

**BMSS Special Interest Group on Stable Isotopes**  
**University of Glasgow**  
**13<sup>th</sup>-14<sup>th</sup> January 2009**

**Organised by the Staff of the Stable Isotope Biochemistry Laboratory, SUERC**



With many thanks to our sponsors:



BMSS ISO-SIG Tuesday, 13<sup>th</sup> January 2009, Hunter Halls East, University of Glasgow

09:20 Welcome

Session I: Protein and Energy Metabolism

Chair:

- 09:30-10:10 Keynote 1: Rob Beynon, Liverpool  
*"Stable isotope strategies for quantitative proteomics"*
- 10:10-10:15 Original Communications (OC, 15 + 5 minutes)
- 10:15-10:35 OC B1 Gary McCaw  
*"Tandem Mass Spectrometry and the Expansion of Newborn Screening"*
- 10:35-10:55 OC B2 Emilie Combet *"Ingested nitrate as a source of luminal nitrosative stress at the human gastro-oesophageal junction"*
- 10:55-11:25 Coffee with sponsors; posters
- 11:30-11:50 OC B3 Simon Eaton *"Hypoglycaemia, poor weight gain and reduced adipose tissue in a premature infant: insights from stable isotopes"*
- 11:50-12:10 OC B4 Eduardo Ferrioli *"Under-reporting of food intake is frequent among Brazilian free-living older persons: a doubly-labelled water study"*
- 12:10-12:30 OC B5 Tom Preston  
*"Measuring the synthetic rate of slow turnover proteins"*
- 12:30- Buffet lunch in the Melville Room; posters

Session 2: Lipids, Hormones and Models

Chair:

- 14:00-14:30 Keynote 2: Muriel Caslake, Glasgow  
*"Lipoprotein Kinetics"*
- 14:35-14:55 OC B6 Alan Hunt *"Dynamic Lipidomics in vitro and in vivo"*
- 14:55-15:15 OC B7 Barbara Fielding *"The use of stable isotopes to investigate tissue-specific fatty acid trafficking in humans, in vivo"*
- 15:15-15:35 OC B8 Leanne Hodson *"Meal linoleate is partitioned to a greater extent in blood lipid fractions than oleate and palmitate"*
- 15:35-16:05 Tea with sponsors; posters
- 16:10-16:30 OC B9 Dave MacFarlane *"Use of stable isotopes to measure abnormalities of fatty acid metabolism in mouse models of fatty liver disease"*
- 16:30-16:50 OC B10 Katherine Hughes *"Development of a new method to measure cortisone production in man"*
- 16:50-17:10 OC B11 Roland Stimson *"Use of 9,11,12,12-[<sup>2</sup>H]<sub>4</sub>-Cortisol Tracer to Quantify Cortisol Release from Subcutaneous and Visceral Adipose Tissue in Man"*

19:00 for 19:30 **BMSS ISO-SIG Dinner** in the Ferguson Room, University of Glasgow

BMSS ISO-SIG Wednesday, 14<sup>th</sup> January 2009, Hunter Halls East, University of Glasgow

Session 3: Carbohydrates, Methods and Instrumentation

Chair:

- 09:00-09:30 Keynote 3: Roel Vonk, Groningen  
*"Stable isotopes and genomic technologies: new adventures"*
- 09:35-09:55 OC B12 Klaus Wutzke *"Effect of alcohol consumption on whole-body protein turnover in healthy adults"*

- 09:55-10:15 OC B13 Laura Hanske “*Urinary biomarkers of habitual dietary non-digestible carbohydrate and protein intake*”
- 10:15-10:45 Coffee with sponsors; posters
- 10:50-11:10 OC B14 Douglas Morrison “*Short chain fatty acid products of carbohydrate fermentation*”
- 11:10-11:50 OC B15 Ken Cook “*Ion Exchange-MS Application for Ionic and Polar Compounds*”
- 11:50-12:00 Close
- 12:00- Buffet lunch in the Melville Room, University of Glasgow
- 14:00- [SIMSUG 2009, 14<sup>th</sup> January 2009, Hunter Halls East, University of Glasgow](#)

### BMSS ISO-SIG Posters

- PO B1 Kay “*LC-MS/MS as a tool for high-throughput multiplexed quantitation of serum proteins using stable isotope labelled peptides*”
- PO B2 Schierbeek “*Simultaneous measurement of both concentration and <sup>13</sup>C enrichment of glutathione and glycine in one single run, using Liquid Chromatography Coupled to Isotope Ratio Mass Spectrometry (LC-IRMS)*”
- PO B3 Abaye “*Blood volume and red cell mass in children with moderate and severe malaria measured by chromium-53 dilution and GC/MS analysis*”
- PO B4 Moerdijk “*A versatile method for stable carbon-isotope (<sup>13</sup>C) analysis of carbohydrates by high-performance liquid chromatography – isotope ratio mass spectrometry*”
- PO B5 Barclay “*Stable Isotope Incorporation into Faecal Bacterial RNA Reflects Predictable Changes in Short Chain Fatty Acids*”
- PO B6 Botting “*The Synthesis of Isotopically Labelled Phytochemicals for Analysis and Metabolic Studies*”
- PO B7 Small “*Production of Complex Metaprobes: <sup>13</sup>C-Labelled Cereals*”
- PO B8 Preston “*Measuring the Liquid Phase Gastric Emptying Rate of Sip Feeds*”
- PO S1 Mander “*Estimation of Gastric Emptying parameters from the <sup>13</sup>C-octanoate breath test using Bayesian hierarchical methods*”
- PO S10 Bass “*Temporal and Spatial Variation in  $\delta^{13}\text{C-DIC}$  and  $\delta^{18}\text{O-DO}$ : a case study using Loch Lomond, Scotland*”
- PO S12 Price “*Evaluation of a new pyrolysis method for analysis of compound specific stable oxygen isotopes*”

## **Keynote 1: Stable isotope strategies for quantitative proteomics**

**Rob Beynon**

*Proteomics and Functional Genomics Group, University of Liverpool*

Proteomics has emerged as a highly sensitive tool for protein identification, applicable to many proteins at the same time. However, we can expect and indeed, observe that identification proteomics will become secondary to the characterisation and quantification of proteins that have previously been identified. Further, an integrated view of transcriptome, proteome and metabolome data will require that we know more than the identity of each protein in a proteome. We can distinguish three types, or phases of proteomics: a) identification proteomics, which is predominantly concerned with gaining the identities of proteins that demonstrate a response of interest, b) characterisation proteomics, which emphasises the gain of further qualitative information on proteins of interest, such as sites of phosphorylation, and c) quantitative proteomics in which the amounts of specific proteins are determined. In my lecture, I will address some recent thinking from our laboratories that has addressed some of these issues and demonstrate the key role that stable isotopes play in these analyses..

**Quantification of proteins by multiplexed isotope dilution.** The more intensely we explore the proteome the more complex it appears. The realisation that there are many more proteins than genes is matched by the difficulties we have in accessing these variants. And, when this first phase of identification and characterisation proteomics is over, we will require new approaches to their quantification. Moreover, if proteomics is to serve systems biology, then we must meet the challenge of quantification and dynamics. In my presentation I will discuss novel approaches that we have developed to the study of proteome dynamics (turnover), proteome simplification (positional signature peptides), absolute quantification (the QconCAT method, in which artificial proteins, encoding proteotypic peptides from many proteins, are synthesised as “designer genes” and expressed as concatenated internal standards) .

**Stable isotope methods for amino acid frequency determination.** I will touch briefly on methods that emphasise the role of mass deficit analyses to assess the frequency of amino acids in peptides. This approach can provide additional information without an increase in experimental complexity, and aid identification/confirmation of peptides.

**Proteome dynamics using stable isotope labelled amino acids as tracers.** An important, if overlooked, aspect of proteome characterization is the definition of the intracellular stability of individual members of the proteome, data that inform systems models and network dynamics, and which allow us to define mechanisms that regulate protein turnover. Global determination of protein stability in the cell is, coupled with absolute quantification of protein abundance defines one half of the protein turnover cycle, with protein synthesis (itself a product of mRNA abundance and translational activity) as the other. Indeed, the failure to fully define the parameters of this cycle may be the most compelling reason for the lack of a strict correlation between the abundance of a protein and its cognate transcript. Metabolic incorporation of stable isotope labeled amino acids, coupled with high resolution separation and mass spectrometry, permit accurate measurement of intracellular stability on a global scale, from which we can infer relationships between intracellular stability and function, and identify factors that dictate the rate at which proteins are sequestered into the degradative apparatus. In this presentation, I will discuss approaches to acquisition of large data sets of protein stability data, particularly in respect of isolated single cells but also in intact animals. Finally, I will discuss some recent data that address structural determinants of protein stability.

