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

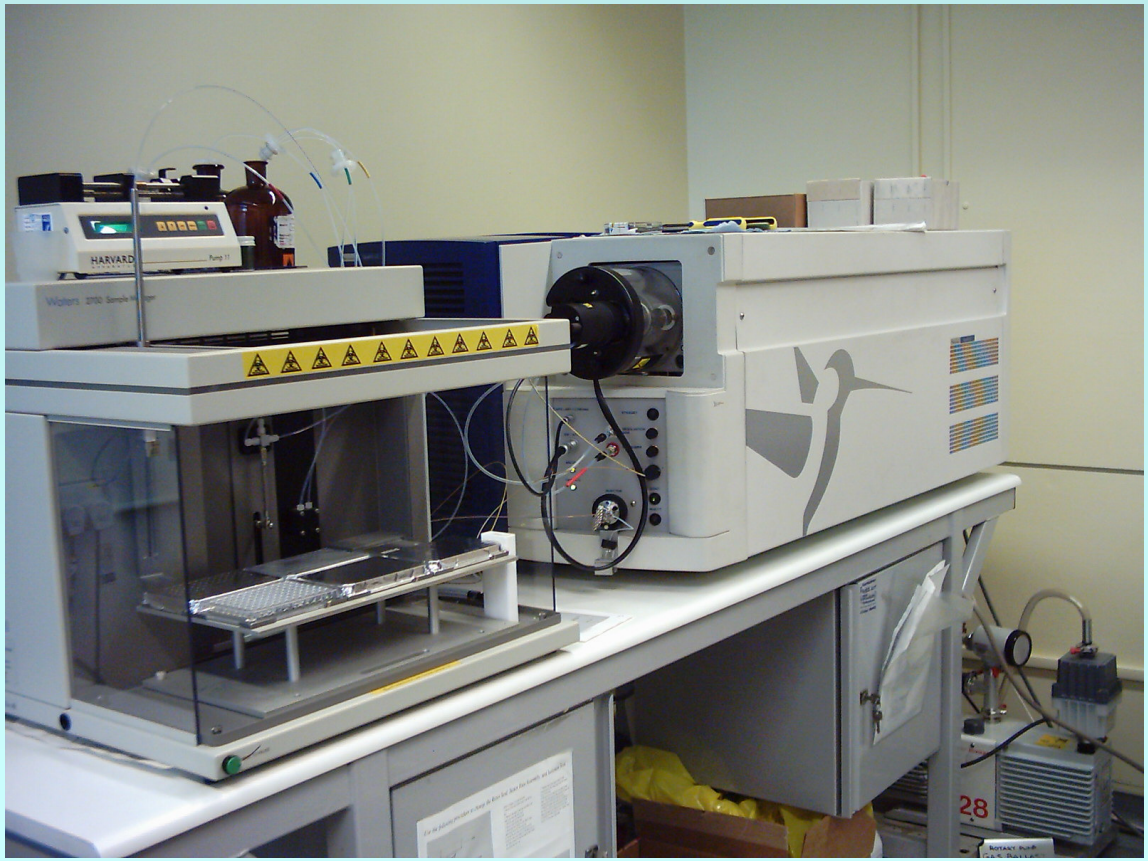
Acylcarnitines: key diagnostic metabolites

