$).

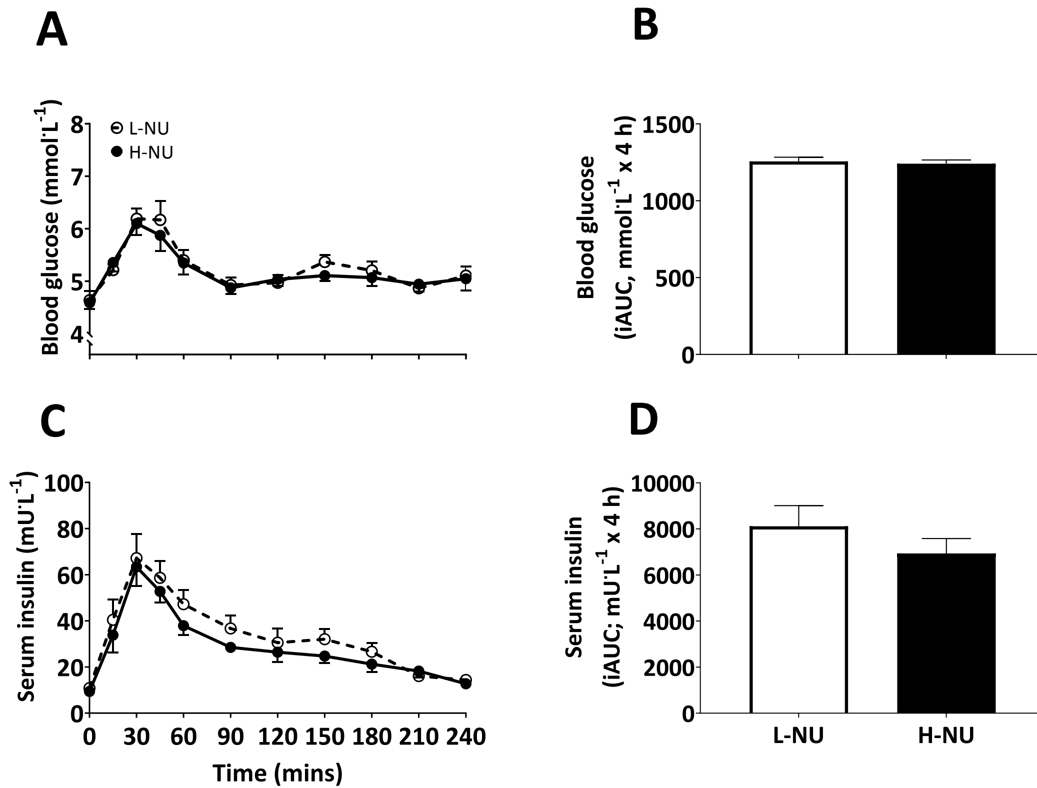


Figure 4.2 **Temporal response and incremental area under the curve of blood glucose (A, B) and serum insulin (C, D) concentrations during the 4 h postprandial period following the consumption of a nucleotide-depleted (L-NU) or nucleotide-rich (H-NU) mycoprotein-based mixed meal in healthy young adults (*n* = 10).** Data were analysed using two-way ANOVAs. Incremental area under the curve (iAUC) data were analysed using paired t-tests. Values are means with SEM represented by vertical bars. Main effects of time, condition, time x condition interaction and paired t-tests (all $P > 0.05$).

4.4 Discussion

We report that the consumption of a high-nucleotide mixed meal transiently (for 12 h) increased serum uric acid concentrations, but did not influence postprandial circulating glucose and insulin concentrations, or an adapted Cederholm insulin sensitivity index, compared with the ingestion of a low nucleotide control meal in healthy young adults.

Higher circulating uric acid concentrations are associated with a variety of inflammatory and/or metabolic disorders, such as gout (122), hypertension (123), type 2 diabetes (125), and metabolic syndrome (124), leading to speculations of a causative role (130). Dietary nucleotides have been implicated as the link between circulating uric acid and such disease progression since daily short-term (5–9 days) ingestion of large quantities (>2 g) (isolated from yeast) has been shown to increase serum uric acid concentrations above clinically acceptable thresholds in healthy adults (131-133). However, it is not clear whether this is the case within the context of a more nutritionally relevant mixed meal. Here, we report a high-nucleotide containing (i.e., 1.7 g) mixed meal increased serum uric acid concentrations within 1 h, and for a duration of 12 h post consumption (see Figure 4.1). This ~12 % rise (peaking at 319 $\mu mol \cdot L^{-1}$) was transient (returning to near basal levels the following day) and not close to clinically significant levels (i.e., ~420 $\mu mol \cdot L^{-1}$ in men, ~360 $\mu mol \cdot L^{-1}$ in pre-menopausal women (95)) in any of the participants. Interestingly, when subdividing our participants based on sex, some differences were evident. Namely, in accordance with the literature (95), males displayed higher postabsorptive serum uric acid concentrations compared with females, and although the absolute rise following the high nucleotide meal was similar between sexes, the higher relative rise amongst females translated to a more persistent elevation (i.e., at 24 h). The mechanism by which nucleotide

rich meal ingestion elevates circulating uric acid concentrations likely relates to increased uric acid production as a metabolic end-product without any appreciable increase in (at least over the immediate postprandial period) urinary clearance. While we did not collect urine samples to confirm this, recent animal data report that experimental alterations in circulating uric acid concentrations are regulated at the level of renal handling (222). However, this work also suggested that the direct mechanism was the level of circulating insulin, which is not consistent with our finding of equivalent postprandial insulin responses across conditions. Future (intervention) studies are warranted to assess whether repeated meals result in persistently and cumulatively (i.e., beyond clinically accepted levels) elevated serum uric acid concentrations, how this affects temporal rates of urinary uric acid clearance and to further probe the mechanisms underpinning nutritional uric acid balance in health and disease.

Making our current data more striking was the observation that serum uric acid concentrations decreased (from 45 min to 4 h post-meal ingestion) under the control condition. This implies that the well documented protective effect of some dietary protein sources per se on circulating uric acid concentrations (shown previously with dairy and some plant-based proteins (223-225)), due to competitive tubular reabsorption and increased uric acid urinary clearance (either directly or indirectly via greater urea production), outweighed any impact of the low nucleotide content (i.e., 0.38 g) of this meal. Since vegan diets have been associated with higher circulating uric acid concentrations (225, 226) — proposed to be attributable to the lack of dairy in the diet — and whilst we did not investigate this, our data imply nucleotide depleted mycoprotein may be a suitable protein choice to manage uric acid levels in these populations.

A potential mechanism by which circulating uric acid concentrations may be causative of declining health status is not clear. Numerous experiments performed in vitro (127, 227-231) have shown that uric acid is a pro-oxidant in the intracellular environment, implying uric acid may contribute to metabolic dysfunction by promoting cellular oxidative stress (130). Here we show that the increase in circulating uric acid concentrations following the nucleotide rich meal was not associated with any impairment in postprandial glucose handling or on an insulin sensitivity index. Though only addressing the acute response to bolus consumption, our data do not support that nutritionally induced acute increases in circulating uric acid concentrations impair markers of metabolic health. Intervention studies addressing the impact of habitual (rather than single meal) dietary nucleotide load on circulating uric acid concentrations and indices of metabolic health will be required to further elucidate the link between circulating uric acid, cellular oxidative stress, and metabolic health.

In conclusion, the ingestion of a nucleotide-rich mixed meal increases serum uric acid concentrations for ~12 h but does not influence postprandial blood glucose or serum insulin concentrations.

Chapter 5:

High dietary nucleotide consumption for one week increases circulating uric acid concentrations but does not compromise metabolic health

5.1 Introduction

Nucleotides are a group of 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) (86). 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 (in the form of RNA and DNA) can vary widely between individuals but is typically 0.1-1 g/day (232, 233). Evidence suggests that nucleotides are a *conditionally-essential* nutrient, with an increased dietary requirement during periods of rapid growth (e.g. most infant feed formulae are enriched with nucleotides; (160, 161)), 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) (162, 163), as well as in recovery from extensive trauma and in intensive care, such as in burns patients (164). Additionally, emerging data imply beneficial effects of nucleotide supplementation on exercise performance and recovery (169).

Despite the requirement for dietary nucleotides, some concerns have been raised regarding high intakes in humans (234). Nucleotides are primarily ingested in the diet as RNA and DNA and subsequently digested and absorbed as pyrimidines and purines (86), 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 (122), hypertension (123) and metabolic syndrome (124), and are a predictor of type 2 diabetes (125), though causal links remain to be established. Although uric acid homeostasis is tightly regulated at the level of hepatic (formation) and renal

(clearance) function (93), short term ingestion of high-dose (> 2 g/day) isolated (or yeast-derived) nucleotides results in acute elevations in circulating uric acid concentrations above clinically acceptable levels (i.e. of > 420 $\mu mol \cdot L^{-1}$) (131-133). 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 (135). 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 (134).

Mycoprotein, a sustainable fungal-derived dietary protein source (63), 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) (42) for commercial products to comply with FAO/WHO/UNICEF recommendations (134, 135). We recently reported that the ingestion of a nucleotide-rich (1.7 g) mycoprotein-containing mixed-meal transiently increased (by 12%) postprandial serum uric acid concentrations in healthy adults for 12 hours compared with ingesting nucleotide depleted mycoprotein (235). 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 over the relationship between circulating uric acid levels and markers of metabolic health (and therefore the potential relevance to disease progression) is not yet available.

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 (IS), glycaemic control and the plasma metabolome).

5.2 Subjects and methods

5.2.1 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 5.1**. 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) (184). Vegetarians, vegans, smokers, and participants taking regular medication or suffering from chronic diseases were excluded. 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 31 kg·m^{-2}. 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 (236). 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).

Table 5.1 **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.

5.2.2 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 mycoprotein (LOW; *n*=10) or a naturally produced, high nucleotide, mycoprotein (HIGH; *n*=10) 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 5.1** 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.

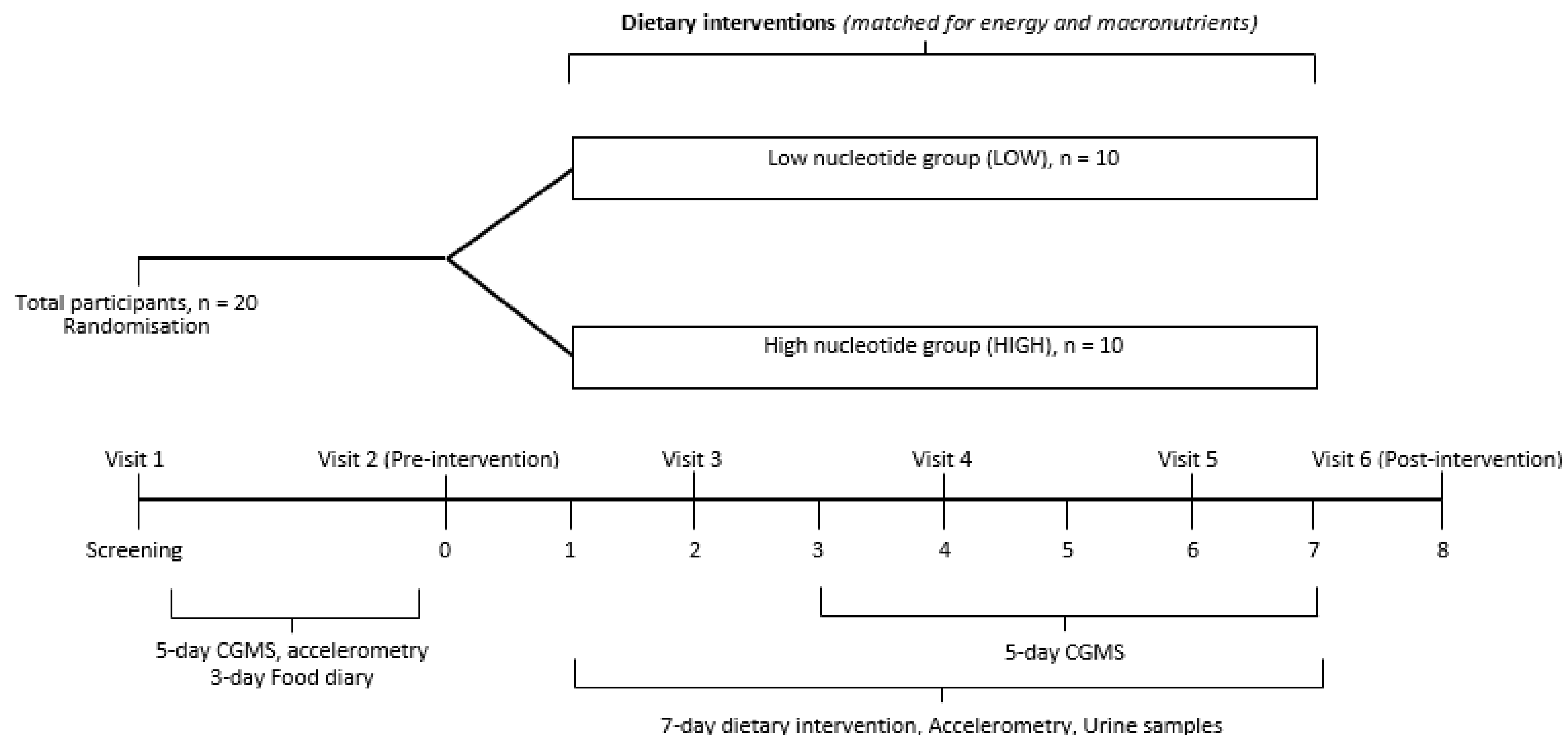


Figure 5.1 **Overview of the experimental protocol.**

5.2.3 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.

5.2.4 Experimental test days

Participants reported to the laboratory at ~08.00 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 hours, 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 (178). 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 rate (RMR), were calculated using the Frayn equations (186). 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 a 2 h period 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 mins following glucose ingestion.

5.2.5 Dietary intervention

Basal metabolic rate (BMR) was estimated using the Henry equations based on age, gender, and weight (187). The IPAQ was used to calculate a physical activity level (PAL) factor (188). 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 department's research kitchen facility. Nutritional information for the two diets is provided in **Table 5.2**. Subjects consumed a diet containing 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, with the exception of 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 in order to track compliance with the dietary intervention, log meal times and provide recipe information/instructions. Thereafter, participants were required to visit the laboratory at ~08.00 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 (using 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 urine of the day, which the participants kept sealed inside a plastic bag and in the fridge until the next time they came to 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.

Table 5.2 **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 the 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 ($g \cdot kg^{-1}$ 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; $P<0.05$)

5.2.6 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 minutes. All tubes were centrifuged at 4° C and 4000 RPM, and aliquoted (one aliquot designated for each of the below analyses) plasma and serum were stored at -80° C.

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 metabolomics 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 in a cost-effective manner (191). Biomarker concentrations quantified by this NMR approach have been shown to be highly consistent with concentrations obtained from standardised clinical chemistry analyses (192). For

a detailed description of the experimental protocol, including sample preparation and NMR spectroscopy please see references (191-193). The data were then 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.

5.2.7 Insulin sensitivity

Five different IS indices (197-201), all validated against the hyperinsulinaemic euglycemic 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 from solely fasting concentrations of glucose and insulin and has been shown to provide a reasonable estimate of hepatic IS (197). 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 sensitivity (199). The Cederholm, OGIS and Gutt indices focus mainly on peripheral IS and muscular glucose uptake by measuring OGTT glucose clearance (198, 200, 201).

5.2.8 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, was 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 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). Days with data for fewer than 70% of the total time-points were also excluded. The remaining data were analysed for glycaemic control (24 h average glucose, glucose area under the curve (AUC) and two-hour postprandial glucose) 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 (202).

5.2.9 Statistical analyses

A power analysis based on the assumption of a 30% increase in serum uric acid concentrations with high-nucleotide mycoprotein consumption (calculated based on an extrapolation from results from a previous study conducted in our lab (48)) was performed 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. 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 detect 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 (n = 142 and n = 79, respectively). 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 calculations 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).

5.3 Results

5.3.1 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 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 summarised in **Table 5.2**. 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. 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 effect; P<0.05). Habitual fibre intake was not different between groups (P>0.05), but increased 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 (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 daily mycoprotein-derived dietary nucleotide intake in both groups are shown in **Table 5.3**. The daily nucleotide load of the mycoprotein consumed in the intervention diets was ~5 fold greater in the HIGH compared with LOW diet (0.89±0.06 g and 4.46±0.29 g in LOW and HIGH, 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 ($P<0.0001$). Remaining foods present in the diets, such as dairy products, cereals and vegetables, were all low in purine nucleotides (<50 mg/100 g). These foods amounted to an estimated additional purine load of 137±7 mg in LOW and 127±5 mg in HIGH ($P>0.05$). 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 (89, 237, 238) and ensured there were no differences in the diets between groups.

