Post mortem investigation of Inherited Metabolic Disease
- the last opportunity for a diagnosis -

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SIDS/SUDI

- Incidence 1:1000 live births
- 25% of deaths in the first year of life
- Precise cause remains unexplained in ~80% of cases
- 3-6% due to inherited metabolic disease
Metabolic causes of SIDS

- Fatty acid oxidation defects
  - e.g. MCAD
- Urea cycle disorders
  - e.g. OTC
- Organic acidurias
  - e.g. MMA, PA, IVA
- Congenital lactic acidosis
  - e.g. PDH, respiratory chain defects
- Carbohydrate disorders
  - e.g. galactosaemia, GSD type I
How can we investigate possible IEM after death?

- Urine - Organic acids
- Eye fluid e.g. 7(OH)octanoate
- Acylcarnitine profiling by MS/MS
  - DBS
  - Bile
  - CSF
- Fibroblast studies
  - DNA – not usually indicated
Acylcarnitines: key diagnostic metabolites

- Acylcarnitines reflect Acyl-CoAs accumulating upstream of a metabolic block – reversible conversion by the action of carnitine acyl transferases.

\[
\text{ACYL-CoA} + \text{carnitine} \xleftrightarrow{\text{ENZYME}} \text{ACYL-carnitine} + \text{CoA}
\]

- Profiles mainly in dried blood spots, plasma, bile & CSF

- History: profiling achieved by a variety of techniques - GC, HPLC, GCMS [>30 mins per sample] - FAB-MS/MS (1990s), and then Electrospray (ESI-MS/MS) (2 mins per sample)
QuattroLC MS/MS
‘Parents of 85’..quantitative profile by stable isotope dilution (8 internal standards*).

- **MS2** – fixed-focus on m/z 85
- **MS1** - SCAN m/z 200 - 520

OUTPUT SIGNAL
‘Parents of 85’ ..quantitative profile by stable isotope dilution.

8 stable isotopically labelled Acylcarnitines added as ‘Internal Standards’

d3-C0 = 37µM
d3-C2 = 9.2µM
d3-C3&4 = 1.8µM
d9-C5 = 1.8µM
d3-C8 = 1.8µM
d9-C14 = 1.8µM
d3-C16 = 3.7µM
How can we investigate after death?

- Consider going straight to fibroblast studies if:
  - No blood / bile taken at PM
  - but strong evidence / family history of IMD
    - e.g. fat deposition in renal tubule cells
  - or pre-mortem samples suggest IMD

- Fibroblasts
  - Flux assays
  - Acylcarnitine profiling
  - Specific enzyme assays e.g. GAI
Establishing Normal Post Mortem Reference Ranges for acylcarnitines

- **Very little data available in literature**
  - One large study ~7000 samples
  - Chace et al 2001 (USA & Canada)

- **BUT**
  - Little hard data on confirmation of “presumed” diagnoses
    - Exception - MCAD
Chace et al 2001 (US & Canada)

- Established reference ranges for a range of acylcarnitine species C0 – C16
- 855 DBS & 30 bile spots

- Very wide reference ranges (µmol/L)
  - C8  0.02-1.03  in DBD
  - C8  0.47 – 24  in bile
    - Contrast DBS for Newborn screening  C8  < 0.3

- Also suggested some diagnostic ratios
  - e.g. C8/C10 in MCAD, C14:1/C12:1 in VLCAD
Chace et al - Findings

- 66 specimens suggested a metabolic disorder
- 23 MCAD (most confirmed by mutation)
- 9 VLCAD (very-long chain acyl-CoA)
- 8 Multiple acyl-CoA dehydrogenase deficiency (MADD)
- 6 CPTII/CACT (carnitine palmitoyltransferase type II)
- 4 Primary carnitine deficiency
- 4 LCHAD/TFP (Long-chain 3-hydroxyacyl-CoA dehydrogenase)
- 3 glutaric acidaemia type I (GAI)
- 4 Isovaleryl-CoA dehydrogenase deficiency
- 5 MMA/PA, MSUD
Data from Sheffield/Bristol/Leicester

