Proceedings of the Nutrition Society (2020), **79**, 373–379 doi:10.1017/S0029665120007016
 First published online 4 June 2020

The Nutrition Society Member-led Meeting was held at the University of Surrey, UK on 9 January 2020

Conference on 'Application of stable isotope techniques in Human Nutrition Research'

Report of a member-led meeting: how stable isotope techniques can enhance human nutrition research

Barbara A. Fielding[1]*, Bruce A. Griffin[1], Wendy Hall[2], Leanne Hodson[3], Rona Antoni[1], A. Margot Umpleby[1], Tracey Robertson[1], Tom Preston[4], Matthew Brook[5,6] and Katherine Pinnick[3]

[1]*Faculty of Health and Medical Sciences, University of Surrey, Guildford, UK*
[2]*Faculty of Life Sciences & Medicine, King's College London, London, UK*
[3]*Oxford Centre for Diabetes, Endocrinology and Metabolism, University of Oxford, Oxford, UK*
[4]*Scottish Universities Environmental Research Centre, Glasgow, UK*
[5]*MRC-Versus Arthritis Centre for Musculoskeletal Ageing Research, University of Nottingham, Nottingham, UK*
[6]*Nottingham NIHR BRC, School of Life Sciences, University of Nottingham, Nottingham, UK*

A Nutrition Society member-led meeting was held on 9 January 2020 at The University of Surrey, UK. Sixty people registered for the event, and all were invited to participate, either through chairing a session, presenting a '3 min lightning talk' or by presenting a poster. The meeting consisted of an introduction to the topic by Dr Barbara Fielding, with presentations from eight invited speakers. There were also eight lightning talks and a poster session. The meeting aimed to highlight recent research that has used stable isotope tracer techniques to understand human metabolism. Such studies have irrefutably shaped our current understanding of metabolism and yet remain a mystery to many. The meeting aimed to de-mystify their use in nutrition research.

Non-alcoholic fatty liver disease: Insulin resistance: Muscle metabolism: Dietary macronutrients: Cell culture

Stable isotopes are present as a minor component of all biological material, and for example, we are perhaps unwittingly ingesting a stable isotope of carbon, known as ^{13}C in all the food that we eat[1]. An atom of ^{13}C, like most stable isotopes in our environment, contains one extra neutron, giving it a higher mass than the more common isotope, which is the basis of the measurement of stable isotope enrichment by MS. There are a number of excellent articles giving more detail on the use of stable isotopes as metabolic tracers[2,3]. Briefly, metabolites for metabolic studies are often available to purchase with one or more of the common isotope atoms replaced by the less common isotope. The whole molecule is referred to as the tracer and the natural version is called the tracee. A subtraction to account for natural abundance is required when calculating the tracer: tracee ratio. Stable isotopes can be administered orally, intraduodenally or intravenously *in vivo*. They are safe to use in human subjects and can even be given to babies, and during pregnancy. Stable isotope tracers are increasingly used in *ex vivo* and cellular models of human metabolism, for which more harmful

Abbreviations: ALP, atherogenic lipoprotein phenotype; DNL, *de novo* lipogenesis; IAA, indispensable amino acids; LDL-C, LDL-cholesterol; NAFLD, non-alcoholic fatty liver disease.

***Corresponding author:** B. A. Fielding, email b.fielding@surrey.ac.uk

radioisotope tracers are being phased-out. The concentration of tracer to be used must be optimised, to provide tracer and tracee concentrations that will be within the sensitivity of measurement.