## ORIGINAL COMMUNICATIONS

### **OC B1: Tandem Mass Spectrometry and the Expansion of Newborn Screening**

**McCaw, G.**

*Scottish Newborn Screening Programme Laboratory, Glasgow*

Newborn screening for inherited metabolic disease began in the early 1960's since when it has become almost universal in developed countries and is rapidly spreading in many developing countries. In that time advances in knowledge and analytical technologies have gradually expanded the range of diseases screened. However, with the introduction of tandem mass spectrometry (MS/MS) to screening over the last 15 years, the potential number of diseases that can be screened for has increased significantly. This technology driven expansion raises a whole range of practical and ethical issues.

## OC B2: Ingested nitrate as a source of luminal nitrosative stress at the human gastro-oesophageal junction

**Combet E<sup>1</sup>, Winter J<sup>1</sup>, Preston T<sup>2</sup>, McColl KEL<sup>1</sup>**

1. Medical Sciences, Faculty of Medicine, University of Glasgow

2. Stable Isotope Biochemistry Laboratory, SUERC, East Kilbride

The incidence of cancer of the proximal stomach has increased markedly over the past 20 years. Unlike cancer of the more distal stomach, it is not related to *Helicobacter pylori* infection and occurs in healthy, non-atrophic, acid secreting stomachs. While the responsible agent remains unidentified, it is likely that environmental factors, such as the diet, play a role in the rising incidence of these cancers. A further risk for oesophageal adenocarcinoma is gastro-oesophageal reflux, where excessive reflux can lead to erosive oesophagitis and columnar metaplasia of the oesophagus squamous mucosa (known as Barrett's oesophagus).

For many years, there has been interest in nitrite as a potential pre-carcinogen for gastric cancer. Saliva contains high concentrations of nitrite derived from the enterosalivary recirculation of dietary nitrate and its reduction by buccal bacteria. Acidification of nitrite in the stomach produces nitrosative species (NO<sup>+</sup>, N<sub>2</sub>O<sub>3</sub>), which can form potentially carcinogenic *N*-nitrosocompounds. Antioxidants such as ascorbic acid protect against this nitrosative chemistry by converting the nitrosative species to nitric oxide. However, nitric oxide diffuses rapidly from the lumen to lipids and adjacent compartments of neutral pH, where it reacts with oxygen to reform further nitrosative species (N<sub>2</sub>O<sub>3</sub>).

Using gas chromatography – ion trap tandem mass spectrometry (GC-MS/MS), we successfully investigated the effect of lipid on the formation of *N*-nitrosocompounds from nitrite in a model of the human stomach. While we successfully demonstrated the inhibitory effect of ascorbic acid on *N*-nitrosation in a single-phase system, *N*-nitrosation was not inhibited by ascorbic acid in presence of 10% lipid. These results indicate that the presence of lipid can markedly alter the protective effects of antioxidants with respect to potentially carcinogenic nitrosative chemistry occurring in the human stomach. *In vivo* studies in healthy and Barrett's oesophagus patients, entailing ingestion of <sup>15</sup>N nitrate and monitoring of *N*-nitrosocompounds formation, confirmed that nitric oxide-derived nitrosative stress takes place in the stomach, as well as the oesophagus during acid reflux events. Meanwhile, the source of the stress was confirmed to be dietary nitrate, due to a 77% enrichment of *N*-nitrosomorpholine with <sup>15</sup>N. Current studies are now attempting to identify whether conjugated bile acids, present in acidic refluxate, can be nitrosated in presence of acid and <sup>15</sup>N nitrite.

## **OC B3: Hypoglycaemia, poor weight gain and reduced adipose tissue in a premature infant: insights from stable isotopes**

***Eaton S<sup>1</sup>, Wells JCK<sup>2</sup>, Coelho R<sup>3</sup>, Symth J<sup>4</sup>, Semple R<sup>5</sup>, O’Rahilly S<sup>5</sup>, Hussain K<sup>3</sup>***

*Departments of <sup>1</sup>Surgery <sup>2</sup>Nutrition and <sup>3</sup>Endocrinology, Great Ormond Street Hospital for Children NHS Trust and the Institute of Child Health, University College London, London, <sup>4</sup>Trevor Mann Baby Unit, Department of Women and Children’s Health, Royal Sussex County Hospital, Brighton , and <sup>5</sup>Department of Clinical Biochemistry, University of Cambridge, Addenbrooke’s Hospital, Cambridge , UK.*

*Background:* Hypoglycaemia (low blood glucose) is common in preterm and intrauterine growth retarded (IUGR) newborns. It can have various manifestations and causes. Stable isotope measurements of total energy expenditure ( $D_2^{18}O$ ; doubly labelled water) and body composition ( $D_2O$  or  $H_2^{18}O$  dilution) have been used in healthy term infants but are not often used in a diagnostic context. We report the utility of these measurements in an infant with hypoglycaemia. |

*Results:* An infant born prematurely with intra-uterine growth retardation had poor weight gain for the first 6 weeks of life. At 6 weeks of age, he developed marked hypoglycaemia, which was not due to hyperinsulinism (a frequent cause of hypoglycaemia in infants) and required a glucose infusion rate of 16 mg/kg/min to maintain normoglycaemia (normal glucose turnover is 3-5 mg/kg/min in term infants, 5-6 mg/kg/min in premature infants). His fasting tolerance was only 5 minutes. At the age of 20 weeks, his weight was 3.6kg (normal weight at 20 weeks for a male infant born at 31 weeks gestation is 5.9kg, and 3.6kg is 3.7 standard deviations away from this). In order to determine whether the inability to increase body weight and maintain normoglycaemia, despite 175 kcal/kg/day energy intake (normal ~ 100kcal/kg/day), was due to a gross increase in energy expenditure, we performed an estimate of total energy expenditure (TEE) over 7 days, and a body composition measurement, using doubly labelled water. This showed that TEE was approximately normal (68kcal/kg/d)<sup>1</sup>, but also revealed that his body fat was extremely low compared with normal reference values for his age (7.4% vs. 19%)<sup>2</sup>. This finding was confirmed by undetectable serum leptin and low serum adiponectin levels (markers of adipose tissue mass) and probably reflects a complete absence of adipose tissue. After insertion of a gastrostomy (feeding tube in the stomach) for continuous feeding, he started to show improved weight gain, such that his weight at 28 weeks was normal (7.4kg). A second doubly-labelled water measurement showed that his TEE was slightly elevated (81.8kcal/kg/d) and that his adiposity had also normalised (28%), in keeping with a normalisation of serum adipokines (leptin and adiponectin). His fasting tolerance (>8h) and was much improved and he showed no further hypoglycaemia.

*Conclusions:* The precise mechanism of this patient’s severe hypoglycaemia is unclear, but we speculate that adipose tissue plays an important role in regulation of blood glucose levels, as suggested from animal models. Measurement of total energy expenditure and body composition was useful to exclude gross hypermetabolism and to define % body fat.

<sup>1</sup>Reichman CA, Davies PS, Wells JCK, Atkin L, Cleghorn G, Shepherd RW: Centile reference standards for total energy expenditure in infants from 1 to 12 months. *Eur J Clin Nutr* 2003; 57: 1060–1067.

<sup>2</sup>Butte NF, Hopkinson JM, Wong WW, Smith EO, Ellis KJ: Body composition during the first 2 years of life: an updated reference. *Pediatr Res* 2000; 47: 578–585.

## **OC B4: Under-reporting of food intake is frequent among Brazilian free-living older persons: a doubly-labeled water study**

***Ferriolli E, Moriguti JC, Lima NKC, Moriguti EKU, Formighieri PF, Pfrimer K, Marchini JS.***

*University of São Paulo, School of Medicine of Ribeirão Preto, Department of Internal Medicine. Av. Bandeirantes, 3900. 14049-900 – Ribeirão Preto – SP, Brazil*