- 2004 – 2007
- ~120 PM Dried Blood Spots
- ~40 Bile samples
- ~20 CSF samples
<table>
<thead>
<tr>
<th></th>
<th>Postmortem DBS (n = 56) Median (Range)</th>
<th>Postmortem BILE (n = 26) Median (Range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TC</td>
<td>215.47 (11.58 – 554.90)</td>
<td>302.51 (51.8 – 1004.)</td>
</tr>
<tr>
<td>C0</td>
<td>141.92 (7.16 – 423.65)</td>
<td>205.36 (5.45 – 533.28)</td>
</tr>
<tr>
<td>C4</td>
<td>5.3 (0.19 – 17.60)</td>
<td>2.63 (0.29 - 20.24)</td>
</tr>
<tr>
<td>C5:1</td>
<td>0.08 (0.01 – 0.35)</td>
<td>0.28 (0.08 - 2.11)</td>
</tr>
<tr>
<td>C5-OH</td>
<td>0.36 (0.04 – 1.2)</td>
<td>0.51 (0.14 - 1.15)</td>
</tr>
<tr>
<td>C8</td>
<td>0.18 (0.02 – 0.86)</td>
<td>0.53 (0 – 51.47)</td>
</tr>
<tr>
<td>C10:1</td>
<td>0.06 (0.02 – 0.24)</td>
<td>0.59 (0 – 50.4)</td>
</tr>
<tr>
<td>C10</td>
<td>0.1 (0.02 – 0.81)</td>
<td>0.59 (0 – 39.01)</td>
</tr>
<tr>
<td>C5-DC</td>
<td>0.16 (0.02 – 0.62)</td>
<td>0.40 (0 – 1.5)</td>
</tr>
<tr>
<td>C14:1</td>
<td>0.09 (0.02 – 0.32)</td>
<td>0.35 (0.03 – 13.23)</td>
</tr>
<tr>
<td>C14</td>
<td>0.18 (0.02 – 0.62)</td>
<td>0.32 (0.05 – 3.61)</td>
</tr>
<tr>
<td>C16</td>
<td>0.74 (0.1 - 2.41)</td>
<td>0.60 (0.09 - 5.52)</td>
</tr>
<tr>
<td>C16-OH</td>
<td>0.045 (0.01 - 0.14)</td>
<td>0.16 (0.03 – 1.98)</td>
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<tr>
<td>C18:1</td>
<td>0.79 (0.14 – 2.89)</td>
<td>0.70 (0.09 - 4.31)</td>
</tr>
<tr>
<td>C8/C10</td>
<td>1.53 (0.33 – 6.3)</td>
<td>0.80 (0 – 3.33)</td>
</tr>
</tbody>
</table>
Typical post mortem bile

C8 = 1.84 µmol/l
C10:1 = 2.7
C10 = 3.7
C12 = 6.8
C12:1 = 6.9
C14:1 = 12.9
C14 = 3.3
C14:1/C12:1 = 1.9
Typical PM DBS

- C0 = 228
- C2 = 120
- C3 = 3
- C4 = 6
- C4(OH) = 2.8
- C6 = 0.8
Unwell from day 3
Brought into hospital - died soon after

- PM showed extensive fatty change –
- No Skin biopsy obtained
- PM blood - only
CPT2 deficiency

C16 = 14.6 µmol/L (<3.4)

C18:1 = 3.4 µmol/L (<2.7)

C14:1 = 1.52 µmol/L (<0.53)
Medium chain acyl-CoA dehydrogenase deficiency MCAD

Natural history of this disease

- Well at birth
- Sudden decompensation during intercurrent infections / fasting during early infancy/childhood
- Hypoglycaemia, hepatomegaly, encephalopathy, seizures
- Easily treated with avoidance of fasting/emergency regimen during infection
Acylcarnitines in neonatal blood spot in MCAD

C8 in the newborn period raised... 1.13µM (ref. <0.30)

DIAGNOSIS ... MCAD
Post mortem sample

C8 ~ 8.4 μm/L (PM <0.86)

C8/C10 = 11.3 (PM <6.3)

Medium Chain Acyl-CoA Dehydrogenase Deficiency
Post mortem sample

BILE

C8 ~ 200µm/L  (PM <52)

C10:0 ~ 35 µm/L

C8/C10 = 5.7  (PM <3.3)

Medium chain acyl-CoA dehydrogenase deficiency
Glutaryl CoA dehydrogenase deficiency (GA1)

LYSINE

TRYPTOPHAN

2-ketoadipate

Glutaryl CoA

Glutaryl carnitine (C5DC)

(3-[OH]G)

(Glutaconyl CoA)

Crotonyl CoA

Acetoacetate
May exhibit: macrocephaly, fronto-temporal atrophy, acute encephalopathic crisis, dystonia, sub-dural haematoma...

Patient

C5-DC (glutaryl carnitine)

Normal
Post mortem sample

Glutaryl-CoA Dehydrogenase Deficiency

C5 DC ~7.2μm/L

PM control <0.62
Post mortem sample

Glutaryl-CoA Dehydrogenase Deficiency

C5 DC ~11.0μm/L
Branched chain amino acid metabolism: 3-KETOTHIOASE DEFICIENCY.

