# Measuring changes in protein expression by targeted quantitative proteomics with repeated reinterrogation of LC-high resolution full scan MS datasets

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# Two Targeted Methods Used in the Kinter Laboratory

A) Selected Reaction Monitoring (SRM) - ThermoScientific TSQ Quantiva

The best targeted method based on specificity, data quality, and ease of processing

But – Need a triple quadrupole instrument (specialized instrument)

B) High resolution accurate mass Selected Ion Monitoring (HRAM or SIM) - ThermoScientific QExactive Plus

Uses an orbitrap instrument (multipurpose instrument)

Universal detector detecting all ions between m/z 300 to1100,

Data processing harder; not as specific, some challenges with low abundance targets

But – data can be re-interrogated forever, new samples only needed for new validation

Reminders

-All methods digest the proteins to peptides with trypsin

-Selected peptides from each protein are ***Targeted*** as quantitative markers of the parent protein

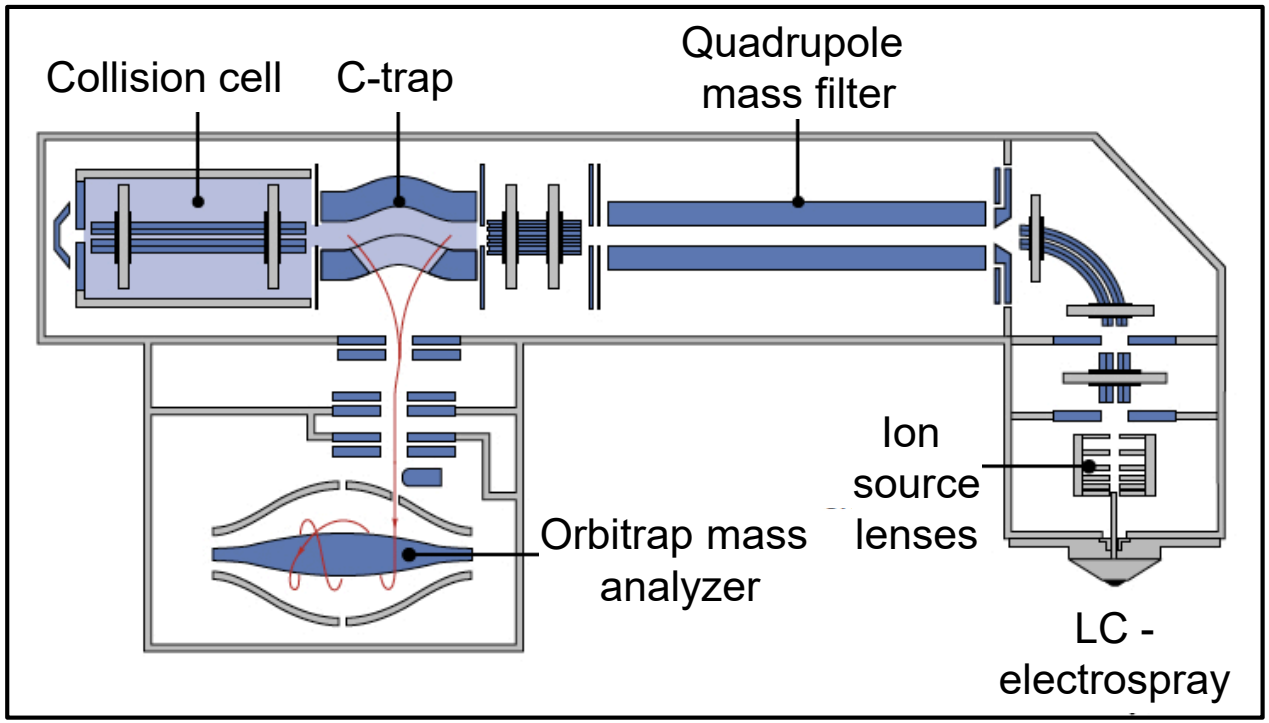
-Liquid chromatography experiments

Retention time plus mass spectrometry is characteristic of identity

Chromatographic peak area is characteristic of amount

-The role of the mass spectrometry is to enhance the ***Specificity*** and ***Sensitivity*** of detection

