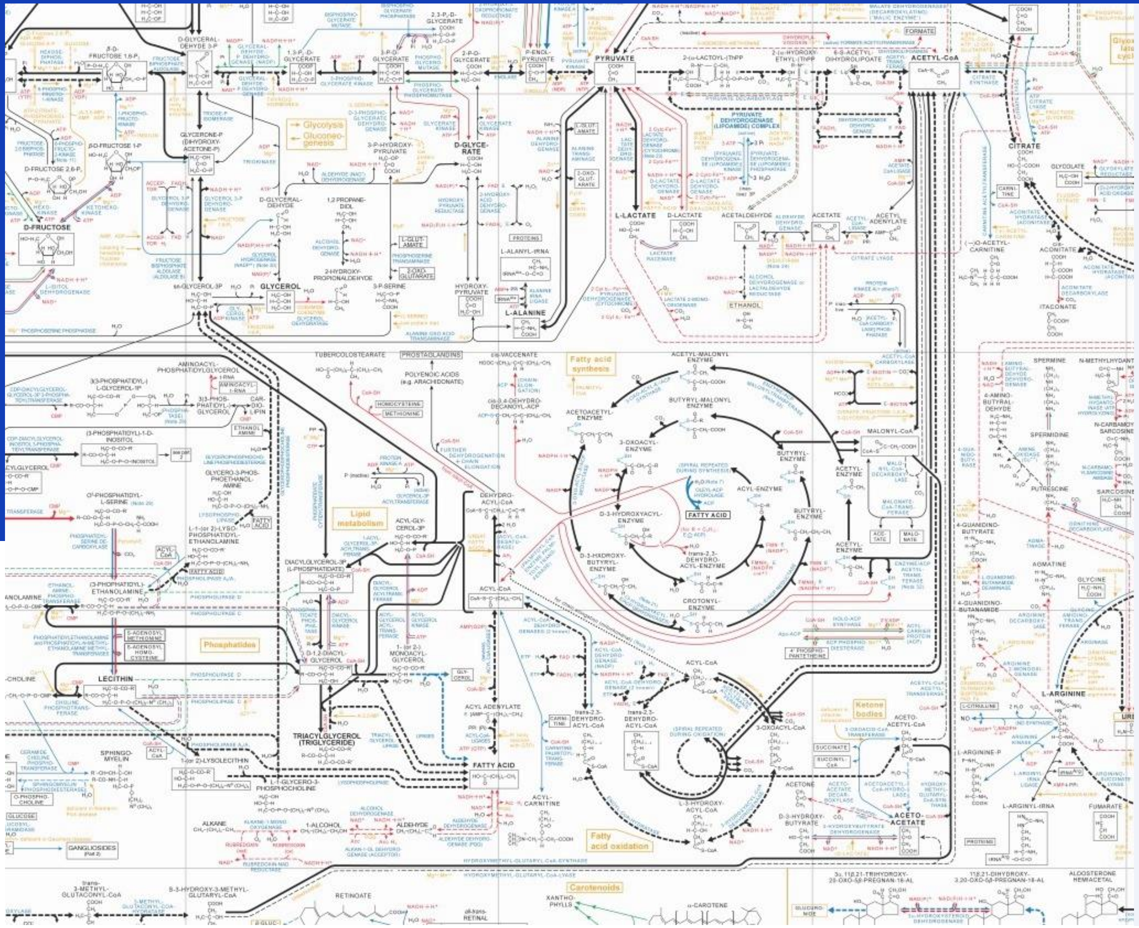
Flux analysis using ^{14}C and AMS

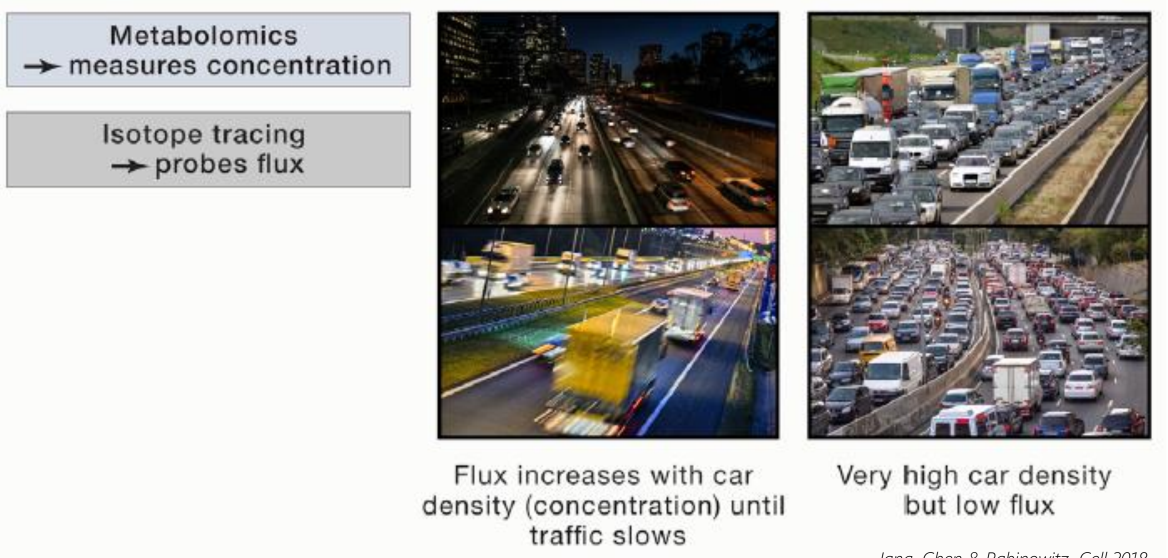


Martine Morrison

Metabolic flux

- Measurement of metabolite concentrations does not tell full story of (disease)biology
  - Concentrations and fluxes do not reliably align