Liver metabolism

Professor Leanne Hodson and Professor Bruce Griffin gave the first talk of the day and they discussed the challenges of tracing fat in and out of the liver. Professor Hodson covered the challenges of measuring liver fat metabolism *in vivo* in human subjects and some of the methodologies used. Stable isotope tracers allowed the fate of fatty acids derived from different sources (adipose tissue, dietary and those synthesised *de novo* in the liver) to be followed through esterification and oxidation pathways, in the fasting and postprandial states. The relevance of the pool size and nutritional state and how this may influence the tracer: tracee ratio were illustrated in studies that administered [U-^{13}C]palmitic acid intravenously as follows. In a fasting study of age- and BMI-matched young men and women, women had higher plasma NEFA concentrations and this resulted in a lower tracer: tracee ratio[4] due to dilution from NEFA arising from adipose tissue lipolysis. In the postprandial state, insulin suppresses adipose tissue lipolysis, leading to a suppression in plasma NEFA concentrations, and the tracer: tracee ratio is consequently increased[5,6]. Using data showing the contribution of different fatty acid sources, including stable isotope labelled [U-^{13}C]palmitic acid in the plasma NEFA pool, to VLDL-TAG, it was highlighted that the way that data are expressed, either as relative or absolute contribution, influences whether there are significant differences between groups[6]. Leanne ended her talk by discussing how stable-isotope tracers have allowed the process of *de novo* lipogenesis (DNL) to be further understood and how specific factors, including insulin resistance[7], sex[8] and nutrients, may influence this pathway[9].

Professor Bruce Griffin continued the theme of tracing liver fatty acids by describing the background to an elegant human dietary intervention study in men with non-alcoholic fatty liver disease (NAFLD)[10]. A moderate elevation of plasma TAG can reduce the particle size of LDL and HDL, which increases the potential of these lipoproteins to cause and prevent cardiovascular atherosclerosis, respectively. These lipid abnormalities, known collectively as an atherogenic lipoprotein phenotype (ALP), can be promoted by dietary free sugars increasing the synthesis, storage and secretion of TAG from the liver as VLDL. Dietary free sugars, including glucose and fructose, most commonly consumed as the disaccharide sucrose, have been implicated in the accumulation of fat in the liver, that leads to NAFLD, and in the production of a TAG-rich VLDL (VLDL1) that promotes the formation of an ALP[11]. The study was designed to elucidate the mechanisms by which dietary free sugars influence the formation of an ALP in men with NAFLD, and low liver fat controls, as defined by magnetic resonance spectroscopy[10]. The original hypothesis stated that men with NAFLD would show an increased propensity to develop an ALP relative to controls, by increasing their production rate and concentration of large, TAG-rich VLDL1, DNL and liver fat, in response to a high *v.* lower sugar diet. Men with NAFLD (*n* 11) and controls (*n* 14) were fed two isoenergetic diets, high or low in free sugars (26 and 6 % total energy) for 12 weeks, in a randomised, crossed-over design. To test the hypothesis, a glycerol stable isotope tracer was administered to fasting participants, which would be then taken up by the liver, become incorporated into secreted VLDL-TAG and with mathematical modelling, enable its production rate to be determined. A stable isotope-labelled amino acid was also administered intravenously to become incorporated into the protein apoB100 in VLDL, to enable the production rate of particle number to be modelled. In this way, stable isotope tracers were used to measure the kinetics of the plasma lipoprotein subclasses VLDL1, VLDL2, IDL, LDL2, LDL3. Ambitiously, systemic NEFA were also traced into VLDL-TAG after intravenous infusion of [U-^{13}C]palmitic acid, and DNL was measured in VLDL-TAG after deuterated water ingestion. The high sugar diet increased the percentage of liver fat in both NAFLD (24·2 *v.* 14·2 %) and controls (3·6 *v.* 1·5 %), relative to the low sugar diet, but the significance of these increases was not maintained after adjustment for a small increase and decrease in body weight, ±2 kg after the high and low sugar diet, respectively. The impact of the high and low sugar diets on lipoprotein kinetics was almost exclusively on the production and catabolism of VLDL-TAG and its subclasses, with only minimal effects on lipoprotein-related apoprotein B. Contrary to the original hypothesis, an effect of the high sugar diet was to channel hepatic TAG into the smaller, usually TAG-depleted VLDL2 subclass in men with NAFLD, but the larger, TAG-rich VLDL1 subclass in the controls, relative to the low sugar diet. These dietary effects could be explained, in part, by differences in the contribution of fatty acids from intra-hepatic stores and DNL, and were all independent of the changes in body weight. In conclusion, stable isotope tracers were instrumental in finding new evidence for the differential partitioning of hepatic TAG into large and small VLDL, as a mechanism to explain how free sugars contribute to NAFLD and the formation of an ALP.