# High Resolution Accurate Mass Selected Ion Monitoring




Hybrid Quadrupole-Orbitrap Mass Spectrometer

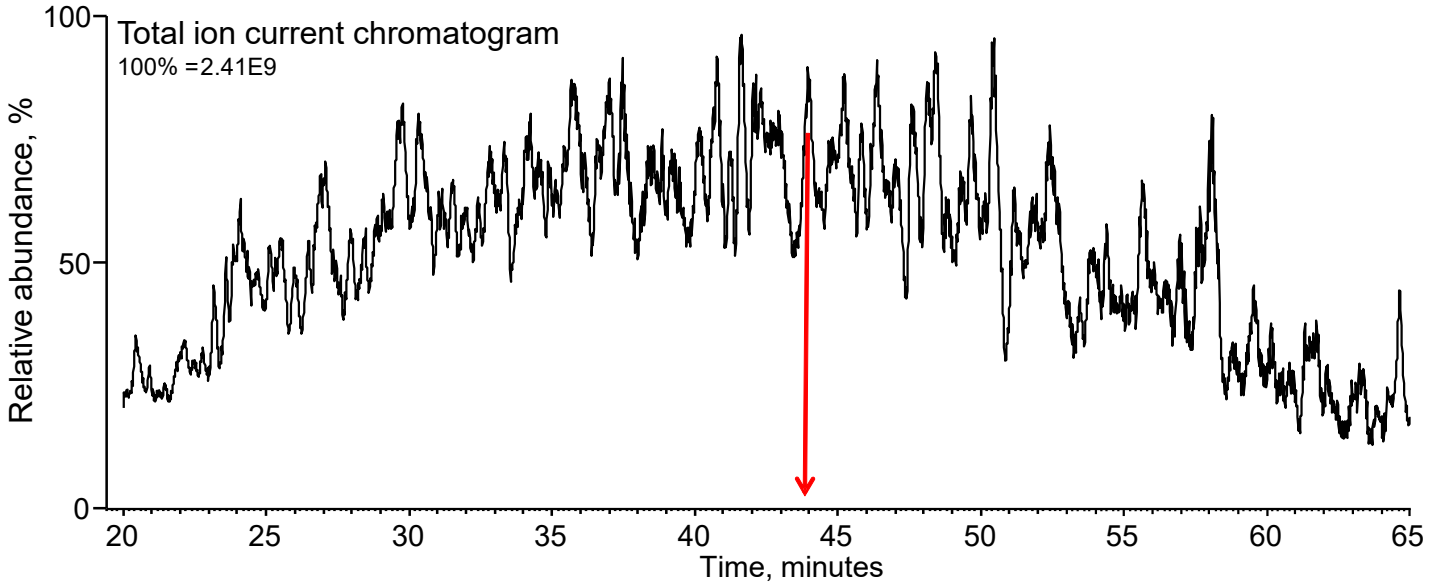
Thermo Scientific QExactive Plus

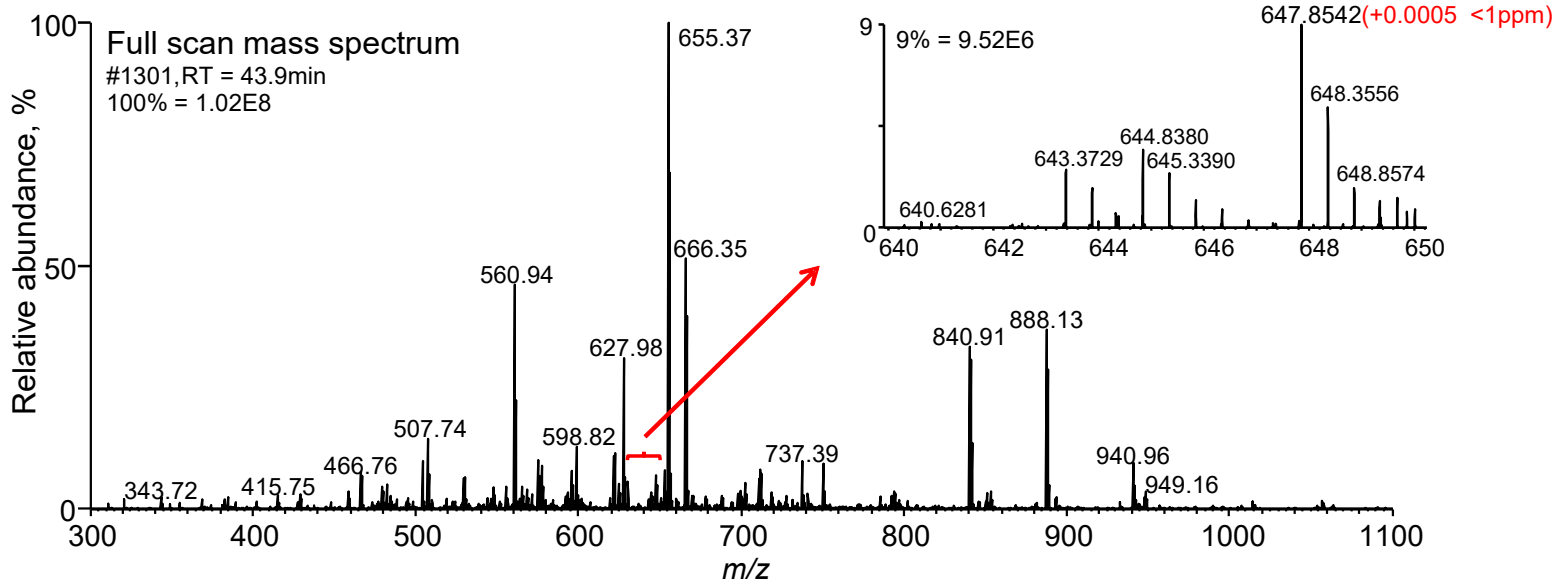
Automated nanoflow C18 capillary column HPLC.
Gradient elution acetonitrile in 0.1% formic acid at a flow rate of 150nL/min.
Full scan mass spectra recorded in the orbitrap from 20-65min. ~80 minute per sample.
Resolution = 280,000, Scanning m/z 300-1100 at 0.9 scan/sec,
2500 total spectra, ~1Mbyte data

Remember: Higher resolution in an orbitrap only requires longer observation times. It *does not discard* ions.