- Metabolic flux analysis
  - Flow of metabolites through a pathway
  - Provides mechanistic understanding of pathway activity
    - Indicator/biomarker of disease state
    - Drug development (which process to target)



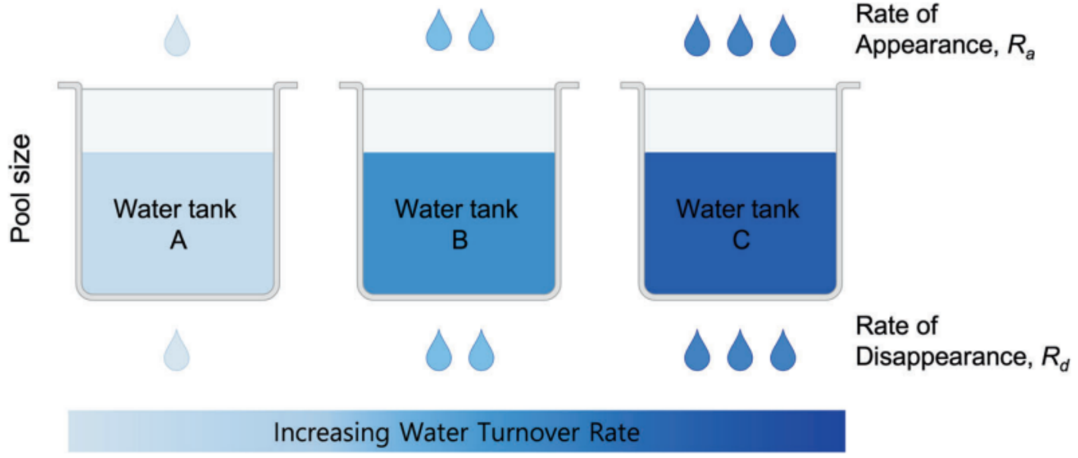
Jang, Chen & Rabinowitz, Cell 2018

Metabolic flux

- Everything in the body turns over at varying rates to achieve overall “dynamic” homeostasis
  - Static (snapshot) information does not reveal the dynamic nature of in vivo metabolism

- A change in pool size (concentration) of any molecule:
  - Result of imbalance between its rates of appearance and disappearance

- Pool size (concentration) can be the same at different rates of appearance and disappearance



^{14}C microtracer approach for flux analysis

- AMS technology can be used to measure metabolic fluxes with very high sensitivity
  - Analysis of ^{14}C => much lower natural abundance than other commonly used isotopes (=lower background)
  - Analysis with AMS is extremely sensitive

- Advantages:
  - Microtracer use → no disturbance of pathway by adding large amounts of precursor
  - Extremely sensitive analysis → can be used for pathways/processes that cannot otherwise be measured
  - Low-dose radioactivity can be applied in humans in early clinical testing stages

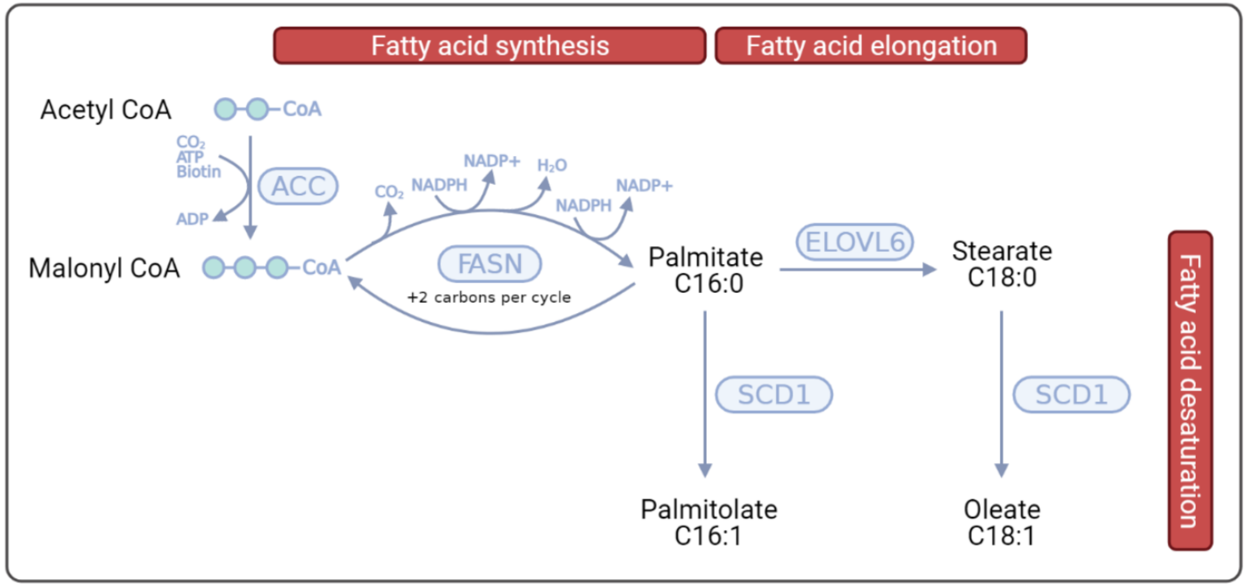
Isotopes commonly used in biological research

	Common Stable	Rare Stable	Very rare Radioactive
Hydrogen	^{1}H Protium (99.985)	^{2}H Deuterium (0.015)	^{3}H Tritium ($< 10^{16}$)
Carbon	^{12}C (98.892)	^{13}C (1.108)	^{14}C (Trace)
Oxygen	^{16}O (99.763)	^{18}O/ ^{17}O (0.02/ 0.037)	^{11}O*