**VALINE**
- 2-ketovalerate
- Isobutyryl-CoA
- Methacrylyl-CoA
- 3-hydroxyisobutyrate
- Methylmalonylsemialdehyde
- Propionyl-CoA
- Methylmalonyl-CoA

**ISOLEUCINE**
- 2-keto-3-methylvalerate
- 2-methylbutyryl-CoA
- C5:1
- C5-OH
- 2-methyl-3-hydroxybutyryl-CoA
- 2-methyl-3-ketobutyryl-CoA

**LEUCINE**
- 2-ketoisocaproate
- Isovaleryl-CoA
- 3-methylcrotonyl-CoA
- 3-methylglutaconyl-CoA
- 3-HMG-CoA

**TCA CYCLE**
- Acetyl-CoA
- Acetoacetate
- Succinyl-CoA
- TCA CYCLE
Symptoms: recurrent episodes of ketoacidosis

Patient

C5:1
C5(OH)

Normal
Post mortem sample

Beta Ketothiolase Deficiency (Twin 1)

C5:1 = 0.6 µm (PM <0.35)
C5(OH) =2.5 µm (PM 0.04 -1.2)
Sample from surviving Twin

C5:1 ~ 0.47 µm(<0.02)
C5:OH ~ 0.83 µm(<0.05)
PM DBS SIDS day 2

C16(OH) = 2.2 µmol/l <0.1
C18:1(OH) = 1.23 µmol/L <0.1
- **Fatty acid oxidation**
  - Myristate = 42%
  - Palmitate = 27%
  - Oleate = 16%
- **Common G1528C LCHAD mutation – not found**
- **LCHAD – 36 (34-114) nmol/mg/min**
- **LC thiolase – 2 (58-110)**
- **HADHB gene c.1292T.C plus c.1301C>T**
- **Mitochondrial Trifunctional Protein deficiency**
<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>Number</th>
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<tbody>
<tr>
<td>Respiratory chain defect</td>
<td>15</td>
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<tr>
<td>Multiple acyl-CoA dehydrogenase defect (severe)</td>
<td>12</td>
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<tr>
<td>Medium-chain acyl-CoA dehydrogenase defect</td>
<td>10</td>
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<tr>
<td>Carnitine palmitoyltransferase deficiency Type II</td>
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<tr>
<td>Very long-chain acyl-CoA dehydrogenase defect</td>
<td>7</td>
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<tr>
<td>Long-chain 3-hydroxyacyl-CoA dehydrogenase deficiency</td>
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<td>Carnitine-acylcarnitine translocase</td>
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<tr>
<td>Mitochondrial trifunctional protein deficiency</td>
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<td>Fumarate hydratase deficiency</td>
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<td>Methylmalonic aciduria</td>
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<td>Zellweger spectrum</td>
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<td>Argininosuccinic aciduria</td>
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<tr>
<td>Carnitine palmitoyltransferase deficiency type I</td>
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<tr>
<td>Glutaric aciduria type I</td>
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<td>Glutathione synthase deficiency</td>
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<td>GSD IV</td>
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<td>Isovaleric acidaemia</td>
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<td>Congenital disorder of glycosylation type 1</td>
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<td>Primary carnitine deficiency</td>
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<td>Pyruvate dehydrogenase deficiency</td>
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<tr>
<td>X-linked adrenoleucodystrophy</td>
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<td>Total diagnoses</td>
<td>83</td>
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<tr>
<td>Total number of post mortem cell lines</td>
<td>1211</td>
</tr>
</tbody>
</table>
Multiple acyl-CoA dehydrogenase deficiency MADD

- Defect of fatty acid & amino acid catabolism
- Severe neonatal / infantile /milder phenotype
- Hypoglycaemia, acidosis, hypotonia, liver disease, cardiomyopathy
The Biochemical defect in MADD

Ketone bodies

Acetyl-CoA

S

SH₂

EFAD

EFAD₂

ETFox

ETF red

Very-long-chain acyl-CoA DH
Medium-chain acyl-CoA DH
Short-chain acyl-CoA DH
Long-chain acyl-CoA DH
Acyl-CoA DH-9

Short/branched-chain acyl-CoA DH
Isobutyryl-CoA DH
Isovaleryl-CoA DH
Glutaryl-CoA DH

Dimethylglycine DH
Sarcosine DH

Fatty acid metabolism

Amino acid metabolism

Choline metabolism

TCA

I

II

III

IV

V

H⁺

ADP

ATP
MADD plasma

Plasma from a child with moderate/severe MADD

MADD or GA2
• Appeared normal at birth
• Sent home on day 1
• Died during car journey home
Post mortem sample

MADD

C4 ~19.2 μm/L (<17.6)

C16:0 ~10 μm/L (<2.4)