# Universal Detection of All peptides Eluting from the HPLC

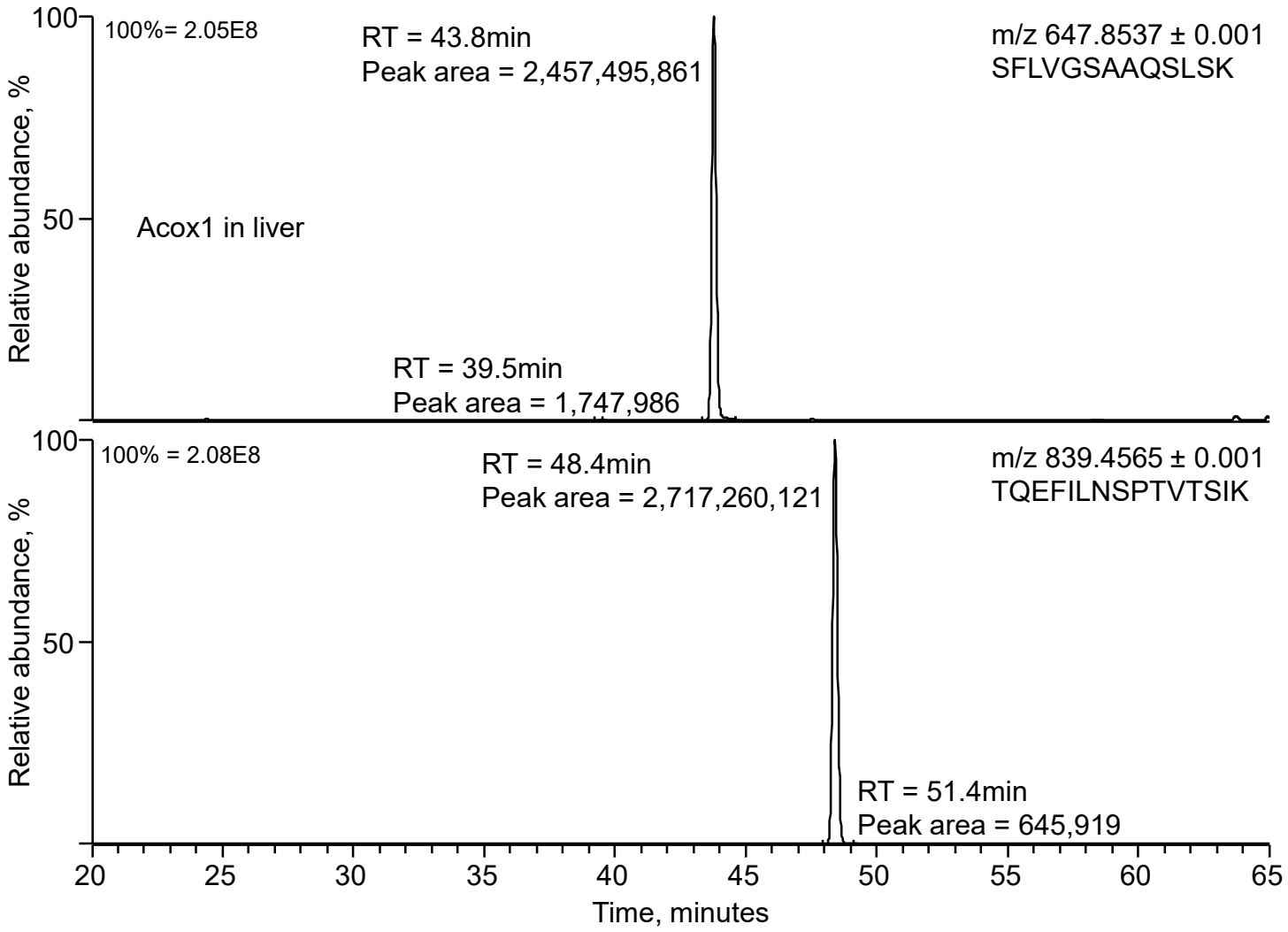
## Mass spectra are used to reconstruct chromatograms



# Specific LC-MS Data is Extracted from the Full Data Set

Specificity: clearly identifiable chromatographic peak unquestionably due to the analyte being measured.



# You Just Need to Know Best *m/z* to Plot (And Validate!!)

Carefully designed based on things we already know about the protein:

1) The amino acid sequence of the protein
2) Databases of detectable peptides
3) Known or calculated retention times
4) mRNA expression data for different tissues to estimate expected amounts

| Acox1 peptides | Peak area, counts | Calculated RT, min | Measured RT, min |
|---|---|---|---|
| NLQAQVSHR | 1,072,395,904 | 26.1 | 24.7 |
| IYDQVQSGK | 1,726,225,792 | 29.5 | 27.0 |
| TEVHESYYK | 994,696,832 | 31.9 | 27.0 |
| A**C**TIAIR | 998,381,376 | 31.9 | 32.0 |
| YAQV**KP**DGTYV**KP**LSNK | 1,232,821,504 | 32.0 | 35.0 |
| AVQAVLR | 875,438,912 | 32.3 | 30.5 |
| LVEIAAK | 1,402,522,368 | 32.8 | 32.0 |
| GLETTATY**DP**K | 1,526,695,680 | 34.2 | 33.7 |
| EIGTHKPLPGITVGDIGPK | 1,644,783,872 | 42.5 | 43.2 |
| DVTLGSVLGR | 803,206,656 | 43.3 | 47.8 |
| **SFLVGSAAQSLSK** | 2,069,009,920 | 44.6 | 44.7 |
| AFTT**W**TANAGIEE**C**R | 502,631,040 | 46.0 | 45.8 |
| GE**C**YGLHAFVVPIR | 1,229,148,032 | 47.3 | 49.7 |
| **TQEFILNSPTVTSIK** | 2,138,268,160 | 47.9 | 49.2 |
| SEPEPQILDFQTQQYK | 952,468,672 | 48.2 | 48.9 |
| TSNHAIVLAQLITR | 1,212,364,800 | 53.1 | 48.5 |
| EVA**W**NLTSVDLVR | 842,518,336 | 55.5 | 55.4 |