The Brazilian population is aging fast. The population aged 60 years or older increased from 5%, in 1960, to 9.1% in 2001. In 2025, Brazil is expected to be the 6<sup>th</sup> country in the world in absolute number of inhabitants aged 60 years or over, with an average life expectancy of 73 years. In a study performed by our group in elderly persons of an urban low-income area, obesity had the alarming prevalence of 28.6% (26% of women and 31% of men). In this study, the mean height (1.57 m) and weight (67.1 kg) of subjects was lower than those of European and North-American populations, and anthropometric measurements were not accurate for the determination of nutritional status. The assessment of food intake is essential for the development of dietetic interventions, but it has been shown that accuracy is low when intake is assessed by questionnaires, the under-reporting of food intake being frequent. Most studies, however, were performed in developed countries and there is no data about the prevalence of under-reporting of food intake in the older population of developing nations, including Brazil. This study was aimed to verify the energy expenditure of independent older Brazilians living in an urban area, through the doubly-labelled water (DLW) method, and to compare the reported energy intake obtained through the application of a validated food frequency questionnaire (FFQ) with the energy expenditure measured by the DLW method. The study was performed in two phases. Initially, 100 subjects aged from 60 to 75 years had their body composition determined by the deuterium oxide dilution method. Subjects were randomly selected among the residents of the area followed by the University Family Health Program. The study was part of a Coordinated Research Program of the International Atomic Energy Agency on lifestyle and energy expenditure of older persons in developing nations, and in this phase a series of measurements were performed, including functional assessments and the application of food intake, quality of life and physical activity questionnaires. In the second phase of the study, five volunteers of each quartile of body fat percentage (20 volunteers in total) had their energy expenditure determined by the doubly labelled water method. Results for this phase will be reported here. Ten of the 20 subjects included in this phase of the study were men and the mean age was  $66.4 \pm 3.5$  years. The mean total energy expenditure was  $2565 \pm 614$  and  $2154 \pm 339$  kcal.d<sup>-1</sup> for men and women, respectively and the Physical Activity Level (PAL) was  $1.58 \pm 0.31$  (range 1.11 – 1.79) and  $1.52 \pm 0.22$  (range 1.3 – 1.94), respectively. Under-reporting of food intake was highly prevalent in this group, with a mean percentage of reported intake in relation to measured energy expenditure of -19.7% and -12.5% in men and women, respectively. The under-report of energy intake tended to increase according to the body percentage fat quartile (-4.7% (1<sup>st</sup> quartile), -15.2% (2<sup>nd</sup> quartile), -21.7% (3<sup>rd</sup> quartile) and -23.5% (4<sup>th</sup> quartile)). In conclusion, the energy expenditure and PAL of independent Brazilian older persons living in an urban area were similar to those previously described for low-active older populations. Under-reporting of food intake was highly prevalent and tended to increase as body fat increased.

## OC B5: Measuring the synthetic rate of slow turnover proteins

**Tom Preston** [T.Preston@suerc.gla.ac.uk](mailto:T.Preston@suerc.gla.ac.uk)

Stable Isotope Biochemistry Laboratory, SUERC, East Kilbride, G75 0QF

Measuring changes in protein synthetic rate using stable isotopes are inherently more sensitive than measuring concentration changes. In the context of the search for early markers of disease, methods to measure the synthetic rate of key proteins are central. Most major body proteins have fractional synthetic rates (FSR)  $<20\% \text{ day}^{-1}$ , below that easily measured by even the most sensitive proteomics tools.

We have previously applied phenylalanine isotopomers to measure changes in plasma protein expression. When conducting longitudinal studies, different isotopomers are used in each wing as tracer recycling may otherwise compromise measurements. Either a large tracer bolus (pool flooding<sup>1</sup>) or a primed, constant intravenous tracer infusion can be used. Protein FSR is calculated from the increase in enrichment with time. Proteins are isolated, hydrolysed and the resulting amino acids are derivatised and their enrichment analysed by single quadrupole GCMS. We have successfully used the pool flooding approach to measure albumin and fibrinogen synthesis in health and disease and their response to feeding<sup>2</sup>. In adults, albumin fractional synthetic rate (FSR) is  $\sim 10\% \text{ day}^{-1}$ , whereas fibrinogen FSR is  $\sim 20\% \text{ day}^{-1}$ .

In chronic inflammatory disease, skeletal muscle wasting is one of the most intractable causes of morbidity and eventually, death. The major contractile proteins such as myosin have FSR of  $\sim 1\% \text{ day}^{-1}$ . Measuring such a low FSR may require large and expensive tracer doses and/or extended *i.v.* infusions. It is also likely to require the most sensitive analysis, replacing quadrupole MS with GC-combustion-isotope ratio MS<sup>3</sup> for <sup>13</sup>C analysis, or for <sup>2</sup>H tracers, GC-pyrolysis-IRMS. Furthermore, use of a large bolus of an essential amino acid has been criticised as it may stimulate muscle protein synthesis. However, extended tracer infusions restrict physical activity and may reduce muscle protein synthesis.

Intrinsic labeling strategies, where non-essential amino acids are labelled *in vivo* from metabolic precursors, are receiving renewed interest. For longitudinal studies, two approaches are envisaged, use of two tracers or use of a single tracer at increasing enrichment in each subsequent study wing. The latter is less appealing as additional tissue biopsies would become necessary to constrain basal enrichment. Both <sup>2</sup>H<sub>2</sub>O and <sup>13</sup>C<sub>6</sub>-glucose can potentially be applied<sup>4,5</sup>. They are economical and both can be given orally, avoiding the complications of *i.v.* infusions. In both cases, alanine is efficiently labelled via its keto acid, pyruvate. Other applications of <sup>2</sup>H<sub>2</sub>O, such as the doubly-labelled water procedure<sup>6</sup>, show that predictable circulating enrichment can readily be achieved over extended periods. The longer term fate of oral <sup>13</sup>C<sub>6</sub>-glucose has been less commonly studied. The optimal pattern of oral doses to achieve steady enrichment in circulating alanine has yet to be established.

<sup>1</sup>Garlick PJ *et al.* (1994) *Am J Physiol* **266**: E2875; <sup>2</sup>Barber MD *et al.* (2000) *Am J Physiol* **279**: E707; <sup>3</sup>Preston T. (1992) *Plant, Cell and Environment*, **15**: 1091-1097; <sup>4</sup>Gardner *et al.*, (2007) *Am. J. Physiol. Gastrointest. Liver Physiol.* **292**:1695-1705; <sup>5</sup>Pascual *et al.* (1998) *J. Nutr.* **128**: 733-739; <sup>6</sup>Moses AWG *et al.*, (2004) *Br J Can.* **90** (5): 996-1002.

## **Keynote 2: Lipoprotein Kinetics**

**Caslake, M J**

*Vascular Biochemistry, Division of Cardiovascular and Medical Sciences, University of Glasgow*

Measurements of plasma lipid and lipoprotein concentrations are static estimates traditionally employed to characterise the disorders of lipoprotein metabolism. However, lipoprotein metabolism is complex and abnormal plasma concentrations can result from alterations in the rates of production, conversion or catabolism of various lipoprotein particles. Advances in gas chromatography – mass spectrometry technology and the widespread availability of relatively inexpensive stable isotopes has seen the increasing use of the endogenous labelling of apolipoproteins with amino acid precursor molecules and triglyceride with precursor glycerol molecules to study lipoprotein kinetics in humans. This approach, coupled with multicompartmental modelling, has provided further clinical understanding of lipoprotein disorders and insight into the mechanism of action of pharmacological agents used to lower circulating levels of lipids and lipoproteins.

## OC B6: Dynamic Lipidomics *in vitro* and *in vivo*

**Alan N Hunt<sup>1</sup> and Anthony D Postle<sup>1,2</sup>**

Schools of <sup>1</sup>Medicine and <sup>2</sup>Chemistry, University of Southampton, Southampton SO17 1BJ, UK.

Stable isotope precursor labelling, combined with tandem electrospray ionisation mass spectrometry (ESI-MS/MS) has recently been exploited to provide dynamic metabolic and mechanistic insights into the complex lipidomes of whole organisms, organs, cells and organelles. It informs lipid biosynthesis and remodelling, intracellular and extracellular lipid transport, lipid-mediated signalling and lipid turnover. In protocols analogous to those in common use to label and track proteome dynamics, the ability to differentiate between newly synthesised and endogenous lipid pools depends upon precursor molecules that can be labelled with stable isotopes and which then generate a diagnostic fragment ion on CID induced fragmentation.. Defined precursor or neutral loss scans of labelled and unlabelled fragments are then compared to segregate each lipid pool and interrogate their temporal relationships.

In whole animals, organ specific properties dictate specific tissue lipid compositions which are characteristic and homeostatically maintained as seen, for example, in highly saturated lung lipids contrasted with highly unsaturated liver lipids. However, transformed cells *in vitro* lose this capacity to maintain whole cell phenotypic lipid characteristics. We have shown their lipidomes are susceptible to dietary alteration but also that some subcellular compartments actively resist change and retain homeostatic control of their lipid compositions. In the endonuclear compartment, for instance, we identified an autonomous pathway for synthesis of phosphatidylcholine (PtdCho) distinct from bulk cell synthesis. We also established that homeostasis mandates an endonuclear PtdCho profile which matches what is seen *in vivo* and is considerably more saturated than that of the whole cell. Endonuclear PtdCho saturation is retained when whole cell PtdCho is manipulated by exogenous polyunsaturated fatty acid, suggesting this specific composition is an essential requirement for sustained cell viability. In contrast, our analyses of the polyphosphoinositide signalling molecule precursor phosphatidylinositol (PtdIns) demonstrate a strikingly different picture. Given the intimate association with cell signalling, PtdIns composition and dynamics reveal a surprising loss of homeostasis *in vitro*. We observe that cells synthesise and utilise a variety of PtdIns compositions suggesting that the predominant molecular species encountered in mammals *in vivo*, stearyl arachidonyl PtdIns, is not essential for cell viability in culture.

