^{14}C-Pathway probing as dynamic biomarker read-out: *De Novo* Lipogenesis using ^{14}C-labeled acetate and Accelerator Mass Spectrometry

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INTRODUCTION

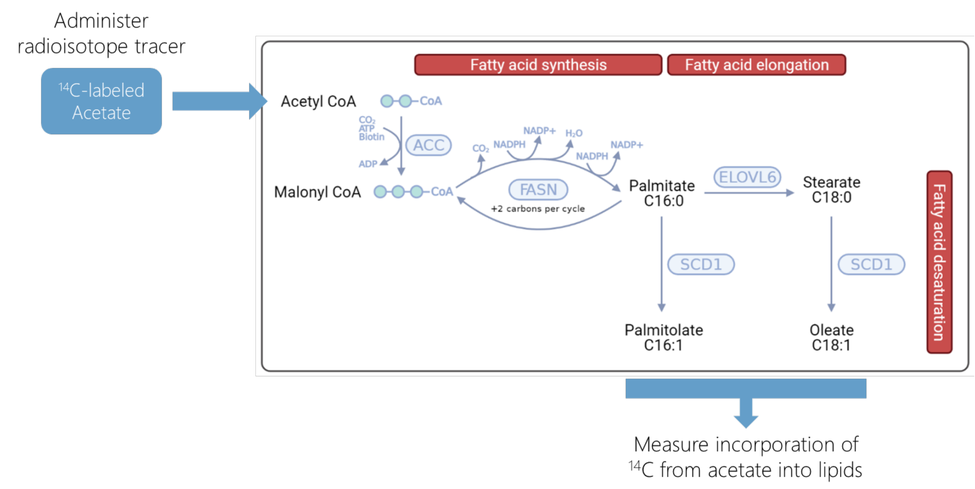
The application of isotopes is a proven concept in biomarker research to measure the dynamics of endogenous processes. Good examples, amongst others, are the administration of D_2O to determine collagen production (analysis of OD-proline in collagen), ^{13}C or ^{2}H labelled amino acid to measure production of specific proteins, ^{41}Ca to monitor changes in bone mineral, etc. to assess the efficacy of interventions. Here we present the use of ^{14}C acetate to investigate *de novo* lipogenesis (DNL) and cholesterol biosynthesis. The advantages of ^{14}C as opposed to ^{13}C are 1) the natural background of ^{14}C is extremely low, and 2) it enables us to harness the extreme sensitivity of Accelerator Mass Spectrometry (AMS).

EXPERIMENTAL

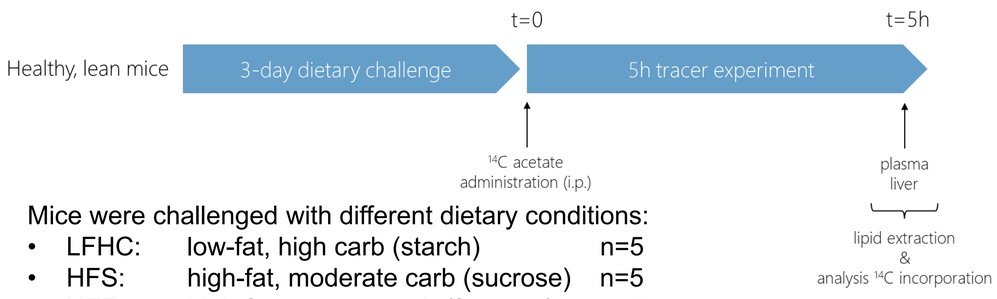
A microtracer equivalent of ^{14}C-acetate was administered to mice (30 Bq) and blood was collected after 5h. Bligh and Dyer extraction was used to measure the total amount of ^{14}C in the plasma lipid fraction. Fatty acids were obtained by saponification of the lipid fraction using 4M KOH followed by extraction with n-hexane. The fatty acids were separated and fractionated by HPLC to obtain a ^{14}C-fatty acid profile by AMS analysis.

In an *ex-vivo* liver (pig) experiment ^{14}C-acetate was administered by portal infusion. Plasma samples were collected up to 4 h and analysed in similar fashion as the mouse plasma samples.