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

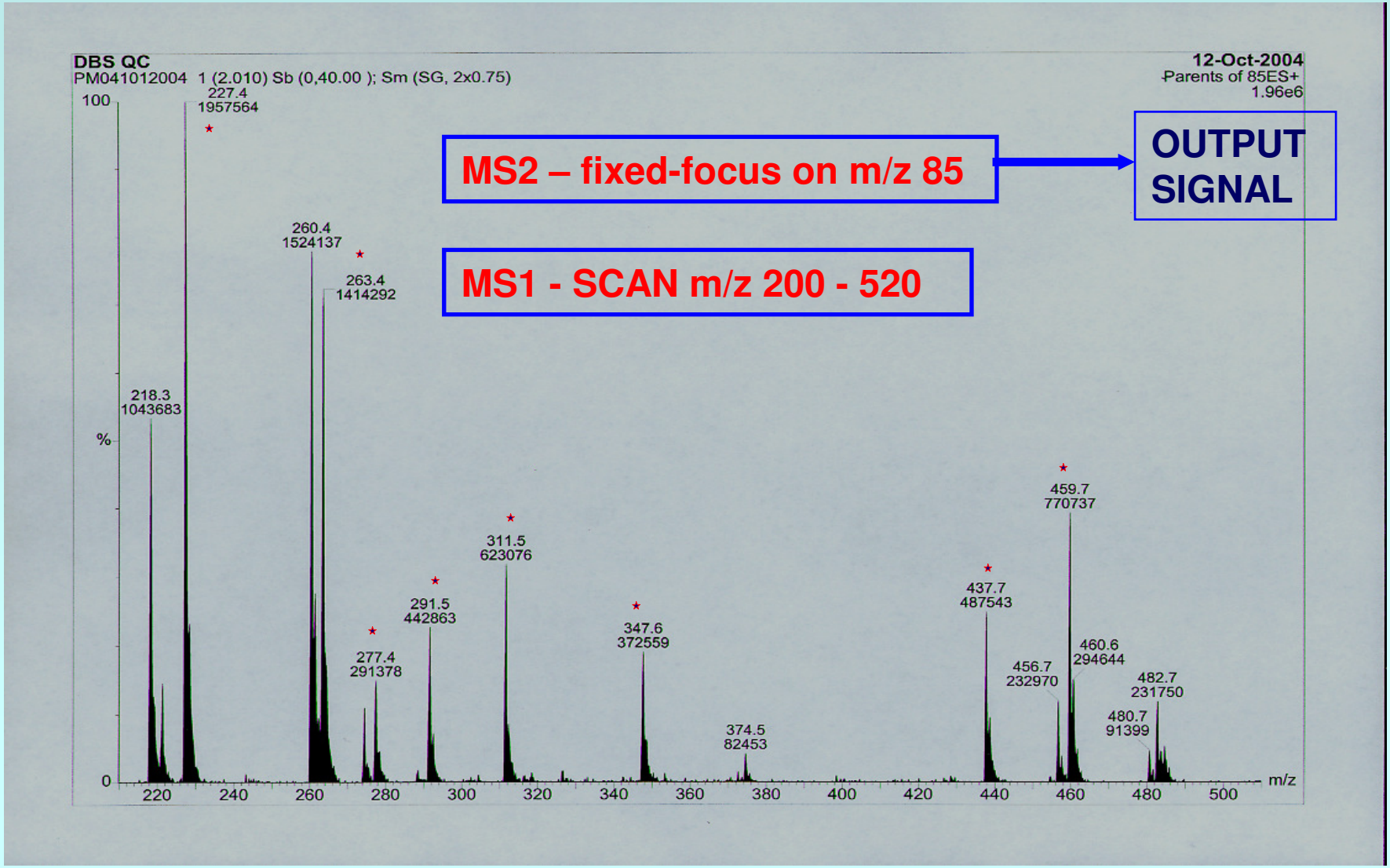
- Profiles mainly in dried blood spots, plasma, bile & CSF

- 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*).