(%) natural abundance = amount of isotope occurring naturally in the atmosphere
*No long-lived radioisotope

NIH National Institute of Diabetes and Digestive and Kidney Diseases

De novo lipogenesis



- De novo lipogenesis (DNL) is an important metabolic pathway in which excess carbohydrates are converted into fatty acids. DNL is strongly regulated by nutritional status (fasted/fed) and macronutrient composition of the diet.
- Deregulation of the DNL pathway is associated with diverse pathological conditions:
  - Metabolic anomalies such as obesity, insulin resistance, non-alcoholic fatty liver disease
  - Cancer
  - Various viral infections

De novo lipogenesis: flux analysis

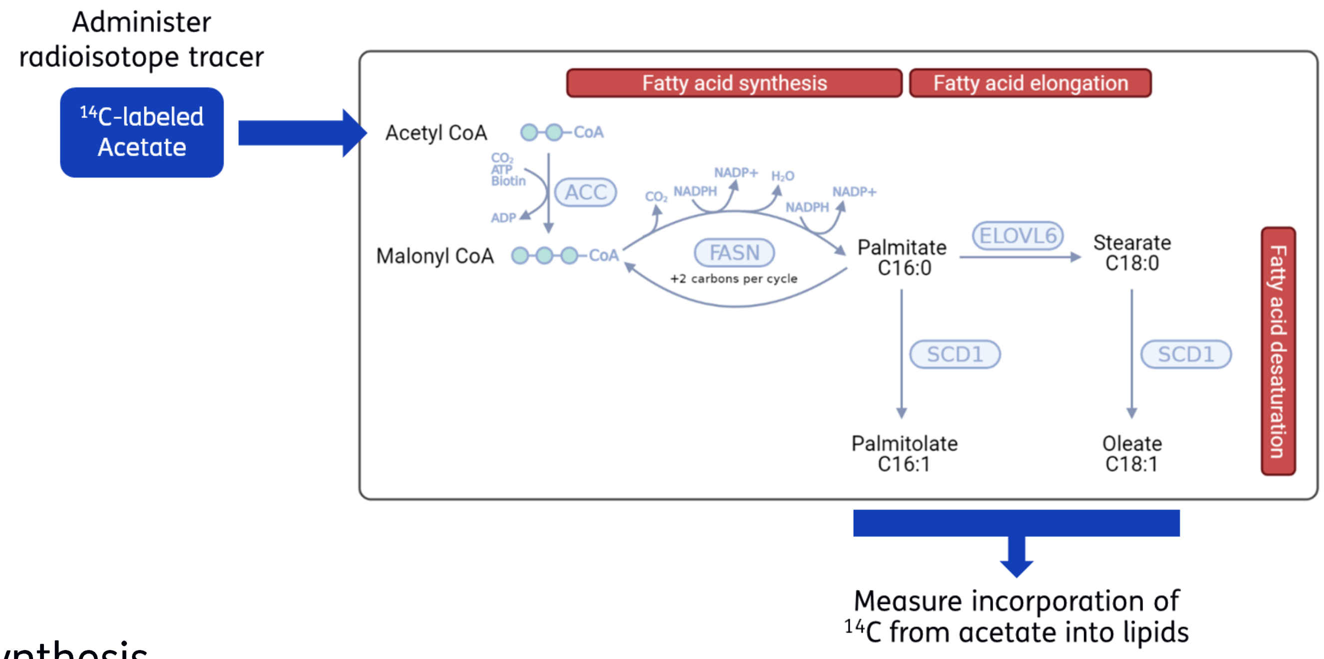
De novo lipogenesis (DNL) flux analysis:

- Administer ^{14}C acetate
- Measure incorporation in lipids

Proof of concept experiments

- Ldlr-/-.Leiden MASH mouse
- *Ex vivo* liver perfusion system

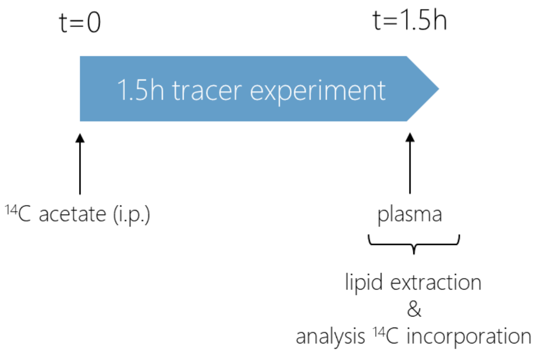
Using same experimental setup → cholesterol synthesis

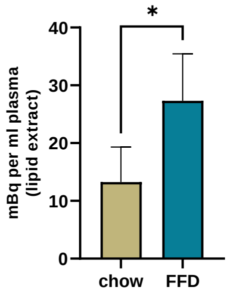


De novo lipogenesis

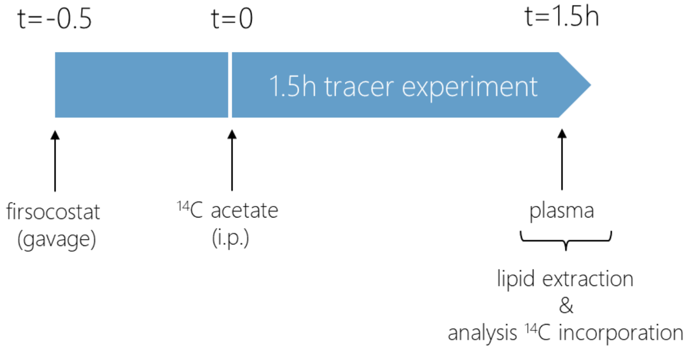
^{14}C from acetate is incorporated into lipid fraction in Ldlr-/-.Leiden MASH mice

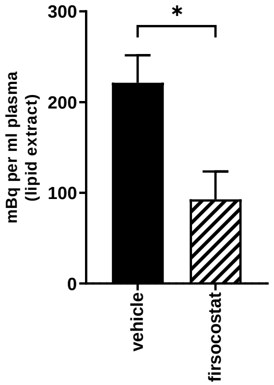
Ldlr-/-.Leiden
MASH model





^{14}C incorporation in lipid fraction is increased in Ldlr-/-.Leiden mice with MASH (FFD) relative to chow.