DE NOVO LIPOGENESIS



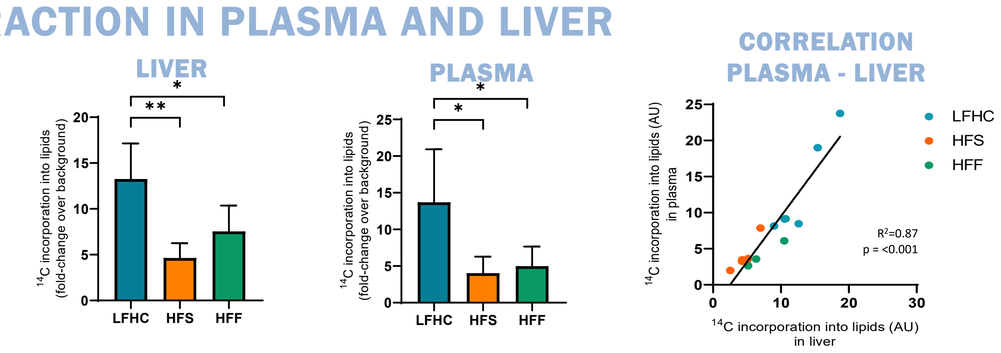
PROOF OF CONCEPT EXPERIMENT



Mice were challenged with different dietary conditions:

- LFHC: low-fat, high carb (starch) n=5
- HFS: high-fat, moderate carb (sucrose) n=5
- HFF: high-fat, moderate carb (fructose) n=5

^{14}C FROM ACETATE IS INCORPORATED INTO LIPID FRACTION IN PLASMA AND LIVER



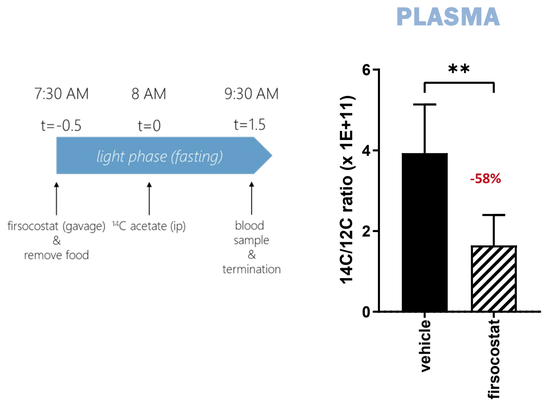
DNL activity is quantifiable in liver tissue and in plasma

- Strong correlation between plasma and liver → plasma can be used as a proxy for liver DNL

Method is sensitive enough to detect dietary effects on DNL activity

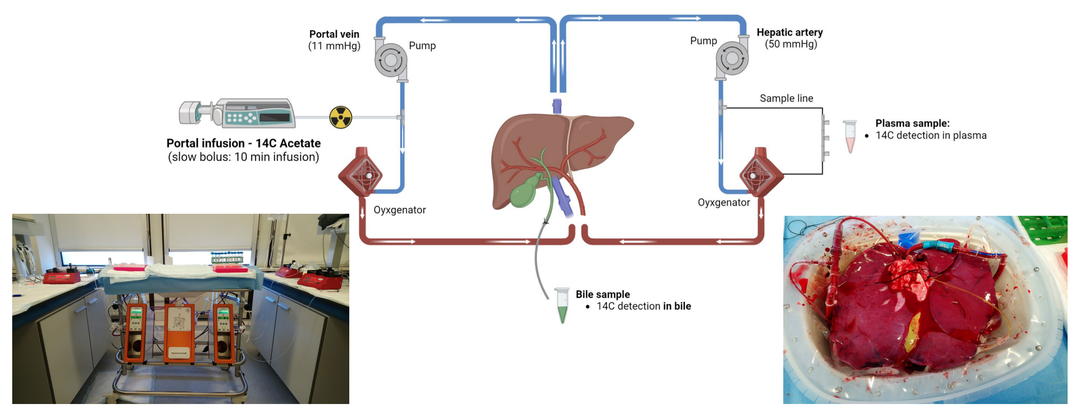
- As expected, DNL activity is highest in the low-fat, high-carb diet group and DNL is suppressed by diets high in fat with fructose or sucrose

