DIAGNOSIS OF FATTY ACID OXIDATION DISORDERS BY MASS SPECTROMETRY

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Outline of talk

- Basic of mitochondrial fatty acid oxidation (FAO)
- Laboratory investigations of FAOD
- New combined FAO rate and probe assay
Fatty acids

- **Short-chain**: 2 to 4 carbon atoms
- **Medium-chain**: 6 to 12 carbon atoms
- **Long-chain**: 14 to 18 carbon atoms
- **Very long-chain**: 20 to 26 carbon atoms
# Nomenclature of Fatty Acids

<table>
<thead>
<tr>
<th>Trivial Name</th>
<th>IUPAC Name</th>
<th>Carboxyl-Reference</th>
<th>ω-Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>palmitic acid</td>
<td>hexadecanoic acid</td>
<td>16:0</td>
<td>16:0</td>
</tr>
<tr>
<td>stearic acid</td>
<td>octadecanoic acid</td>
<td>18:0</td>
<td>18:0</td>
</tr>
<tr>
<td>oleic acid</td>
<td>9-octadecenoic acid</td>
<td>18:1 Δ⁹</td>
<td>18:1 (ω-9)</td>
</tr>
<tr>
<td>linoleic acid</td>
<td>9,12-octadecenoic acid</td>
<td>18:2 Δ⁹,¹²</td>
<td>18:2 (ω-6)</td>
</tr>
<tr>
<td>linolenic acid</td>
<td>9,12,15-octadecenoic acid</td>
<td>18:3 Δ⁹,¹²,¹⁵</td>
<td>18:3 (ω-3)</td>
</tr>
</tbody>
</table>
Incidence of FAOD

- CUD: 1 in ~40,000 (Japanese, similar to ~100,000 in other countries)
- MCAD: 1 in ~10,000 to 20,000
- VLCAD: 1 in ~75,000
- LCHAD: 1 in ~75,000
- TFP: 1 in ~100,000
- Others: less than 1 in ~100,000

All FAOD: 1 in ~14,000 to as high as 1 in ~4000

Congenital Hypothyroidism: 1 in ~3300

PKU: 1 in ~16,000

American College of Medical Genetics. *Newborn Screening: Toward a Uniform Screening Panel and System*. Final Report, March 8, 2005
Approach to investigations of FAOD

• Pre-symptomatic screening through expanded newborn screening programs
• Laboratory investigations of symptomatic patients
• Prenatal diagnosis
• Postmortem diagnosis
Combined oxidation rate and acylcarnitine profiling into one culture experiment
Methodologies

- Skin fibroblast cultures fed with $^{2}\text{H}_{31}$-palmitate
- Measurement of deuterium labeled acylcarnitines in culture medium using an ESI-MS-MS method
- Measurement of deuterated water (DHO) formation resulted from overall mitochondrial fatty acid β-oxidation using an isotope ratio mass spectrometer
Skin fibroblasts are cultured until confluence is reached.

Subculture into culture plate

Leave to adhere overnight

Replace with medium contained Deuterium labelled fatty acids and L-carnitine

72 to 96 hours

Analyze deuterium labelled acylcarnitine species by MS/MS
Fig. 1. Box & whisker plot showing the percentages of control median of deuterated water enrichment (2H2O) in control, FAOD and other IMD cell lines incubated at 37°C in humidified 5% CO2 and 95% air for 96 hours with 0.2 mM 2H31-palmitate and 0.4 mM L-carnitine in DMEM no glucose, without serum supplemented, in the presence of 0.4% defatted BSA and 2 mM L-glutamine. Red line shows 60% of the control median (60.9 ppm/mg protein/96 h).