Dietary fat

Recent consensus on the causative role of LDL in the development of cardiovascular atherosclerosis[12] confirms what was already well established from a totality of evidence for the cholesterol hypothesis. It also strengthens the evidence for the guidelines to restrict the intake of SFA to no more than 10 % of total energy[13,14], since the association between SFA and CVD is mediated largely through the capacity of SFA to raise serum LDL-cholesterol (LDL-C). However, the cholesterol-raising capacity of SFA is influenced by many factors, including macronutrient substitution, the

https://doi.org/10.1017/S0029665120007016 Published online by Cambridge University Press

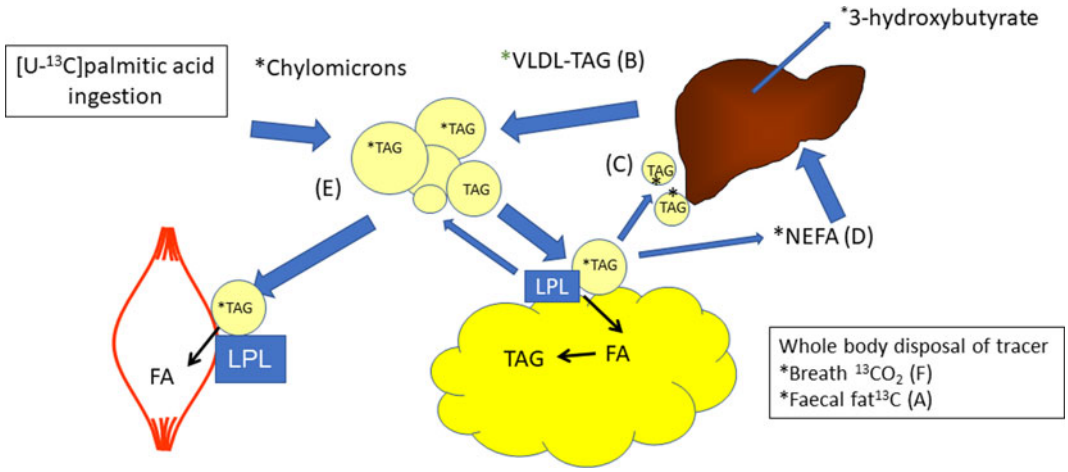

Fig. 1. (Colour online) A single stable isotope can trace multiple routes of saturated fat disposal. ^{13}C labelled palmitic acid is absorbed by enterocytes in the gut and secreted into the lymph before appearing in the blood in chylomicron TAG. The accessible route of the tracer is marked with asterisks. Incomplete absorption of SFA by the gut will be measured in faecal fat (A). The liver secretes VLDL-TAG containing the tracer (B), mainly via the uptake of chylomicron remnants (C) or uptake of NEFA, spilled over (D) from the lipoprotein lipase (LPL)-mediated hydrolysis of chylomicron-TAG. The measurement of tracer in the plasma TAG pool (E) will represent exposure of tissues and organs to palmitic acid from the meal, due to its presence in chylomicron- and VLDL-TAG. The total percent dose of administered tracer oxidised in all tissues will be determined from measurements of breath $^{13}CO_2$ (F).