Versions:

1. ~20 starting peptides from FASTA sequence
2. Validated underlined finalists
3. Two bold peptides

Validation steps

a) collision induced dissociation spectra
b) detection of expected fragment ions
c) proper retention time

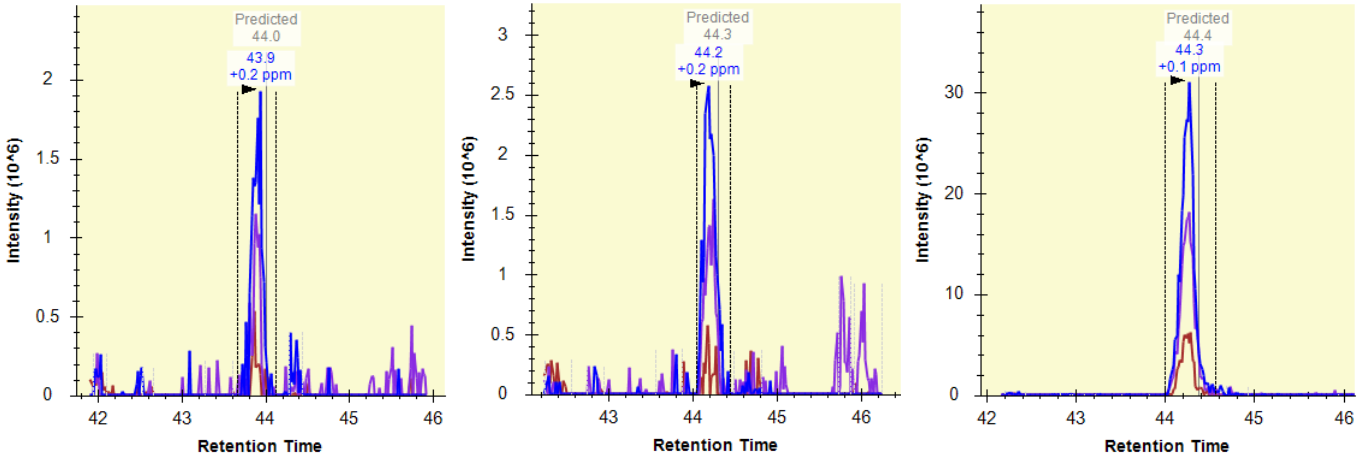
Add to Skyline database of validated peptides and relative retention times

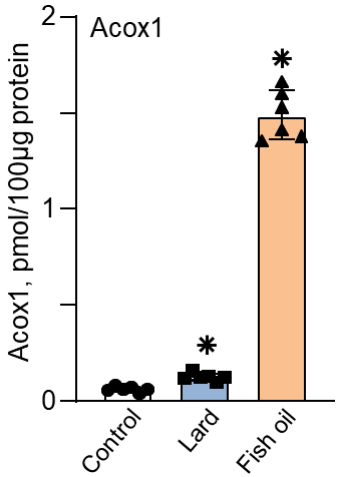
Looking for the best flyers

Estimation of absolute protein quantities of unlabeled samples by selected reaction monitoring mass spectrometry. Ludwig, Claassen, Schmidt, Aebersold. *Mol Cell Proteomics,* 2012

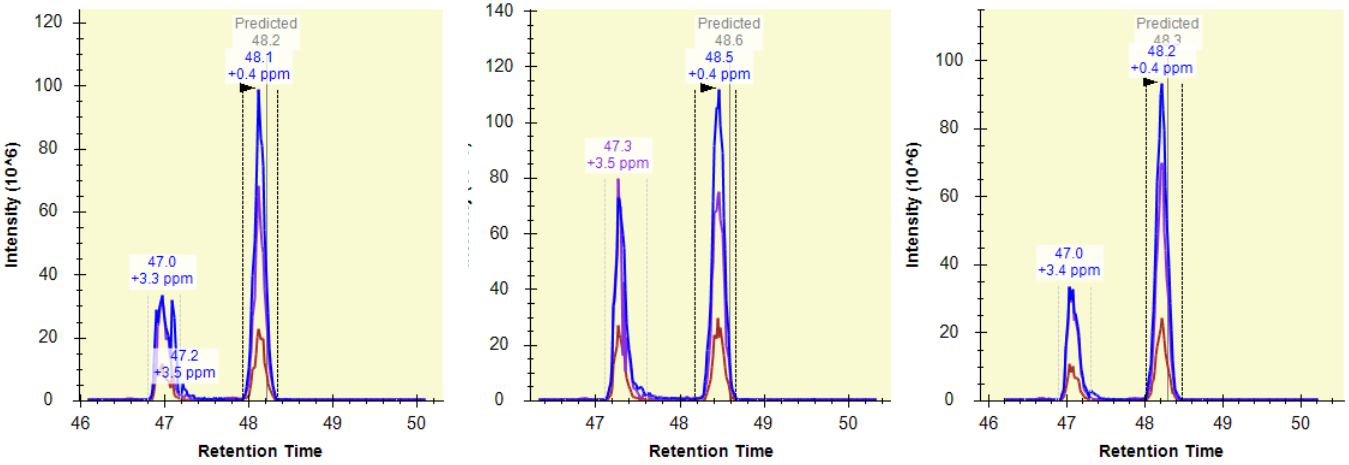
# What Do the Data Look Like?