The benign nature of stable isotopes has allowed us to extend lipidome labelling to whole animals including human subjects, and accessibility of plasma has permitted appraisal in particular of the dynamics of extracellular PtdCho transport and turnover. This approach has provided significant insight into whole organism lipidomics in health and disease. For example, comparative analyses have revealed quite striking differences in PtdCho turnover and transport between human and commonly used rabbit models questioning the use of the latter in informing atherosclerosis research.

Several points emerge from our observations: (i) eukaryote lipids are spatially segregated at both micro and macro levels, (ii) *in vivo* there is an effective and tight homeostasis of lipidome composition, (iii) *in vitro* some membranes compositions retain tight homeostatic control - "lipostats" - while others are "plastic" and (iv) we have only scratched the surface of lipidomic complexity within organisms but the methodologies outlined offer an enticing prospect for future advances in the understanding of lipid metabolism.

## **OC B7: The use of stable isotopes to investigate tissue-specific fatty acid trafficking in humans, *in vivo***

**Fielding BA, Hodson L and McQuaid S**

*Oxford Centre for Diabetes, Endocrinology and Metabolism, University of Oxford, Churchill Hospital, OX3 7LJ*

The technique of arterio-venous difference is a powerful method for studying tissue metabolism *in vivo*. Using this technique, measurements of the concentrations of metabolites in arterial and venous samples are combined with blood flow to calculate metabolic flux. Over a number of years, we have used the technique to catheterise the venous drainage of the subcutaneous abdominal depot and studied the net fluxes of non-esterified fatty acids (NEFA) and triacylglycerol (TG) across the tissue {Frayn, 1997 #503}. We have shown that adipose tissue fatty acid flux in man is precisely regulated according to the metabolic needs of the body. More recently, we have combined the technique of arterio-venous difference with stable isotope tracers of fatty acids which has enabled us to more precisely calculate adipose tissue fatty acid trafficking.

Here we describe a protocol to specifically compare endogenous and exogenous (meal) fatty acid metabolism in subcutaneous adipose tissue which we have applied to subjects with metabolic dysregulation and recently used to compare the metabolism of different adipose tissue depots.

In order to compare the metabolism of abdominal and femoral adipose tissue the following protocol was used. The superficial epigastric vein was cannulated to sample the venous effluent of subcutaneous abdominal adipose tissue, and a branch of the superior saphenous vein was cannulated to sample from the femoral adipose tissue depot. Arterial blood was sampled from an arterialised hand vein. Twelve healthy non-obese volunteers were studied. In order to simultaneously study endogenous and exogenous fatty acids, [<sup>2</sup>H<sub>2</sub>]palmitic acid was infused intravenously and [U-<sup>13</sup>C]palmitic acid was given as part of a test meal. Adipose tissue blood flow in both sites was measured by the <sup>133</sup>xenon washout technique.

Adipose tissue blood flow was similar in subcutaneous abdominal and femoral adipose tissue after an overnight fast, but was significantly lower in the femoral depot in the postprandial period. There was significantly greater extraction of [U-<sup>13</sup>C]palmitate in plasma TG (representing chylomicron/ meal TG) across the subcutaneous adipose tissue but no significant differences between sites for direct uptake of [<sup>2</sup>H<sub>2</sub>]palmitic acid, representing endogenous systemic plasma fatty acids. To further examine the metabolism of endogenous fatty acids, we calculated the adipose tissue extraction of [<sup>2</sup>H<sub>2</sub>]palmitate in plasma TG, representing systemic fatty acids incorporated into very low density lipoprotein (VLDL) by the liver. We detected no significant difference between the two depots.

The data suggest that subcutaneous abdominal adipose tissue is more metabolically active than femoral adipose tissue in terms of the removal of dietary fatty acids. These findings could have implications for the disposal of dietary fatty acids according to body fat distribution. The combination of stable isotope tracers with arteriovenous difference methodologies provide a potent technique to study adipose tissue depots in humans, *in vivo*.

## **OC B8: Meal linoleate is partitioned to a greater extent in blood lipid fractions than oleate and palmitate.**

***Hodson L, McQuaid SE, Fielding BA***

*Oxford Centre for Diabetes, Endocrinology and Metabolism, University of Oxford, Churchill Hospital, OX3 7LJ.*

The fatty acid composition of blood lipids is becoming increasingly used as a biomarker of dietary intake but few studies have comprehensively compared the acute metabolic fate of specific fatty acids *in vivo*. We hypothesised that different classes of dietary fatty acids would be variably partitioned in metabolic pathways and that this would become evident over 24 h.

We traced the fate of fatty acids using equal amounts of [U-<sup>13</sup>C]linoleate, [U-<sup>13</sup>C]oleate and [U-<sup>13</sup>C]palmitate given in a test breakfast meal in twelve healthy subjects (6 males). The concentrations of the tracers in plasma chylomicron-triacylglycerol (TG) tended to be highest for oleate and lowest for linoleate. This pattern held but became more pronounced in plasma non-esterified fatty acid (NEFA) and very low-density lipoprotein (VLDL)-TG ( $P \leq 0.01$  and  $P \leq 0.02$  for [U-<sup>13</sup>C]oleate vs both [U-<sup>13</sup>C]palmitate and [U-<sup>13</sup>C]linoleate for NEFA and VLDL-TG respectively). There was significantly more [U-<sup>13</sup>C]linoleate than the other two tracers in plasma cholesteryl ester and phospholipid (PL). We calculated the contribution of meal fatty acids to the respective fractions using the isotopic enrichment values in the different lipid fractions. At 24 h, 10 % of plasma PL-linoleate originated from the breakfast test meal. This was significantly greater than for oleate and palmitate (both  $3 \pm 0.3$  %,  $P < 0.05$ ). This pattern was also true for erythrocyte PL fatty acids.

The marked rapid incorporation of linoleate from a single meal into blood PL fractions may explain why linoleate is a useful biomarker of dietary intake when compared to oleate and palmitate.

## OC B9: Use of stable isotopes to measure abnormalities of fatty acid metabolism in mouse models of fatty liver disease

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Non-alcoholic fatty liver disease is characterised by the accumulation of intrahepatic triglyceride (TG) in individuals who do not consume an excess of alcohol. Factors which influence its progression from simple fat accumulation (steatosis) to inflammation (steatohepatitis) and liver fibrosis are poorly understood. In mice, a methionine-choline deficient diet (MCDD) causes steatohepatitis, weight loss and hepatic insulin resistance. In contrast, mice fed a choline-deficient, methionine supplemented, diet (CDD) develop fatty liver without steatohepatitis, hepatic insulin resistance or weight loss. We hypothesised that differences in liver and adipose fatty acid metabolism underlie the contrasting predisposition to steatohepatitis and hepatic insulin resistance with CDD versus MCDD, and used stable isotope methodologies to measure fatty acid metabolism in vivo.

Male C57Bl6 mice aged 14 weeks were fed CDD, MCDD, or supplemented control diet (CS) for 2 weeks. In one cohort of animals (n=7-8/group), jugular venous cannulae were placed on day 11, and a <sup>13</sup>C<sub>4</sub> palmitate infusion undertaken on day 14 following a 4 hour fast. Enrichment of plasma palmitate with tracer (M+4) was measured by GCMS and the rate of appearance (Ra) of palmitate calculated by isotope dilution. The Ra of free fatty acids (FFA) was determined by correcting for the proportion of plasma FFA composed of palmitate. The enrichment of TG-palmitate with tracer in tissue relative to plasma was also measured. In a separate experiment (n=8/group) <sup>13</sup>C<sub>2</sub>-acetate dietary labelling was used to quantifying *de novo* lipogenesis (DNL) using mass isotopomer distribution analysis (MIDA). Results are means±SEM for CS, CDD and MCDD respectively. \*P<0.05, \*\*P<0.01, \*\*\*p<0.001 by one way ANOVA.