differential effects of individual SFA and food source, and lastly, significant inter-individual variation in the LDL-C response to changes in SFA intake. While variance in dietary compliance may undoubtedly contribute to the variation in response to intervention studies, there is evidence for innate differences in the metabolic handling of SFA and regulation of cholesterol homeostasis, especially between the liver and gut, to explain individual variation in LDL-C. Professor Bruce Griffin and Dr Barbara Fielding described an ongoing collaborative study between the Universities of Surrey, Reading and Imperial College London, which was designed to investigate the mechanisms to explain variation in serum LDL-C response to SFA[15]. The original hypothesis stated that inter-individual variation in serum LDL-C to reduced intake of SFA will be explained by variation in the efficiency of the absorption of dietary SFA, and relative stimulation of LDL receptors. This effect will be mediated by a variable combination of inputs from the gut microbiota, bile acid composition, gut permeability and SCFA, and apoprotein-E genotype. The first task in testing this hypothesis was to reproduce variation in serum LDL-C previously observed in large intervention trials in response to changes in the intake of SFA[16,17], and to select hyper and hypo-LDL-C responders for further metabolic and genetic phenotyping. A group of healthy middle-aged men (n 106) were fed two diets in sequence; first, a high SFA diet (SFA 18 % total energy) followed by a lower SFA diet (SFA 5 % total energy). Hyper and hypo-LDL-C responders (n 18 × 2) have been selected for metabolic and genetic phenotyping after a high (18 %) SFA diet followed by a low (10 %) SFA diet for 4 weeks each. These diets were consumed within the homes of free-living participants, by the substitution of 40 g habitual fat, while maintaining their habitual diet. Metabolic assessments were conducted at the end of the two diets, with statistical comparisons between hyper- and hypo-responsive subgroups ongoing. During these metabolic visits, participants undertook stable isotope postprandial studies to compare metabolic responses to acute saturated fat ingestion. Two hundred milligrams of [U-^{13}C]palmitic acid was given in a warmed chocolate milkshake with serial blood and breath samples taken for the following 480 min (Fig. 1). Stool samples were taken for 48 h following the ingestion of the isotope to measure absorption (dose administered–dose recovered). Plasma samples will be analysed by GC-isotope ratio MS to measure the incorporation of the tracer in a number of fatty acid pools: (1) plasma TAG, as a measure of plasma residence time/exposure of dietary SFA after a meal; (2) NEFA as a measure of fatty acid spill over and metabolism; and (3) 3-hydroxybutyrate, a ketone body and proxy for hepatic fatty acid oxidation. Combined with measurements from other analytical platforms, these data will reveal the multifaceted determinants underlying known variations in LDL-C responses to saturated fat.

Insulin resistance measurements

Insulin resistance is the key metabolic abnormality in diabetes, metabolic syndrome and other metabolic diseases. Measuring the degree of insulin resistance and how it changes in response to treatment is an important tool. Professor Margot Umpleby (University of Surrey,

https://doi.org/10.1017/S0029665120007016 Published online by Cambridge University Press

UK) gave an account of the pros and cons of different methods of measuring insulin resistance. Although insulin affects many metabolic pathways, techniques for measuring insulin resistance focus on glucose metabolism. There are several methods for measuring whole-body insulin sensitivity ranging from a simple static test such as Homeostatic model assessment, which is based on fasting insulin and glucose concentrations[18], to a dynamic test such an intravenous glucose tolerance test which requires the analysis of the glucose response with the minimal model, a complex mathematical model[19].

A euglycaemic hyperinsulinaemic clamp is considered to be the gold standard technique for measuring insulin sensitivity[20]. Insulin is infused for 2 h at a constant rate to achieve insulinaemia. Glucose is infused to prevent a fall in glucose concentration and maintain euglycaemia. The amount of glucose infused during the final 30 min is a measure of the insulin-induced deficit in glucose in the extracellular fluid due to both a decrease in hepatic glucose production rate (Ra) and an increase in glucose uptake (Rd). It does not provide a separate measure of the insulin sensitivity of glucose Ra and Rd. To do this, a continuous infusion of a stable isotope of glucose is incorporated into the protocol. Because hepatic glucose Ra is very sensitive to insulin, it must be measured using a very low dose of insulin, whereas glucose Rd is measured with a much higher dose of insulin. This necessitates using a two-step insulin clamp (a low dose and a high dose)[21].