5.3.2 Physical activity

Physical activity data are shown in **Table 5.4**. 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 $P>0.05$).

5.3.3 Serum and urine uric acid concentrations

Figure 5.2 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 starting to decrease (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).

5.3.4 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 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). 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 5.3**. 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 2) 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 5.4)**.

5.3.5 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 difference 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).

5.3.6 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 contribution of fat and carbohydrate oxidation to total energy expenditure in both the fasted and fed state are displayed in **Figure 5.5**.

5.3.7 Nuclear magnetic resonance (NMR) based metabolomics

Table 5.5 lists the 224 metabolites measured by NMR metabolomics. One participant in the HIGH group was excluded from the metabolomics analysis due to being an extreme outlier (presented non-plausible changes of over 300% in most metabolites between measurements; 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 5.6** and **Table 5.6** 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 $mmol \cdot L^{-1}$; $P<0.05$). Similarly, apolipoprotein A1 decreased by 7.5 % in LOW (from 1.24±0.05 to 1.14±0.04 $mmol \cdot L^{-1}$) but only by 2.7 % in HIGH (from 1.26±0.04 to 1.22±0.04 $mmol \cdot L^{-1}$; $P<0.05$) and the mean diameter for HDL particles had a slight decrease of 1.26 % in LOW, whilst remaining constant in HIGH ($P<0.05$).

Table 5.3 **Daily mycoprotein-derived dietary nucleotide intake (in grams) and nucleotide composition of dry mycoprotein (%)**

	LOW	HIGH
CMP (g / % mycoprotein dry wt)	-	0.32 / 0.62
UMP (g / % mycoprotein dry wt)	0.12 / 0.26	0.22 / 0.44
GMP (g / % mycoprotein dry wt)	0.10 / 0.21	0.14 / 0.28
TMP (g / % mycoprotein dry wt)	0.21 / 0.46	1.53 / 3.00
CDP (g / % mycoprotein dry wt)	0.09 / 0.19	0.43 / 0.84
UDP (g / % mycoprotein dry wt)	0.02 / 0.07	0.17 / 0.34
CTP (g / % mycoprotein dry wt)	0.21 / 0.47	1.62 / 3.18
ADP (g / % mycoprotein dry wt)	0.01 / 0.02	0.04 / 0.08
TTP (g / % mycoprotein dry wt)	-	0.02 / 0.04
ITP (g / % mycoprotein dry wt)	0.13 / 0.29	-
ATP (g / % mycoprotein dry wt)	-	0.01 / 0.01
Total nucleotides (g / % mycoprotein dry wt)	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 5.4 **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.
Abbreviations: LOW, low nucleotide mycoprotein group; HIGH, high nucleotide mycoprotein group.

Table 5.5 **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 lipids 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
XXL-VLDL-TG	Triglycerides in chylomicrons and extremely large 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

XL-HDL-P	Concentration of very large HDL particles	**Lipoprotein Particle 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
L-HDL-L	Total lipids in large HDL	**Fatty Acids and 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
S-HDL-FC	Free cholesterol in small HDL	**Glycolysis Related 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
HDL2-C	Total cholesterol in HDL2	**Branched-chained 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
TG/PG	Ratio of triglycerides to phosphoglycerides	**Fluid Balance and Inflammation**	
PC	Phosphatidylcholine and other cholines	Crea	Creatinine
SM	Sphingomyelins	Alb	Albumin
TotCho	Total cholines	Gp	Glycoprotein acetyls, mainly a1-acid glycoprotein

Table 5.6 **Significant NMR-based metabonomics features identified using either Significant 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

LOW, low nucleotide mycoprotein group; HIGH, high nucleotide mycoprotein group
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.

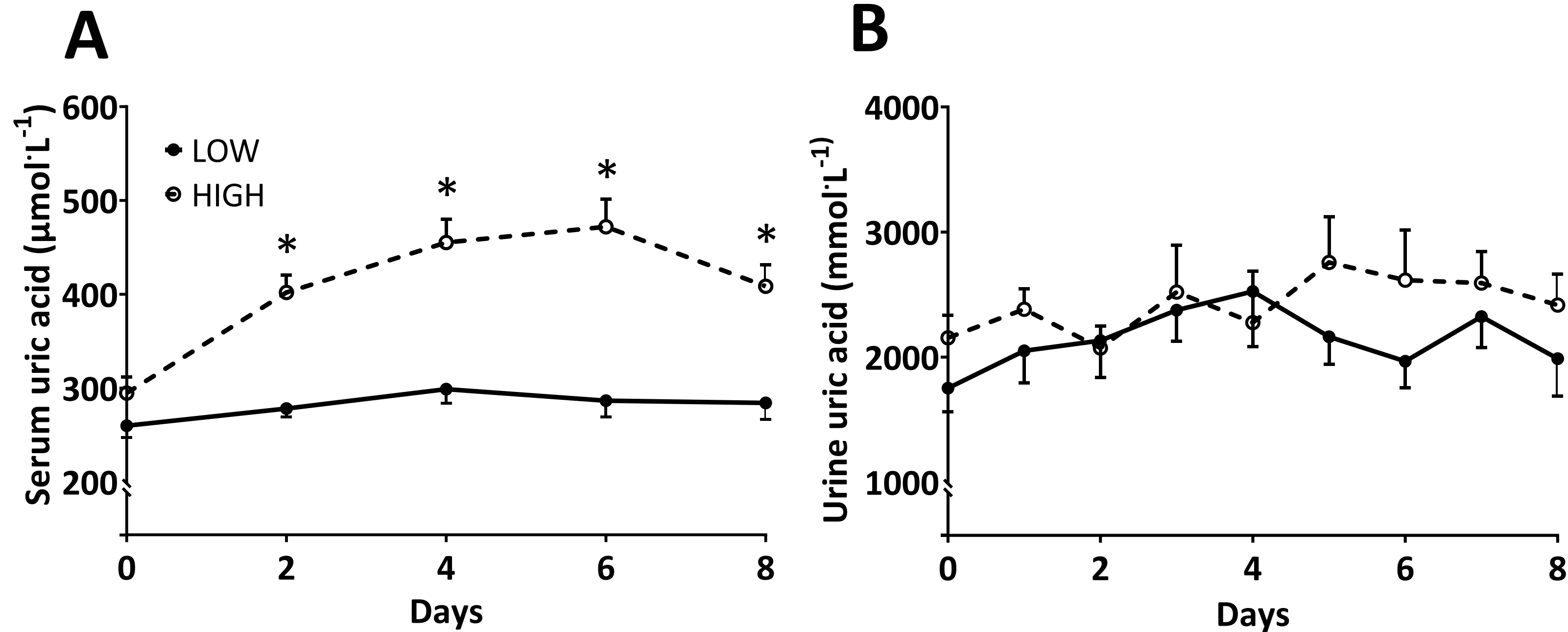


Figure 5.2 **Serum (A) and urine (B) uric acid concentrations throughout a one-week fully controlled dietary intervention with either a low-nucleotide mycoprotein (LOW) or a high-nucleotide mycoprotein (HIGH).** Serum samples were collected on days 0, 2, 4, 6 and 8 of the intervention, and urine samples 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

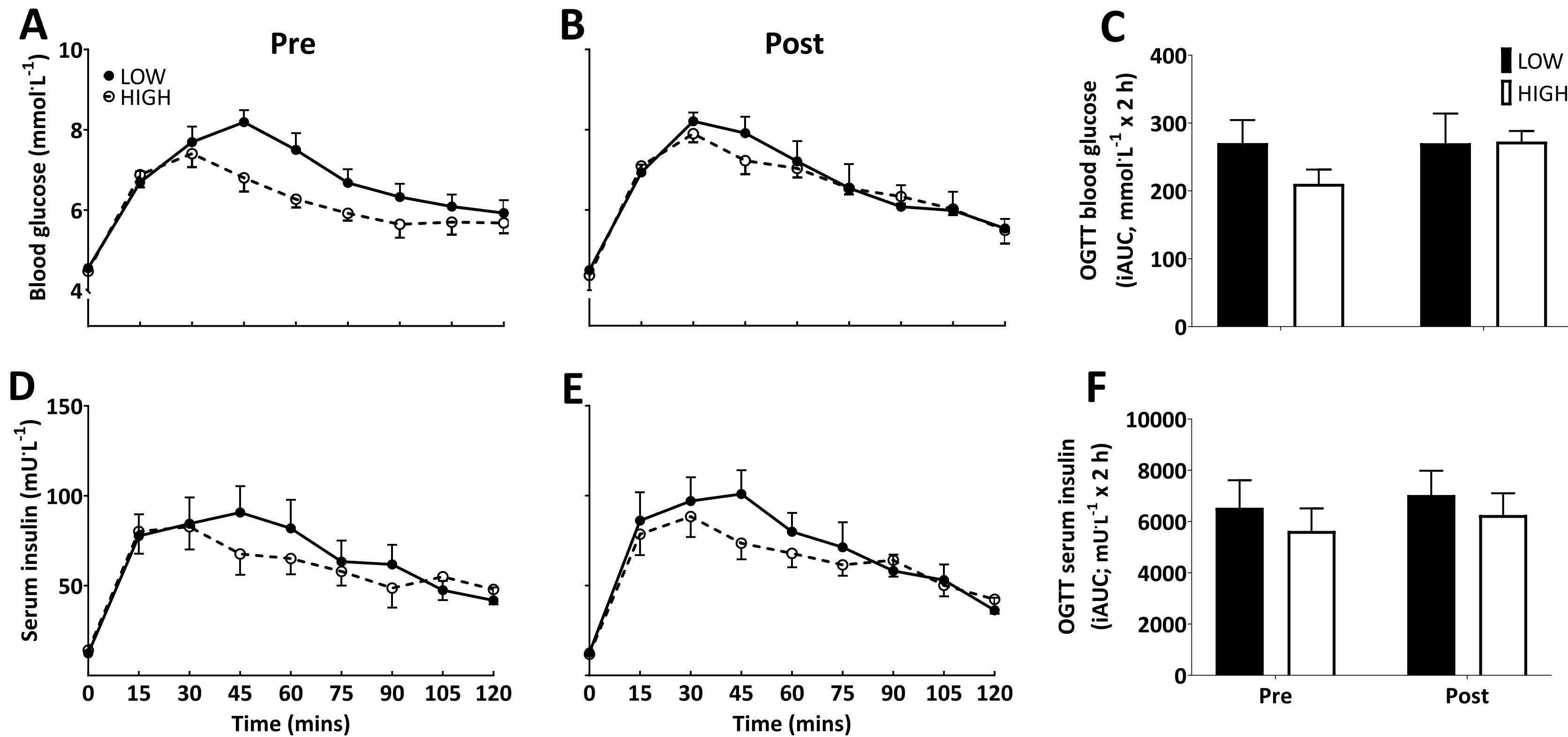


Figure 5.3 **Blood glucose (A, B and C) and serum insulin (D, E and F) concentrations during oral glucose tolerance test (OGTT) on days 0 (A and D) and 8 (B and E) of a one-week fully controlled dietary intervention with either a low-nucleotide mycoprotein (LOW) or a high-nucleotide mycoprotein (HIGH).** 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.

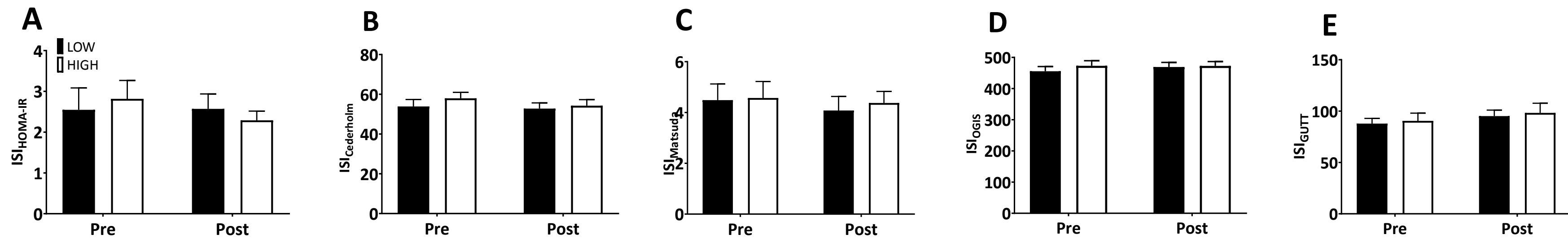


Figure 5.4 **Insulin sensitivity indices (A: HOMA-IR, B: Cederholm, C: Matsuda, D: OGIS and E: Gutt) calculated with the blood glucose and serum insulin concentrations measured fasting and during oral glucose tolerance tests (OGTT) pre and post a one week fully controlled dietary intervention with either 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)

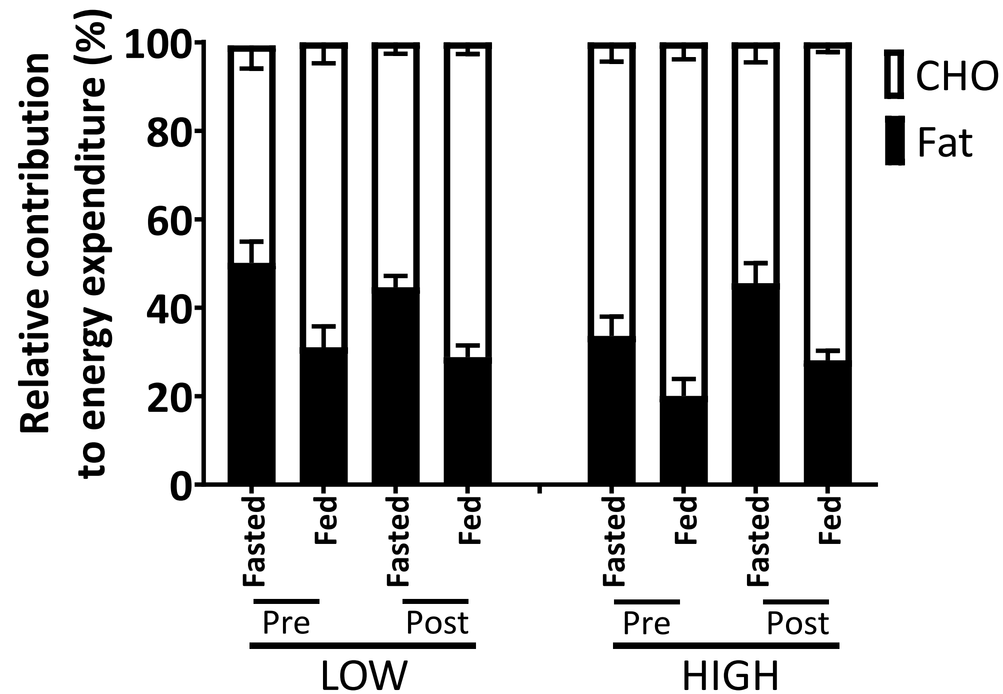


Figure 5.5 **Relative contribution of fat and carbohydrate oxidation rates to energy expenditure calculated via indirect calorimetry using the Frayn equations, in the fasted and CHO-fed states, pre- and post- a one-week fully controlled dietary intervention with either a low-nucleotide mycoprotein (LOW) 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 $P>0.05$).