CDD induced liver steatosis while MCDD produced a centrilobular steatohepatitis. MCDD but not CDD led to weight loss (body weight 28.89±0.89 vs 26.79±0.52 vs \*\*\*21.63±0.53 grams), with reduced subcutaneous and perigonadal adipose depots. Plasma FFAs were increased in the MCDD group (P=0.05) but no difference was found in the Ra of FFA between groups (74±12 vs 81±7 vs 91±12 μmol/kg/min). MIDA of hepatic TGs showed increased absolute DNL synthesis rates only in the MCDD group (1.41±0.33 vs 2.16±0.35 vs \*\*3.61±0.16 μmol/day). No difference was found in the absolute contribution of plasma FFA to liver or subcutaneous adipose TG (5.8±1.7 vs 3.9±0.8 vs 6.3±1.1 μmoles and 2.6±0.9 vs 2.6±0.7 vs 3.9±0.3 μmoles respectively). The rate of incorporation of newly synthesised fatty acids into plasma TGs (using MIDA) was reduced in the CDD and MCDD groups (0.11±0.02 vs \*\*0.05±0.01 vs \*\*0.03±0.01 μmol/day), but there was no significant reduction in absolute incorporation of plasma FFA into plasma TGs (0.31±0.09 vs 0.24±0.05 vs 0.11±0.05 μmoles). Reduced hepatic TG export was assessed using intravenous tyloxapol and was reduced in MCDD mice only (data not shown).

Steatohepatitis in MCDD is associated with a greater increase in DNL and a profound impairment of hepatic TG export compared with CDD. Reduced fatty acid release from the liver, rather than enhanced peripheral lipolysis, appears to explain weight loss on MCDD. Progressive steatohepatitis and hepatic insulin resistance in MCDD rather than CDD is likely due to toxic effects of intracellular FFAs in the liver.

## OC B10: Development of a new method to measure cortisone production in man.

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**Background:** The isozymes of 11 $\beta$ -hydroxysteroid dehydrogenase (11 $\beta$ HSD 1&2) interconvert the glucocorticoids cortisol (F) and cortisone (E). Deficient inactivation of F by the type 2 isozyme causes hypertension and enhanced regeneration of F by 11 $\beta$ HSD1 is implicated in the metabolic consequences of obesity. Using the stable isotope tracer 9,11,12,12 d<sub>4</sub>-cortisol, regeneration of F by 11 $\beta$ -reductase activity has been measured across the splanchnic circulation and adipose tissue. E production (11 $\beta$ -dehydrogenase activity) has not been quantified previously by stable isotope tracer dilution.

**Aim:** To develop a method to measure E production in vivo using the stable isotopically labelled tracer 1,2 d<sub>2</sub>-cortisone (d<sub>2</sub>E).

**Methods:** Analysis of endogenous and isotopically-labelled steroids was performed using a Thermo TSQ Quantum Discovery liquid chromatograph mass spectrometer. Chromatographic separation was optimised using a Biphenyl Allure column (38°C) and mobile phase of methanol: ammonium acetate (5mM)(60:40, isocratic 0.5ml/min). Ionisation was performed in positive electrospray mode, and mass transitions determined using MS/MS (spray voltage 3.25kV; tube lens 21V (d<sub>2</sub>E), 22V (E), 23V (d<sub>2</sub>F); source temperature 400°C; collision gas pressure 1.5mTorr). A calibration curve was plotted by calculating the peak area ratio of analyte versus that of internal standard (11 $\alpha$ -cortisol) against the amount of added analyte. An enrichment curve with fixed proportions of tracer to tracee was prepared to allow calculation of the rate of appearance of cortisone. To establish if the deuteriums on d<sub>2</sub>E exerted a primary isotope effect on enzymatic kinetics, HEK293 cells transfected with h11 $\beta$ -HSD1 were incubated with E or d<sub>2</sub>E (2 $\mu$ M; 5h) and production rates of F or d<sub>2</sub>F compared. HEK293/h11 $\beta$ -HSD1 cells were also incubated (6-24h) with a fixed concentration of [<sup>3</sup>H]<sub>2</sub>E (5nM) and increasing concentrations of E or d<sub>2</sub>E (95-4995nM). In healthy men, pharmacokinetics for d<sub>2</sub>E were derived in healthy volunteers (with Ethical Approval) by measuring plasma d<sub>2</sub>E following bolus injection (141 $\mu$ g), and cortisone production rate was calculated from enrichment of cortisone with d<sub>2</sub>E-tracer, steady state d<sub>2</sub>E infusion (105 $\mu$ g/h, loading dose 76 $\mu$ g).

**Results:** The mass transitions of protonated E, d<sub>2</sub>E, F and d<sub>2</sub>F were  $m/z$  361-163, 363-165, 363-121 and 365-122 respectively. The protonated molecular ions of the d<sub>2</sub>E and d<sub>2</sub>F differed from E and F by 2 mass units, whereas the product ions of d<sub>2</sub>F differed by only one due to loss of deuterium following fragmentation across the steroidal B ring. Incubation of HEK293/h11 $\beta$ -HSD1 cells with d<sub>2</sub>E or E resulted in a time-dependent production of d<sub>2</sub>F or F respectively (0.4 $\pm$ 0.2vs1.0 $\pm$ 0.8 pmol/min/10<sup>5</sup> cells). A significant isotope effect on competition with [<sup>3</sup>H]<sub>2</sub>E was not observed (d<sub>2</sub>EvsE:  $V_{max}$ 1.8 $\pm$ 0.6vs2.2 $\pm$ 0.8 pmol/min/10<sup>5</sup> cells; apparent  $K_m$ 2.3 $\pm$ 1.0vs2.8 $\pm$ 1.3 $\mu$ M). In vivo d<sub>2</sub>E t<sub>1/2</sub> was 48.9mins and V<sub>d</sub> was 39.5L, compared with t<sub>1/2</sub> 28mins<sup>1</sup> previously reported for E. Whole body E production was 21.5 $\pm$ 5.1nmol/min at a prevailing circulating concentration of F of 246.7 $\pm$ 30.0nM. This is comparable with results previously calculated from non-steady state kinetics using a d<sub>4</sub>F tracer (24.1nmol/min, adjusted for substrate concentration and V<sub>d</sub><sup>2</sup>). Clearance of d<sub>2</sub>E was 0.27L/min, similar to that of cortisol (0.28L/min)<sup>2</sup>.

**Conclusion:** We have developed a method of measuring cortisone production in steady state using the stable isotope tracer 1,2 d<sub>2</sub>E. Tissue-specific cortisone production can now be measured with this method allowing further understanding of the contribution of the 11 $\beta$ -HSDs to glucocorticoid homeostasis in obesity and hypertension.

**References:** 1. Peterson JCI 1957;36(9):1301. 2. Andrew JCEM 2002;87:277.

## OC B11: Use of 9,11,12,12-<sup>[2]H</sup><sub>4</sub>-Cortisol Tracer to Quantify Cortisol Release from Subcutaneous and Visceral Adipose Tissue in Man

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**Background** Circulating cortisol is regulated by the hypothalamic-pituitary-adrenal axis, however cortisol is also regenerated from cortisone by the enzyme 11 $\beta$ -hydroxysteroid dehydrogenase type 1 (11 $\beta$ -HSD1), which is highly expressed in liver and adipose tissue. In obesity, 11 $\beta$ -HSD1 mRNA and activity are increased *in vitro* in subcutaneous and visceral adipose tissue biopsies. However, the contribution of 11 $\beta$ -HSD1 to cortisol release from adipose tissue independent from adrenal production, and its effect on portal vein cortisol concentrations, has not previously been quantified *in vivo*. We previously developed 9,11,12,12-<sup>[2]H</sup><sub>4</sub>-cortisol as a stable isotope tracer to quantify 11 $\beta$ -HSD1 *in vivo* and have now used this tool to quantify tissue-specific release of cortisol from liver and adipose tissue.

**Methods** Six healthy men underwent 9,11,12,12-<sup>[2]H</sup><sub>4</sub>-cortisol infusions with simultaneous sampling of arterialised and superficial epigastric veins. Four men with stable chronic liver disease and a transjugular intrahepatic porto-systemic shunt *in situ* underwent identical tracer infusion with simultaneous sampling from the portal, hepatic, and arterialised peripheral veins. 9,11,12,12-<sup>[2]H</sup><sub>4</sub>-Cortisol is converted to the inactive 9,12,12-<sup>[2]H</sup><sub>3</sub>-cortisone due to loss of the 11 $\alpha$ -<sup>2</sup>H, which is subsequently regenerated by 11 $\beta$ -HSD1 into 9,12,12-<sup>[2]H</sup><sub>3</sub>-cortisol. Dilution of d<sub>4</sub>-cortisol by d<sub>3</sub>-cortisol is a specific measure of *in vivo* 11 $\beta$ -HSD1 activity. Blood was analysed by liquid chromatography-tandem mass spectrometry with electrospray ionisation to quantify abundances of protonated ions of cortisol (precursor/product  $m/z$  363 $\rightarrow$ 121), <sup>[2]H</sup><sub>3</sub>-cortisol (366 $\rightarrow$ 121), <sup>[2]H</sup><sub>4</sub>-cortisol (367 $\rightarrow$ 121), cortisone (361 $\rightarrow$ 163) and <sup>[2]H</sup><sub>3</sub>-cortisone (364 $\rightarrow$ 164).