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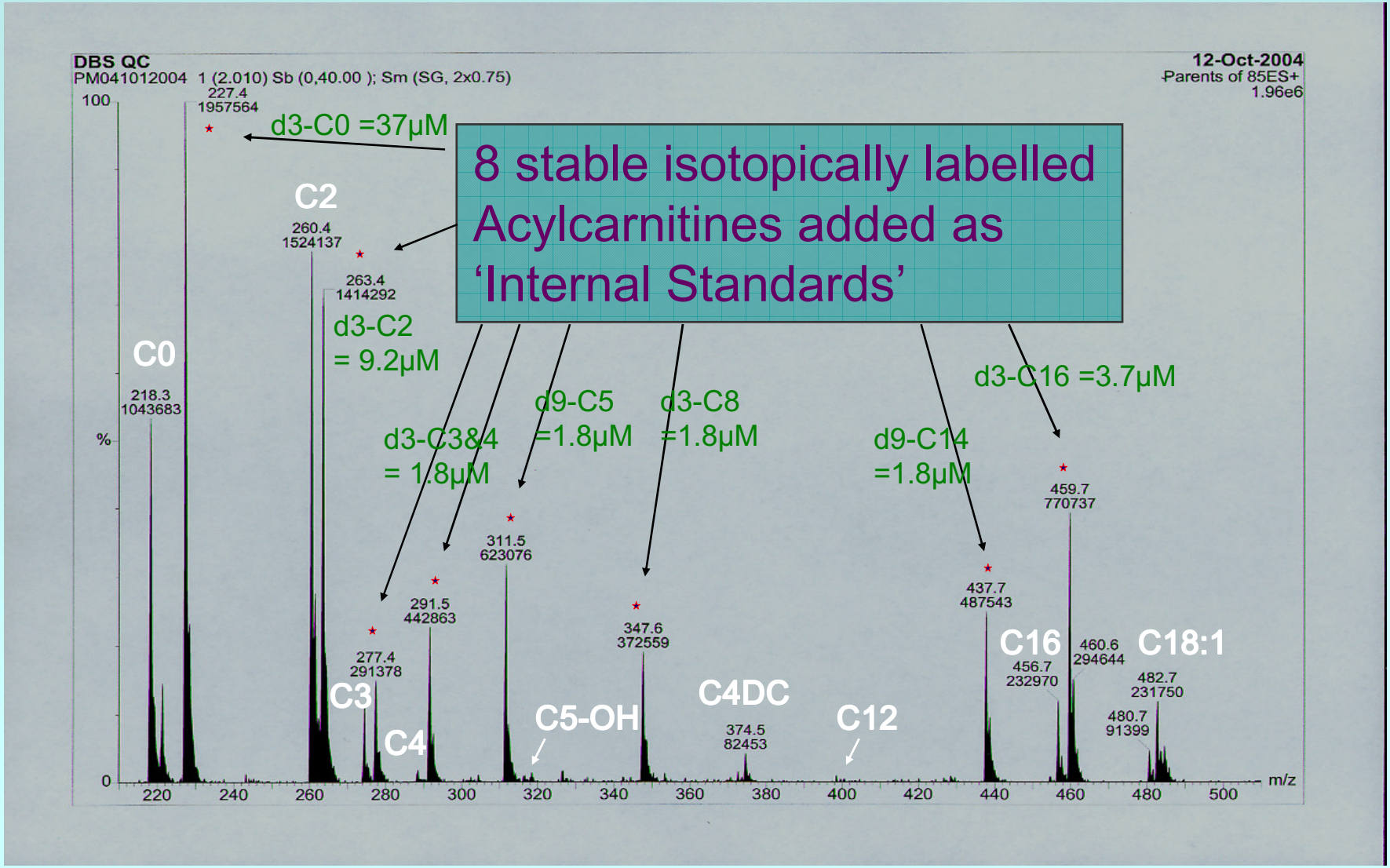


How can we investigate after death?