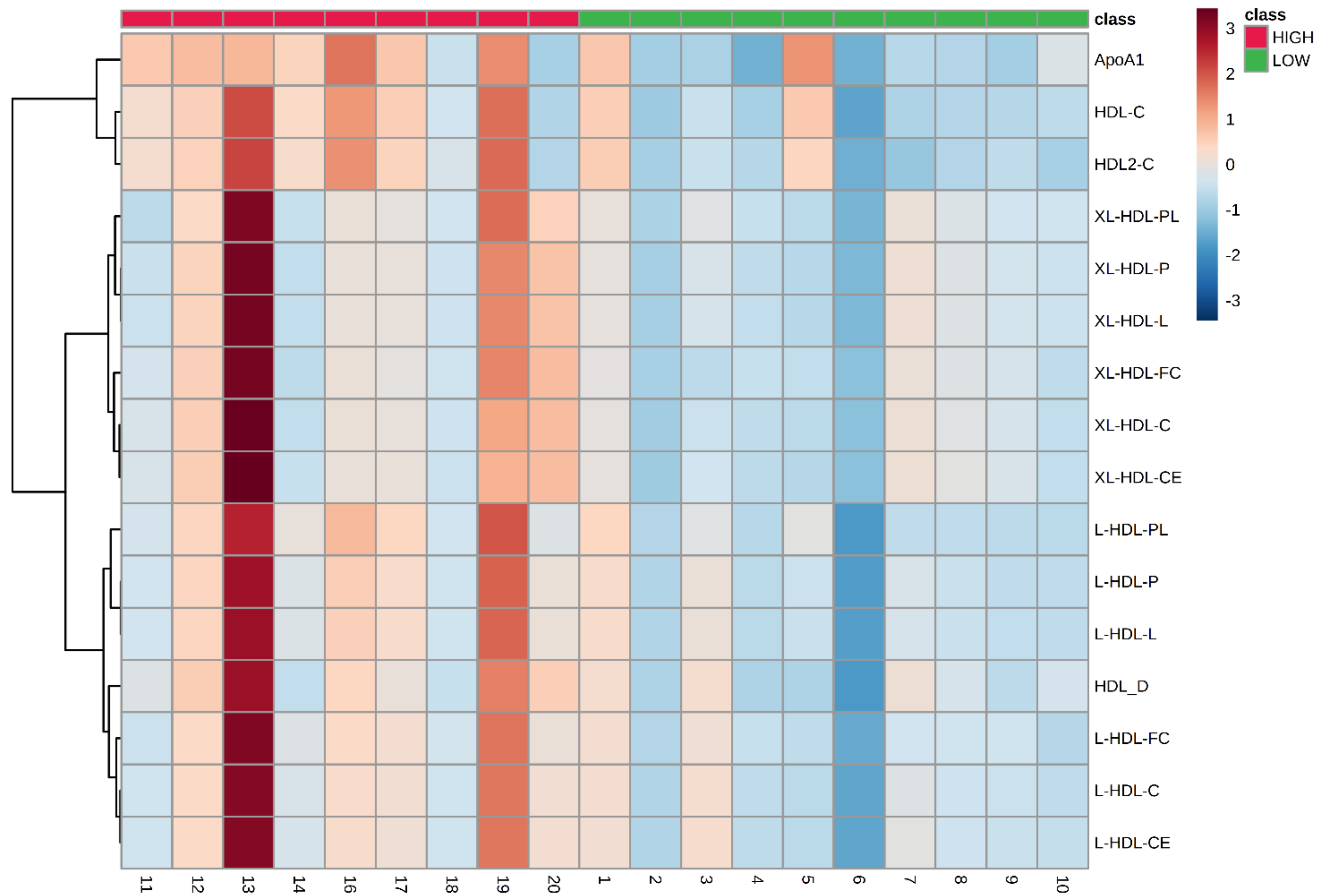


Figure 5.6 **Heat map representing the metabolomics measures which suffered the significant changes between pre- and post- a one-week fully controlled dietary intervention with either a low-nucleotide mycoprotein (LOW) or a high-nucleotide mycoprotein (HIGH), as calculated by the Δ change for each participant.** Participants in LOW are represented in green and participants in HIGH are shown in red.

5.4 Discussion

We investigated the impact of consuming high-nucleotide mycoprotein, as opposed to a nucleotide-depleted mycoprotein, as the major source of dietary protein at lunch and dinner for one week, during a fully controlled, energy and macronutrient balanced dietary intervention on serum uric acid concentrations, IS, glycaemic control and plasma lipid composition. We report that the high nucleotide intake led to a gradual and sustained increase in serum uric acid concentrations, to a magnitude above clinically relevant thresholds. We did not observe associated changes in indices of whole-body IS or 24 h free-living glycaemic control. Additionally, we found minor effects of the nucleotide intervention upon the plasma lipidome.

Studies carried out in the 1960s and 70s established a causal link between dietary nucleotide intake and increased serum uric acid concentrations (131-133). Daily, short-term (5-9 days), ingestion of large quantities (> 2 g) of nucleotides isolated from yeast resulted in rises in serum uric acid concentrations above clinically accepted thresholds (i.e., ~420 $\mu mol \cdot L^{-1}$ in men, ~360 $\mu mol \cdot L^{-1}$ in pre-menopausal women (95)). Recently, we have also shown that the consumption of a single high-nucleotide (1.7 g) mixed meal increased serum uric acid concentrations for 12 hours, however not to clinically significant levels (235). Here we examined whether repeatedly ingesting nucleotide-rich mixed meals for 1 week 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. We applied a nutritional intervention, which was identical in both groups except for the nucleotide composition of mycoprotein (0.89 and 4.46 g of mycoprotein-derived nucleotides, in the control and intervention groups, respectively; nucleotide load in the control group was

similar to average nucleotide intakes (232, 233)), with no differences in energy or macronutrient consumption between groups (see Table 5.2) to young adults (who were well matched across groups; see Table 5.1). We report that a one-week fully controlled mixed diet containing a high-nucleotide mycoprotein as the major source of dietary protein at lunch and dinner (~ 4.5 g daily nucleotide intake) led to gradual and steady increases in serum uric acid concentrations. This ~60 % rise peaked at clinically relevant levels (472 $\mu mol \cdot L^{-1}$) after 5 days undertaking the diet. Our findings are in line with the previous work, and we have shown that daily high nucleotide intake, even when incorporated into mixed meals, leads to a cumulative increase in serum uric acid concentrations, which after three to five days become clinically significant.

Interestingly, after 7 days on the diet, serum uric acid concentrations decreased (while still staying elevated from baseline) to concentrations under clinically relevant thresholds (~409 $\mu mol \cdot L^{-1}$). Uric acid is a waste product of purine catabolism and is mostly excreted via the urine (the kidneys eliminate two-thirds and the gastrointestinal tract eliminates one-third of the total uric acid excreted) (239). As such, we collected morning urine samples every day to assess changes in urine uric acid concentrations and, consequently, urinary uric acid excretion. We did not find any changes in urine uric acid concentrations throughout the intervention, nor differences between groups, suggesting that rises in serum uric acid concentrations did not lead to increased urinary excretion. However, collecting 24 h urine for the seven days of the intervention might have provided greater insight into the total daily excretion of uric acid. It is possible that an increased urine output would have led to increased excretion of uric acid without an increase in uric acid concentrations in urine samples. Alternatively, it is also possible that the timing of the collected urine samples did not coincide with the

timing of increased uric acid excretion (e.g. increased uric acid excretion in urine in the first few hours after a meal). Conversely, other, unidentified, mechanisms might be responsible for the decrease in serum uric acid concentrations between day 6 and day 8 of the intervention. A sudden increase in dietary nucleotide intake might lead to a transient rise in serum uric acid concentrations, which subsides after an adaptation period. After that, it is conceivable that dietary nucleotides might be more efficiently utilised in the production of nucleic acids (e.g. DNA, RNA, ATP) instead of catabolised, resulting in lower accumulation of uric acid. Further investigations are required to confirm if this decreasing trend persists after a longer period of high nucleotide intake. Moreover, the relevance of uric acid as a biomarker of metabolic health, and/or its role in disease progression still require investigation. In the present study, we also sought to investigate whether an increase in postabsorptive serum uric acid concentrations would lead to associated impairments in other markers of metabolic health.

Higher circulating uric acid concentrations have been shown to be associated with a variety of inflammatory and/or metabolic disorders, such as gout (122), hypertension (123), type 2 diabetes (125), and metabolic syndrome (124), leading to speculations of a causative role (130). The mechanisms by which circulating uric acid concentrations might cause a decline in metabolic health status are not clear. *In vitro* experiments have shown that uric acid is a pro-oxidant in the intracellular environment, implying uric acid may contribute to metabolic dysfunction by promoting cellular oxidative stress (127, 227-231). Hyperuricemia has also been suggested to promote insulin resistance and associated hyperinsulinaemia (240, 241). Meanwhile, hyperinsulinaemia can lead to hyperuricaemia by reducing renal excretion of uric acid (222). We have previously shown that the acute increase in circulating uric acid concentrations

following a nucleotide-rich meal was not associated with any impairment in postprandial glucose handling or on indices of IS (235). However, whether this is also the case following repeated intake of high nucleotide mixed meals and when postprandial serum uric acid is above clinically relevant thresholds was not yet known. In the present study, despite clinically significant increases in serum uric acid concentrations, we did not observe any changes in glycaemic control nor indices of liver or peripheral IS. Since epidemiological studies have shown total (postprandial) hyperglycaemia and/or the prevalence of (postprandial) hyperglycaemic excursions over the day to be better predictors of longer-term cardio-metabolic health (203), we applied continuous glucose monitoring throughout the study period which captures free-living blood glucose concentrations throughout the day. However, whether we looked across the entire day or focussed on postprandial periods, we did not observe any impact of the high-nucleotide intervention. As such, our results clearly show that despite repeated consumption of high nucleotide meals for one-week leading to a cumulative increase in serum uric acid concentrations, measures of IS and glycaemic control remained unaffected.

An interesting additional finding was that fasting blood glucose concentrations were lowered in both groups throughout the intervention. We had previously identified a trend for a decrease in fasting blood glucose concentrations during a week of substituting animal products for mycoprotein as the main protein source for lunch and dinner (236). As a recognised clinical marker of IS, this suggests mycoprotein consumption, regardless of its nucleotide content, may support metabolic health, although, as we previously discussed (236), it is difficult to explain why this was observed in the absence of effects on calculated IS and/or 24 h glucose control. It is possible that mycoprotein consumption specifically

altered glucagon sensitivity (potentially due to mycoprotein's high fibre content) (206), or induced early improvements in β-cell function (207), but clearly, this warrants further research.

Earlier studies demonstrated that 3-8 weeks of mycoprotein consumption reduces total plasma cholesterol and plasma LDL concentrations thereby resulting in improved HDL/LDL ratios (52, 53). By applying a novel NMR-based quantitative and targeted metabonomics approach (208), we previously found that merely one week of substituting the consumption of meat/fish for lunch and dinner by mycoprotein considerably modulated the plasma lipidome, however, unlike in past studies, we found the reduction in cholesterol was ubiquitous across lipoprotein species, and therefore did not impact lipoprotein ratios (236). In the present study, using the same NMR-based metabolomics approach, we found much smaller differences between groups on the plasma metabolome. There were no differences between groups for 208 of our 224 targets (see Table 5.6 and Figure 5.6). As such, the effects of mycoprotein intake on the plasma metabolome were largely the same, regardless of the nucleotide content of the diet. We found differences in the lipid 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, specifically, all of these decreased in our low-nucleotide (control) group, but did not decrease in the high-nucleotide group. It is unclear what were the mechanisms that led to these coordinated reductions in HDL cholesterol in the low-nucleotide group, whilst remaining stable in the high-nucleotide group, considering there were no differences between groups regarding any other parameters of dietary intake or physical activity. However, it is clear that these results do not show any adverse effects of high nucleotide consumption on plasma lipid profile.

In conclusion, twice-daily consumption of high-nucleotide mycoprotein for one week led to sustained increases in serum uric acid concentrations (above clinically relevant thresholds), but no associated deleterious effects on IS, glycaemic control and plasma lipid composition.

Chapter 6:

Oral supplementation with dietary nucleotides with or without ribose does not influence skeletal muscle ATP content, muscle fuel utilisation during exercise, exercise performance or recovery from exercise

6.1 Introduction

Energy for all types of biological functions, including exercise, is provided chemically by adenosine triphosphate (ATP). In skeletal muscle, contraction is sustained by the hydrolysis of ATP by myosin ATPase (138). Skeletal muscle has the capacity to rapidly modulate the rate of energy production and changes in fuel utilisation in response to exercise. Resting intramuscular stores of ATP are small and can be depleted by maximal or prolonged exercise, but a plethora of metabolic pathways, such as the ATP-phosphagen system and anaerobic glycolysis, drive the generation of ATP, promoting the restoration of intramuscular ATP levels (139).

Muscle fatigue has been defined as the failure to maintain an expected force, leading to a reduced performance of a certain task (140). Voluntary contraction and fatigue are complex mechanisms, where energy supply and deficiency play a major role. Metabolic changes at fatigue can depend on the type of exercise performed but a common denominator is the decreased capacity to generate ATP paired with high ATP turnover, leading to increased catabolism of the nucleotide pool in the muscle cell (142). During prolonged exercise (i.e. an exercise intensity which can be sustained for 30 to 180 mins, corresponding to 60 to 85 % of maximal oxygen consumption), fatigue is primarily brought about by glycogen depletion, but the exact biochemical mechanisms underpinning this are still unclear (138). This glycogen depletion typically coincides with the depletion of ATP, even when other fuel sources are abundant (242), suggesting a mechanistic link between glycogen depletion and a compromised rate of ATP regeneration (243-246).

Nucleotides are a group of molecules comprising a nitrogenous base, ribose or deoxyribose and one or more phosphate groups, used widely within human

metabolism, namely in the structure of nucleic acids and energy-rich molecules (e.g. ATP) (86). Cells with high energy requirements (such as muscle cells) and/or with vigorous DNA replication (such as immune and intestinal cells) present with an increased turnover of nucleotides (247). Nucleotides are synthesised by the body, but in certain circumstances, exogenous nucleotides might become semi-essential. Evidence supports an increased dietary requirement for nucleotides during periods of rapid growth (e.g. infancy; (160, 161)) and stress, such as certain disease and recovery states (162-164). Muscular exercise is one of the most common states of physiological stress and emerging data imply beneficial effects of dietary nucleotide supplementation on exercise performance and recovery (165-171, 174). Dietary nucleotide supplementation has been shown to reduce post-exercise stress hormone response (166, 167), improve markers of immune health (169, 170), and result in improvements to muscular strength (165), force production (171) and time to exhaustion (169). It has been suggested that dietary nucleotides might positively affect energy production and oxidative stress in the mitochondria but studies have not yet examined this (175). Increased production of ATP modulated by dietary nucleotide intake prior to submaximal moderate-to-high-intensity exercise might lead to improved exercise performance, potentially by sparing muscle glycogen. Additionally, these potential benefits to energy production might also lead to improved recovery following glycogen depletion.

Ribose, a pentose monosaccharide that makes up the structure of nucleotides, has been proposed as a limiting factor in the production of ATP. Hellsten and colleagues observed that one week of frequent intense training was sufficient to lower resting muscle adenine nucleotide concentrations and, importantly, that oral intake of ribose after training enhanced the rate of adenine nucleotide

resynthesis, probably by increasing the rate of phosphoribosyl pyrophosphate (PRPP) synthesis (critical for *de novo* nucleic acid synthesis) (177). As such, dietary ribose (like dietary nucleotide) intake might modulate fuel utilisation and improve energy production and exercise performance in a depleted state, as well as assist skeletal muscle cells in recovery from fatigue; but further research to establish these effects is necessary.

Mycoprotein, a sustainable fungal-derived dietary protein source, is naturally rich in nucleotides (~ 10 g per 100 g dry weight), but heat-treated during production to reduce nucleotide content (to under 2 g per 100 g dry weight). We have shown that nucleotide-rich mycoprotein consumption leads to acute and short-term (7 days) rises in serum uric acid concentrations due to increased nucleotide availability and breakdown (235).

In this study, we investigated the impact of the twice-daily ingestion, for 2 weeks, of a nucleotide-rich mycoprotein drink, with or without added ribose, in resting skeletal muscle ATP and glycogen concentrations and in endurance exercise muscle metabolism and performance. Additionally, we examined the effects of the consumption of the same drink (with extra added carbohydrate) on muscle ATP and glycogen concentrations and exercise performance in acute recovery from a fatigued state.

6.2 Subjects and methods

6.2.1 Participants and medical screening

Twelve healthy, recreationally active, young adults (age: 22±1 y; BMI: 23±1 $kg \cdot m^{-2}$; male = 5 and female = 7) participated in the present study. Subjects' characteristics and habitual diet details are presented in **Table 6.1**. 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. Smokers and participants taking regular medication or suffering from chronic diseases were excluded. Participants included were recreationally active (partook in regular exercise or sport at a non-competitive level, two to five days a week), not endurance-trained, normotensive, and had a BMI between 18.5 and 30 $kg \cdot m^{-2}$. All participants were informed of the study's purposes, procedures and risks, and provided written informed consent. This study was conducted at the Nutritional Physiology Research Unit, Department of Sport and Health Sciences, St. Luke's Campus, University of Exeter, between July of 2018 and February of 2020, and was approved by the University of Exeter's Sport and Health Sciences Ethics Committee (Ref No: 171206/B/04) in accordance with the Declaration of Helsinki. This project was registered at ClinicalTrials.gov (NCT03659890).