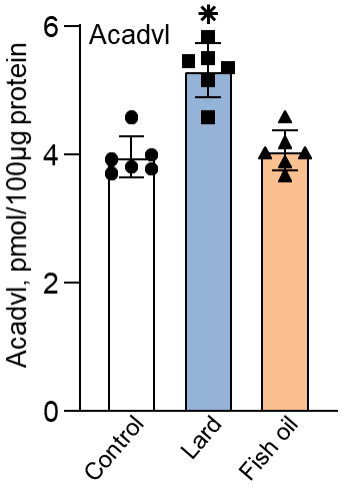
## Acox1 SFLVGSAAQSLSK, m/z 647.8537




## Acadvl GIVNEQFLLQR, m/z 658.8697




Control diet

45% Lard diet

45% Fish Oil diet

# What Do the Data Look Like?

| | x17 s63008 control | x28 s63010 | x16 s63007 | x29 s63011 | x30 s63012 | x18 s63009 | y49 s63017 45% lard | y39 s63015 | y48 s63016 | y34 s63013 | y38 s63014 | y50 s63018 | z70 s63024 45% fish oil | z57 s63020 | z58 s63021 | z56 s63019 | z69 s63023 | z68 s63022 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Bsa in at 8pmol/60µg total protein | | | | | | | | | | | | | | | | | | |
| LVTDLTK | 3.888E+09 | 4.256E+09 | 4.362E+09 | 4.242E+09 | 4.404E+09 | 3.673E+09 | 4.028E+09 | 4.146E+09 | 3.782E+09 | 4.710E+09 | 4.032E+09 | 3.821E+09 | 3.580E+09 | 3.970E+09 | 4.193E+09 | 4.078E+09 | 3.993E+09 | 3.890E+09 |
| YLYEIAR | 4.756E+09 | 4.714E+09 | 4.923E+09 | 4.852E+09 | 5.149E+09 | 4.445E+09 | 5.097E+09 | 5.043E+09 | 4.569E+09 | 4.574E+09 | 4.658E+09 | 4.978E+09 | 4.854E+09 | 5.272E+09 | 4.900E+09 | 4.493E+09 | 4.917E+09 | 4.362E+09 |
| LVNELTEFAK | 5.194E+09 | 5.641E+09 | 5.915E+09 | 5.348E+09 | 5.648E+09 | 5.482E+09 | 5.766E+09 | 5.989E+09 | 5.119E+09 | 5.437E+09 | 5.532E+09 | 5.309E+09 | 5.917E+09 | 5.815E+09 | 5.143E+09 | 4.681E+09 | 5.410E+09 | 5.224E+09 |
| geomean | 4.580E+09 | 4.837E+09 | 5.027E+09 | 4.793E+09 | 5.040E+09 | 4.473E+09 | 4.910E+09 | 5.003E+09 | 4.456E+09 | 4.893E+09 | 4.701E+09 | 4.657E+09 | 4.685E+09 | 4.956E+09 | 4.728E+09 | 4.410E+09 | 4.736E+09 | 4.459E+09 |
| | | | | | | | | | | | | | | | | | | |
| Acox1 | | | | | | | | | | | | | | | | | | |
| SFLVGSAAQSLSK | 2.253E+07 | 2.497E+07 | 1.619E+07 | 2.057E+07 | 2.755E+07 | 2.899E+07 | 4.517E+07 | 5.922E+07 | 4.318E+07 | 4.042E+07 | 3.278E+07 | 4.512E+07 | 4.984E+08 | 5.212E+08 | 4.992E+08 | 4.669E+08 | 5.537E+08 | 5.093E+08 |
| TQEFILNSPTVTSIK | 1.998E+07 | 2.066E+07 | 1.633E+07 | 2.036E+07 | 2.684E+07 | 2.521E+07 | 4.991E+07 | 6.028E+07 | 4.148E+07 | 4.782E+07 | 3.740E+07 | 4.299E+07 | 5.815E+08 | 5.073E+08 | 5.043E+08 | 4.319E+08 | 5.859E+08 | 6.092E+08 |
| geomean | 2.122E+07 | 2.271E+07 | 1.626E+07 | 2.047E+07 | 2.719E+07 | 2.703E+07 | 4.748E+07 | 5.975E+07 | 4.232E+07 | 4.397E+07 | 3.501E+07 | 4.404E+07 | 5.384E+08 | 5.142E+08 | 5.017E+08 | 4.491E+08 | 5.695E+08 | 5.570E+08 |
| pmol/100µg total P | 0.062 | 0.062 | 0.043 | 0.057 | 0.072 | 0.080 | 0.129 | 0.159 | 0.126 | 0.120 | 0.099 | 0.126 | 1.529 | 1.380 | 1.412 | 1.354 | 1.600 | 1.662 |
| | | | | | | | | | | | | | | | | | | |
| | average | 0.063 | | | | | average | 0.126 | | | | | average | 1.49 | | | | |
| | sd | 0.013 | | | | | sd | 0.019 | | | | | sd | 0.13 | | | | |
| | rsd | 20% | | | | | rsd | 15% | | | | | rsd | 8% | | | | |
| | | | | | | | r/control | 2.02 | | | | | r/control | 23.8 | | | | |
| | | | | | | | ttest | 9.8E-05 | | | | | ttest | 9.5E-07 | | | | |
| Acadvl | | | | | | | | | | | | | | | | | | |
| IFEGANDILR | 1.262E+09 | 1.410E+09 | 1.411E+09 | 1.406E+09 | 1.447E+09 | 1.524E+09 | 2.075E+09 | 1.963E+09 | 1.716E+09 | 1.667E+09 | 1.744E+09 | 1.848E+09 | 9.502E+08 | 8.587E+08 | 1.072E+09 | 8.724E+08 | 9.480E+08 | 9.087E+08 |
| GIVNEQFLLQR | 1.288E+09 | 1.439E+09 | 1.437E+09 | 1.470E+09 | 1.435E+09 | 1.556E+09 | 2.232E+09 | 2.143E+09 | 1.875E+09 | 1.695E+09 | 1.893E+09 | 2.003E+09 | 2.286E+09 | 2.179E+09 | 2.474E+09 | 1.902E+09 | 2.159E+09 | 2.002E+09 |
| geomean | 1.275E+09 | 1.424E+09 | 1.424E+09 | 1.438E+09 | 1.441E+09 | 1.540E+09 | 2.152E+09 | 2.051E+09 | 1.794E+09 | 1.681E+09 | 1.817E+09 | 1.924E+09 | 1.474E+09 | 1.368E+09 | 1.629E+09 | 1.288E+09 | 1.431E+09 | 1.349E+09 |
| pmol/100µg total P | 3.70 | 3.92 | 3.77 | 3.99 | 3.80 | 4.58 | 5.83 | 5.45 | 5.35 | 4.57 | 5.14 | 5.49 | 4.18 | 3.67 | 4.58 | 3.89 | 4.02 | 4.02 |
| | | | | | | | | | | | | | | | | | | |
| | average | 3.96 | | | | | average | 5.31 | | | | | average | 4.06 | | | | |
| | sd | 0.32 | | | | | sd | 0.43 | | | | | sd | 0.31 | | | | |
| | rsd | 8% | | | | | rsd | 8% | | | | | rsd | 8% | | | | |
| | | | | | | | r/control | 1.34 | | | | | r/control | 1.026 | | | | |
| | | | | | | | ttest | 1.4E-04 | | | | | ttest | 0.590 | | | | |