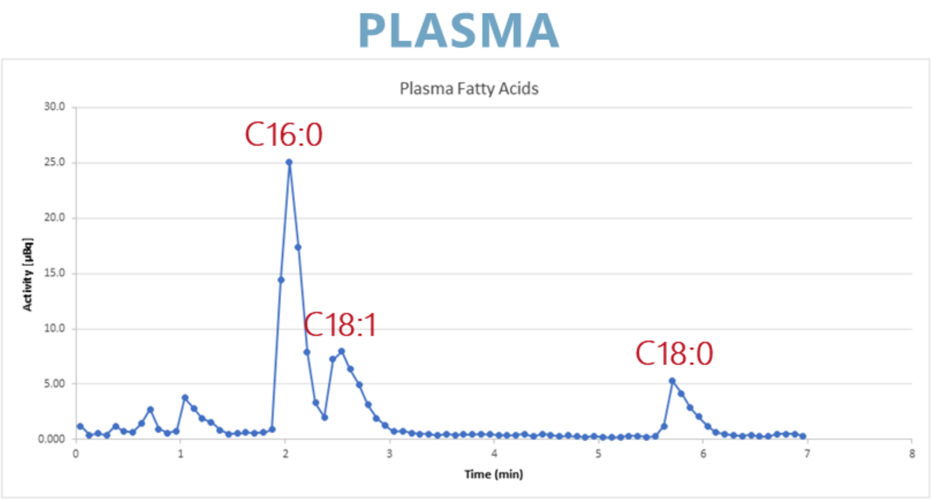


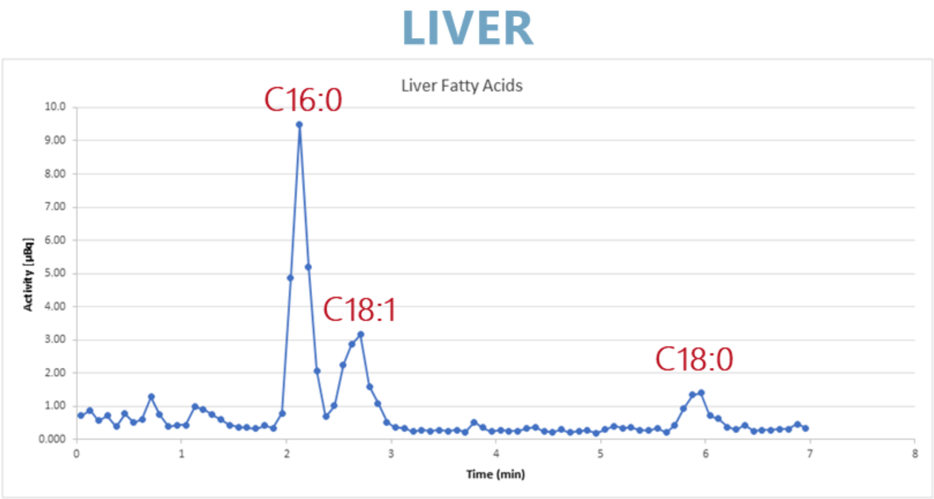


DNL inhibitor firsocostat (inhibits ACC), strongly reduces ^{14}C incorporation in plasma lipid fraction as expected.

De novo lipogenesis

^{14}C fatty acids in plasma and liver reflect de novo lipogenesis activity



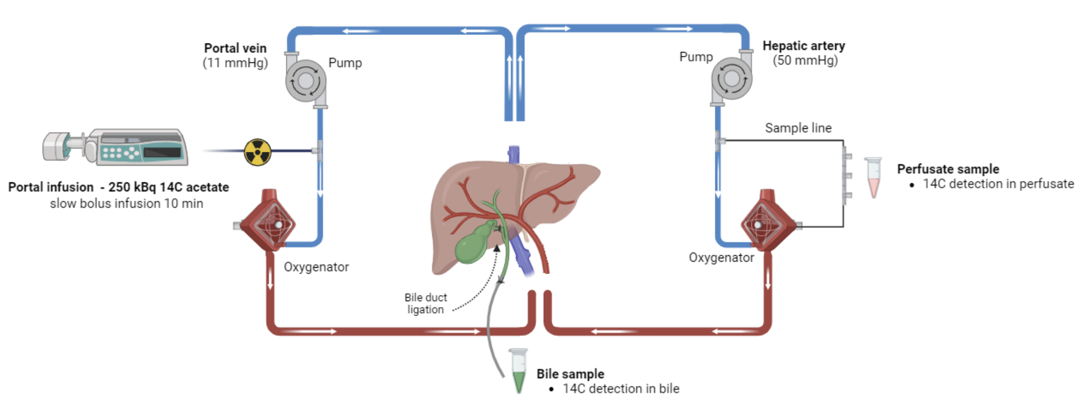


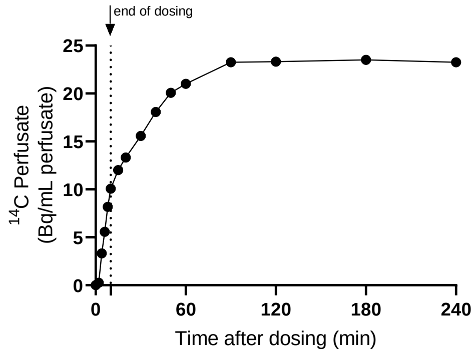
Fatty acid profiling analysis (LC/MS) combined with AMS enrichment analysis showed that the ^{14}C signal from acetate is predominantly found in palmitate (C16:0, the primary end product of DNL) and in lesser amounts also in fatty acids that result from further processing of palmitate (C18:0 and C18:1) 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.