INHIBITION OF ^{14}C INCORPORATION BY DNL INHIBITOR

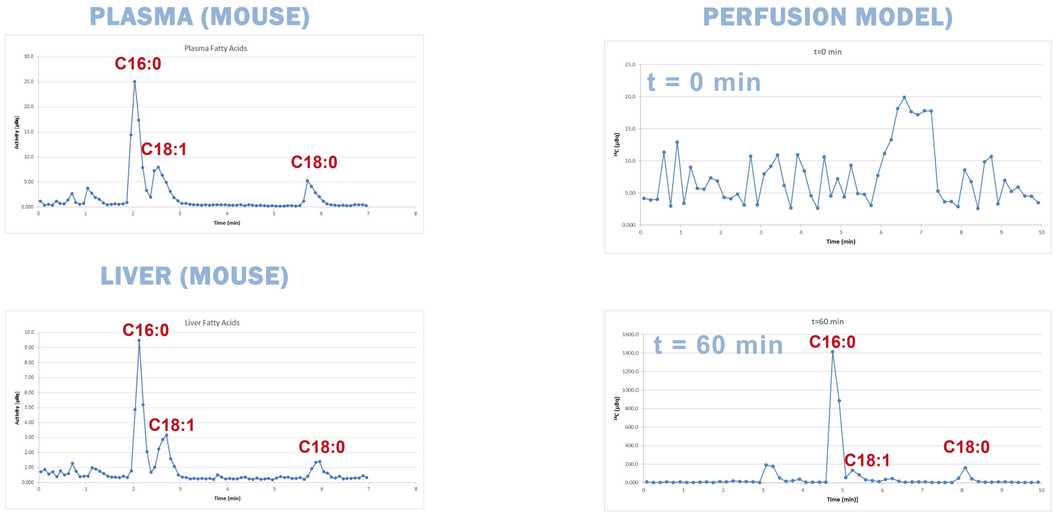


Treatment with the DNL inhibitor firsocostat (ACCi) resulted in a strong reduction of ^{14}C incorporation into total plasma lipids as expected.

EX VIVO LIVER PERFUSION MODEL



LC-MS-AMS CONFIRMED INCORPORATION OF ^{14}C IN FATTY ACIDS



Subsequent fatty acid profiling analysis (quantitation by AMS, identification by LC/MS) and enrichment analysis of the different fractions collected showed that the ^{14}C signal from acetate is predominantly found in palmitate (C16:0, the primary end product of DNL) and in lesser amounts also in fatty acids that result from further processing of palmitate (elongation and/or desaturation: C18:0 and C18:1) thus confirming that the observed incorporation of ^{14}C from acetate into the lipid fraction of plasma and liver is indeed a reflection of DNL.

CONCLUSIONS

The administration of ^{14}C-acetate, *in vivo* and *ex vivo* models, followed by AMS analysis results in the incorporation of the ^{14}C label in the primary fatty acid products of DNL (including elongation and desaturation). The results of the experiments with different diets and the administration of a DNL inhibitor agree very well with the expected outcome, thus proving the method allows quantitative measurement of DNL activity. Latest experiments also showed the incorporation of the ^{14}C label in cholesterol. The ^{14}C-label in the liver perfusion model was excreted in bile. Identification of the metabolites containing the label is in progress.

The next step is to apply this approach in a study with human volunteers. Extrapolation of the amount of ^{14}C administered in the mouse experiments (30 Bq) suggests that a dose of about a few µCi should be sufficient to measure DNL activity in human volunteer studies. This amount of radioactivity is the same order of magnitude as for drug microtracer studies, and thus would not require any dosimetry study to be applied in a clinical setting.

The administration of ^{14}C-labeled substrate followed by isolation of the specific ^{14}C-end-product of a metabolic pathway, either by extraction of UPLC isolation, can be applied generically to quantify the dynamics of enzymatic processes. The principle can be used in many other applications, not only for conversion of small molecules but also the de novo synthesis rate of specific proteins by administering a ^{14}C labelled amino acid. Isolation of the target protein can be achieved with, for example, immunoaffinity capture technologies.

Advantages of this approach are:

1) It is a generic biomarker technology to measure the dynamics of metabolic pathways (fluxes), protein production, or other processes with very high specificity.
2) Accelerator mass spectrometry is among the most sensitive analysis techniques enabling the measurement of biomarkers at extremely low concentrations.
3) Due to the extremely low and *de facto* constant background of ^{14}C the measured end-product concentration is not superimposed on inter and/or intra subject background variation. Theoretically, this should increase the statistical power of study outcomes.