- 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

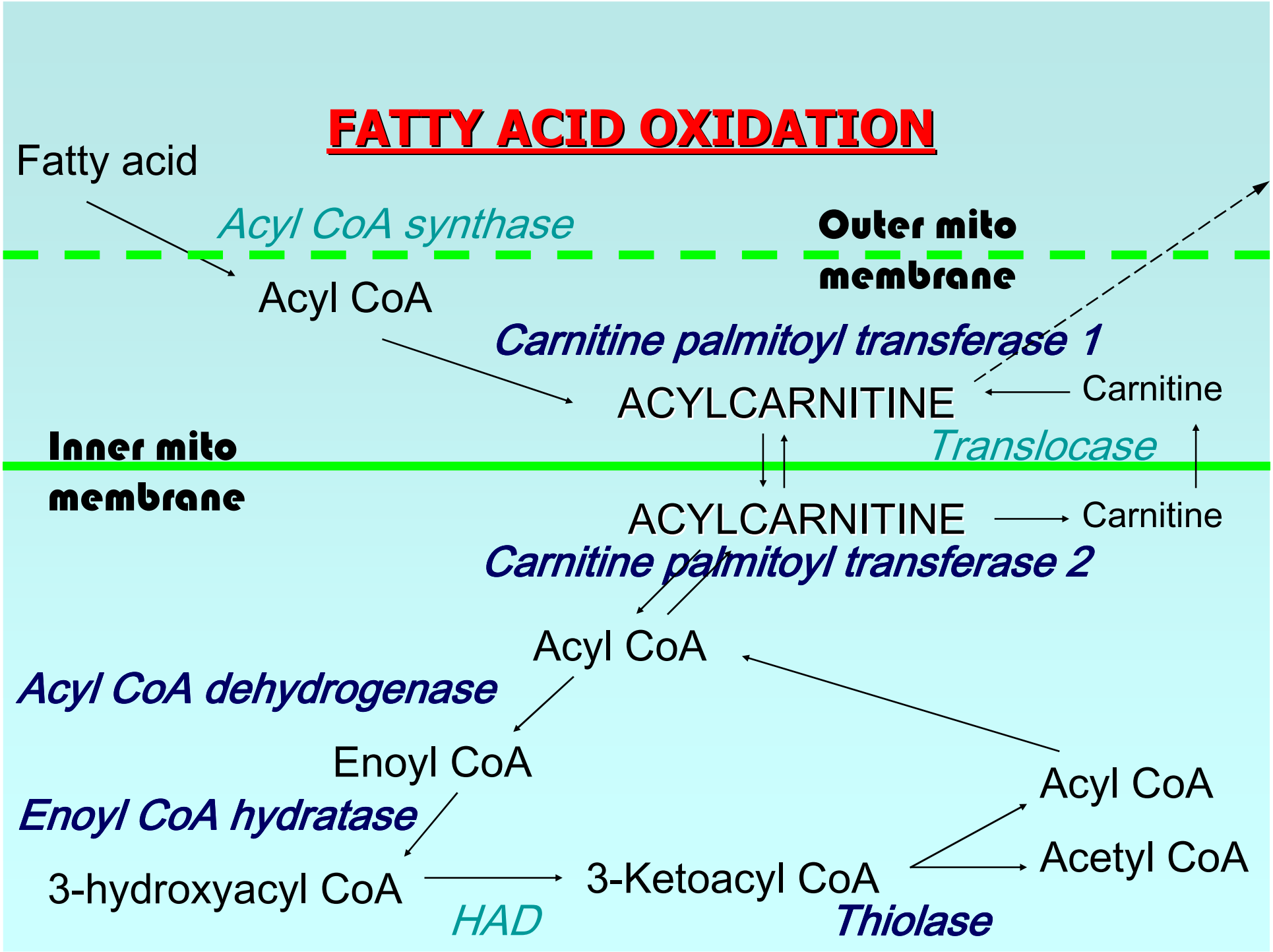
- 66 specimens suggested a metabolic disorder
- 23 MCAD (most confirmed by mutation)
- 9 VLCAD (very-long chain acyl-CoA)
- 8 Multiple acyl-CoA dehydrogenase deficiency (MADD)
- 6 CPTII/ CACT (carnitine palmitoyltransferase type II)
- 4 Primary carnitine deficiency
- 4 LCHAD/TFP (Long-chain 3-hydroxyacyl-CoA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