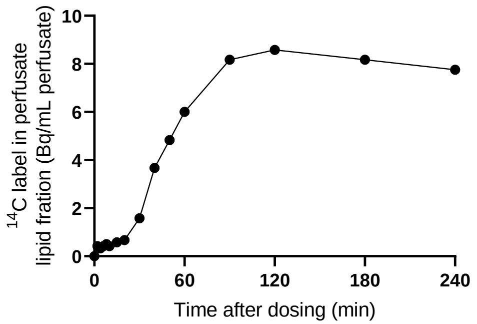
TNO innovation for life

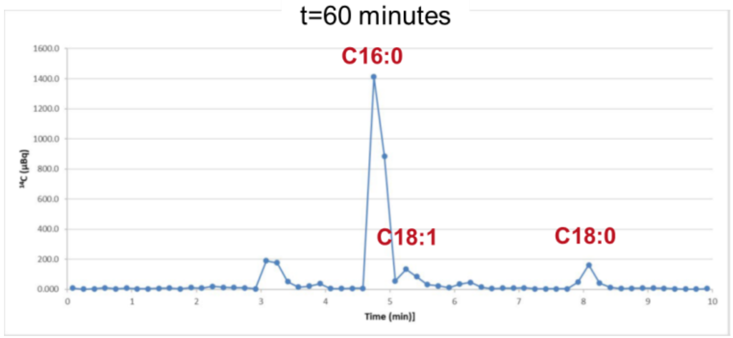
De novo lipogenesis: ex vivo liver perfusion

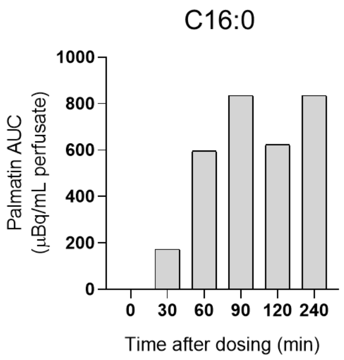
^{14}C from acetate is incorporated into lipid fraction, fatty acids, cholesterol and bile in ex vivo liver

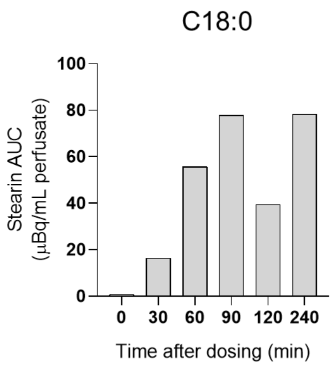


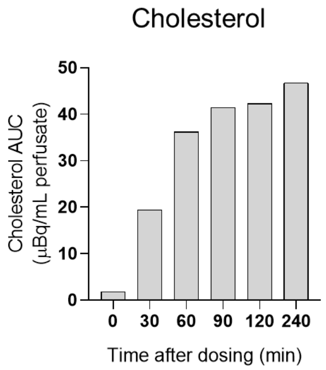


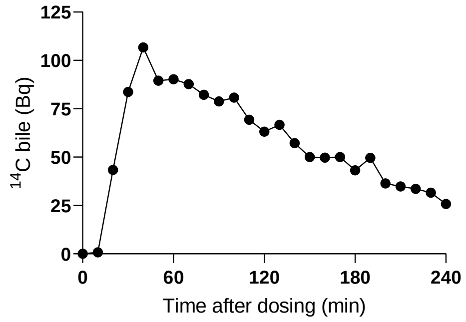












Other application examples (ongoing work)

Preclinical proof of concept studies

- HDL functionality / reverse cholesterol transport
- Muscle protein synthesis & breakdown (combined ^{14}C and D_2O analysis)

First clinical demonstrator

- Glucose metabolism (DNL, conversion to fructose)

HDL functionality

Reverse cholesterol transport:

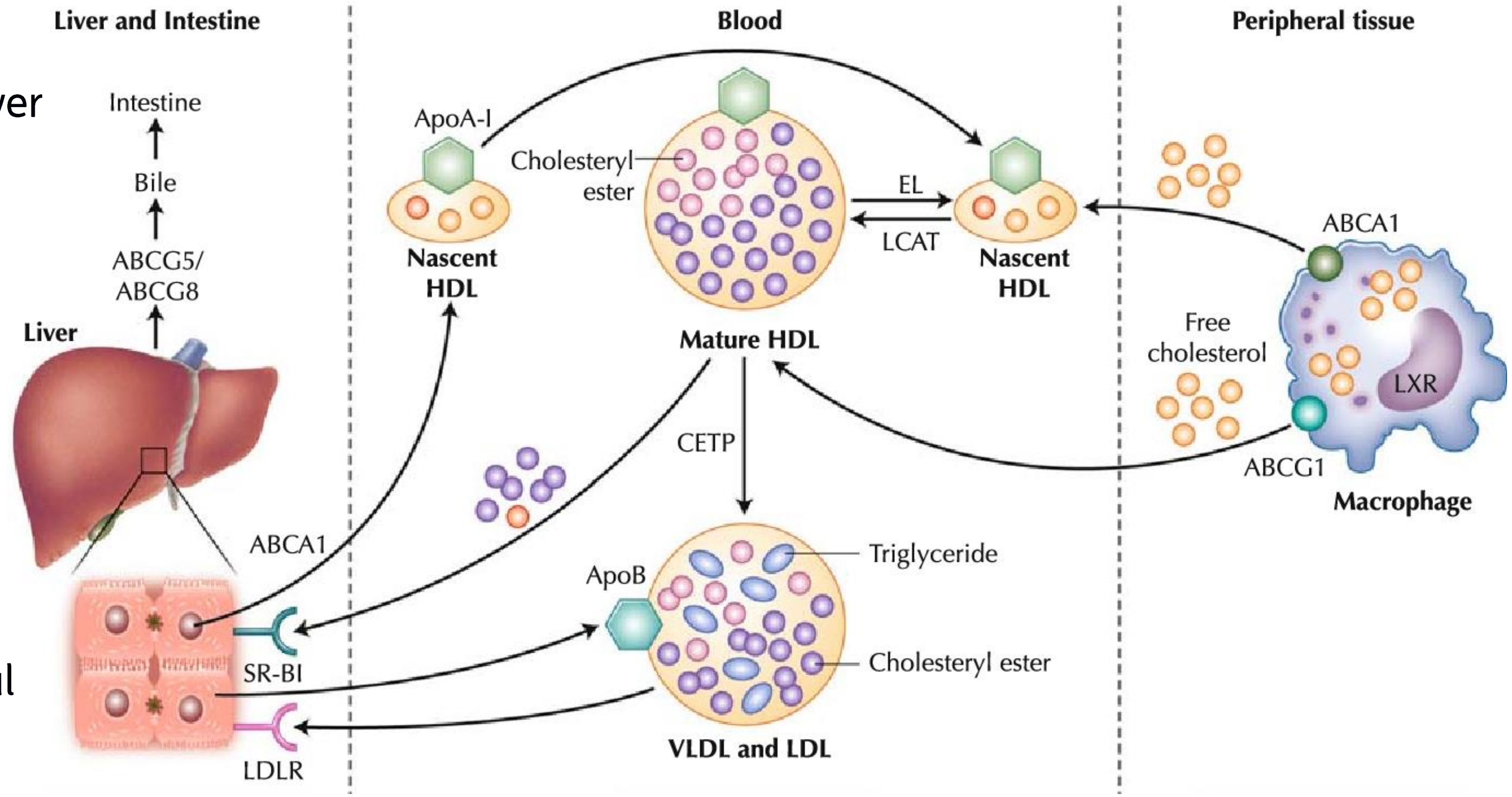
- HDL lipoproteins clear cholesterol from peripheral tissues and return it to the liver to allow its excretion via bile
- Lowers CVD risk

CETP transfers cholesterol from HDL to (V)LDL particles (return to periphery)

- Increases CVD risk

It was long thought that <u>amount</u> of HDL cholesterol was main determinant of CVD risk, now known that <u>functionality</u> is critical