Table 6.1 **Participants' characteristics and habitual diet**

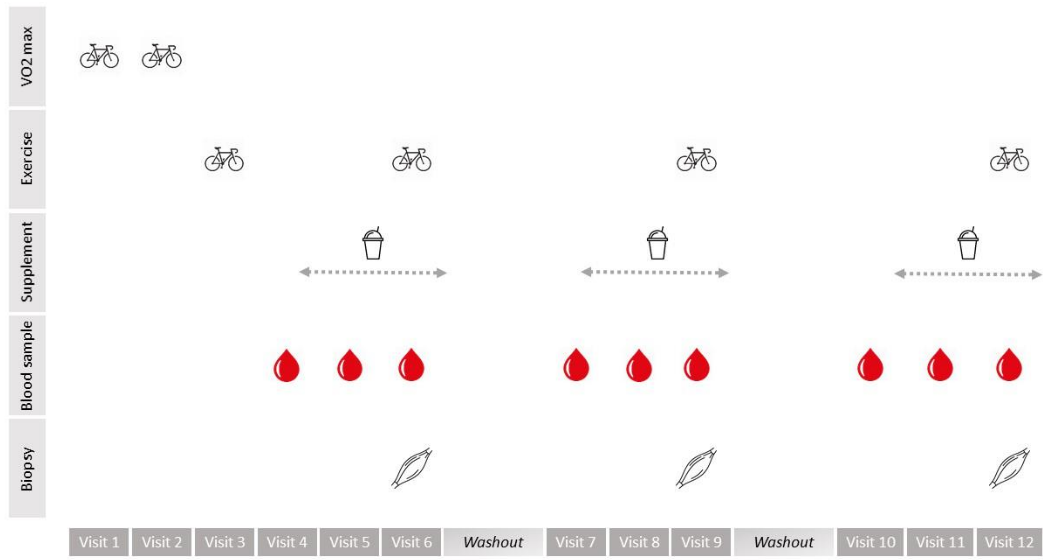
Sex	7 F / 5 M
Age (y)	22±1
Height (cm)	171±2
Body mass (kg)	67±3
BMI (kg·m^{-2})	23±1
Body fat (% of body mass)	14±3
Lean mass (kg)	58±3
$\dot{V}O_2$ max (ml·kg^{-1}·min^{-1})	37±1
Energy (MJ/day)	8.7±1.1
Energy (kcal/day)	2059±254
Protein (g/day)	116±16
Protein (g·kg^{-1} body weight)	1.7±0.3
Protein (% total energy)	22.7±1.8
Carbohydrate (g/day)	218±29
Carbohydrate (% total energy)	42.9±3.5
Fat (g/day)	81±13
Fat (% total energy)	34.4±2.4
Fibre (g/day)	25±2

6.2.2 Experimental protocol

The present study was a randomised, controlled, double-blind crossover design trial, with participants being randomly allocated into the order in which they received three dietary supplements for a two-week period each (counterbalanced to account for order effects). Participants were asked to consume 1.1 g·kg^{-1} bw of either nucleotide-depleted mycoprotein (CON) or high-nucleotide mycoprotein

(NUC and RIB) and 600 mg·kg^{-1} bw of either dextrose (CON and NUC) or ribose (RIB) daily, divided into two identical drinks, for 14 consecutive days, followed by an experimental test day on day 15.

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 the order in which they received the three conditions by using an online randomiser (http://www.randomization.com/). **Figure 6.1** shows an overview of the study design. Before starting supplementation and data collection, participants were asked to complete a 3-day food diary to assess their habitual dietary intake (Table 6.1), 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).



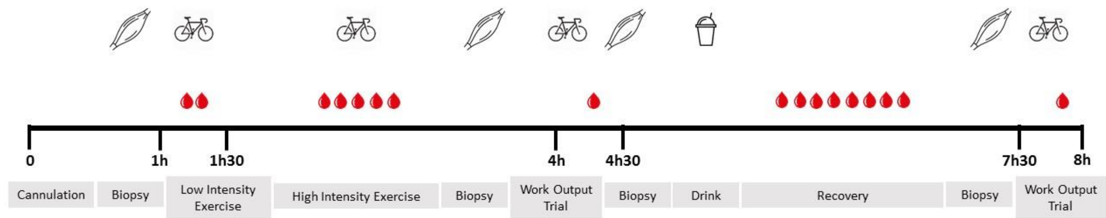


Figure 6.1 **Overview of the study design (A) and the study test days (B).**

6.2.3 $\dot{V}O_2$ max testing and confirmation

After health screening and providing consent, participants reported to the lab to complete a continuous incremental $\dot{V}O_2$ max test on an electronically braked cycle ergometer (Lode Excalibur Sport, Lode, Netherlands). Participants began cycling at a power output approximate to their body weight (generally 60 – 80 W) and the resistance was then increased by 20 – 40 W every 3 mins until volitional exhaustion. Measurements of oxygen consumption ($\dot{V}O_2$) were collected using a facemask and the Metamax 3B (MM3B) portable indirect calorimetry system (Cortex, Leipzig, Germany) throughout the test and were used to determine each participant's maximal oxygen output ($\dot{V}O_2$ max). A $\dot{V}O_2$ max confirmation test was performed at least two days following the first test, to assure that the maximum had indeed been reached.

6.2.4 Exercise protocol familiarisation

Two days after the $\dot{V}O_2$ max confirmation test (or later), participants returned to the lab, after refraining from intense exercise for 24 h and following a fast of at least 4 hours, to be familiarised with the exercise protocol to be completed during the experimental test days. Participants began cycling at a power output corresponding to 40-50% of their $\dot{V}O_2$ max. They cycled at this intensity for 10 mins and measurements of oxygen and carbon dioxide consumption were collected for the final 3 mins. At the end of the 10 mins, the power output automatically increased to correspond to 70-75% of the participant's $\dot{V}O_2$ max. The participants were instructed to keep their revolutions at a minimum of 70 and to cycle until volitional fatigue and for at least 1 hour continuously. If the subject was unable to cycle continuously for an hour, they were excluded from participation in the study. Expired gases were collected for the last three minutes

of each 15 min period. When participants reached fatigue and could not keep cycling above the indicated revolutions, they were permitted a 5 min break, after which they got back on the ergometer until achieving fatigue again. This was repeated until participants were not able to keep cycling for over 2-3 mins (usually after 3 or 4 bouts). This method has been previously shown to induce almost complete muscle glycogen depletion and a substantial depletion of muscle ATP content (248).

After the glycogen depleting protocol (EXH), participants rested for 15 mins before being familiarised with a work output test whereby the achieved work output in 15 mins was recorded while cycling on the electronically braked ergometer set into hyperbolic mode (cycling cadence dictates work output). This test has previously been shown to have a lower CV than time trial based performance tests (249) and to be sensitive to starting muscle glycogen content (248).

6.2.5 Supplementation periods

Participants reported to the laboratory at ~08.00 before beginning each supplementation cycle. A fasted venous blood sample was collected from an antecubital vein. Participants were provided with a supply of two weeks of supplements for the current condition and with a document to log the times of the intake of the beverages, in order to track compliance. The participants came back to the lab on days 6 and 8 of the supplementation cycle, again at ~08.00, where fasted venous blood samples were collected.

On their first supplementation cycle, participants were asked to write down everything they consumed for the 24 hours before the experimental test day (i.e. everything they ate and drank on day 14 of the supplement) and were given a

standardised meal for dinner. Participants were then instructed to repeat everything they had consumed and to maintain the same meal timings, on days 14 of the remaining two conditions.

6.2.6 Experimental beverages

Participants were provided with individual daily doses of the supplementation (CON, NUC or RIB) and with a bottle of energy-free flavouring (Jordan's Skinny Syrups, Skinny Mixes LLC, GoodWest Industries Inc., PA, USA). Subjects were given a blender and instructed to mix the supplement with water and flavouring, and to divide the total daily amount into two identical doses and consume one in the morning and the other in the afternoon. The nutritional content of the supplemental drinks and participants' daily mycoprotein-derived dietary nucleotide intake is displayed in **Table 6.2**. The mycoprotein dose was calculated taking into account the dietary nucleotide load to allow for a potential 20 % increase in resting muscle ATP concentrations, and the low bioavailability of dietary nucleotides (~ 5-10 %) (250). The ribose dose was based on a previous study by Hellsten et al (177). The dose was divided into two daily drinks to increase absorption and it was administered for 2 weeks in line with previous dietary nucleotide supplementation studies (165, 168, 169, 171).

Experimental test day beverages were identical to the drinks consumed during the supplementation periods but had an added extra 0.7 $g{\cdot}kg^{-1}$ bw of dextrose. This dextrose dose was added to support post-exercise glycogen resynthesis at a suboptimal level, to allow for potential beneficial effects between conditions during recovery (147). The experimental drinks were prepared the evening before the experimental trial. The mycoprotein and dextrose/ribose were assimilated with 400 mL water and 40 mL of artificial energy-free flavouring, blended for

approximately 2 min, topped up with water to make a total final beverage volume of 500 mL, which was then equally divided into two bottles and refrigerated overnight. Double blinding of the drinks was achieved by having a different researcher from the individual running the trial prepare the drinks in an opaque bottle ready for consumption. The participants were asked which drink they believed to have consumed at the end of each test visit. None of the participants was able to guess their supplement order correctly.

Table 6.2 **Daily nutritional content of the supplemental drinks**

	CON	NUC	RIB
Components			
Mycoprotein (g dry wt)	74±3	74±3	74±3
Dextrose (g)	41±2	41±2	-
Ribose (g)	-	-	41±2
Energy and macronutrients			
Energy (kJ)	1747±71	1747±71	1747±71
Energy (kcal)	415±17	415±17	415±17
Protein (g)	33±1	33±1	33±1
Carbohydrate (g)	48±2	48±2	48±2
Fat (g)	10±0	10±0	10±0
Fibre (g)	19±1	19±1	19±1
Total nucleotides (g / % mycoprotein dry wt)	1.45 / 1.96	6.53 / 8.83	6.53 / 8.83
CMP (g / % mycoprotein dry wt)	-	0.46 / 0.62	0.46 / 0.62
UMP (g / % mycoprotein dry wt)	0.19 / 0.26	0.33 / 0.44	0.33 / 0.44
GMP (g / % mycoprotein dry wt)	0.16 / 0.21	0.21 / 0.28	0.21 / 0.28
TMP (g / % mycoprotein dry wt)	0.34 / 0.46	2.22 / 3.00	2.22 / 3.00
CDP (g / % mycoprotein dry wt)	0.14 / 0.19	0.62 / 0.84	0.62 / 0.84
UDP (g / % mycoprotein dry wt)	0.05 / 0.07	0.25 / 0.34	0.25 / 0.34
CTP (g / % mycoprotein dry wt)	0.35 / 0.47	2.35 / 3.18	2.35 / 3.18
ADP (g / % mycoprotein dry wt)	0.01 / 0.02	0.06 / 0.08	0.06 / 0.08
TTP (g / % mycoprotein dry wt)	-	0.03 / 0.04	0.03 / 0.04
ITP (g / % mycoprotein dry wt)	0.21 / 0.29	-	-
ATP (g / % mycoprotein dry wt)	-	0.01 / 0.01	0.01 / 0.01

Abbreviations: CON, control condition; NUC, high nucleotide condition; RIB, high nucleotide + ribose condition.; 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

6.2.7 Experimental test days

On day 15 of each supplementation cycle, participants reported to the laboratory around 07.00-08.00, after an overnight fast and from refraining from intense exercise and alcohol consumption for at least 24 hours, to undertake three identical experimental test days. A cannula was inserted into an antecubital vein of one arm for venous blood sampling and a fasted venous blood sample was collected. A baseline muscle biopsy sample was collected from a randomised leg (counterbalanced between participants for dominant and non-dominant). Muscle biopsies were collected from the mid-region of the vastus lateralis (approx. 20 cm above the patella) with a modified Bergström suction needle under local anaesthesia (2% lidocaine). All biopsy samples were immediately frozen in liquid nitrogen, removed from the needle, and stored at -80°C until subsequent analysis. Right after the first biopsy, an incision was made (~ 1-2 cm down, towards the patella) and covered, in preparation for the second biopsy, which was collected immediately after the glycogen depleting exercise protocol, whilst still sitting on the ergometer. The participants were again fitted a facemask for the measurement of expired gases for periodic bouts of 3 mins and started the glycogen depleting exercise protocol described previously (EXH). At the end of their last high-intensity exercise bout, participants were instructed to lean back to the bed (which was placed behind the ergometer) and to rest the biopsied leg on the handle of the bike. A biopsy was taken immediately after cessation of exercise. The first work output test (PERF) was performed after the exercise biopsy, and after the incision for the third biopsy had been made. The third biopsy

was collected right after the performance test, whilst the participant laid in bed (similar to the first one). Following this, the participant was given the first experiment beverage and allowed to recover for three hours. The second drink was consumed 60 mins later. The subjects laid in bed for the recovery period but were not allowed to sleep. After the recovery period, a fourth and final muscle biopsy was collected and a second work output test was performed (REC). Venous blood samples were collected throughout the experimental protocol at the following time points: t = 10 of the low-intensity exercise; t= 15, 30, 45 and 60 of the glycogen depleting exercise protocol; at volitional fatigue (just before the exercise biopsy); t = 0, 30, 60, 90, 120, 150 and 180 of recovery; and immediately after each of the performance tests.

6.2.8 Plasma and serum collection and analyses

Twenty µl of whole blood were immediately analysed for glucose and lactate (Biosen C-Line GP+, EKF Diagnostics, Cardiff, UK). Three mL of blood were collected into LH (lithium heparin) plasma tubes (Becton Dickinson, Franklin Lakes, New Jersey, USA) and immediately centrifuged. The remaining 2 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 minutes. All tubes were centrifuged at 4° C and 4000 RPM, and aliquoted (one aliquot designated for each of the below analyses) plasma and serum were stored at -80° C. 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).

6.2.9 Skeletal muscle tissue analyses

Muscle samples were immediately snap-frozen in liquid nitrogen after removal from the leg. Biopsy samples were freeze-dried and stored at -80º C. Freeze-dried muscle was dissected free of visible blood and connective tissue, powdered and used for the determination of muscle glycogen, using the spectrophotometric method of Harris (180). Muscle nucleotides (ATP and IMP), as well as phosphocreatine (PCr) and creatine, were quantified by ion-pair reversed-phase chromatography with a gradient elution protocol with UV detection (181) using a Flexar FL HPLC system (PerkinElmer, Waltham, MA, USA).

6.2.10 Statistical analyses

A power analysis based on findings from Hellsten et al (177) was performed and determined that 12 participants were needed to provide a power of 80% and a 95% CI. Recruitment and testing were ended once the trial was fully recruited, whilst accounting for a 30% dropout rate, according to the a priori power calculation.

All data are expressed as means ± standard errors (SEM). The three conditions 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 detect individual differences. Additionally, for glucose and lactate, incremental Area Under the Curves (iAUC) were calculated and a one-way ANOVA was performed to detect any significant effect of condition. All calculations were performed using GraphPad Prism version 7.0 (GraphPad Software, San Diego, California, USA).

6.2 Results

6.3.1 Respiratory exchange ratio and carbohydrate and fat oxidation

The RER and the relative contribution of CHO and fat oxidation to total energy expenditure over the low-intensity exercise and the first 60 mins of EXH are presented in **Figure 6.2**. As expected, CHO oxidation increased and fat oxidation decreased from the low to the high-intensity exercise, reversing again throughout the 60 mins of EXH (time effect, $P<0.0001$), but there were no differences between conditions.

6.3.2 Muscle metabolite concentrations

Skeletal muscle metabolite (glycogen, ATP, IMP, free creatine, PCr and total creatine) concentrations at rest, at exhaustion following exercise at 70 % $\dot{V}O_2$ max, after a 15 min performance test and after a 3 hour recovery period are presented in **Table 6.3**. There were no differences between conditions at rest for any of the measures (all $P>0.05$). There was a trend for IMP to increase by ~ 27 % with EXH (time effect, $P=0.05$) but no changes were detected in ATP (time, condition and time x condition interaction effects; all $P>0.05$). Muscle glycogen and PCr concentrations are displayed in **Figure 6.3**. From similar resting values, muscle glycogen (297±24, 305±19 and 272±10 mmol·kg^{-1} dw in CON, NUC and RIB, respectively, $P>0.05$) and PCr (103±8, 101±9 and 96±7 mmol·kg^{-1} dw in CON, NUC and RIB, respectively, $P>0.05$) decreased by ~ 20 and ~ 40 %, respectively, during EXH (time effects; both $P<0.05$). On the other hand, free creatine increased by ~ 50% (from 50±5, 54±3 and 51±6 to 86±5, 68±4 and 76±9 mmol·kg^{-1} dw in CON, NUC and RIB, respectively; time effect, $P<0.05$). No significant differences between conditions were identified for glycogen, ATP, IMP,

free and total creatine (condition and time x condition interaction effects; all $P>0.05$). For PCr, a difference between conditions was found in recovery (time x condition interaction effect; $P<0.05$), with the RIB condition displaying a faster and higher increase in PCr concentrations after exhaustive exercise than NUC and CON, and with CON also showing a superior replenishment than NUC. Finally, a trend ($P=0.08$) for different glycogen depletion levels between conditions during EXH was observed.