**Results** Significant 9,12,12-<sup>[2]H</sup><sub>3</sub>-cortisol release was observed from subcutaneous adipose tissue (8.7  $\pm$  3.3 pmol/min/100g adipose tissue). Splanchnic 9,12,12-<sup>[2]H</sup><sub>3</sub>-cortisol release (8.0  $\pm$  1.7 nmol/min) was accounted for entirely by the liver; release of cortisol from the viscera into portal vein was not detected.

**Conclusions** Cortisol is released from subcutaneous adipose tissue by 11 $\beta$ -HSD1 in humans, and increased enzyme expression in obesity is likely to increase local glucocorticoid signalling and contribute to whole-body cortisol regeneration. However, visceral adipose 11 $\beta$ -HSD1 activity is insufficient to increase portal vein cortisol concentrations and influence intra-hepatic glucocorticoid signalling.

## Keynote 3: Stable isotopes and genomic technologies: new adventures

**Roel J Vonk, Marion G Priebe, Tao He, and Han Roelofsen**

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Stable isotopes are widely used in biomedical research. The *classical technologies* are the tracer technology and mass-enrichment. We will illustrate this by presenting our research on lactose intolerance. We developed the *in vivo* lactase activity test (lactose digestion index; Vonk et al. *Eur.J.Clin.Invest.* 2001;31:226-233). Applying this test in a healthy Chinese population in Sichuan province, P. R. China, we observed that the healthy population had sufficient small intestinal lactase activity to digest one serving of milk. Furthermore not all Chinese with similar lactose digestion index showed similar symptoms; some were asymptomatic. It turned out that a second factor is involved, presumably the colonic production and removal rate of short-chain fatty acids, which are formed by the colonic microbiota from undigested lactose (He et al. *Eur.J.Clin.Invest.* 2008;38:541-547). New areas of stable isotope applications are in the areas of *proteomics and metabolomics*. We will illustrate this with our proteomic biomarker research related to insulin resistance. Proteomic approaches proved to be successful in finding strongly expressed biomarkers in easy accessible bio-fluids such as plasma and urine. However, finding low-abundance biomarkers related to chronic diseases in these fluids is difficult, because the fluids have a complex composition, a high intra- and inter-individual variability and the dynamic range of proteins present is high.

Sample complexity and dynamic range can be reduced by applying suitable fractionation and filtration techniques, but this might lead to loss of information. Targeted proteomic approaches are therefore urgently needed. The development of insulin resistance and the interrelated diabetes type 2 is a multi-organ process, in which crosstalk between adipose tissue, pancreas, muscle and liver occurs. The adipose tissue could be pivotal, because it is an active endocrine organ involved in storage and release of energy, and also involved in the regulation of energy metabolism in other organs via secretion of peptide hormones (adipokines). Especially visceral adipose tissue has been implicated in the development of diseases. Factors secreted by the stromal-vascular fraction may modulate adipokine secretion by adipocytes. Therefore, we first focus on **adipose tissue** secretome rather than on the **adipocyte** secretome. We identified many secreted proteins including regulatory, extracellular matrix, immune system-related and degradation proteins (Alvarez-Llamas et al. *Mol Cell Proteomics.* 2007 6:589-600). Subsequently, we developed a novel method called **Comparison of Isotope Labeled Amino acid Incorporation Rates (CILAIR)**, to detect secretome changes in adipose tissue induced by e.g. hormones (Roelofsen et al., *Mol Cell Proteomics.* 2008 *in press*). By comparing incorporation rates of <sup>13</sup>C-labeled lysine in the presence and absence of insulin (60 nM), we found that the synthesis rates of proteins involved in endoplasmic reticulum stress response and in extra-cellular matrix remodeling were affected by insulin.

The adipose tissue secretome can be considered as a pool of potential biomarkers traceable in plasma. Our novel proteomic approach, CILAIR, enables the analysis of inter-organ crosstalk and therefore has great potential to lead to the discovery of biomarkers of diabetes. In addition it will provide new insights in the underlying mechanisms of this disease.

## OC B12: Effect of alcohol consumption on whole-body protein turnover in healthy adults

***Wutzke KD, Bruns G, Wigger M***

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**Introduction:** Alcohol abuse is one of most harmful and most costly drug use problems worldwide [1]. However, little is known about short-term changes of the protein metabolism in response to socially consumed amounts of alcohol.

**Aim of the Study:** To investigate the whole-body protein turnover, either before or after continuous, moderate ethanol-induced oxidative stress by red wine consumption over a relatively short period in healthy subjects.

**Material and Methods:** In the first test procedure, 12 healthy adults (6 male, 6 female, age: 23-28 years, body mass index: 21-29 kg/m<sup>2</sup>) received an individual regular diet over 10 days (protein supply: 1.0-1.4 g/kg/day). After 8 days, a <sup>15</sup>N-labelled yeast protein (88.8 atom-%) was administered in a dosage of 4.2 mg/kg body weight. Urine and faeces were collected over 48 hours. Thereafter, in the second test procedure, the subjects consumed 0.4 ml ethanol /kg/day equivalent to 3.3 ml red wine /kg/day (dry Bordeaux, 12.0 vol-% ethanol) together with dinner over a 10-day period. Both the test procedures were repeated under identical conditions. The <sup>15</sup>N-enrichment was measured by isotope ratio mass spectrometry (Tracer mass 20-20<sup>TM</sup>, SerCon, Crewe, U.K.). Whole-body protein turnover rates were calculated by using a three compartment model [2].

**Results:** The whole-body protein turnover and the net protein gain either without or with red wine consumption amounted to 3.74±0.6 vs. 3.51±0.6 g/kg/day (p= 0.16) and -0.15±0.3 vs. 0.12±0.4 g/kg/day (p= 0.13), respectively. The faecal <sup>15</sup>N-excretion amounted to 4.3 and 4.5 %, respectively. Red wine consumption led to decreased but not statistically different whole-body protein turnover rates.

**Discussion:** <sup>15</sup>N-labelled yeast protein was found to be an appropriate and reliable tracer substance for measuring whole-body protein parameters in men by using a three compartment model. Moderate alcohol consumption does not induce significant short-term changes in the whole-body protein turnover of healthy adults. Our combination of measuring the protein flux after red wine consumption together with food intake is a novelty.

### **References:**

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[2] Wutzke KD, Lorenz H: The effect of L-carnitine on fat oxidation, protein turnover and body composition in slightly overweight subjects. *Metabolism* 2004;53:1002-6.

## OC B13: Urinary biomarkers of habitual dietary non-digestible carbohydrate and protein intake\*

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Non-digestible carbohydrates (NDC) pass into the large intestine, where they become available for bacterial degradation. The main products of colonic carbohydrate fermentation are short-chain fatty acids (SCFA)<sup>1</sup>, which have been associated with several beneficial effects in the gut<sup>2</sup>. In contrast, products of colonic fermentation of non-digestible proteins, branched chain fatty acids (BCFA) and phenols<sup>3,4</sup>, may be deleterious to human health<sup>5</sup>. In the present study, the relationship between the urinary output of SCFA, BCFA and phenols and habitual dietary intake of fibre and protein was investigated.

20 healthy subjects were asked to record their diet for 2 days. A 24 h urine sample was collected. Urinary nitrogen, creatinine, SCFA (acetate, propionate, butyrate), BCFA (2-methylbutyrate, 3-methylbutyrate, 2-methylpropionate) and phenols (phenol and *p*-cresol) were analysed by GCMS. BCFA were quantified by chemical internal standards. SCFA and phenols were quantified by isotope dilution using deuterated internal standards.

Median (range) habitual dietary intake of protein and fibre<sup>6</sup> was 65 (43-116) and 16 (9-45) g/d, respectively. Dietary protein intake was positively correlated with urinary excretion of nitrogen ( $R^2=0.8$ ,  $p<0.001$ ) and 2-methylpropionate ( $R^2=0.6$ ,  $p<0.01$ ). Participants were allocated by their dietary fibre intake into different groups: low (<12 g/d); moderately low (12-18 g/d), moderately high (18-25 g/d) and high intake (>25 g/d). Fibre intake was positively correlated with urinary excretion of acetate ( $R^2=0.4$ ,  $p=0.1$ ), SCFA ( $R^2=0.4$ ,  $p=0.09$ ) and VFA ( $R^2=0.5$ ,  $p<0.05$ ) whilst molar concentration of 3-methylbutyrate ( $R^2=0.7$ ,  $p<0.01$ ) and BCFA per total VFA ( $R^2=0.6$ ,  $p<0.05$ ) decreased with high fibre intake. Urinary acetate excretion was significantly higher in groups with a moderately high and high fibre intake ( $364\pm 289$  and  $427\pm 183$   $\mu\text{mol}/24$ , resp.) compared to a low fibre intake ( $187\pm 22$   $\mu\text{mol}/24$  h  $p<0.05$ ). Similar results have been observed for SCFA excretion, molar concentration of acetate and SCFA per total VFA and excretion relative to creatinine output. Molar concentration of 3-methylbutyrate and BCFA per total VFA were significantly lower when fibre intake was high ( $p<0.05$ ). As a consequence, acetate:3-methylbutyrate and SCFA:BCFA ratios were positively correlated with fibre intake ( $R^2=0.7$ ,  $p<0.01$  and  $R^2=0.6$ ,  $p<0.01$ , resp.). Significantly higher acetate:3-methylbutyrate and SCFA:BCFA ratios were observed for a fibre intake of more than 12 and 18 g/d, resp.