- Functionality can be assessed by flux approach



HDL functionality: flux analysis

Administer ^{14}C-cholesterol nanoparticles

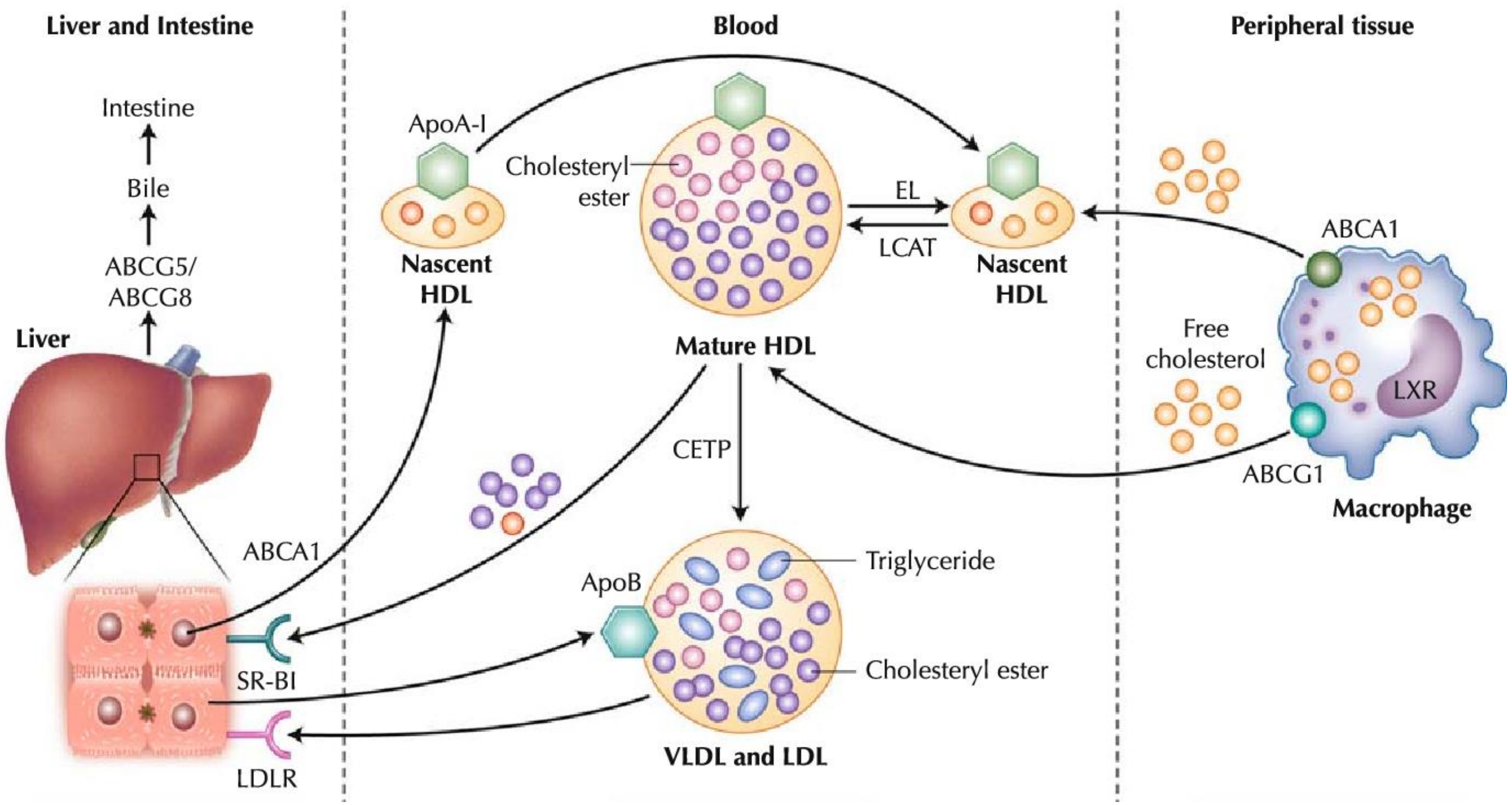
- Taken up by vascular macrophages

Measurement of ^{14}C-cholesterol in:

- Plasma HDL
- Plasma (V)LDL
- Liver
- Feces

Applied in preclinical study

- Analyses ongoing, first results promising!



Muscle protein turnover

Imbalance in protein turnover:

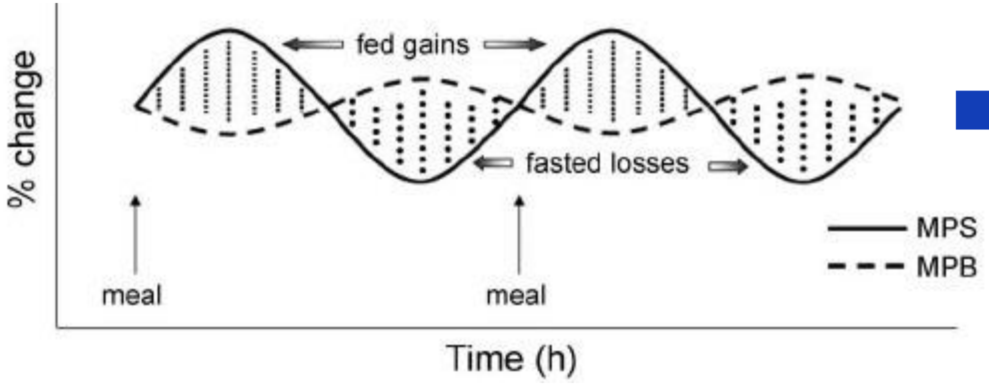
- Reduced muscle protein synthesis
- Increased muscle protein breakdown

Muscle atrophy:

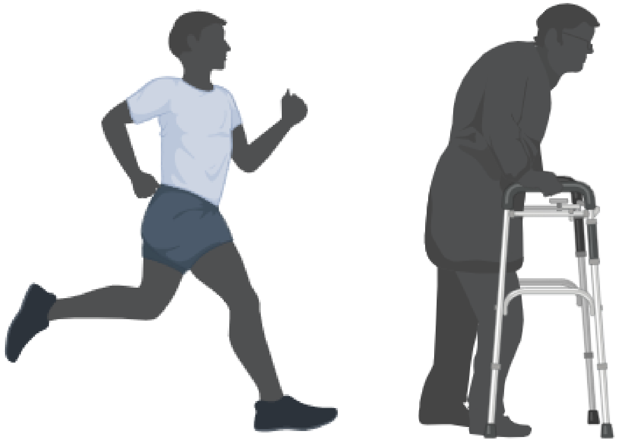
- Loss of muscle mass
- Associated with increased adverse outcomes
- Caused by: immobilisation (e.g. hospital admission), ageing, obesity, cancer (cachexia)

For design & mechanistic understanding of treatments: need to know effects on turnover