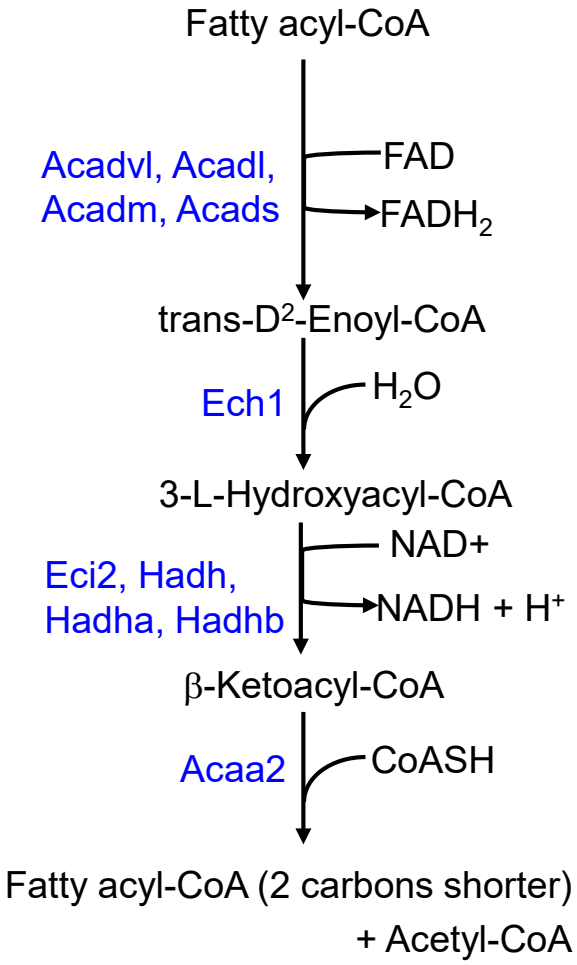
-Statistically significant differences at 30%

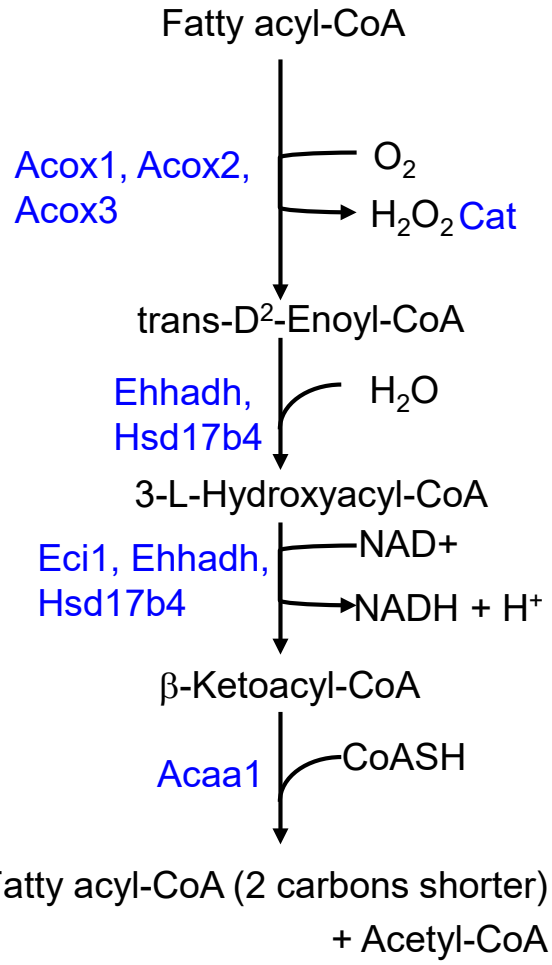
-Acox1 is low abundance protein in the heart

for comparison Gapdh = 30pmol/100µg total protein (~500x measured in the same run)