Higher intake of dietary fibre was correlated with colonic carbohydrate fermentation, indicated by an increase in urinary acetate excretion. Furthermore, the formation of bacterial protein degradation products, such as 3-methylbutyrate, was reduced by high fibre intake. The ratio of acetate:3-methylbutyrate was revealed as a putative biomarker for colonic carbohydrate fermentation in the present study. Since different NDC increase, proportionally, the production of specific SCFA<sup>1</sup>, the quotient of SCFA:BCFA is proposed as a more general biomarker to predict habitual dietary fibre intake. Using these urinary biomarkers could improve colonic health research and strengthen dietary survey work.

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## **OC B14: Short Chain Fatty Acid Products Of Carbohydrate Fermentation**

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Short chain fatty acids (SCFA) are the products of bacterial fermentation of non-digestible carbohydrates (NDCs) that enter the large intestine in man. The main SCFA anions produced through bacterial fermentation of NDCs are acetate, propionate and butyrate. Lactate is also produced through fermentation of some NDCs. The ratio of SCFA production is approximately 60:20:20 for acetate:propionate:butyrate respectively. However, the proportional production of SCFA varies across NDC type and the composition of the human gut microbiota. Together these dictate the site, rate and extent of SCFA production from NDC.

There is growing interest in the physiological consequences of SCFA production. In the Western world, with adequate/excessive nutritional intake, there is only a minor contribution from SCFA to overall energy balance. Recent focus has shifted towards the properties of each individual SCFA both locally and systemically. Locally butyrate acts as an anti-inflammatory and potential anti-neoplastic agent and may contribute to the protective effect of dietary fibre in colorectal cancer. Acetate is a major precursor to butyrate production through bacterial interconversion (or cross-feeding). Systemically acetate acts as an energy source for peripheral tissues and may promote lipogenic and cholesterologenic pathways in the liver. Propionate may inhibit the effects of acetate in the liver. In addition, there is much current interest in the satiating effects of SCFA, propionate in particular, through the interaction of SCFA with anorectic gut hormones which reduce food intake.

Whilst many of the physiological properties of SCFA have been demonstrated in vitro or in animal models, human data is much less convincing and often contradictory. One of the main reasons for the lack of convincing human data is that human intervention trials suffer from the heterogeneity of NDC available to promote SCFA production and that no methods are available to measure SCFA production in large intervention or epidemiological studies. Biomarkers that assess the quantity and quality of NDC that becomes bioavailable are necessary. However, the "holy grail" in dietary fibre and SCFA research in humans remains a simple protocol to determine the flux of individual SCFA in vivo that can be applied in large studies. Such methodology would allow stratification of intervention outcomes against SCFA production, which in turn can be related to NDC type and gut microbiota composition.

Recent advances which allow targeted modification of SCFA production will be discussed to illustrate the potential satiating effects of propionate. In the clinical setting, butyrate delivery to the colon is valued to moderate inflammation and cancer initiation. The development of flux protocols is challenging but our recent data has demonstrated that isotopic approaches, that are non-invasive and applicable to large prospective studies, are possible.

## **OC B15: Ion Exchange-MS Application for Ionic and Polar Compounds**

***Cook, Ken***

Dionex UK Ltd

Ionic and polar compounds such as organic acids, carbohydrates, nucleotides and amino acids are difficult to separate on traditional reverse phase columns. Ion exchange is a far better separation process, however the salt eluents are incompatible with mass spectrometers. Here we describe an ion exchange system which can give good separation of these polar compounds with on-line desalting to allow MS detection. This technique will allow studies of key metabolites which do not separate on traditional reverse phase and may often be isobaric.

## POSTER PRESENTATIONS

### **PO B1: LC-MS/MS as a tool for high-throughput multiplexed quantitation of serum proteins using stable isotope labelled peptides**

**Richard Kay, Chris Barton, Phil Teale, Steve Pleasance**

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The quantitation of proteins using mass spectrometry is fast evolving into a powerful tool for clinical and pharmaceutical applications. We describe high throughput approaches for quantifying both high and medium abundance serum proteins using tandem mass spectrometry in conjunction with stable isotope labelled internal standard peptides. Multiple high abundant, and clinically relevant apolipoproteins were quantified using a multiplexed MRM based approach and demonstrated high concordance with well established immunological techniques. A similarly high throughput MRM method was developed for quantitation of a medium abundant protein which is both clinically important and a biomarker of recombinant therapeutic protein drug abuse in sports.

## **PO B2: Simultaneous measurement of both concentration and <sup>13</sup>C enrichment of glutathione and glycine in one single run, using Liquid Chromatography Coupled to Isotope Ratio Mass Spectrometry (LC-IRMS)**

**Schierbeek H, Rook D, Te Braake Fwj, Dorst Ky, Voortman G, Goudoever JB.**

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A novel method using liquid chromatography coupled to isotope ratio mass spectrometry (LC-IRMS) was developed for simultaneous measurement of <sup>13</sup>C-glutathione as its dimeric form (GSSG) and its precursor [1-<sup>13</sup>C]glycine in neonatal erythrocytes. After transformation of GSH into GSSG, both the intra-erythrocytic concentration as well as the <sup>13</sup>C-isotopic enrichment of GSSG and glycine were determined using only 150 µL of whole blood. Preterm infants received a primed continuous infusion of [1-<sup>13</sup>C]glycine during 6 hours. The results show that using LC-IRMS, the concentration (range of µmol/mL) can be reliably measured using cycloleucine as internal standard with a precision better than 0.1 µmol/mL. In addition, the <sup>13</sup>C-isotopic enrichment measured in the same run gave reliable values with excellent precision (SD < 0.3 ‰) and accuracy (measured between 0 and 5 APE). With this new developed method the fractional synthesis rate (FSR) and the absolute synthesis rate (ASR) of glutathione in premature infants can be determined in one single run. Successfully used in a clinical study, this method opens up a variety of kinetic studies with relatively low administration of tracer infusates, reducing the total cost of the study design. In addition, only minimal sample volumes are required, enabling studies even in extremely small subjects, such as preterm infants as illustrated in this study.

## **PO B3: Blood volume and red cell mass in children with moderate and severe malaria measured by chromium-53 dilution and GC/MS analysis**

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**Introduction:** Fluid volume changes in malaria are complex. Estimating fluid status in sick children with malaria is very challenging. Previous methods for analysis of blood and red cell volume have used radio-isotopes. Recently, a stable isotope chromium method has been described.<sup>1</sup>We applied this technique to measure blood and red cell volume in children with malaria in Gabon, W. Africa.

**Subjects:** 19 children (15 mos to 7 yrs), with malaria: 10 (3M: 7F) with severe malaria versus 9 (3M: 6F) with moderate disease. The study conducted at the Albert Schweitzer Hospital, Lambaréné, Gabon & St George's, University of London. Approval was obtained from the ethics committees of Albert Schweitzer Hospital & the Gabonese Ministry of Health with parental consent

**Methods and findings:** Blood was labelled with <sup>53</sup>Cr-chromate, re-injected, then sampled 30 minutes later. <sup>53</sup>Cr content of pre- and post-injection samples was measured by GC/MS of the chromium- trifluoroacetylacetone chelate using a <sup>50</sup>Cr ISD. Blood and red cell volumes were calculated from isotopic dilution in 15 of the 19 children; in four insufficient signal mitigated analysis. Normalised mean blood volume for the whole group was 59 ± 26 ml/kg, close to predicted values, but red cell volume was reduced (181 vs 269 ml; p=0.01) commensurate with reduced haematocrit. Severe cases tended to have a lower haematocrit and lower red cell volumes than moderate cases (not significant; p=0.33) but similar blood volumes. In one case, we analysed sequential samples over 42 days and estimated the rate of <sup>53</sup>Cr disappearance as 1.7%/day (equivalent half-life: 38 days).