6.3.3 Blood glucose and lactate concentrations during exercise

The concentrations of glucose and lactate in whole blood in the post-absorptive state, during low-intensity exercise, at 15 min intervals throughout the first 60 min of exercise, at the point of exhaustion and after a 15 min work output test are shown in **Figure 6.4**. Blood glucose concentrations were maintained from baseline, during low-intensity exercise and throughout the first 60 min of exercise at ~ 4.1 $mmol \cdot L^{-1}$ and declined to ~ 3.4 $mmol \cdot L^{-1}$ at the point of exhaustion in all visits, with no differences detected between conditions (Fig. 6.4A). In all visits, blood lactate concentrations increased from rest to ~ 2.3 $mmol \cdot L^{-1}$ during exercise, then declined to ~ 1.3 $mmol \cdot L^{-1}$ at the point of exhaustion, again, with no differences apparent between conditions (Fig. 6.4B). After PERF, blood glucose and lactate concentrations did not change and were not different between conditions (all $P>0.05$).

6.3.4 Blood glucose and lactate concentrations during recovery

The concentrations of glucose and lactate in whole blood during a 3 hour recovery period after exhaustive exercise and consuming the recovery drinks, and after a 15 min recovered work output test are shown in **Figure 6.5**. Blood glucose

concentrations increased in CON and NUC from ~ 3.4 mmol·L^{-1} before the consumption of the first recovery drink, peaking at ~ 5.7 mmol·L^{-1} after 90 minutes (30 minutes after second drink), but remained unchanged in RIB (*P*<0.0001 for time, condition and interaction effects; Fig. 6.5A). In all visits, blood lactate concentrations remained stable during recovery at ~ 0.8 mmol·L^{-1} (*P*>0.05; Fig. 6.5B). After REC, blood glucose concentrations decreased to ~ 3.2 mmol·L^{-1} in CON and NUC, and to ~ 2.7 mmol·L^{-1} in RIB (*P*<0.0001 for time and condition effects *P*<0.05 for an interaction effect). Meanwhile, blood lactate concentrations increased to 1.9±0.3, 2.3±0.3 and 1.4±0.1 mmol·L^{-1} in CON, NUC and RIB, respectively (*P*<0.0001 for a time effect; *P*<0.05 for condition and interaction effects).

6.3.5 Exercise performance

Work outputs (kJ) achieved in PERF and REC, as well as the difference between tests, are presented in **Figure 6.6**. Work output was not different between conditions in the PERF (*P*<0.05). In REC, work output was significantly different between groups (*P*=0.01), mainly driven by it being greater in the NUC condition (96±9 kJ) than in the RIB condition (83±8 kJ; *P*=0.01). The difference between the work output in REC and the work output in PERF was 12±5, 24±6 and 10±7 % in CON, NUC and RIB, respectively, but this was not significantly different (*P*<0.05).

6.3.6 Serum uric acid concentrations

Figure 6.7 displays serum uric acid concentrations throughout the intervention. There were no differences in fasting serum uric acid concentrations between conditions at baseline (307±17 μmol·L^{-1} in CON, 315±20 μmol·L^{-1} in NUC and

305±18 $\mu mol \cdot L^{-1}$ in RIB; $P>0.05$). Serum uric acid concentrations remained unchanged in CON but increased in both NUC and RIB (time and condition effects, $P<0.0001$; time x condition interaction effect; $P<0.0005$), peaking on day 6 (400±31 $\mu mol \cdot L^{-1}$ in NUC and 432±31 $\mu mol \cdot L^{-1}$ in RIB; $P>0.05$) and remaining elevated on days 8 (376±29 $\mu mol \cdot L^{-1}$ in NUC and 420±34 $\mu mol \cdot L^{-1}$ in RIB; $P>0.05$) and 15 (363±31 $\mu mol \cdot L^{-1}$ in NUC and 426±41 $\mu mol \cdot L^{-1}$ in RIB; $P>0.05$). There was a trend for an increase in serum uric acid concentrations in the CON condition on day 6 (400±31 $\mu mol \cdot L^{-1}$, $P=0.05$) but not in any other time point. In addition to serum uric acid concentrations in NUC and in RIB being higher than in CON throughout the intervention, serum uric acid concentrations in RIB were higher than in NUC on days 8 and 15 (both $P<0.05$).

Table 6.3 **Skeletal muscle metabolites at rest, at exhaustion following exercise at 70 % $\dot{V}O_2$ max, after a 15 min performance test and after a 3 hour recovery period**

	Rest			70% $\dot{V}O_2$ max			Post-performance test			Recovery		
	CON	**NUC**	**RIB**	**CON**	**NUC**	**RIB**	**CON**	**NUC**	**RIB**	**CON**	**NUC**	**RIB**
Glycogen	297±24	305±19	272±10	224±19	219±18	228±11	229±16	222±18	211±13	252±11	248±18	246±13
ATP	25±2	24±2	25±2	26±2	21±3	26±2	23±2	23±2	28±2	23±2	20±2	25±2
IMP	4.0±0.3	4.1±0.3	4.1±0.3	5.5±0.6	5.5±1.3	4.6±0.4	4.7±0.6	4.5±0.4	4.6±0.4	4.1±0.4	4.8±0.9	4.4±0.4
Creatine	50±5	54±3	51±6	86±5	68±4	76±9	51±4	53±6	55±5	49±4	49±4	56±4
PCr	103±8	101±9	96±7	63±8	61±7	60±7	99±8	91±13	113±8	92±8	75±10	91±10
Total creatine	153±10	155±11	148±8	148±10	129±7	136±8	150±8	144±11	168±10	141±9	123±8	147±12

All values are means ± S.E.M. and expressed as mmol·kg^{-1} dry muscle
Abbreviations: CON, control condition; NUC, high nucleotide condition; RIB, high nucleotide + ribose condition.

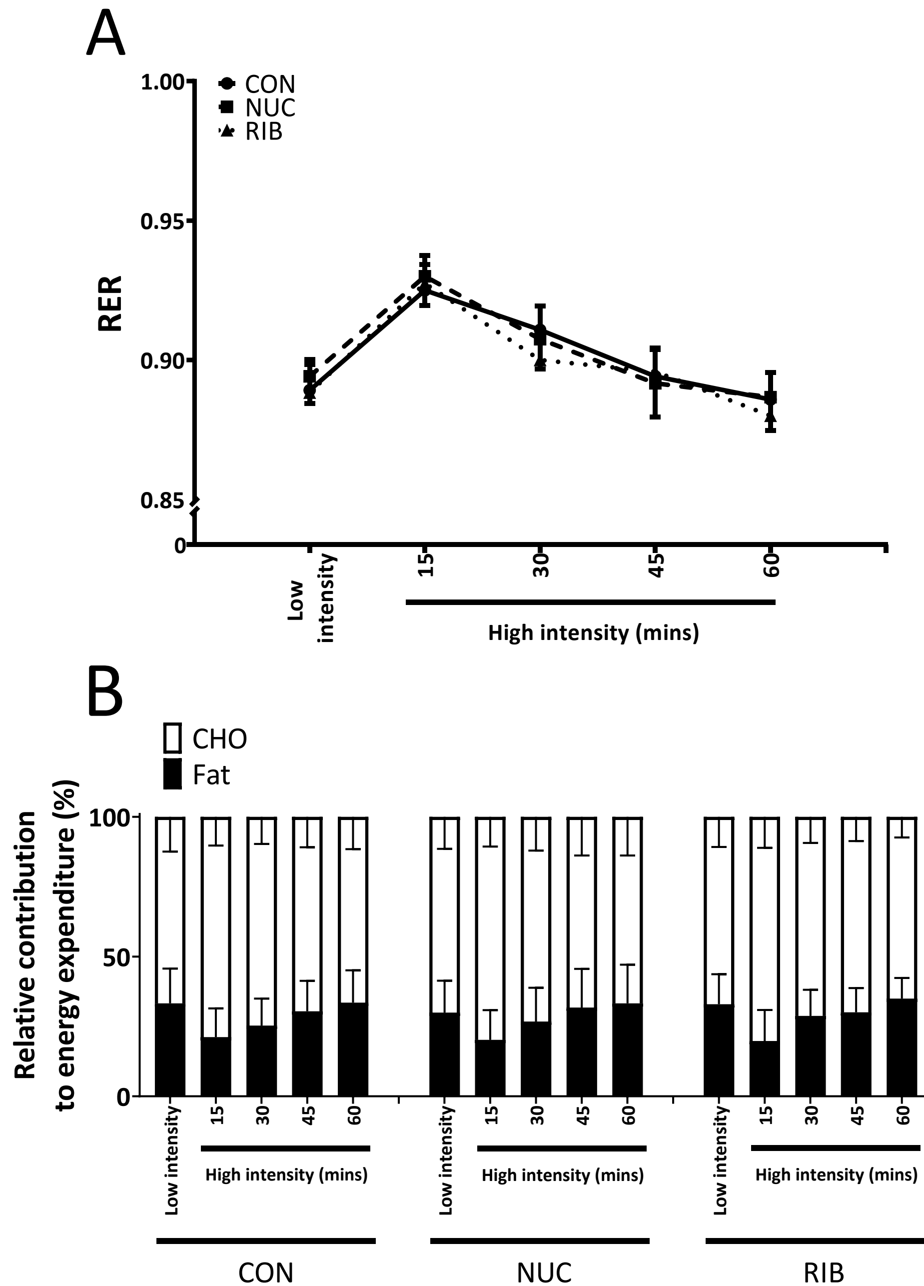


Figure 6.2 **Respiratory exchange ratios (RER; A) and relative contribution of fat and carbohydrate oxidation to energy expenditure (B) during low-intensity exercise (~ 45 % of $\dot{V}O_2$ max) and at 15 min intervals throughout the first 60 min of exercise at ~ 70% of $\dot{V}O_2$ max, after consuming 1.1 g·kg^{-1} bw of either nucleotide-depleted mycoprotein (CON) or high-nucleotide mycoprotein (NUC and RIB) and 600 mg·kg^{-1} bw of either dextrose (CON and NUC) or ribose (RIB) daily, divided into two identical drinks, for 14 days.** There were significant time effects (*P*<0.0001), but there were no differences between conditions (all *P*>0.05).

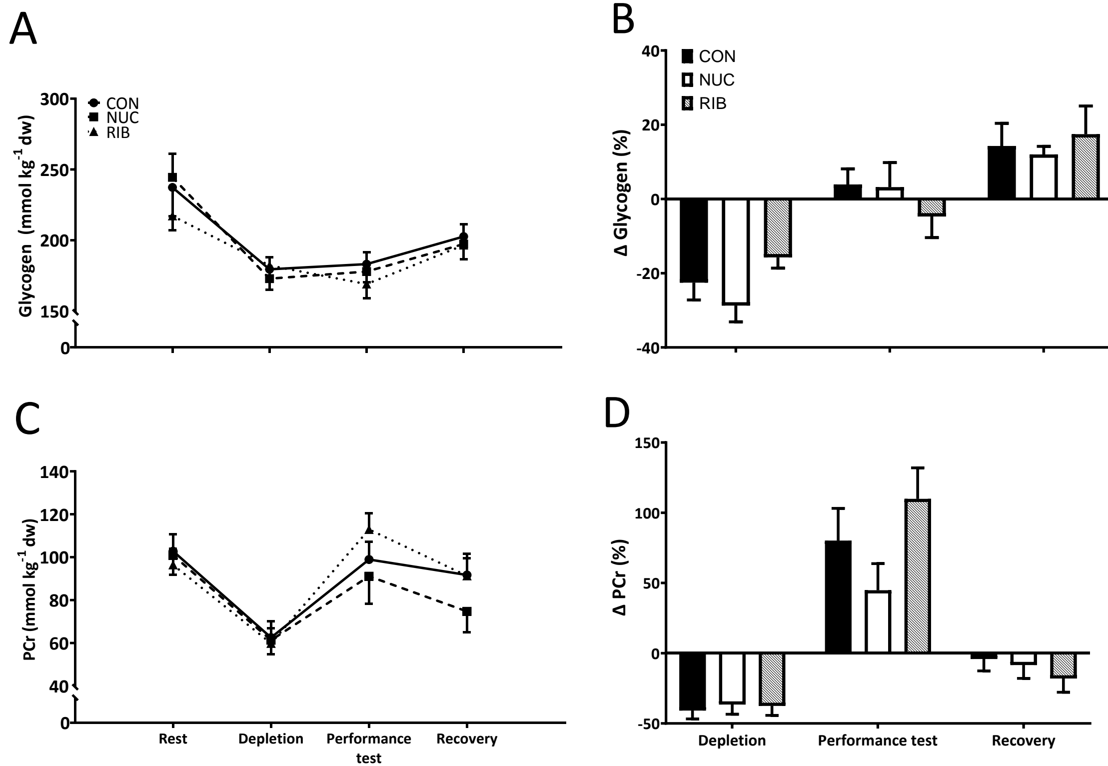
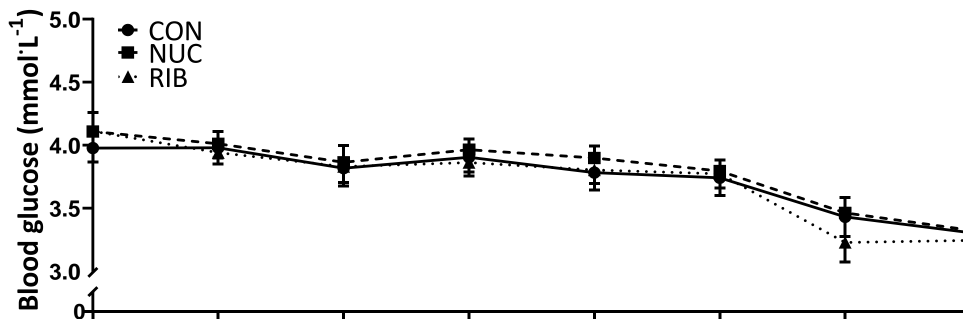


Figure 6.3 **Muscle glycogen (A) and PCr (C) concentrations at rest, at exhaustion following exercise at 70 % $\dot{V}O_2$ max, after a 15 min performance test and after a 3 hour recovery period, as well as muscle glycogen and PCr utilisation and recovery (B and D), after consuming 1.1 g·kg^{-1} bw of either nucleotide-depleted mycoprotein (CON) or high-nucleotide mycoprotein (NUC and RIB) and 600 mg·kg^{-1} bw of either dextrose (CON and NUC) or ribose (RIB) daily, divided into two identical drinks, for 14 days.** There were significant time effects (*P*<0.05), but there were no differences between conditions (all *P*>0.05).

A



B

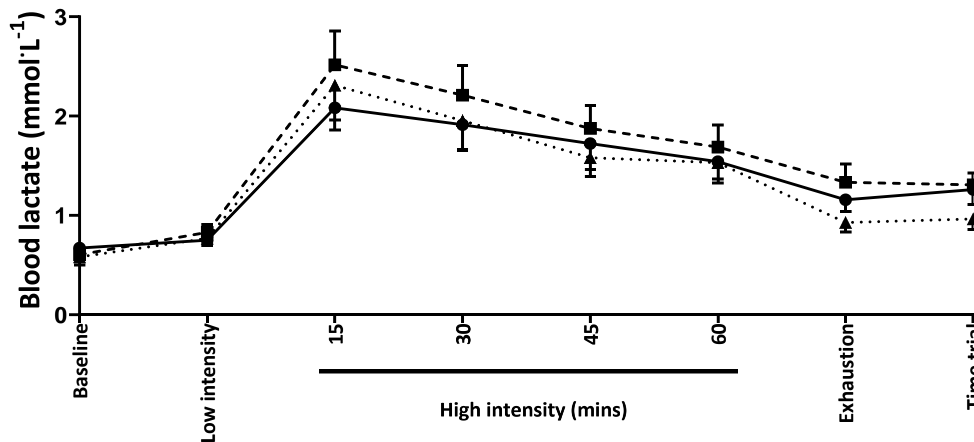


Figure 6.4 **Blood glucose (A) and lactate (B) concentrations during low-intensity exercise (~ 45 % of $\dot{V}O_2$ max), at 15 min intervals throughout the first 60 min of exercise at ~ 70% of $\dot{V}O_2$ max, at the point of exhaustion and after a 15 min work output test, after consuming 1.1 g·kg^{-1} bw of either nucleotide-depleted mycoprotein (CON) or high-nucleotide mycoprotein (NUC and RIB) and 600 mg·kg^{-1} bw of either dextrose (CON and NUC) or ribose (RIB) daily, divided into two identical drinks, for 14 days.** There were significant time effects, with glucose declining at exhaustion and lactate increasing during exercise at ~ 70% of $\dot{V}O_2$ max and decreasing at exhaustion ($P<0.05$), but there were no differences between conditions (all $P>0.05$).