Biomedical Mass Spectrometry Laboratory, CUHK
**Control at 0 hour, in d31-palmitate**

Internal standards:
- 221 (d3-acetyl-);
- 249 (d3-butyryl-);
- 305 (d3-octanoyl-);
- 361 (d3-dodecanoyl-);
- 418 (d3-palmitoyl-)

(methyl acylcarnitine esters)
Mass spectra of typical acylcarnitine profiles using 2H31-palmitate as substrate for feeding cultured fibroblasts incubated at 37°C in humidified 5% CO2 and 95% air for 96 hours with 0.5 mM L-carnitine in DMEM no glucose, without serum supplemented, in the presence of 0.4% defatted BSA and 2 mM L-glutamine. **Diagnostic intermediate acylcarnitines found in each FAOD are bold.** Control skin fibroblast cell line; C3-, propionyl-; C4:0-, butyryl-; C6:0-, hexanoyl-; C8:0-, octanoyl-; C10:0-, decanoyl-; C12:0-, dodecanoyl-; C16:0-, palmitoyl-.
CUD at 96 hour, in $d_{31}$-palmitate

Internal standards:
- 221 ($d_3$-acetyl-)
- 249 ($d_3$-butyryl-)
- 305 ($d_3$-octanoyl-)
- 361 ($d_3$-dodecanoyl-)
- 418 ($d_3$-palmitoyl-)

(methyl acylcarnitine esters)
**CPT-ID** at 96 hour, in $d_{31}$-palmitate

Internal standards:
- 221 ($d_3$-acetyl-)
- 249 ($d_3$-butyryl-)
- 305 ($d_3$-octanoyl-)
- 361 ($d_3$-dodecanoyl-)
- 418 ($d_3$-palmitoyl-)

(methyl acylcarnitine esters)
S192 Pal car A2 96h
ACYLH1946 1 (4.003) Sm (Mn, 2x0.75); Sb (1.40.00)

CPT-IID/CACTD at 96 hour, in d$_{31}$-palmitate

Internal standards:
221 (d$_{3}$-acytethyl-);
249 (d$_{3}$-butyryl-);
305 (d$_{3}$-octanoyl-);
361 (d$_{3}$-dodecanoyl-);
418 (d$_{3}$-palmitoyl-)
(methyl acylcarnitine esters)
**SCHADD** at 96 hour, in d$_{31}$-palmitate

Internal standards:
- 221 (d$_3$-acetyl-)
- 249 (d$_3$-butyryl-)
- 305 (d$_3$-octanoyl-)
- 361 (d$_3$-dodecanoyl-)
- 418 (d$_3$-palmitoyl-)

(methyl acylcarnitine esters)
Internal standards:
221 (d₃-acetyl-);
249 (d₃-butyryl-);
305 (d₃-octanoyl-);
361 (d₃-dodecanoyl-);
418 (d₃-palmitoyl-)
(methyl acylcarnitine esters)
VLCADD at 96 hour, in d$_{31}$-palmitate

Internal standards:
- 221 (d$_3$-acetyl-);
- 249 (d$_3$-butyryl-);
- 305 (d$_3$-octanoyl-);
- 361 (d$_3$-dodecanoyl-);
- 418 (d$_3$-palmitoyl-)

(methyl acylcarnitine esters)
LCHADD/TFPD at 96 hour, in d_{31}-palmitate

Internal standards:
221 (d_{3}-acetyl-);
249 (d_{3}-butyryl-);
305 (d_{3}-octanoyl-);
361 (d_{3}-dodecanoyl-);
418 (d_{3}-palmitoyl-)
(methyl acylcarnitine esters)
**Strengths and weaknesses**

- All FAO disorders studied could be differentiated from the control and non-FAOD IMD groups using the combined functional assay.
- The present method requires only one culture experiment, is relatively simple and non-radioactive.
- Acylcarnitine profile is similar between CUD and control, however total amount of acylcarnitine intermediates produced can be used as a specific marker for CUD.
- Identical profiles observed between CACT and CPT-II or LCHAD and MTP (alternative substrates needed, Roe DS et al Mol Genet Metab 2006).
- Not many laboratories equipped with cell culture facilities, isotope ratio mass spectrometer and ESI-MSMS.
Carnitine deficiency, systemic primary CDSP or carnitine uptake defect CUD

- CUD is the most common FAOD in Japanese, and probably also in Chinese
- recurrent hypoketotic hypoglycaemia encephalopathy, failure to thrive, sudden death
- carnitine-responsive cardiomyopathy with or without weakness
- very low serum and tissue carnitine concentrations, decrease renal reabsorption of carnitine
- laboratory investigations: serum and urine carnitine, radioactive carnitine uptake assay in fibroblast culture and OCTN2 mutation analysis
Differentiation of carnitine uptake defect from normal control and other FAOD based on total acylcarnitines production in cultured cells