- Can be assessed by flux approach



Net effect on muscle mass

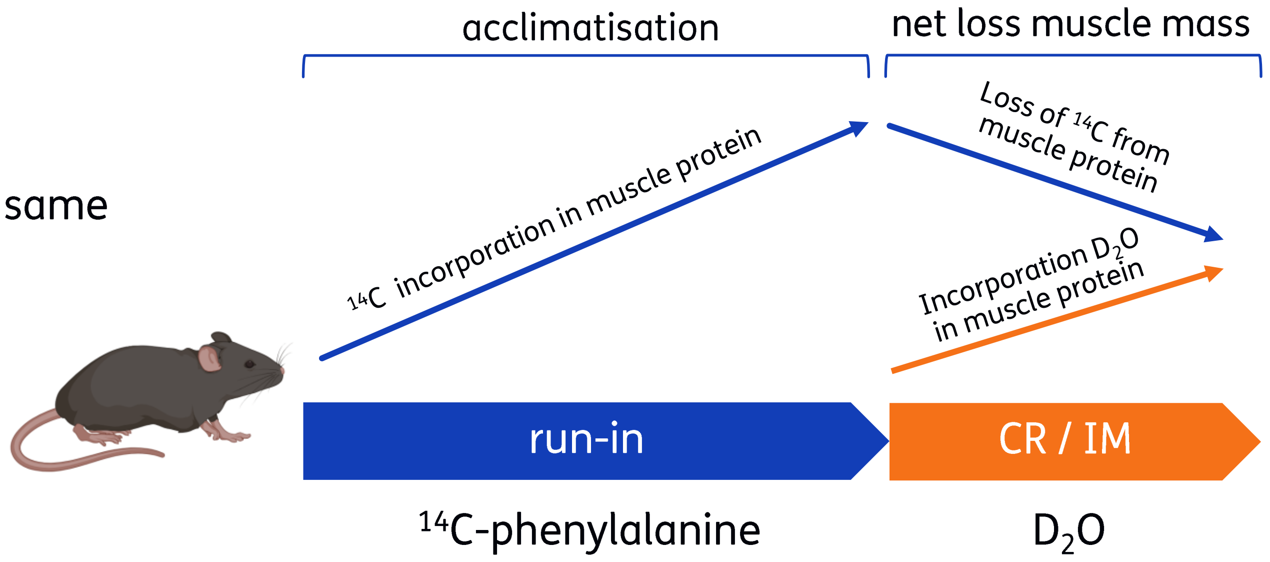


Muscle protein turnover: flux analysis

Flux analysis of muscle protein turnover:

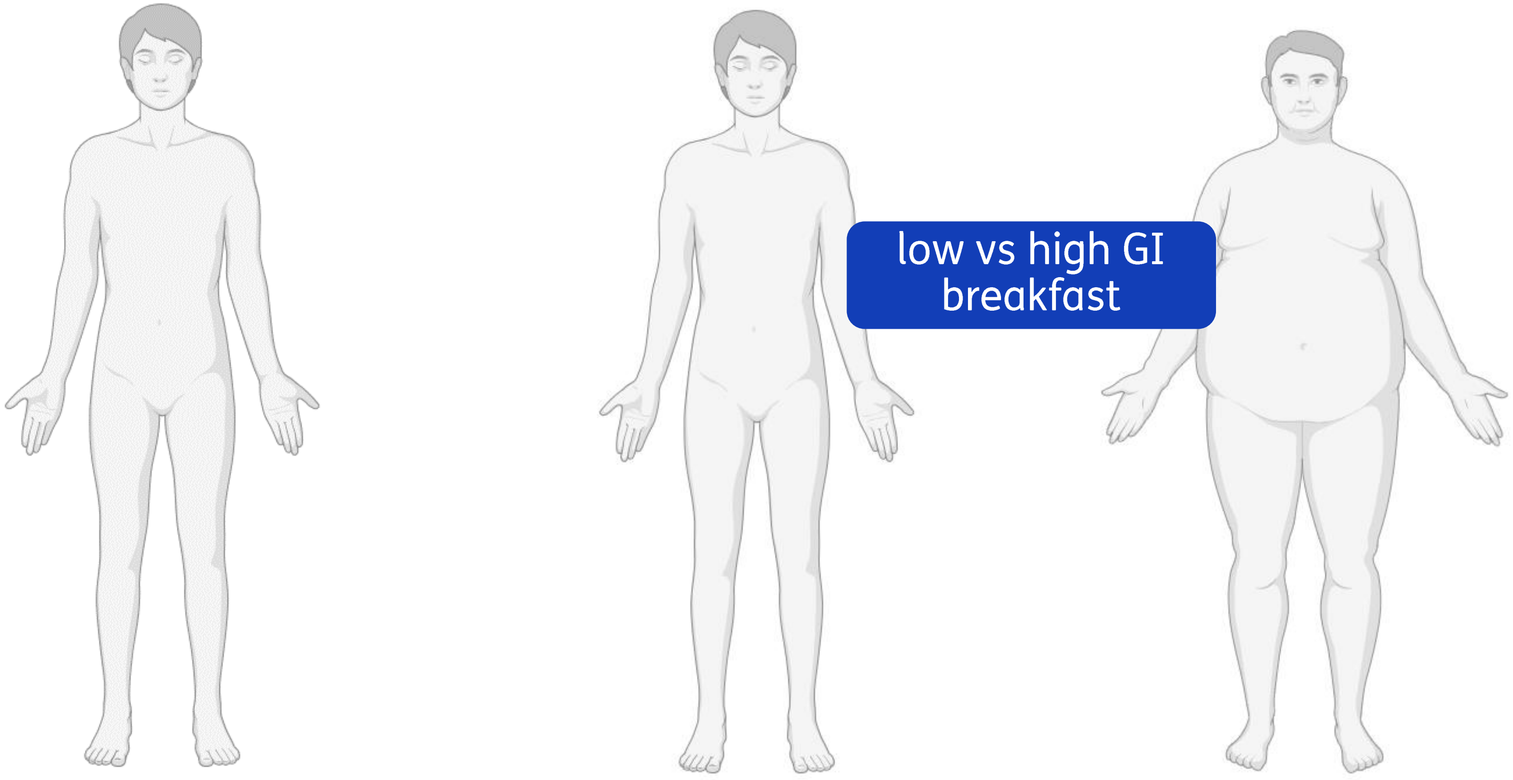
- Combined ^{14}C and D_2O approach
- Allows assessment of synthesis and breakdown in same experiment
- Applied in preclinical study (muscle atrophy in caloric restriction & immobilisation model)

→ data analysis ongoing



Clinical demonstrator

First clinical demonstrator for microtracer flux approach



^{14}C-labelled
low kcal sweetener

^{14}C-Glucose

Low kcal sweetener (vs glucose reference):

- Caloric value (exhaled CO_2)
- Mass balance

Lean vs obese & high vs low GI breakfast

- Conversion glucose → fructose
- *De novo* lipogenesis from glucose
- Forearm glucose disposal (IR)

Also: plasma biobank from ^{14}C-glucose labelled subjects → potential future analyses