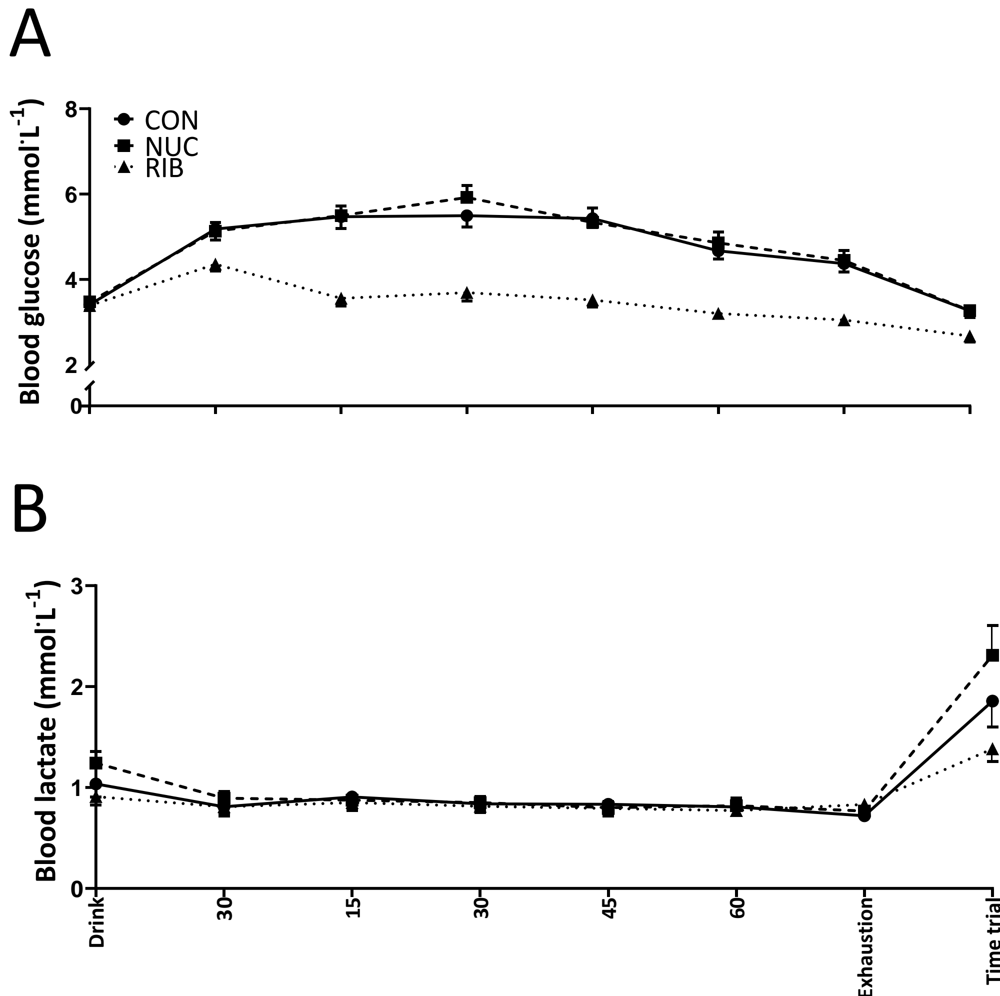


Figure 6.5 **Blood glucose (A) and lactate (B) concentrations during a 3 hour recovery period after exhaustive exercise and consuming a recovery drink, and following a 15 min recovered work output test, after consuming 1.1 g·kg^{-1} bw of either nucleotide-depleted mycoprotein (CON) or high-nucleotide mycoprotein (NUC and RIB) and 600 mg·kg^{-1} bw of either dextrose (CON and NUC) or ribose (RIB) daily, divided into two identical drinks, for 14 days.** Blood glucose concentrations increased in CON and NUC but remained unchanged in RIB (P<0.0001 for time, condition and interaction effects). In all visits, blood lactate concentrations remained stable during recovery (P>0.05). After the performance test, blood glucose concentrations decreased to a larger degree in RIB than in CON and NUC (P<0.0001 for time and condition effects P<0.05 for an interaction effect). Blood lactate concentrations increased differently between groups (P<0.0001 for a time effect; P<0.05 for condition and interaction effects).

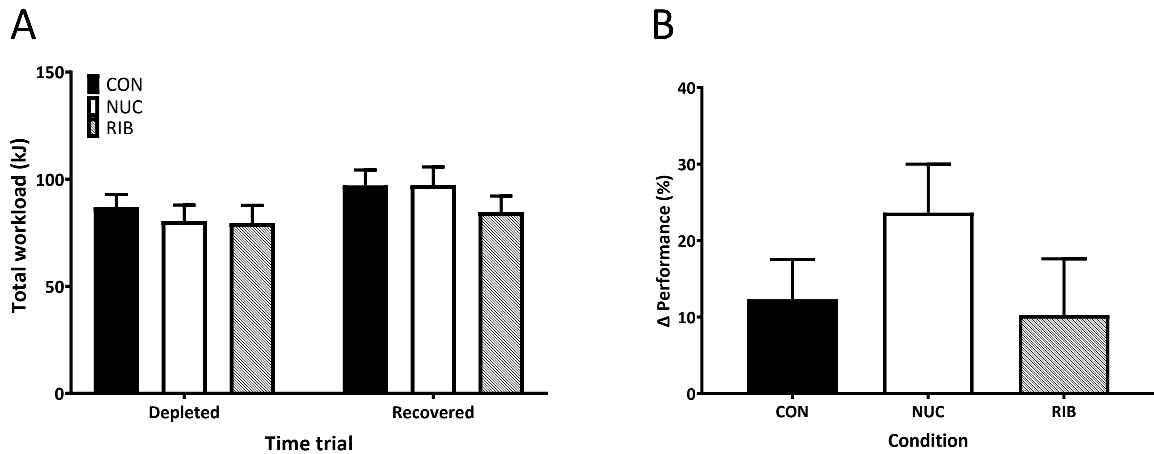


Figure 6.6 **Work output generated during a 15 min "all-out" exercise performance trial immediately following exhaustive exercise at ~ 70% of $\dot{V}O_2$ max and after a 3 hour recovery period (A) and the % change in work output between the two trials (B), after consuming 1.1 g·kg^{-1} bw of either nucleotide-depleted mycoprotein (CON) or high-nucleotide mycoprotein (NUC and RIB) and 600 mg·kg^{-1} bw of either dextrose (CON and NUC) or ribose (RIB) daily, divided into two identical drinks, for 14 days.** Work output was not different between conditions immediately following exhaustive exercise at ~ 70% of $\dot{V}O_2$ max ($P<0.05$). After a 3 hour recovery period, work output was significantly different between groups ($P=0.01$), mainly driven by it being greater in NUC than in RIB. The % change in work output between the two trials was not different between conditions ($P<0.05$).

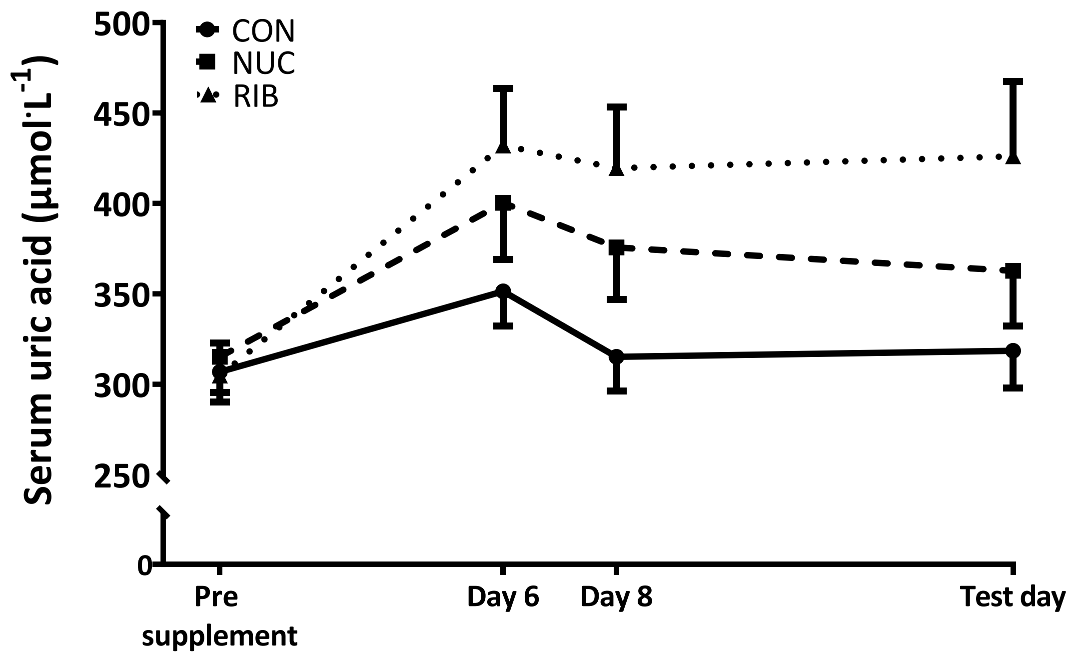


Figure 6.7 **Fasted serum uric acid concentrations before, on day 6, day 8 and after 14 days of consuming 1.1 g·kg^{-1} bw of either nucleotide-depleted mycoprotein (CON) or high-nucleotide mycoprotein (NUC and RIB) and 600 mg·kg^{-1} bw of either dextrose (CON and NUC) or ribose (RIB) daily, divided into two identical drinks.** Serum uric acid concentrations remained unchanged in CON, but increased in both NUC and RIB (time and condition effects, P<0.0001; time x condition interaction effect; P<0.0005), peaking on day 6 and remaining elevated on days 8 and 15. In addition to serum uric acid concentrations in NUC and in RIB being higher than in CON throughout the intervention, serum uric acid concentrations in RIB were higher than in NUC on days 8 and 15 (both P<0.05).

6.3 Discussion

We investigated the impact of the twice-daily ingestion, for 2 weeks, of a nucleotide-rich mycoprotein drink, with or without added ribose, on resting skeletal muscle ATP and glycogen concentrations and endurance exercise muscle metabolism and performance. Additionally, we examined the effects of the consumption of the same supplement (complemented with extra carbohydrate) on muscle ATP and glycogen concentrations and exercise performance in acute recovery from a fatigued state. We report that nucleotide and ribose supplementation did not result in increased resting muscle ATP or glycogen concentrations and, accordingly, we did not observe any changes in muscle metabolism during exhaustive exercise nor improvements in exercise performance in a depleted state. We found no differences in ATP and glycogen replenishment during acute recovery from a fatigued state nor in exercise performance post 3 h acute recovery.

The effect of dietary nucleotide supplementation on exercise performance, recovery and exercise modulated immune responses has been investigated in a small number of recent studies. Beneficial effects have been found for measures of muscular strength and force production (165, 171), markers of immune function and physiological stress (166-170), and time to exhaustion in endurance exercise (169). The exact mechanisms responsible for these effects have not yet been investigated and remain subject to debate. The currently available studies were performance/output-driven, and therefore various mechanistic hypotheses can be developed. One such hypothesis is that dietary nucleotides might exert positive effects in mitochondrial energy production, oxidative stress regulation and DNA repair (175). In the present study, we aimed to investigate the impact of dietary nucleotides in skeletal muscle fuel utilisation and energy production.

We conducted, for the first time, a detailed investigation of muscle metabolism on resting skeletal muscle, as well as on exercised muscle, during a glycogen depleting exercise, and finally, during a short period of recovery from depletion. We demonstrated that increased dietary nucleotide intake did not lead to increased skeletal muscle ATP and glycogen concentrations in the resting state. Additionally, we did not observe any changes in muscle metabolism measures during low-intensity exercise nor during high-intensity glycogen depleting exercise and, subsequently, dietary nucleotide supplementation did not lead to improvements in exercise performance on a depleted state. Finally, dietary nucleotides ingested immediately after exhaustive exercise did not produce benefits in ATP and glycogen resynthesis in the acute recovery from an exhausted state, nor in exercise performance post 3 h recovery.

A previous study demonstrated that providing participants with sublingual nucleotides for 14 days increases time to exhaustion during an incremental running exercise protocol to exhaustion (168). Furthermore, animal studies suggest dietary nucleotide intake can lead to increased liver glycogen (251) and improved fatty acid metabolism (252), which could result in exercise performance benefits. Additionally, a more recent study in mice demonstrated that the ingestion of dietary nucleotides significantly increased forced swimming time, enhanced lactate dehydrogenase activity and hepatic glycogen levels, and delayed the accumulation of blood urea nitrogen and blood lactate after 30 days of treatment (176). Improvements in oxidative stress biomarkers and antioxidant enzymes, as well as an increase in mitochondrial energy metabolic enzyme activities in the skeletal muscles of these mice, were also observed. These findings suggested dietary nucleotide intake might beneficially impact energy

production and muscle fuel utilisation, however, we were not able to observe this in the present study.

Manipulating skeletal muscle ATP content and measuring muscle ATP kinetics during exercise is a difficult undertaking, as the complex nature of the skeletal muscle energy systems provides various methodological challenges (e.g. difficult to measure ATP kinetics in real-time during exercise). Muscle ATP stores are very small and remain relatively stable, even when the ATP demand increases by more than 1000 fold, and a variety of metabolic pathways coordinate to maintain the required rates of ATP resynthesis. In the present study, we were unable to detect a decrease in skeletal muscle ATP concentrations after exhaustive exercise. Therefore, we could not test the hypothesis that ATP was limiting to performance and that nucleotide supplementation would play a role in ATP recovery post fatiguing exercise. Employing new techniques to alter muscle ATP concentrations and to monitor changes in muscle fuel ATP kinetics might be necessary to unravel the mechanisms underpinning the beneficial effects of dietary nucleotides in exercise performance. Exercise protocols comprising repeated sessions of high-intensity or maximal exercise might result in more substantial ATP depletion. Meanwhile, monitoring ATP kinetics *in vivo*, in real-time, using non-invasive magnetic resonance spectroscopy (MRS) and magnetic resonance imaging (MRI) could be feasible due to the improving sensitivity and efficiency of these techniques (253). Finally, stable isotope tracer methodologies could be useful to measure incorporation of dietary nucleotides into muscle ATP (254).

Alternatively, it is possible that potential nucleotide-induced improvements in exercise performance are not attributable to changes at the level of energy production, but instead are a result of systemic effects, such as the attenuation

of physiological stress responses which has been observed in previous studies. Ostojic and colleagues (169) suggested that the ergogenic effects of dietary nucleotides could be due to enhanced peripheral transportation of oxygen, however, we did not find any changes in oxygen consumption during the glycogen depleting protocol and we did not collect oxygen consumption data throughout the performance tests. We cannot exclude the possibility that others factors, which have not yet been investigated, are responsible for the ergogenic effects of dietary nucleotides, such as a potential effect on central nervous system fatigue mechanisms (255). Finally, the dose and regime of supplementation in this and previous studies have varied, which might, at least in part, account for the disparate results.

In addition to a thorough examination of the effects of dietary nucleotide intake, we investigated the impact of consuming dietary ribose on muscle metabolism and exercise performance. Dietary ribose intake has been shown to enhance the rate of adenine nucleotide resynthesis after intense intermittent exercise (likely by increasing the rate of PRPP synthesis) (177). We did not observe any changes in muscle metabolite concentrations nor any improvements in exercise performance in the RIB condition. As previously mentioned, we failed to detect any changes in ATP after our exercise protocol and, as such, it is unlikely that we would observe the same effects in recovery from exercise reported by Hellsten et al, who induced a 15 – 20 % reduction in resting adenine nucleotides after high-intensity exercise training. Hellsten and colleagues' novel finding only became evident 72 hours after the intense exercise, and stood in contrast with a previous study which did not find any effects of oral ribose supplementation on repeated maximal exercise and *de novo* ATP resynthesis after 24 hours (256).

Blood glucose in the 3-hour recovery period did not increase with the consumption of the RIB drinks, in contrast with a ~ 70 % peak increase in CON and NUC. Dietary ribose has been shown to produce asymptomatic, mild hypoglycaemia of short duration. Despite the RIB recovery drinks including an added extra 0.7 $g{\cdot}kg^{-1}$ bw of dextrose, this was not sufficient to elevate participants' blood glucose. However, there were no associated differences in skeletal muscle glycogen replenishment in the 3-hour recovery. It is conceivable that these low circulating glucose concentrations led to impaired exercise performance in the recovery performance test. Future studies looking at acute recovery from a depleted state might benefit from not using dextrose as a control, or from adding higher quantities of dextrose or sucrose to the recovery supplement, in order to prevent differences in blood glucose responses.