-18 samples = 25hrs LC-MS time + data analysis time

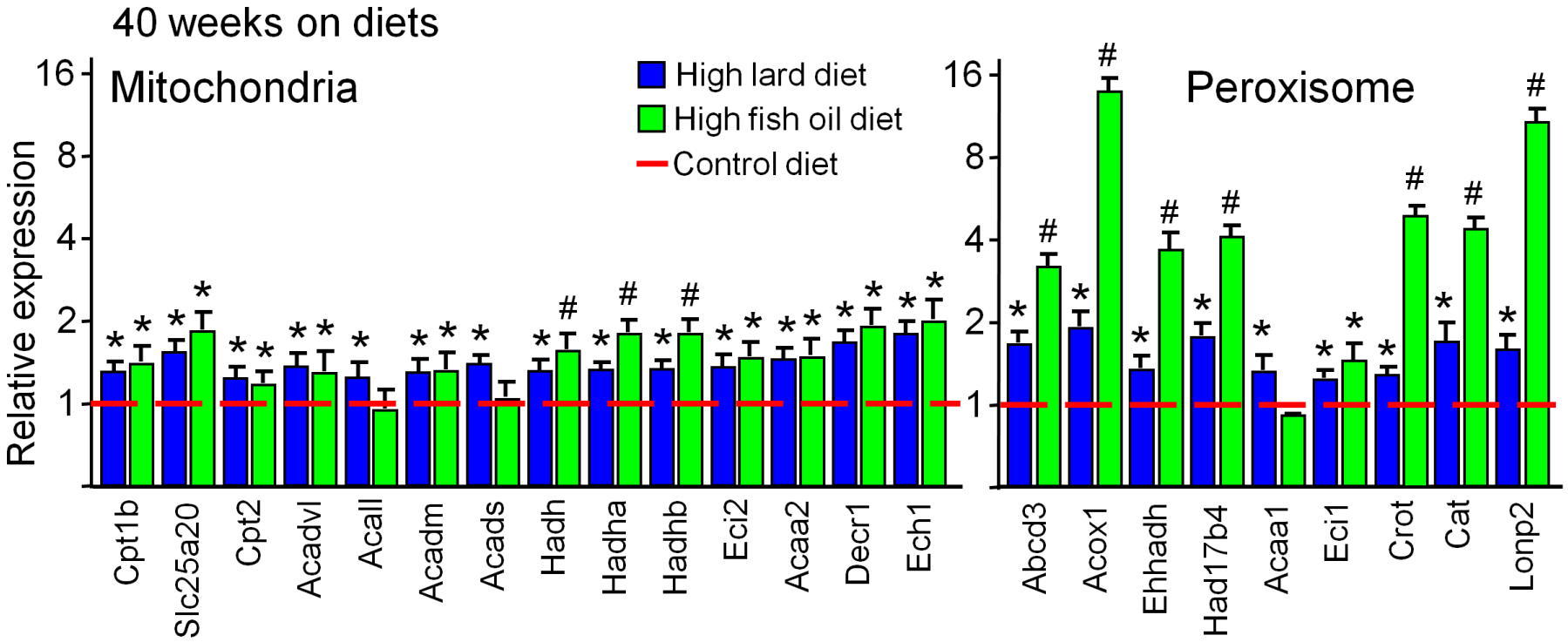
# What about Lipid Metabolism?
# A Panel of Assays for Both Mitochondrial and Peroxisomal Beta-Oxidation is Readily Built