**Conclusions:** 1) <sup>53</sup>Cr-labelled red cells may be used to estimate blood and red cell volumes and is applicable to situations such as childhood disease and use in resource-constrained settings, 2) Inverse matrix analysis can be used to unravel isotopomer distribution, 3) Blood volume is relatively well-preserved in children with malaria, although red cell mass is rapidly depleted.

**Future:** A larger sample size with a non-parasitised control group is proposed.

**Reference:** Veillon C. et al., 1994; Clin. Chem., 40: 71-73.

## **PO B4: A versatile method for stable carbon-isotope ( $^{13}\text{C}$ ) analysis of carbohydrates by high-performance liquid chromatography – isotope ratio mass spectrometry**

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We developed a method to analyze stable carbon isotope ( $^{13}\text{C}/^{12}\text{C}$ ) ratios in a variety of carbohydrates using high-performance liquid chromatography - isotope ratio mass-spectrometry (HPLC-IRMS). Chromatography is based on strong anion-exchange columns with low strength NaOH eluents. An eluent concentration of 1 mM resulted in low background signals and good separation of most of the typical plant neutral carbohydrates. We also show that more strongly bound carbohydrates such as acidic carbohydrates can be separated by inclusion of  $\text{NO}_3^-$  as an inorganic pusher ion in the eluent. Analysis of neutral carbohydrate concentrations and their stable carbon isotope ratios is shown for plant materials and marine sediment samples both at natural abundance and for  $^{13}\text{C}$ -enriched samples. The main advantage of HPLC-IRMS analysis over traditional gas-chromatography based methods is that no derivatisation is needed resulting in simple sample treatment and an improved accuracy and reproducibility.

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## **PO B5: Stable Isotope Incorporation Into Faecal Bacterial Rna Reflects Predictable Changes In Short Chain Fatty Acids**

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Products of the metabolic activity of unculturable bacteria may regulate the host immune responses in inflammatory bowel disease (IBD). Indirect markers of bacterial metabolism, such as short chain fatty acids (SCFA) are confounded by factors such as motility and colonocyte function. Stable isotope probing (SIP) is a novel technique that can link metabolic activity with species identity in unculturable bacteria (1).

The aim of the study was to validate stable isotope incorporation into bacterial RNA and compare it with patterns of SCFA production in human faecal samples. Four fresh stool samples from 4 healthy individuals were prepared as 20% (w/v) slurries in a phosphate buffer. 30ml aliquots, in triplicate, were supplemented with either oligofructose, pectin, L-rhamnose or no carbohydrate. All samples were spiked with 50mg of <sup>13</sup>C urea at time zero and incubated for 4 hours at 37°C before snap freezing. SCFA's were extracted and measured by gas chromatography. Bacterial RNA was purified and relative <sup>13</sup>C enrichments were measured by liquid chromatography isotope ratio mass spectrometry (LC-IRMS).

Addition of carbohydrate significantly increased production of acetate (C2) propionate (C3) and butyrate (C4) ( $p < 0.05$  vs. no carbohydrate) for all treatments except that pectin did not increase propionate nor did L-rhamnose increase butyrate production. SCFA profiles (% of total SCFA) showed significant changes in the relative proportion of C2-C4 depending on carbohydrate support, with L-rhamnose producing significantly more propionate and significantly less acetate and butyrate. RNA were enriched in all samples indicating bacterial sequestration of tracer. Incorporation of <sup>13</sup>C was significantly higher in pectin and L-rhamnose supported media and there was a correlation between propionate production and RNA enrichment ( $R^2 = 0.444$   $p < 0.017$ ).

Stable isotope incorporation into bacterial RNA can be achieved using simple tracers of *de novo synthesis*. LC-IRMS has adequate sensitivity to detect small changes in <sup>13</sup>C incorporation not afforded by centrifugal methods. There was a significant association between <sup>13</sup>C enrichment of RNA and SCFA profiles. Probing with group specific oligo-nucleotide probes will yield information on the relative changes in bacterial metabolic activity in patients with IBD.

1. Barclay et al JPGN 2008;46:486-95

## **PO B6: The Synthesis of Isotopically Labelled Phytochemicals for Analysis and Metabolic Studies**

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### **Isoflavone Phytoestrogens**

One of the initial problems with phytoestrogen research was the lack of availability of pure standards of the individual phytoestrogens, and their metabolites, which caused problems for analysis. It is particularly important to have good internal standards for analytical programmes, such as surveys of food or epidemiological studies, with large numbers of biological samples, when accuracy and reproducibility are vital. Two FSA funded projects at St Andrews were intended to address this problem. During these projects a range of isoflavones (and the related lignans) were synthesised containing three or more carbon atoms as their heavy, but non-radioactive, isotopes. Use of these new standards has resulted in significant improvements in the sensitivity, accuracy and reproducibility of the analytical methods, which has proved especially important in studies on large populations where the aim is to search for correlations between health outcomes, such as cancer incidence, and diet. The isoflavones are also known to occur in modified forms. In plant material isoflavones exist as glycosides and in humans they are metabolised to glucuronide conjugates. To be able to carry out analysis of these species <sup>13</sup>C-labelled versions are also required. Recently an improved method has been successfully developed for the synthesis of these conjugates, which gave superior yields to existing literature methods.

### **Glucosinolates**

There is convincing evidence from both epidemiological and animal studies that consumption of broccoli and other cruciferous vegetables is associated with a decreased risk of cancers of the gastrointestinal and respiratory tracts, which has been attributed to the high content of glucosinolates in these vegetables. However, there are many unanswered questions regarding human exposure to glucosinolates and their bioavailability and metabolism. Isotopically labelled glucosinolates, and their metabolites, were synthesised for use as internal standards for analysis and for metabolic studies in rats to search for new biomarkers of exposure (in collaboration with the Macaulay Institute and the Robert Gordon University).

## PO B7: Production of Complex Metaprobes: <sup>13</sup>C-Labelled Cereals

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Digestion and fermentation of starchy foods can be measured using stable isotopes. Stable isotopes are non-hazardous and are ideal for studies in humans. As all elements of organic matter occur naturally with stable isotopes, foodstuffs can readily be enriched to act as metabolic tracers. Stable isotope-labelled tracers have been termed metaprobes<sup>1</sup>. Simple metaprobes are extrinsically labeled tracers produced by chemical synthesis. Complex metaprobes are intrinsically labelled during biosynthesis. The ideal metaprobes are foodstuffs with tracer molecules incorporated during biosynthesis. The rate and extent of metaprobe metabolism in the body follows that of unlabelled material and can be monitored using blood, urine and breath sampling. Two approaches have been used to label plants during photosynthesis: taking gas-tight transparent enclosures to the field<sup>2</sup>; growing crops in controlled environment chambers<sup>3</sup>. We have applied <sup>13</sup>C-labelled wheat flour prepared by the latter method<sup>4</sup> in carbohydrate nutrition studies in children<sup>5</sup>. The current study concerns production of <sup>13</sup>C-labelled cereals in controlled environment chambers on a larger scale and their application in human nutrition studies.

<sup>1</sup>Young VR & Ajami AM (2001) Metabolism 2000: the emperor needs new clothes, *Proceeding of the Nutrition Society* **60** (1): 27-44

<sup>2</sup>Palata JA (2001) Source/sink interaction in crop plants *in Stable Isotope techniques in the study of biological and functioning of ecosystems* EDS Unkovich M, Pate J, McNeill A & DJ Gibbs, Current Plant Science and Biotechnology in Agriculture **40**, Chp 8, pp145-165

<sup>3</sup>Svejcar TJ, Boutton TW & Trent JD (1990) Assessment of carbon allocation with stable carbon isotope labeling *Agron J* **82**: 18-21

<sup>4</sup>Harding M, Coward WA, Weaver LT, Sweet JB & Thomas JE (1994) Labelling wheat flour with <sup>13</sup>C, *Isotopenpraxis* **30**, 1-8

<sup>5</sup>Christian MT, Amarri S, Franchini F, Preston T, Morrison DJ, Dodson, B, Edwards CA & Weaver LT (2002) Modeling <sup>13</sup>C-breath tests to determine site and extent of starch digestion and fermentation in infants, *J Pediatric Gastroenterology and Nutrition* **34**, 158-164

## **PO B8: Measuring the Liquid Phase Gastric Emptying Rate of Sip Feeds**

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The assimilation of sip feeds can be delayed by slow gastric emptying (GE). In the context of peri-operative nutrition, timely uptake is advantageous. A simple non-invasive breath test has been developed to assess liquid phase GE. The test uses sodium 1-<sup>13</sup>C-acetate as tracer\*. Here we show that this test to measure the half emptying time of liquid feeds is readily performed on surgical patients. It imposes no hazard due to ionising radiation and can be performed remotely, away from an installed facility.

\*Braden *et al.*, 1995, *Gastroenterology* **108**: 1048-1055