In this study, the glycogen depleting exercise protocol used resulted in only 20% depletion, despite previously having been shown to elicit over 80% glycogen depletion (179). This might explain why we did not observe a change in ATP concentrations – without glycogen depletion, ATP depletion would not be expected to occur either. A slightly lower rate of glycogen depletion would have been expected due to participants having exercised at a lower intensity for an identical length of time (70 vs 75 % $\dot{V}O_2$ max for ~ 90 mins), however, it is difficult to explain the degree of difference in responses (179). It is possible that this was due to the untrained status of the participants in the present study (37 vs 48 $ml{\cdot}kg^{-1}{\cdot}min^{-1}$ $\dot{V}O_2$ max), leading to lower tolerance to both peripheral and central fatigue (257). Another factor that might have led to the unexpected outcomes of the exercise protocol in the present study is the high number of female participants we recruited (7 out of our 12 participants). Past studies exclusively recruited male participants and it is well documented that premenopausal women have a

significantly greater ability to oxidize fat during exercise than men (258-260). We anticipated that if this was the case, it would result in longer exercise durations for the female participants, finally resulting in similar glycogen depletion, however, this does not seem to have occurred. Indeed, RER and relative contribution of carbohydrate for energy expenditure were slightly lower than anticipated, despite displaying the same decreasing trend throughout the exercise protocol. It can be argued that the glycogen depleting exercise performed in the present study was not suited for the recruited population and that in the future other protocols should be adopted, potentially involving decreased exercise durations and short repeated high-intensity bursts.

Dietary nucleotide intake has been shown to increase serum uric acid concentrations (131-133) and, recently, we found that high-nucleotide mycoprotein consumption leads to both acute (235) and sustained increases in serum uric acid concentrations (above clinically relevant thresholds) during a week of twice-daily intake. Both in previous investigations and the present study, concentrations appear to peak after 5 days of increased intake, decreasing later (after 7 and 14 days) but without returning to baseline concentrations after one to two weeks. We had previously wondered if this decrease could be associated with the incorporation of dietary nucleotides into body fuel stores (e.g. muscle ATP), however, it may be the case that dietary nucleotides are mostly metabolised and excreted, and this decrease is simply a reflection of the body adapting to a higher dietary nucleotide load. Dietary ribose consumption has also been shown to increase serum uric acid concentrations after 7 days of consuming 20 g per day, however, these returned to baseline after 14 days (261). In the RIB condition, participants consumed double this amount daily (~41 g) and the effects of the simultaneous consumption of nucleotides and ribose apparently

compounded, resulting in an increase superior and longer-lasting than the one observed in the NUC condition. Epidemiological and observational studies have reported that serum uric acid concentrations positively correlate with the development of a variety of metabolic diseases (122-125). We have previously failed to establish a causal link between high serum uric acid and impaired insulin sensitivity, glycaemic control and/or lipid metabolism, however longer duration investigations are required to further elucidate this relationship.

In conclusion, twice daily ingestion, for 2 weeks, of a nucleotide-rich mycoprotein drink, with or without added ribose, does not influence skeletal muscle ATP content, muscle fuel utilisation during exercise, exercise performance or recovery from exercise.

Chapter 7:

General discussion

7.1 Overview of the findings

The present thesis focused on two main topics. Firstly, the effect of mycoprotein and dietary nucleotide consumption on metabolic health; namely on insulin sensitivity, glycaemic control, the plasma lipidome and serum uric acid in healthy young individuals. Secondly, the impact of dietary nucleotides and ribose on resting muscle ATP and glycogen concentrations and on endurance exercise metabolism and performance, as well as on muscle ATP and glycogen concentrations and exercise performance in acute recovery from a fatigued state, in healthy and untrained young adults.

It was found that substituting meat and fish for mycoprotein twice daily for 1 week did not modulate whole-body insulin sensitivity or glycaemic control but considerably impacted upon the plasma lipidome, the latter primarily consisting of a coordinated reduction in circulating cholesterol-containing lipoproteins. It was also observed that the ingestion of a nucleotide-rich mixed-meal increased serum uric acid concentrations for ~12 h and that one week of twice-daily consumption of nucleotide-rich meals increased postabsorptive serum uric acid concentrations above clinically relevant thresholds. However, these did not influence postprandial blood glucose or serum insulin concentrations and were not associated with any deleterious effects on peripheral insulin sensitivity or daily glycaemic control and plasma lipid composition. Finally, twice-daily ingestion, for 2 weeks, of a nucleotide-rich mycoprotein drink, with or without added ribose, did not impact resting skeletal muscle ATP and glycogen concentrations and did not modulate muscle fuel utilisation during exercise, exercise performance or recovery from exercise.

7.2 Mycoprotein and metabolic health

The potential of non-animal-derived, sustainably produced dietary proteins to support metabolic health is a pressing research focus, due to mounting evidence suggesting that dietary protein consumption above the current RDAs might be beneficial (4-7, 9, 10, 182) and due to growing concerns regarding the environmental impact of animal-derived protein sources (1). The findings presented in this thesis and previous research into the metabolic effects of mycoprotein intake support the use of mycoprotein as a sustainable dietary protein source that can be incorporated into the daily diet without compromising short-term metabolic health and facilitating rapid and beneficial changes to the plasma lipidome. However, there are many avenues of mycoprotein research yet to be explored and a few new questions prompted by the results discussed throughout this thesis.

Contrary to our hypothesis, the mycoprotein intervention did not change indices of whole-body insulin sensitivity or 24 h free-living glycaemic control. However, it did lead to lower fasting blood glucose concentrations. As a recognised clinical marker of insulin sensitivity, this result is encouraging that mycoprotein consumption may support metabolic health, but it is difficult to explain why this was observed in the absence of effects on calculated insulin sensitivity under OGTT conditions and 24 h glucose control. Importantly, the effect of mycoprotein consumption on glucose kinetics is still unclear. Previous studies have demonstrated that the presence of viscous polysaccharides can slow down the rate of appearance of glucose after a meal (83). This might be because soluble fibre can increase the viscosity of a meal, slowing its digestion and absorption in the small intestine. β-glucan, which corresponds to two-thirds of the fibre in mycoprotein, has previously been shown to modulate glycaemia and

insulinaemia when consumed with a high carbohydrate load (84). As previously discussed, mycoprotein contains 25 g of fibre per 100 g of dry weight, a fibrous chitin-glucan matrix that is 89% insoluble. The β-glucan present in mycoprotein is largely insoluble, as opposed to the β-glucan usually found in cereal grains, such as oats and barley. It is unclear if the intake of insoluble vs soluble β-glucan results in different metabolic effects, as insoluble fibres generally have no effect on postprandial glycaemia or insulinaemia (262). Though the chitin-glucan matrix is insoluble and not viscous, chitin is likely to undergo alkaline deacetylation to produce the viscous polysaccharide chitosan at some point in the gastrointestinal tract. In turn, this may confer resistance to the flow induced by gastrointestinal motility, reducing contact time in the small intestine and resulting in slower gastric emptying and, consequently, nutrient absorption (85). Another potential mechanism is that the presence of fibre causes accelerated GIP and GLP-1 response, leading insulin to be released and resulting in greater glucose handling (263). Alternatively, SCFAs derived from the fermentation of soluble fibre and resistant starch in the large intestine may play a role in glucose handling, potentially by activating AMPK and causing liver glucose output to be suppressed (212, 264). Future studies should focus on these mechanisms to further elucidate a potential role of mycoprotein intake in glucose homeostasis. However, the data present in this thesis also stresses the requirement to evaluate whether acute and more mechanistic findings concerning the regulation of postprandial blood glucose control translate to longer-term indices of metabolic health. To optimise the wellbeing and health of individuals, as well as more widespread societal benefits (environmental, financial and health-related), it is the long-term effects of mycoprotein which are, ultimately, the most relevant.

A limitation of the study described in Chapter 3 was the relatively short duration of the nutritional intervention, potentially precluding us observing a more gradual beneficial response to mycoprotein. This was necessary, due to the tightly controlled nature of this study. Longer duration interventions, in a less controlled, free-living context, are now required to clarify the impact of habitual mycoprotein consumption on wider measures of metabolic health. Population-level, epidemiological studies, might be applied in the future to describe the impact of mycoprotein consumption over months/years in a large number of people. For instance, it is possible that there is a causal link between blood lipid profile and insulin resistance, with changes in the former influencing the latter and, as such, the onset of benefits in blood lipids might precede improvements in insulin sensitivity. Furthermore, looking at different daily or weekly protein doses would be useful to determine optimal intakes, in order to clarify recommendations, as this is important to inform public policy and consumer-targeted communications. Importantly, the research conducted in the course of the present thesis was applied to healthy, young participants. In the future, further research will be needed to address the effects of mycoprotein consumption in various more metabolically compromised populations, namely in individuals with prediabetes or (type 2) diabetes, as changes in insulin sensitivity and glucose control may simply have been too subtle to detect in healthy individuals. Previous studies into the effects of mycoprotein consumption on blood lipid profiles have observed larger effects in hypercholesteraemic participants (52, 53, 57), and it is conceivable that mycoprotein might have beneficial effects in insulin-resistant individuals too. It is also important to note that certain populations might be more resistant to adopting alternative protein sources into their habitual diets (e.g., older adults, men, certain ethnic and patient groups) and, coincidently, these

populations are often the ones more at risk of metabolic disease. As such, we should often assess the value of our interventions, who they will likely benefit and how can we communicate with the general population in order to effect real change in eating behaviours and, consequently, health status.

Given that both in this study, and in previous studies investigating mycoprotein's impact in the plasma lipid profile (52, 53), the energy and macronutrient contents of the diets were similar across groups, it makes sense that responsibility for the observed shift might be attributable to mycoprotein's fibre content (26 v. 34 g daily in the control and the intervention groups, respectively, in this instance). *In vitro* colonic models have shown that mycoprotein and its purified dietary fibre are fermentable, capable of producing SCFAs (81). Both mycoprotein and purified mycoprotein dietary fibre produce increased propionate and butyrate at the expense of acetate, and increasing colonic propionate production inhibits the incorporation of plasma acetate into cholesterol (82). Colonic SCFAs are largely absorbed into the colonocytes where part of the SCFAs are used as an energy source and are immediately oxidised (265). The remaining SCFAs are transported to the liver via the portal circulation where a further fraction is metabolised. Only the SCFAs that pass the liver and escape splanchnic extraction end up in the peripheral circulation. As a result, SCFA concentrations in plasma and serum are considerably lower than in the lumen of the colon and SCFA production is difficult to measure *in vivo* (265). Despite this, the findings of this thesis included a considerable (40%) increase in plasma acetate following one week of mycoprotein consumption. This is in line with previous work showing the acute consumption of other dietary fibres leading to increased postprandial serum acetate concentrations (218), and that fibre (e.g. oat- or bran-rich diets) induced reductions in circulating cholesterol are also associated with increases

in blood acetate (219). It is possible but not clear whether acetate as an *in vivo* end product of mycoprotein bacterial fermentation, mediated the plasma cholesterol-lowering effect, either by inhibiting cholesterol synthesis or by other unknown mechanisms (such as reduced cholesterol absorption or increased peripheral clearance). Furthermore, it is important to ponder whether the *type*, rather than simply the amount, of dietary fibre contained within mycoprotein may, at least in part, explain the beneficial effects of mycoprotein consumption on circulating cholesterol concentrations. Unfortunately, the design of this study (not fibre-matched across groups) does not allow for the disentanglement of the interesting potential effects of fibre quantity *v.* type and this avenue should be pursued in future research.

After the publication of the study described in Chapter 3, similar investigations were also published by Crimarco et al (266), on the effects of consuming a different plant-based alternative protein source (containing CHO and protein derived from sources such as pea, beans, potatoes and brown rice; Beyond Meat, Los Angeles, CA, USA) as opposed to animal-based protein on a variety of health factors, such as fasting serum trimethylamine-N-oxide (TMAO), blood lipids, glucose and insulin. TMAO is a gut microbe-dependent metabolite (267) which has been suggested as an early biomarker of non-alcoholic fatty liver disease (NAFLD), CVD, type 2 diabetes and metabolic syndrome (268-270). However, the mechanisms underlying these relationships are not well understood, and Mendelian randomisation studies suggest both causality (in case of type 2 diabetes) as well as confounding or reverse causality (in CVD) might be involved (271). Nevertheless, the findings of this study were in line with the results described in the present thesis, with the plant-based protein intake leading to improvements in several cardiovascular disease risk factors, including LDL-

cholesterol (and TMAO). The authors credited these cholesterol-lowering effects with the lower saturated fat and higher fibre and plant protein in their plant-based condition. However, in this paper, the effects of the intervention were less robust (only LDL-C, HDL-C and triglycerides were measured and the changes observed were not as considerable) and the study was less well-controlled (participants were asked to consume ≥2 servings per day of the protein products provided and diet recall interviews were conducted, but the diets administered were not fully controlled). The decline in cholesterol concentrations using Beyond Meat products was of a lesser magnitude than what was observed with mycoprotein, despite the intervention lasting for a longer period of time, and it can be speculated that this was due to the differences in fibre intake between both studies (28 g per day with Beyond Meat and 34 g with mycoprotein). It is also important to note that this is the sole study investigating the health effects of the Beyond Meat plant-based protein products, compared with now a bank of literature looking at the impact of mycoprotein in blood lipid profile. However, this study too lends support to the previous argumentation on the role of fibre (both present in mycoprotein and Beyond Meal) in modulating blood lipid profile, potentially by increasing gut SCFA production. Data from this study and the present thesis support increasing fibre intake as a strategy to improve markers of metabolic health (specifically blood lipid profile) and position mycoprotein and other plant-based alternative protein sources as a practical and simple way of accomplishing this. Furthermore, given the strive of various populations to reduce animal-derived protein consumption, and the cross-sectional (272) and randomised control trial (273) indications that vegetarian diets are capable of conferring benefits with respect to ischemic heart disease, obesity or type-2

diabetes, it is of relevance for the present and future work to assess what dietary protein sources may be used to facilitate such diets.

In Chapter 3, a decrease in plasma DHA and n-3 fatty acid concentrations was observed, presumably due to a low dietary load of these lipids (usually present in fish, seaweed, eggs and nuts – none of these included in the controlled intervention). However, plasma concentrations of these fatty acids can substantially vary according to the individual's most recent meal and do not accurately reflect long-term dietary consumption (220). It is possible to assess omega-3 status via analysis of erythrocyte fatty acids, a measurement that reflects longer-term intakes over approximately the previous 120 days (220). The proposed "omega-3 index" reflects the content of EPA plus DHA in erythrocyte membranes, expressed as a percentage of total erythrocyte fatty acids (274, 275). We found that the daily intake of mycoprotein led to rapid, clear and physiologically significant decreases in lipoprotein lipid content; however, unlike in some previous studies, this reduction was across the board and did not result in a more beneficial HDL:LDL ratio (as HDL was also reduced). It is conceivable that by ensuring a higher intake of essential fatty acids, such as DHA and omega-3, we could have maintained participants' HDL concentrations and achieved an increased and more desirable HDL:LDL ratio. Future dietary intervention studies assessing mycoprotein's effects in metabolic health, principally fully controlled dietary interventions, should guarantee adequate consumption of essential fatty acids to optimise beneficial effects to lipid profile.

The participants in Chapter 3 habitually consumed 1.5 $g{\cdot}kg^{-1}$ of protein per day. However, the intervention diet provided only 1.2 $g{\cdot}kg^{-1}$ of protein per day, resulting in an approximate 20 % decrease in habitual protein intake. Whilst the chosen protein intake for this study is aligned with the habitual intake of the UK

population as a whole, the participants recruited being younger adults and, many of them, health-conscious Sport Science and Medicine students, had a higher habitual protein intake. High-protein diets have been shown to improve glycaemic control (204) and we cannot fully discard the possibility that the drop in protein intake obscured any potential changes in insulin sensitivity or glycaemic homeostasis. In the future, the research questions being asked and the population being studied should inform the determination of the optimal amount of mycoprotein and total protein content of intervention diets. A high protein mycoprotein-based diet will undoubtedly be a high fibre diet. Both high protein and high fibre intake have been shown to lead to higher satiety and to induce higher levels of dietary thermogenesis (10, 54). As such, these types of diets might be particularly useful in the study of the effect of mycoprotein in satiety and weight loss and/or maintenance.

The intersection of mycoprotein's effects on metabolic health and satiety is an exciting future field of study. Mycoprotein consumption has been shown to induce an acute thermogenic response, similar to that seen following the ingestion of other (animal) protein sources (48), and therefore would presumably contribute to overall daily energy expenditure during a weight loss regimen. Additionally, mycoprotein and most mycoprotein-containing products have a low energy density. The consumption of foods with a low energy density is positively associated with reduced *ad libitum* energy intake and positive weight management outcomes (276). The substitution of high-energy-density foods for mycoprotein-containing products may be an effective tool to manipulate the energy density of a meal or diet. As a low-energy-density, high-protein food source, mycoprotein would also have theoretical value in a diet aimed at maintaining protein intake in an effort to retain lean tissue during an energy deficit.