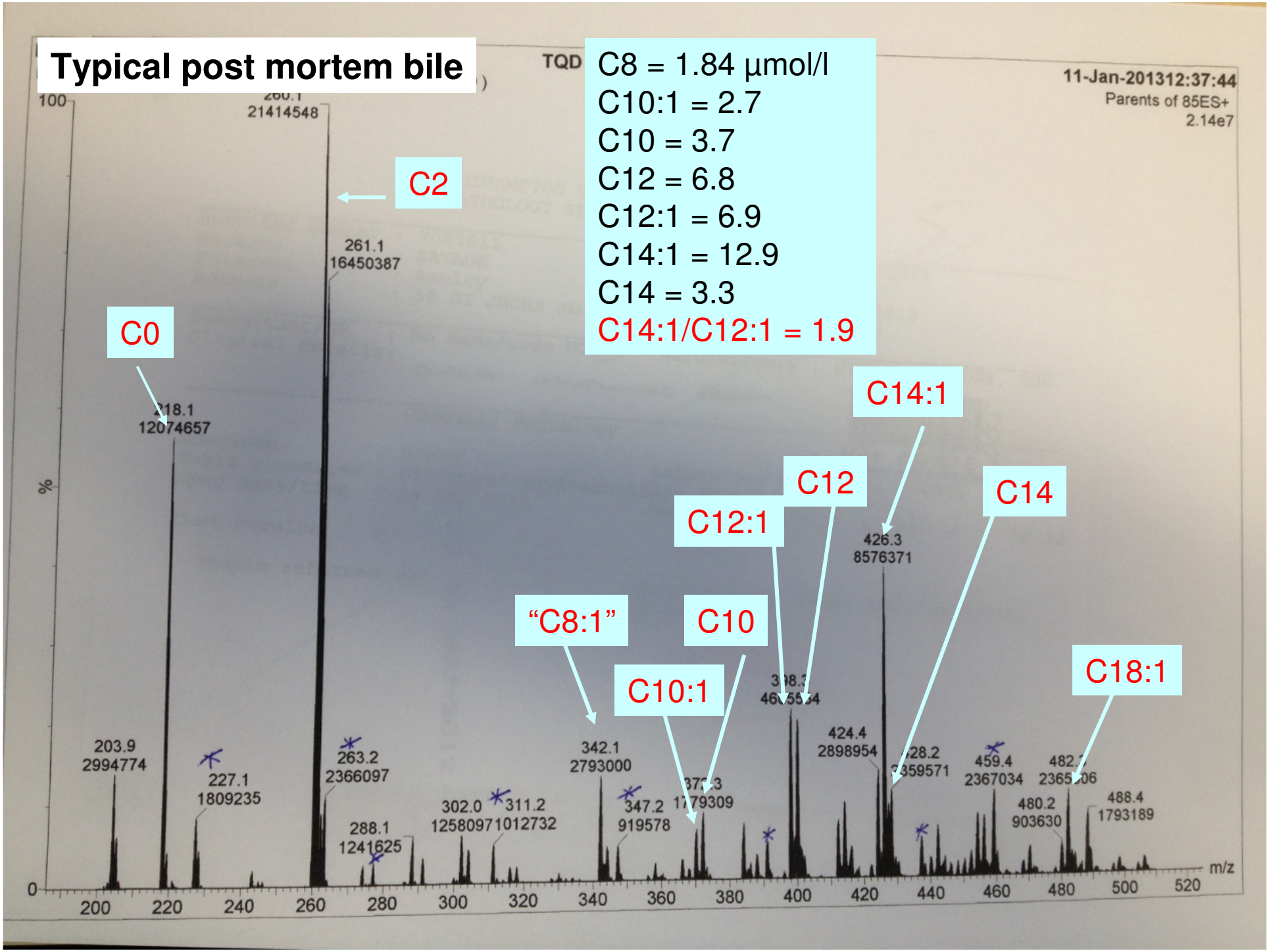
Data from Sheffield/Bristol/Leicester

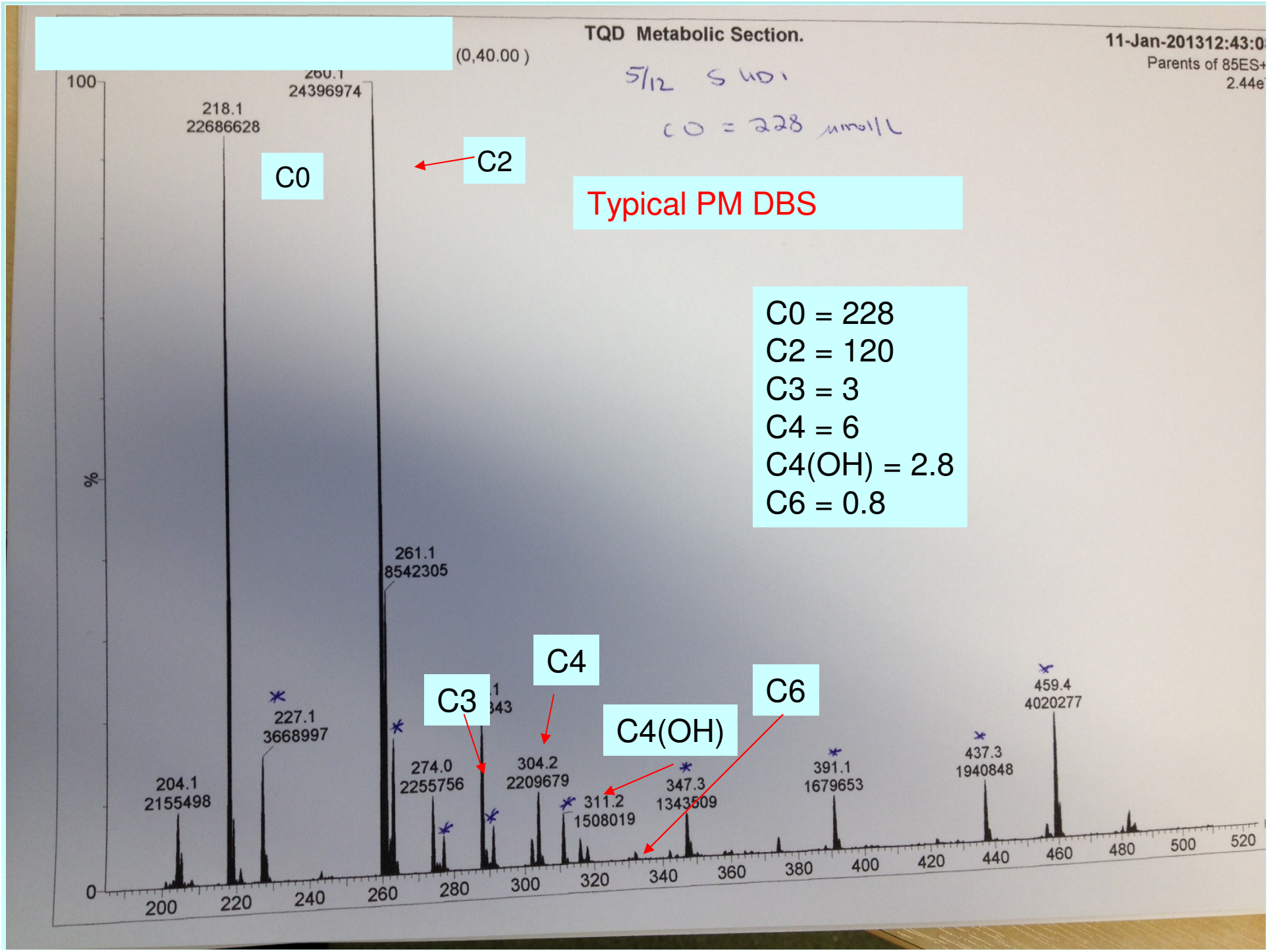
- 2004 –2007
- ~120 PM Dried Blood Spots
- ~40 Bile samples
- ~20 CSF samples