Acetyl-CoA → Krebs cycle

# Peroxisomal Beta Oxidation is Highly Inducible with the High Fish Oil Diet

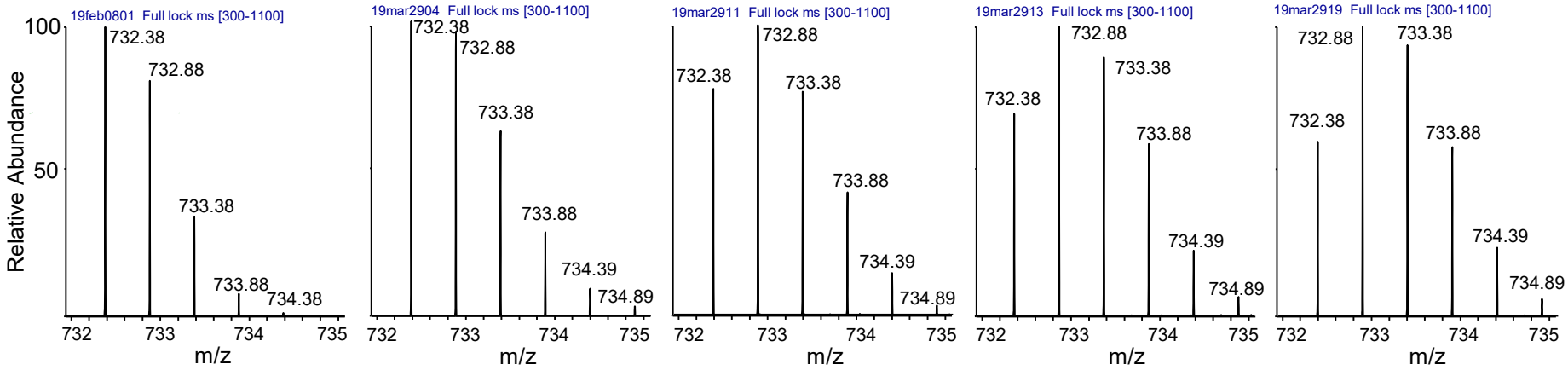


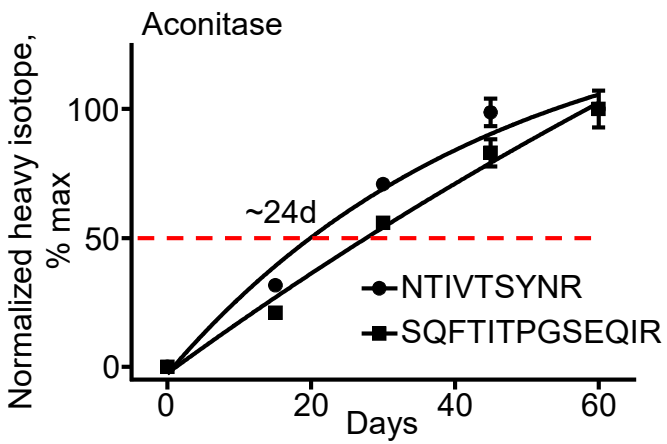
# Targeted Methods Used to Measure Protein Synthesis Rates

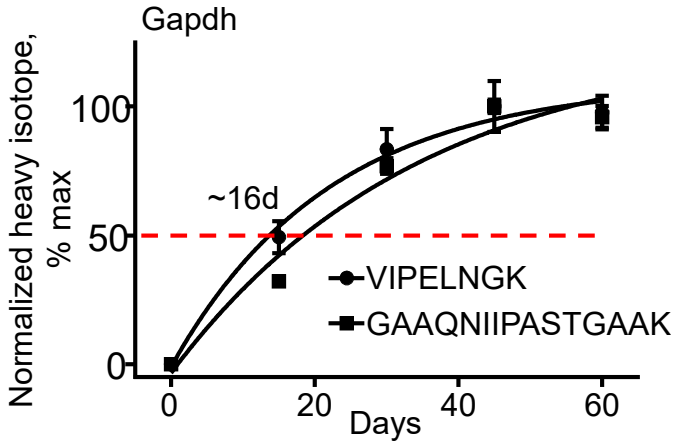
Deuterium labeling of newly synthesized protein

Priming dose of $D_2O$ (8% body water) then constant 4% $D_2O$ in drinking water (mike's calculations, about $10 per mouse per month)

## Aconitase peptide SQFTITPGSEQIR

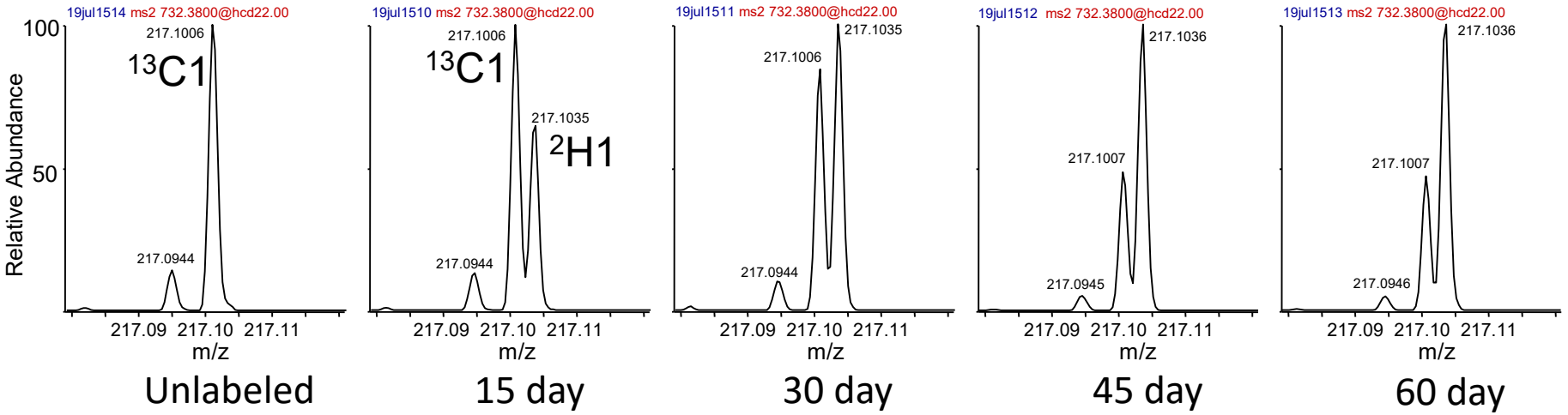


# We are Developing Parallel Reaction Monitoring (PRM) for a Clearer Look at Deuterium Incorporation

Aconitase peptide SQFTITPGSEQIR, b2 = 216.1



# Summary and Future Directions

High resolution of the orbitrap mass spectrometer produces a dataset with huge potential for targeted quantitative analyses

Overall goal is to expand discovery capabilities of our targeted approach by dramatically increasing the number of assays and panels

Currently have about 2000 validated peptides (~400 proteins) with accurate m/z tags + relative retention times in our database

- On-going project to add 600 new mitochondrial proteins
- Follow-up with addition of peroxisomal proteins (200?)
- Make better use of the results of other collaborations to routinely add those results to our system

In the longer term, we would like to develop processing tools for a more automated system to efficiently query these large sets of proteins for quantitative measurements

*Questions??*