There is growing interest in measuring the insulin sensitivity of hepatic glucose Ra and glucose Rd following a mixed meal. The difficulty with this method is that hepatic glucose Ra, glucose appearance from the meal (Ra_{meal}) and glucose disposal vary with time, and computationally this is challenging. Most researchers use the dual tracer technique: one glucose tracer is infused intravenously to measure hepatic Ra and a different tracer is added to the meal to measure oral glucose appearance[22]. Computational errors are reduced if the ratio of hepatic glucose Ra and iv tracer is kept constant. To do this, the tracer infusion rate is decreased when the meal is given. The triple tracer technique is considered to be the gold standard method[23]. This requires a third tracer, which is infused at a rate to mimic the Ra_{meal}. This adds considerably to the cost of the study. Comparison with the dual tracer technique method has shown the triple tracer technique method outperforms it only marginally.

To measure insulin sensitivity, the data generated by these methods can be incorporated into the oral glucose minimal model[24]. This generates a measure S_I^P (effect of insulin to inhibit glucose production) and S_I^D (effect of insulin to stimulate glucose disposal). Although more physiological than the clamp technique, this method requires the expertise of a mathematical modeller. This technique was used in a study described by Dr Tracey Robertson (University of Surrey, UK) in order to trace the metabolic fate of potato starch. To fully understand the effects of chilling and reheating a starchy carbohydrate meal on the postprandial glycaemic response, potatoes were grown in an atmosphere of $^{13}CO_2$ to intrinsically label the starch ^{13}C atoms as the potatoes grew (IsoLife, Wageningen, The Netherlands). On two occasions, participants consumed either a freshly cooked or microwave-reheated mashed potato meal, consisting of [U^{13}C]potato (>98 % enrichment of starch carbons), diluted to 5 % with non-labelled potato mixed with butter and received a [$^2H_2$]glucose infusion to enable glucose flux modelling. Intrinsic labelling of fresh food, such as potatoes, allows examination of the effects of changes to the food matrix by food processing techniques. The study found a reduced postprandial insulin response following the reheated meal, with no effect on endogenous glucose production, suggesting enhanced hepatic insulin sensitivity as a possible mechanism of action. It was hypothesised that these effects may be a consequence of the formation of two types of resistant starch, RS3 and RS5, in the reheated meal. *In vitro* laboratory work is planned to investigate this further.

Dietary protein

Professor Tom Preston (University of Glasgow, UK) gave an account of the use of intrinsic stable isotope labelling of plants using 'in-house' techniques. With a large portion of the population in low- and middle-income countries reliant on plant protein and increasing calls in the West to moderate animal-source protein intake, there is rising awareness of the need to meet amino acid requirements from plant-based protein sources. The FAO recommends the use of digestible indispensable amino acid score to assess protein quality as opposed to protein digestibility corrected amino acid score, wherever possible[25]. The digestible indispensable amino acid score was introduced to describe ileal digestibility of individual indispensable amino acids (IAA), preferably assessed in human volunteers. In response to the FAO call, a dual tracer method was proposed that applies differently-labelled 'test' and 'standard' proteins within the same mixed meal that can be fed to human volunteers. Appearance in the circulation of individual IAA from the test protein is then compared with that from the standard protein. Since both these protein sources should be intrinsically labelled with stable isotope tracers, a low quantity of deuterium was chosen to label test legumes, which were grown from seed to maturity on modest quantities of heavy water. The standard protein used was in the form of spirulina whole cells (*Arthrospira platensis*) which is available commercially at high ^{13}C-enrichment. Comparison with IAA appearance from the standard protein avoids the need for ileal intubation as the tracer enrichment ratio of individual free IAA in the circulation will reflect that at their site of production, the terminal ileum. A plateau feeding protocol was adopted to minimise the number of blood samples taken for mass spectrometric analysis. The first publication[26] described the use of the dual tracer method and compared IAA bioavailability in two legumes in a small group of adult volunteers in South India. This study also showed that a common processing