Post mortem reference ranges

	Postmortem DBS (n = 56) Median (Range)	Postmortem BILE (n = 26) Median (Range)
TC	**215.47** (11.58 – 554.90)	**302.51** (51.8 – 1004.)
C0	**141.92** (7.16 – 423.65)	**205.36** (5.45 – 533.28)
C4	**5.3** (0.19 – 17.60)	**2.63** (0.29 - 20.24)
C5:1	**0.08** (0.01 – 0.35)	**0.28** (0.08 - 2.11)
C5-OH	**0.36** (0.04 – 1.2)	**0.51** (0.14 - 1.15)
C8	**0.18** (0.02 – 0.86)	**0.53** (0 – 51.47)
C10:1	**0.06** (0.02 – 0.24)	**0.59** (0 – 50.4)
C10	**0.1** (0.02 – 0.81)	**0..59** (0 – 39.01)
C5-DC	**0.16** (0.02 – 0.62)	**0.40** (0 – 1.5)
C14:1	**0.09** (0.02 – 0.32)	**0.35** (0.03 – 13.23)
C14	**0.18** (0.02 – 0.62)	**0.32** (0.05 – 3.61)
C16	**0.74** (0.1 - 2.41)	**0.60** (0.09 - 5.52)
C16-OH	**0.045** (0.01 - 0.14)	**0.16** (0.03 – 1.98)
C18:1	**0.79** (0.14 – 2.89)	**0.70** (0.09 - 4.31)
C8/C10	**1.53** (0.33 – 6.3)	**0.80** (0 – 3.33)


FATTY ACID OXIDATION
Fatty acid
Acyl CoA synthase
Outer mito membrane
Acyl CoA
Carnitine palmitoyl transferase 1
ACYLCARNITINE
Carnitine
Inner mito membrane
Translocase
ACYLCARNITINE
Carnitine
Carnitine palmitoyl transferase 2
Acyl CoA
Acyl CoA dehydrogenase
Enoyl CoA
Acyl CoA
Enoyl CoA hydratase
Acetyl CoA
3-hydroxyacyl CoA
3-Ketoacyl CoA
HAD
Thiolase