DBS

C5 ~ 13.7 μm/L (<3.4) leucine, isoleucine

C5 DC ~ 9.6 μm/L (<0.62) lysine

C14 ~ 1.8 μm/L (<0.62)
Tritium release from labelled $[9,10-^3H]$ substrates

$[9,10-^3H]$Myristic acid (C14:0)
CH$_3$ CH$_2$ CH$_2$ CH$_2$ CH$_2$* CH$_2$* CH$_2$ CH$_2$ CH$_2$ CH$_2$ CH$_2$ CH$_2$ COOH

$[9,10-^3H]$Palmitic acid (C16:0)
CH$_3$ CH$_2$ (CH$_2$)$_3$ CH$_2$ CH$_2$* CH$_2$* CH$_2$ CH$_2$ CH$_2$ CH$_2$ CH$_2$ CH$_2$ CH$_2$ COOH

$[9,10-^3H]$Oleic acid (C18:1)
CH$_3$ (CH$_2$)$_5$ CH$_2$ CH$_2$ CH* CH* CH$_2$ CH$_2$ CH$_2$ CH$_2$ CH$_2$ CH$_2$ CH$_2$ COOH
Confirmation in fibroblasts MW

- Myristate = 3%
- Palmitate = 3%
- Oleate = 2%
- % of simultaneous normal controls
- Consistent with severe MADD
% residual activity for M/P/O for various FAOD’s

Pattern recognition in FAO

Severe MADD
Two subsequent prenatals on amniotic fluid / cultured amniocytes
- 1 affected
- 1 unaffected
MK 14/12/04

- Sudden death at 3 days
- PM findings
  - gross deposition of fat in liver
  - fat deposition in renal tubules
MK 14/12/04

- Fatty acid oxidation flux in cultured fibroblasts (% of controls)
  - Myristate 5%
  - Palmitate 1%
  - Oleate 8%
  - Octanoate 196%
    - β-oxidation is intact for medium chain substrates which are independent of the carnitine cycle (CPTI, CPTII, CAT)
% residual activity for M/P/O for various FAOD’s

Pattern recognition in FAO

Carnitine cycle defects
CPTI, CPTII, CAT
Fibroblast Acylcarnitine Profiling

- Non-radioactive methodology
- Easier analysis of end product (MS/MS)
- Improved specificity
Principle of method

- Plate fibroblasts into multi-well plates
- Settle overnight
- Add substrate
  - Fatty acid plus carnitine
    - e.g. 200\(\mu\)m/L palmitate, 400 \(\mu\)m/L carnitine
- Incubate for 72-96 hours
- Analyse acylcarnitine profile of medium on MS/MS
- Adjust for fibroblast protein concentration
Data from 45 patient cell lines showing abnormal acylcarnitine species in each of 9 fatty acid oxidation disorders / phenotypes

(Controls n = 146)
Acylcarnitine profiling in fibroblasts from MK

- No increase in any acylcarnitine species
  - No abnormality of β-oxidation spiral!

- ?? Defect of getting long-chain acylcarnitine into mitochondria
  - i.e. CPT I, CPT II, CAT
Data from 45 patient cell lines showing abnormal acylcarnitine species in each of 9 fatty acid oxidation disorders / phenotypes

(Controls n = 146)

CPTII & CAT have high C16 & low C5/C16 ratio
% residual activity for M/P/O for various FAOD’s

Pattern recognition in FAO

Carnitine cycle defects

CPTI

FAO Disorders

Myristate
Palmitate
Oleate
<table>
<thead>
<tr>
<th>Acylcarnitine</th>
<th>Patient MK</th>
<th>Controls (n=70) Mean ± 2 SD</th>
<th>Positive CPTI controls N=5</th>
</tr>
</thead>
<tbody>
<tr>
<td>C16</td>
<td>0.06</td>
<td>0.15 – 1.25</td>
<td>0.07; 0.15; 0.08; 0.16</td>
</tr>
<tr>
<td>C5/C16 ratio</td>
<td>15.5</td>
<td>0.13 – 1.01</td>
<td>6.1; 3.6; 5.1; 2.3</td>
</tr>
</tbody>
</table>
Family of MK

- Subsequent baby tested positive for CPTI
- Low long-chain fat diet
- MCT supplementation with ↑ carbohydrate
- Avoidance of fasting
- Emergency regimen when unwell

- Infant doing fine!
Advantages of fibroblasts

- Easy to obtain and grow
  - Post mortem, repeat assays, storage,

- Less subject to secondary factors
  - deterioration, nutrition, clinical state

- Flux assays (intact cells)
  - overall measure of many pathways using labelled substrates

- Specific enzyme assays e.g. CPTI, CPTII, CAT
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