Matthew Peach – ROKOS Internship

**Using stable isotope tracers to investigate de Novo Lipogenesis in health and disease (and other current approaches for investigating human metabolism)**

Over the summer, I spent time working in the Metabolic Research Group at the Oxford Centre for Diabetes, Endocrinology and Metabolism (OCDEM). This is a department of the Radcliffe Department of Medicine based at the Churchill Hospital, Headington.

**Research Area**

My supervisor, Prof. Leanne Hodson, is primarily interested in intrahepatic fat storage and how changes in lipid metabolism can lead to pathologies such as NAFLD (Non-Alcoholic Fatty Liver Disease) which can predispose individuals to type-2 Diabetes Mellitus and cardiovascular disease. NAFLD is characterised by an abnormal accumulation of fat in the liver (known as steatosis) due to non-alcohol related factors and is estimated to affect around 25% of the world population. This accumulation of fat is thought to be partly due to an increase in lipid synthesis in the liver – otherwise known as *De novo lipogenesis* (DNL). By administering a stable isotope tracer (for instance D₂O) and monitoring its incorporation into the products of DNL, an approximate rate of liver DNL can be attained.

![A schematic overview of the procedure for measuring the rate of DNL using a D₂O. The deuterium is incorporated into the products of DNL – fatty acyl CoAs. The major product of DNL is palmitoyl CoA which is esterified with glycerol to form TAG (liver fat). Palmitoyl CoA can undergo further processing such as elongation and desaturation (oxidative insertion of double bonds). GC-MS can detect and quantify both the undeuterated and deuterated species. Since deuteration of methyl palmitate (the derivative of palmitoyl CoA detected by GC-MS) could have only occurred during DNL, the ratio of the](attachment:image.png)

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A schematic overview of the procedure for measuring the rate of DNL using a D₂O. The deuterium is incorporated into the products of DNL – fatty acyl CoAs. The major product of DNL is palmitoyl CoA which is esterified with glycerol to form TAG (liver fat). Palmitoyl CoA can undergo further processing such as elongation and desaturation (oxidative insertion of double bonds). GC-MS can detect and quantify both the undeuterated and deuterated species. Since deuteration of methyl palmitate (the derivative of palmitoyl CoA detected by GC-MS) could have only occurred during DNL, the ratio of the
deuterated to undeuterated methyl palmitate in the sample (normalised to the enrichment of the individual’s water pool) gives an approximate rate of DNL.

TAG - Triacylglycerol; NADPH – reduced nicotinamide adenine dinucleotide; DNL – de novo lipogenesis; VLDL – very low-density lipoprotein; GC-MS – gas chromatography-mass spectrometry

The Hodson group can alter experimental conditions such as the diet of participants, medications or amount of exercise to see how the rate of DNL changes. They can also monitor how the products of DNL may be altered (i.e. through elongation, saturation etc).

A schematic showing some of the pathways that can lead to an increase in liver DNL. This diagram is somewhat complex, but the take home message is that metabolites and hormones can activate signalling pathways which increase the expression of genes implicated in DNL. This diagram doesn’t include the ways that some metabolites can (in)directly activate or inactivate enzymes implicated in DNL. Schematic taken from ‘De novo lipogenesis in liver health and disease: more than just a shunting yard for glucose’ Sanders et al (2016)

During my time in the group, I had the opportunity to get involved with the sample processing and analysis from start to finish. I have outlined the process below:

- Six individuals were given a daily dose of D₂O for 6 weeks. They were given a loading dose of 300g per day for one week and a maintenance dose of 70g per day for the remaining weeks. This should have resulted in an estimated enrichment of 3% (i.e. 3% of total body water pool was deuterated).
- Every week samples of blood, urine and saliva were taken. Urine and saliva were self-collected while the blood was taken by trained university nurses.
- The blood was immediately centrifuged, the resulting plasma fraction was aspirated, and preservatives were added.
- Plasma samples underwent density gradient ultracentrifugation to separate out the lipoprotein fractions. This enabled the VLDL fraction to be easily isolated.
- The VLDL fraction then underwent a Folch Extraction to isolate the triacylglycerol. Briefly, the Folch Extraction involves adding the VLDL into a solution of chloroform:methanol (2:1 v/v). The sample is spun in an ultracentrifuge which produces a biphasic system – the lower, chloroform phase contains the TAG (and other lipid), while the upper, methanol phase contained water soluble molecules.
- The lower phase was extracted and dried under nitrogen. The samples were reconstituted in chloroform and underwent a two stage, solid phase extraction (SPE) using a C-18 column. In the first stage, the samples were eluted with chloroform which separated out the TAG/CE (cholesterol esters) from the remaining lipid. In the second stage, the TAG/CE eluate was run through a column again but this time using hexane as an eluent. This separated the TAG from the remaining CE.
- The TAG fraction was heated with sulphuric acid and methanol to produce fatty acid methyl esters (FAMES)
- The FAMES are dissolved in chloroform were analysed using a GC-MS (Gas Chromatography – Mass Spectrometry) device. The parent ion of interest in methyl palmitate (m/z 270)
- The peaks of the parent ion (m/z 270) and the deuterated, daughter ions (m/z 271 and m/z 272) were integrated.
- This allowed the calculation of a TTR value (Tracer-Tracee ratio). This is a measure how label much label has been incorporated into the fatty acids.
- TTR values are normalised to plasma water enrichment for the individual concerned and are reported as a ‘percentage contribution of the tracer to DNL’. This is a measure of the rate of hepatic DNL in individuals.
- Plasma water enrichment was measured using IRMS (isotopic ratio mass spectrometry). This technique reports a quantity called a ‘delta value’ however this can be converted into TTR. Saliva and urine samples were also analysed to determine whether measured enrichments were comparable to plasma.


I am midway through analysing the data and will be presenting the results at the Metabolic Research Group lab meeting this coming autumn.

What did I learn from this experience?

I decided to complete my ROKOs internship at OCDEM because I wanted to explore whether I would enjoy a career in research. Being embedded in a research group for nine weeks gave me a feel of what a working in research would be like. Through talking to early career researchers in the group, I gained an insight into the career progression within research, and both the rewarding and challenging aspects of the job.

The Metabolic Research Group has a wide range of interests in the field of metabolic research and I am grateful that the group allowed me to gain ‘hands on’ experience with myriad of equipment and experimental techniques. I gained experienced using GC-MS, IRMS, solid phase extraction, density gradient ultracentrifugation, clinical chemistry analysers, immunostaining with confocal microscopy, TIRF microscopy, patch clamp techniques, sample preparation for electron microscopy, western
blotting, ELISA, BCA assays, qPCR, lipolysis assays, transfection techniques and luciferase assays to measure promoter activity within a DNA construct. In addition, I did some work in tissue culture (including changing media and splitting cells). As human volunteers are used extensively, it was also interesting to learn about some of the ethical and legal consideration when designing a study.

I also had the opportunity to shadow a clinical biochemist at the John Radcliffe Hospital. I was able to have a tour around the lab and sit in a weekly clinical meeting where the specialty registrars, consultants and clinical scientists discussed current cases. It was interesting to see how lab techniques which I had been using at OCDEM were equally applicable for the diagnosis of metabolic disorders.

However, one of the most important things I learned from my internship is the importance of being in a research group that has a good working environment. Such environments appear to stem from those in charge and Prof Hodson (the principal investigator) places great importance on the happiness and welfare of her team. A lovely feature of the working day was the 11am coffee and cake break where everyone would sit together and have a chat. She is also very keen on the professional development of her team and is always willing to mentor them when it comes to career progression. As a result of this experience, I will bear in mind the importance of the supervisor when selecting my fourth-year research project and possibly a PhD project in the future.

I wish to thank Pembroke College for awarding me this generous scholarship. An enormous thank you also goes to the Metabolic Research Group at OCDEM for allowing me to be a part of their department over the summer. A special mention must be reserved for Prof. Leanne Hodson, Dr Sion Parry, Dr Anne Clark, Dr Andrea van Dam, Tom Cornfield, Matt Hutchinson, Elspeth Johnson and Jo Gamwell – their hospitality, mentoring and cake has made this an immensely enjoyable and useful experience.

Below – some images from my time at OCDEM.

A human pancreatic islet which I stained using antibodies conjugated to fluorophores and imaged using a confocal microscope. The islet consists of cells which produce the hormones insulin and glucagon which regulate blood glucose levels. Blue is the insulin stain (insulin producing cells), green is the glucagon (glucagon producing cells), red is chromogranin A (a protein involved in hormone secretion so found in both types of cells) and the multicoloured image is the merge of all three.

A close-up of the merge.
One of the GC-MS machines. This is used for identifying the deuterated and undeuterated methyl palmitate.

The IRMS machine

The confocal microscope

The confocal microscope
An ultracentrifuge used to isolate the VLDL fraction

The tissue culture section of the lab