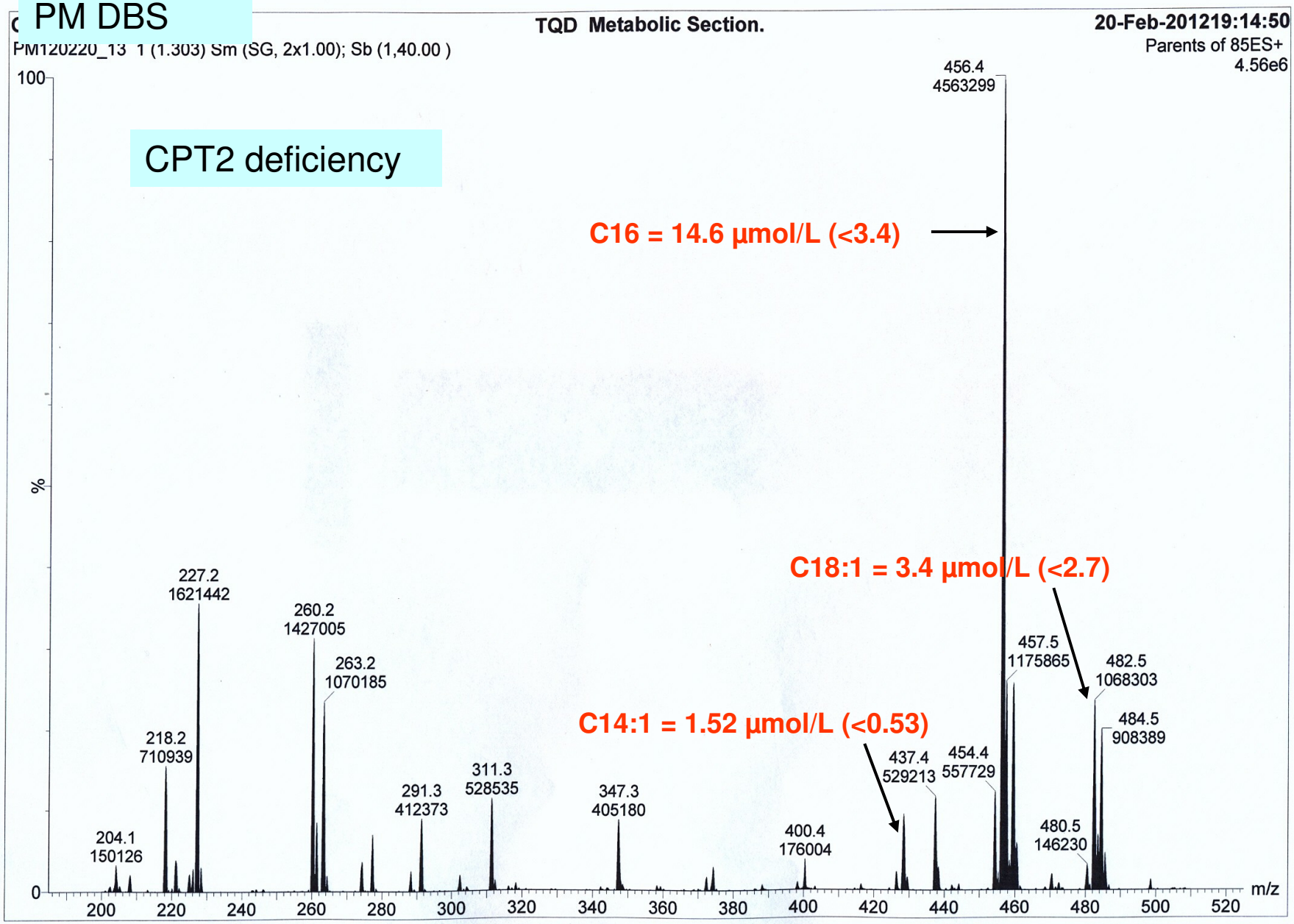

Typical post mortem bile
C8 = 1.84 µmol/l
C10:1 = 2.7
C10 = 3.7
C12 = 6.8
C12:1 = 6.9
C14:1 = 12.9
C14 = 3.3
C14:1/C12:1 = 1.9
TQD
11-Jan-201312:37:44
Parents of 85ES+
2.14e7
100
%
0
m/z
C0
C2
C14:1
C12
C12:1
C14
"C8:1"
C10
C10:1
C18:1
261.1
16450387
218.1
12074657
203.9
2994774
227.1
1809235
263.2
2366097
288.1
1241625
302.0
12580971012732
311.2
342.1
2793000
347.2
919578
424.4
2898954
426.3
8576371
459.4
2367034
480.2
903630
488.4
1793189
200
220
240
260
280
300
320
340
360
380
400
420
440
460
480
500
520


TQD Metabolic Section.
(0,40.00)
11-Jan-201312:43:0
Parents of 85ES+
2.44e
5/12 S 401
C0 = 228 µmol/L
Typical PM DBS
C0
C2
C3
C4
C4(OH)
C6
C0 = 228
C2 = 120
C3 = 3
C4 = 6
C4(OH) = 2.8
C6 = 0.8
100
%
0
218.1
22686628
260.1
24396974
261.1
8542305
227.1
3668997
204.1
2155498
274.0
2255756
304.2
2209679
311.2
1508019
347.3
1343509
391.1
1679653
437.3
1940848
459.4
4020277
200
220
240
260
280
300
320
340
360
380
400
420
440
460
480
500
520