https://doi.org/10.1017/S0029665120007016 Published online by Cambridge University Press

product, dehulled mung bean, led to a 9 % improvement in digestibility, likely due to the removal of anti-nutritional factors concentrated in the seed hull. The high digestibility of the standard protein (85 %) had been demonstrated using a test meal containing deuterated free amino acids. Intrinsic labelling using free amino acids in the diet can label chicken and egg. These animal-source proteins showed high digestibility, which compared well with literature values in animals and with intubation or faecal digestibility measurements in human subjects[27]. Subsequently, the negative effect of some dietary components on protein digestion, in this case black tea polyphenols, was demonstrated[28]. When prepared by pressure cooking, the bioavailability of the limiting IAA, lysine, was shown in a group of healthy young children to be 65 % in mung bean and lower than rice, millet or egg protein. It was argued that a small quantity of animal protein may be necessary when complementary feeds are formulated[29]. Further studies in healthy adults compared the digestibility of three common pulses prepared by commonly-used pressure cooking, also showed lysine digestibility to be modest at 60–63 %[30]. Preparation techniques such as extrusion can improve plant protein digestibility through improved bioaccessibility and reduced anti-nutrient content. The bioavailability of protein from chickpea and pea protein following extrusion has recently been shown to compare with that of animal-source protein in moderately malnourished children[31], implying that food preparation can be optimised when formulating foods containing plant protein in future nutritional interventions in populations receiving marginal diets.

Muscle metabolism

Matthew Brook (University of Nottingham, UK) presented the application of stable isotope tracers to measure muscle protein metabolism and the impacts of protein/amino acid feeding. Skeletal muscle is crucial for movement and plays many essential roles in whole-body metabolism. Maintaining skeletal muscle throughout life is therefore of great importance, with muscle wasting as a result of ageing, disease or illness having detrimental effects on quality of life[32,33]. Fundamentally, muscle mass is controlled by the balance between muscle protein synthesis and muscle protein breakdown, with stable isotope tracers enabling the dynamic regulation of these processes to be measured. Typically, isotopically labelled amino acids, e.g. [$^{13}C_6$] phenylalanine and [$^{13}C_2$]leucine are utilised in arterio-venous balance or direct incorporation techniques. Arterio-venous balance involves amino acid tracer infusion into the whole body or across a limb, with the rate of disappearance (R_d) of the amino acid tracer from the arterial pool providing a proxy for synthesis, with the rate of appearance (R_a) of the tracee in the venous pools (thereby diluting the tracer) providing that of breakdown. Arterio-venous balance calculations can therefore provide estimates of both synthesis and breakdown, as well as *de novo* synthesis of intermediary metabolites and substrate oxidation across tissues or limbs (when combined with blood flow and CO_2 measurements)[34,35]. These techniques have demonstrated that in the absence of dietary protein, muscle protein breakdown > muscle protein synthesis, resulting in negative net balance and protein loss. Importantly, this is reversed upon the consumption of protein (i.e. muscle protein synthesis > muscle protein breakdown) resulting in positive net balance and highlighting the diurnal balance of fasted fed cycles that maintain muscle mass in healthy, weight-bearing individuals. Despite providing many key insights into the regulation of muscle balance in health and disease, a lack of muscle specificity remains (particularly at the whole-body level) with amino acid released from other tissues[36]. As such, direct incorporation techniques provide a gold standard in determining fractional synthesis rates (i.e. muscle protein synthesis), in which the rate of tracer incorporation can be determined by collecting muscle biopsies over two time points; techniques that have defined much of understanding of nutritional regulation of muscle protein synthesis. For instance, in responses to a protein bolus, muscle protein synthesis is robustly (+100 %) yet transiently (90–120 min) increased, returning to baseline despite elevated plasma amino acid concentrations[37]. This demonstrated the muscle full response, in which the muscle becomes refractory to additional protein and is maximal upon the consumption of about 20 g high-quality protein[38]. Furthermore, with the IAA (particularly leucine) components of a protein driving anabolic responses[39], low doses of leucine-enriched IAA or leucine itself can produce robust increases in muscle protein synthesis equivalent to 20 g whole protein[40], potentially providing therapeutic interventions. Finally, the use of ^2H_2O, also written as D_2O (also known as heavy water or deuterium oxide) provides longer-term measures (from days up to months) of muscle protein synthesis and therefore may hold promise in determining the efficacy of long-term nutritional interventions[41].