The effects of mycoprotein on satiety are also of particular interest. It has been shown previously that protein sources differ in their capacity to affect satiety (36). For example, gelatin protein provided as a single meal (277) or as a primary protein source over a 36-hour experimental period (36) was reported to suppress appetite to a greater extent when compared with isonitrogenous milk protein equivalents, which the authors suggested may be related to the effects of amino acid composition in the central nervous system. Differences in sensory characteristics, such as greater viscosity and creaminess, may also play a role in increasing satiety and reducing energy intake (278-281). It is possible that various metabolites associated with the partial fermentation of dietary fibres may explain the potent appetite-suppressing effect of mycoprotein (62). For example, the SCFA propionate has been shown to induce secretion of PYY and GLP-1 in humans in studies of acute ingestion and may, in part, explain the short-term appetite-regulating effects of some dietary fibres (78). Both mycoprotein and mycoprotein-derived dietary fibre promote the production of propionate, but the relevance of this mechanism in explaining the effects on appetite regulation remains to be fully elucidated (81). Irrespective of the mechanism, the effects of mycoprotein on satiety and thermogenesis, along with the high-protein/low-energy content of mycoprotein, position this food source as an intriguing approach to support a diet (under *ad libitum* conditions) aimed at weight loss or maintenance. Also worthy of note, diets with a lower glycaemic index have independently been shown to improve weight maintenance following weight loss during energy restriction (30). The capacity of mycoprotein to lower the glycaemic load of a meal or habitual diet adds an additional line of inquiry about its potential utility in weight management. Decreased body weight in overweight and obese individuals has been shown to result in a variety of beneficial effects on metabolic

health, such as improved insulin sensitivity and lipid profile (282-284). Controlled, longer-term laboratory weight loss studies comparing mycoprotein with other protein sources are warranted. Well-designed studies will be required to disentangle which of the potential beneficial effects of these dietary interventions can directly be attributed to mycoprotein intake or indirectly, due to mycoprotein enabled weight loss.

On the other hand, whether mycoprotein can provide an effective and sustainable source of dietary protein to support healthy (and active) ageing remains to be investigated. While the potential usefulness of mycoprotein is promising, the development of age-related anabolic resistance presents a challenge. It would be expected that a relatively large dose of mycoprotein would be required to maximally stimulate the muscle protein synthetic response in older adults (285). Given that older adults generally display a reduced appetite compared with younger adults, and paired with the potent satiating effect of mycoprotein, it would follow that consuming a sufficient amount of mycoprotein per meal (or over repeated meals to obtain daily intakes) may be challenging. Future research is warranted to establish whether mycoprotein could be used to support optimal rates of muscle protein synthesis while avoiding a negative energy balance in older adults (and/or individuals at risk of malnourishment), thereby having potential as part of a viable strategy to support healthy ageing. Studies are being undertaken in this area and during my PhD I had the opportunity to be involved in this exciting and pressing research topic. A variety of products to support healthy aging and prevent sarcopenia are available in the market and it is likely that in the near future there will be a push for plant-based alternatives, which can be used both by vegans and by environmentally-conscious individuals. Mycoprotein has the potential to satisfy this need.

7.3 Dietary nucleotides, serum uric acid and metabolic health

The findings from Chapters 4 and 5 of this thesis clearly illustrate the relationship between an increased dietary nucleotide intake and a consequent rise in serum uric acid concentrations. However, this thesis was not able to establish a causal link between the elevated serum uric acid concentrations resulting from increased dietary nucleotide intake, and insulin resistance or other impairments to metabolic health. Additional, longer-term investigations in this area are necessary. It is possible that a high (purine) nucleotide diet results in persistent elevated serum uric acid concentrations, and that these elevated concentrations need months, or even years, to impair normal metabolic processes such as insulin sensitivity.

The existing research on the metabolic effects of elevated serum uric acid concentrations is replete with contradictory findings. Epidemiological studies have associated high uric acid concentrations with increased risk of cardiovascular mortality, and specifically as a risk factor of aortic disease-related mortality (286, 287). Animal studies have demonstrated a relationship between hyperuricemia and the development of hypertension, believed to develop through increased vasoconstriction and arteriolar wall thickening (126). This served as a rationale for several studies investigating the effect of allopurinol (a uric acid lowering drug) treatment on blood pressure. Findings from allopurinol trials have been conflicting, with some studies, namely in hypertensive adolescents (288-290), identifying a blood pressure-lowering effect whilst others, generally in adults and individuals with chronic kidney disease (CKD) (291) not observing any benefits. However, despite this, uric acid concentrations have been shown to predict the development of CKD, diabetic kidney disease (DKD) and atherosclerosis (292-294). These confusing findings bring into question the specific role of uric acid in disease development; is uric acid a risk factor of

metabolic dysfunction or simply a marker? Recently, studies using Mendelian randomisation (295), a research method that uses genetic variants as natural experiments to provide evidence about potential causal relations between modifiable risk factors and disease, have attempted to answer this question. Using 28 loci which have been shown to be associated with high uric acid (296), these studies have found that increased uric acid levels are a risk factor for gout, but not for any of the other diseases studied, such as atherosclerosis and diabetes, serving only has a marker and not a causal agent (297-299). Despite the wealth of data investigating the effects of elevated serum uric acid, there is not a firm answer as to the extent of uric acid's role in disease development. Recent findings seem to indicate that uric acid merely acts as a marker of metabolic dysfunction (except in the case of gout). If this is indeed the reality, we need to ponder if promoting the lowering of circulating uric acid concentrations in individuals who do not suffer from gout is of any benefit, considering the protective effects uric acid has been shown to have in a variety of immune and neurological disorders (101-103, 105, 106).

Longer dietary interventions manipulating serum uric acid concentrations to establish or reject its role as a causal agent of metabolic dysfunction are difficult to conduct both due to logistical reasons (controlled dietary interventions are expensive and require a large commitment from participants) and due to ethical reasons (if increased serum uric acid concentrations have indeed a deleterious effect in metabolic health, studies might result in long-term harm). Mendelian randomisation is less prone to confounding factors and to reverse causation, which are an issue in epidemiological studies, providing a way to infer causation without having to recur to randomised controlled trials, which sometimes, like in this instance, might not be ethical to conduct (295, 300). However, Mendelian

randomisation studies can still be prone to their specific types of confounding and, despite their advantages versus epidemiological studies, the causal inference still requires caution. Additionally, the interpretation of null Mendelian randomisation studies is challenging, especially in the common situation of a weak association between the gene and the biomarker (295, 300). A combination of Mendelian randomisation studies, longitudinal studies (looking at serum uric acid concentrations and the development of metabolic disease over years/decades (301)) and medium-term RCTs is likely the best feasible approach to further clarify the metabolic effects of uric acid. Further research into the mechanisms of disease development, namely in widespread metabolic impairments such as insulin resistance and elevated blood pressure, will also be able to elucidate this relationship, and might potentially demonstrate that increased serum uric acid concentrations associated with disease states are often a consequence as opposed to a cause (e.g. hyperinsulinaemia can lead to hyperuricaemia by reducing renal excretion of uric acid (302)).

The potential protective effect of nucleotide-depleted mycoprotein in reducing serum uric acid concentrations should also be investigated in the future. Interventions conducted in hyperuricaemic vegan populations, namely patient populations presenting with gout, insulin resistance or elevated blood pressure, could investigate the potential of including mycoprotein in the diet to reduce serum uric acid concentrations, in the same way dairy has been shown to do. However, it is worth noting that, whilst this might be relevant for the prevention of gout, in the case of metabolic health, the value of these investigations is dependent on serum uric acid concentrations being found to have a causal role in disease development.

In Chapter 5, morning urine samples were collected every day to assess changes in urine uric acid concentrations and, consequently, urinary uric acid excretion. The kidneys are responsible for the elimination of two-thirds of the uric acid excreted, whilst the remaining one-third is eliminated by the gastrointestinal tract (239). Defective renal handling of uric acid has been identified as a pathophysiologic factor underpinning hyperuricemia and gout, however, the molecular mechanisms of renal uric acid transport are still incompletely understood (303). No changes in urine uric acid concentrations were found throughout the intervention, nor differences between groups, suggesting that rises in serum uric acid concentrations did not lead to increased urinary excretion. However, it is possible that increased urine output would have led to increased excretion of uric acid without an increase in uric acid concentrations in urine samples. Alternatively, it is also possible that the timing of the collected urine samples did not coincide with the timing of increased uric acid excretion (e.g. increased uric acid excretion in urine in the first few hours after a meal). Therefore, collecting 24 h urine for the seven days of the intervention might have provided greater insight into the total daily excretion of uric acid. More specifically, collecting and analysing urine samples separately for the whole 24 h could provide more insight into the dynamics of renal handling of uric acid throughout the day/in response to meals. It can also be speculated that dietary intake of nucleotides over a certain level might lead to a disproportional increase in the excretion of uric acid via the gastrointestinal tract and, as such, measuring uric acid concentrations in faecal samples might be as (or more) important as in urine samples. As such, the investigation of the mechanisms of renal handling and gastrointestinal excretion of uric acid should not be neglected in further studies.

An extensive protocol of urine and faecal sample collections might provide further insight into how the body adapts to an increased dietary nucleotide intake.

Also in Chapter 5, fasting blood glucose concentrations displayed a lowering trend throughout the intervention week in both groups. Thus, it appears that the glucose-lowering effect of mycoprotein occurs irrespectively of its nucleotide content. These results stand in stark contradiction to the hypothesis that high nucleotide intake would lead to impaired glucose handling. Additionally, coordinated reductions in HDL cholesterol were observed in the low-nucleotide group, whilst remaining stable in the high-nucleotide group. It is unclear what mechanisms would lead to these findings, considering there were no differences between groups regarding any other parameters of dietary intake or physical activity. However, it is clear that these results do not show any adverse effects of high nucleotide consumption on plasma lipid profile.

Finally, from a practical point of view, the industrial processes required to reduce the nucleotide content of mycoprotein are costly and environmentally demanding (42). Not requiring them would further improve mycoprotein's sustainability credentials, by further reducing water usage and greenhouse gases emissions. Clarifying the role of dietary nucleotide-related increases in serum uric acid in metabolic health could potentially provide a scientific basis for alterations to this process. Intermediate levels of RNA/nucleotide depletion during mycoprotein production might be the key compromise between both ensuring optimal health outcomes and reducing the environmental impact of the process. The Food Standards Agency (FSA) has required detailed feeding studies on the effects of a revised product to approve an alteration to the specifications of mycoprotein, in terms of the levels of RNA present in the final product. As such, future research utilising mycoprotein products with different levels of nucleotides (resulting from

different degrees of depletion) will be able to provide the research community, the FSA, the producers and, ultimately, the wider population with more data on the feasibility of producing a safe, more cost-effective and sustainable product.

7.4 Dietary nucleotides and ribose on muscle metabolism and exercise performance

Research into the effects of dietary nucleotides and ribose in muscle metabolism and exercise performance is still very limited, with findings often being contradictory and difficult to interpret, as it was the case in the present thesis. Despite this, a beneficial effect of dietary nucleotides in energy production should not yet be ruled out. This is still an emerging field of research with a lot of room for additional lines of enquiry.

An apparent small improvement in exercise performance during a 15 min work output test after a 3 hour recovery period when nucleotides were provided in the recovery drink was observed (although not statistically different vs the control group). This would be in line with a previous study that had shown that providing participants with sublingual nucleotides for 14 days increased time to exhaustion during an incremental running exercise protocol (to exhaustion) (169). Animal studies suggest possible effects of dietary nucleotides that could be beneficial for exercise performance, such as increased liver glycogen (251) and improved fatty acid metabolism (252). Additionally, a more recent study in mice demonstrated that the ingestion of dietary nucleotides significantly increased forced swimming time, enhanced lactate dehydrogenase activity and hepatic glycogen levels, and delayed the accumulation of blood urea nitrogen and blood lactate after 30 days of treatment (176). Improvements in oxidative stress biomarkers and antioxidant enzymes, as well as an increase in mitochondrial energy metabolic enzyme activities in the skeletal muscles of these mice were also observed. It is also possible that any improvements in exercise performance as a result of dietary nucleotide supplementation are not attributable to changes at the level of energy production, but instead are a result of other systemic effects. Dietary nucleotides

have been shown to improve markers of immune function and physiological stress, such as leading to lower circulating cortisol levels, after high-intensity exercise. This effect of attenuation of physiological stress during exercise in performance is not well understood. The free testosterone:cortisol ratio (FTCR) is often used to study overtraining in a variety of sports by representing the balance between anabolic and catabolic activity (304). It can be speculated that a decrease in cortisol levels would consequently lead to an increased FTCR, which is positively associated with exercise performance (305-308), hence potentially improving certain performance measures such as time to exhaustion and work produced. Another mechanism by which dietary nucleotides might have an ergogenic effect is by enhancing peripheral transportation of oxygen, so future studies might benefit from collecting oxygen consumption data throughout performance tests (169). Finally, we cannot exclude the possibility that other factors, which have not yet been investigated, are responsible for the beneficial effects of dietary nucleotides, such as a potential effect on central nervous system fatigue mechanisms, and these warrant future research.

A lot of the research into the potential effects of dietary nucleotides has been performed *in vitro* or in animal studies. These findings, whilst not directly applicable to humans, can provide evidence for potential new areas of study and generate new hypothesis to later be tested on *in vivo* human studies. Explorative mechanistic studies investigating nucleotides' effects are a current popular research topic and future research will be crucial to unravel all the physiological processes with a potential to be modulated by dietary nucleotides. Topics such as the potential effects on central nervous system fatigue mechanisms mentioned previously will benefit from more extensive animal and *in vitro* research, which in the future might inform further human research.

An important consideration, briefly mentioned in Chapter 6 and relevant for the study of the effect of nucleotides in muscle metabolism, pertains to the manipulation of skeletal muscle ATP contents and the measurement of muscle ATP kinetics during exercise. Muscle ATP concentrations are very small and remain relatively stable, even when the ATP demand increases by more than 1000 fold during maximal contraction to contractile failure. As such, muscle ATP is not considered an energy store but an essential requirement for optimal cell function. This makes muscle ATP difficult to modulate, even through intense exercise protocols. A few studies have shown that daily high-intensity exercise for a week leads to a reduction of ~ 25 % in resting muscle ATP (177). Future studies looking at the effects of dietary nucleotides and ribose in recovery from intense exercise might want to start with an exercise programme spanning multiple days, to ensure ATP depletion, and potentially rely on dietary carbohydrate manipulation to reduce glycogen concentrations. Regarding the quantification of ATP replenishment in the muscle, different technologies, such as the measurement of mitochondrial ATP synthesis using magnetic resonance spectroscopy might provide further insight (309). Developing new techniques, both to alter muscle ATP concentrations and to reliably measure small shifts in muscle fuel ATP kinetics might ultimately be necessary to unravel the mechanisms underpinning the beneficial effects of dietary nucleotides in exercise performance.

As previously discussed, the glycogen depleting exercise performed in the present thesis might have not been suited to the studied population and, as such, in the future other protocols should be adopted, potentially involving decreased exercise durations and short repeated high-intensity bursts. Future studies looking at acute recovery from a depleted state might also benefit from not using

dextrose as a control or, alternatively, from adding higher quantities of dextrose or sucrose to the recovery supplement, in order to prevent differences in blood glucose responses.

The study described in Chapter 6 was a very ambitious and thorough physiological investigation which aimed to describe the effects of dietary nucleotides and ribose in exercise metabolism and performance in great detail. Whilst I was able to answer some of the questions with confidence (i.e. what is the effect on fasting glycogen/ATP), for others this was not possible (due to an unsatisfactory glycogen and ATP depletion). As such, conclusions from these findings have to be taken carefully, but lessons learned from this experience can hopefully provide useful insights when setting up future research projects.

7.5 Summary and conclusions

In summary, this thesis has shown that substituting meat and fish for mycoprotein at lunch and dinner for one week does not modulate whole-body IS or 24-h free-living glycaemic control but considerably impacts upon the plasma lipidome. As such, mycoprotein can be incorporated into the daily diet without compromising short-term metabolic health and facilitating rapid and possibly beneficial changes to the plasma lipidome.

This thesis has demonstrated that the ingestion of a nucleotide-rich mixed meal increases serum uric acid concentrations for ~12 h, but does not influence postprandial blood glucose or serum insulin concentrations. Accordingly, twice-daily consumption of high-nucleotide mycoprotein for one week leads to sustained increases in serum uric acid concentrations (above clinically relevant thresholds), but not to any associated deleterious effects in insulin sensitivity, glycaemic control and plasma lipid composition.

Finally, it was shown that twice-daily supplementation, for 2 weeks, with a nucleotide-rich mycoprotein drink, with or without added ribose did not result in any changes in ATP and glycogen concentrations, nor endurance exercise performance. In acute recovery from a fatigued state, dietary nucleotides had a potential subtle beneficial effect in exercise performance, without modulating ATP or glycogen concentrations. Substituting dextrose for ribose in the recovery period did not result in any benefits and led to a decrease in performance, likely modulated by a decrease in blood glucose availability.

The findings from this thesis provide novel insights into the role of mycoprotein as a promising alternative protein source, the physiological effects of nucleotide intake and their impact on uric acid concentrations and metabolic health, as well as in exercise metabolism and endurance performance.

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