- 2 (HK)
- 1 (Germany)
- 1 (Netherlands)
- 3 (Taiwan)
- 2 (Genetic Repository, Coriell)*
- 22 (control human fibroblasts)
- 10 (other FAOD fibroblasts)
Oxidation of d31-palmitate in Human Skin Fibroblast Cultures

Mann-Whitney U test  p<0.05
Acylcarnitine Profiles in Human Skin Fibroblast Culture Medium

Mean acylcarnitine intermediates (nmol/mg protein/96h)

- C4:0-carnitine
- C6:0-carnitine
- C8:0-carnitine
- C10:0-carnitine
- C12:0-carnitine
- C14:0-carnitine
- C16:0-carnitine

Control

CUD

Asian Pacific Conference of Chromatography & Mass Spectrometry
Total Acylcarnitines in Human Skin Fibroblast Culture Medium

95% CI Mean of total acylcarnitine intermediates

Mann-Whitney U test  p<0.001

Conclusions

• Total amount of acylcarnitine intermediates produced (C4:0 to C16:0 even-chain acylcarnitines) under the standardized culture conditions can be used as a specific marker for CUD.

• This method is relatively simple and may replace the radio-active uptake assay for the diagnosis of carnitine uptake defect.
Clinical symptoms suggestive of FAOD

Routine biochemistries: ↑ NH₃, LFT, CK, lactate, ↓ glucose

Urine

Organic acids, acylglycines

Free carnitine, acylcarnitines

Abnormal and characteristic

Confirmed by tissue enzyme assay or mutation study

In vitro fibroblast functional assay – combined FAO rate and AC profiling

Amniotic fluid

Inconclusive

Prenatal Diagnosis

SIDS

Normal AC profile, TC>9.3 & ²H₂O enrichment>55

FAOD unlikely

Normal AC profile, TC<9.3 & ²H₂O enrichment<109

Respiratory chain or non-FAO disorders

Confirmed by tissue enzyme assays or mutation study

Unidentified or new disorders

Normal AC profile, ²H₂O enrichment<55

Defects: CPT-1, CPT-II/CACT, MAD, SCAD, MCAD, VLCAD, LCHAD/TFP, SCHAD

Confirmed by tissue enzyme assays or mutation study

No

Normal AC profile, TC>9.3 & ²H₂O enrichment<55

CUD, confirmed by mutation study of OCTN2

No

Normal AC profile, TC>9.3 & ²H₂O enrichment>55

Unidentified or new disorders

Urine Serum/plasma/DBS

Prenatal Diagnosis

Amniotic fluid

SIDS
Acknowledgments

Thank you for your attention

- Laboratory for Genetic Metabolic Diseases, Academic Medical Centre, The Netherlands
- Prof RJA Wanders
- Dr J Ruiter
CACT (Carnitine:Acylcarnitine Translocase) deficiency

- a mitochondrial carrier protein as one of the components of the carnitine cycle
- catalyzes a reversible exchange of carnitine and acylcarnitine across the mitochondrial membranes
- typical presentations include seizures, progressive liver and/or heart failure, coma
- hypoketotic hypoglycaemia, hyperammonemia, lactic acidosis, elevated CK and transaminases
- dicarboxylic aciduria
- low free and total plasma carnitine, increase in acylcarnitines to free carnitine ratio
- high plasma long chain acylcarnitines concentration
Prenatal diagnosis for a family with a case of CACT deficiency

• The proband was a male baby who died suddenly on day one after birth

• Postmortem examination was suggestive of FAOD, mutation analysis confirmed a diagnosis of CACT deficiency

• Aminotic fluid was received during a second pregnancy
Oxidation of D31-palmitate in Human Aminocyte Cultures

Mean ± 2 SE Enrichment (ppm/mg protein/96h)

Control

Fetus

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A column chart titled "Acylcarnitine profiles in human aminocyte culture medium" shows the mean of acylcarnitine intermediates (nmol/mg protein/96 h) for Control and Fetus (prenatal) groups. The chart compares different acylcarnitine species, including PALC4, PALC6, PALC8, PALC10, PALC12, PALC14, and PALC16.
Conclusions

- Combined functional assay for FAO revealed normal FAO rate and unremarkable acylcarnitine profile
- The fetus was unaffected
- Mutation analysis confirmed a carrier status of CACT deficiency