Cell culture

Dr Katherine Pinnick explained how stable isotopes can be applied to cell culture systems to examine cellular metabolic pathways in adipocytes. Adipocytes possess the capacity to synthesise new fatty acids from non-lipid precursors; a process termed DNL. To assess which substrates are used for DNL in the developing adipocyte, Katherine described how primary human preadipocytes were cultured in the presence of ^{13}C-labelled substrates. Over the course of 2 weeks, the cells differentiated into mature adipocytes. During this time, new fatty acids were synthesised by DNL and stored as TAG in lipid droplets. GC-MS was then performed to measure ^{13}C incorporation into the newly synthesised lipids. Using this approach, glucose, pyruvate and glucogenic amino acids, such as glutamine, were all found to contribute to the synthesis of new fatty acids by DNL[42]. In the second part of the talk, stable isotopes were used to examine fructose metabolism in adipocytes. ^2H_2O

https://doi.org/10.1017/S0029665120007016 Published online by Cambridge University Press

labelling of the culture medium showed that total DNL was comparable in cells cultured with either glucose alone or an equivalent concentration of glucose:fructose (50:50). Despite no difference in the total TAG content between the two sugar treatments, notable differences in TAG fatty acid composition were observed. The end product of DNL, palmitate, can itself be subject to further modification by elongase and desaturase enzymes. Katherine went on to discuss how the fatty acid composition data suggest some of these fat-modifying enzymes may be differentially regulated by glucose and fructose. Overall, the findings presented show that stable isotopes can be used *in vitro* as highly sensitive tools to gain a mechanistic understanding of complex metabolic pathways and to complement human nutrition studies.

In summary, the meeting illustrated how stable isotope techniques can reveal what is 'beneath' static measurements of plasma glucose and lipid concentrations after stresses such as acute meal intake or diet, and obesity-associated metabolic dysfunction in human subjects. Examples were given of studies that used single or multiple stable isotope tracers, according to the question(s) being asked. In contrast to studies that used stable isotopes to address issues related to a Western diet, it was also demonstrated that such techniques can aid the measurement of human protein requirements and determine amino acid bioavailability from plant proteins for populations on a marginal diet. It was also shown that amino acids stable isotope tracers can unravel determinants of skeletal muscle synthesis/breakdown to further our understanding of interventions that could allow maintenance of a healthy muscle mass. Stable isotope techniques are also useful in cell models, and human primary preadipocytes were used as an example. Published work as well as ongoing studies were presented, illustrating how stable isotope techniques can be used to enhance nutrition and metabolic research. It was also highlighted that these techniques can be used alongside 'omics' platforms to give powerful insights into metabolism.

Acknowledgements

The organising committee would like to thank the Nutrition Society for the opportunity to host this member-led meeting, and for providing organisational help.

Financial Support

The Nutrition Society provided financial support for the meeting. The University of Surrey acknowledges the financial support of the studies described, from the following BBSRC awards: BB/G009899/1, BB/P010245/1 and BB/R00028X/1. Leanne Hodson is a British Heart Foundation Senior Research Fellow in Basic Science and acknowledges support from the BHF: FS/11/18/28633 and FS/15/56/31645 and BBSRC awards: BB/N005600/1, BB/N015665/1.

Conflict of Interest

None.

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https://doi.org/10.1017/S0029665120007016 Published online by Cambridge University Press