Unwell from day 3
Brought into hospital - died soon after

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

PM DBS



CPT2 deficiency

C16 = 14.6 µmol/L (<3.4)

C18:1 = 3.4 µmol/L (<2.7)

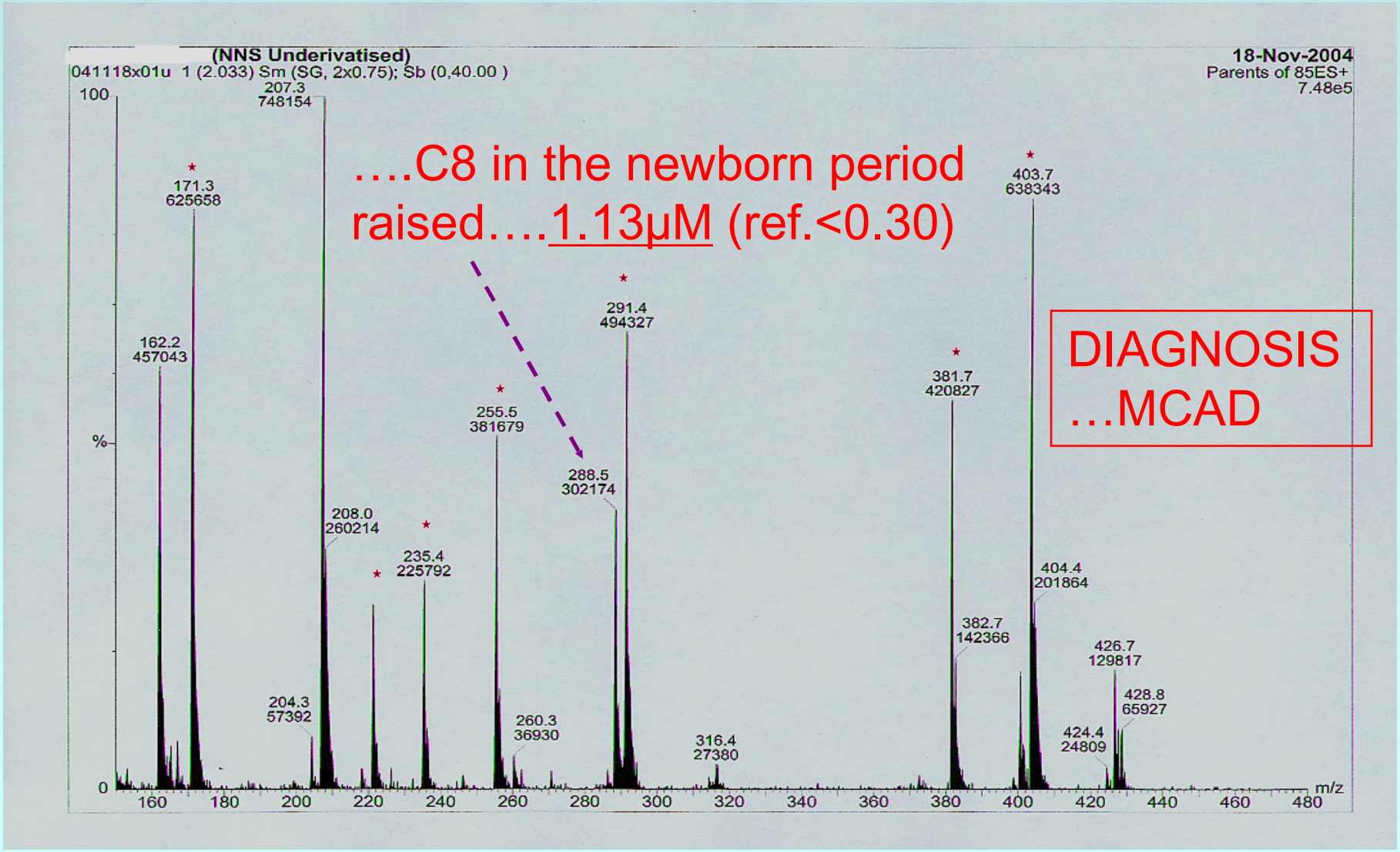
C14:1 = 1.52 µmol/L (<0.53)

Medium chain acyl-CoA dehydrogenase deficiency MCAD

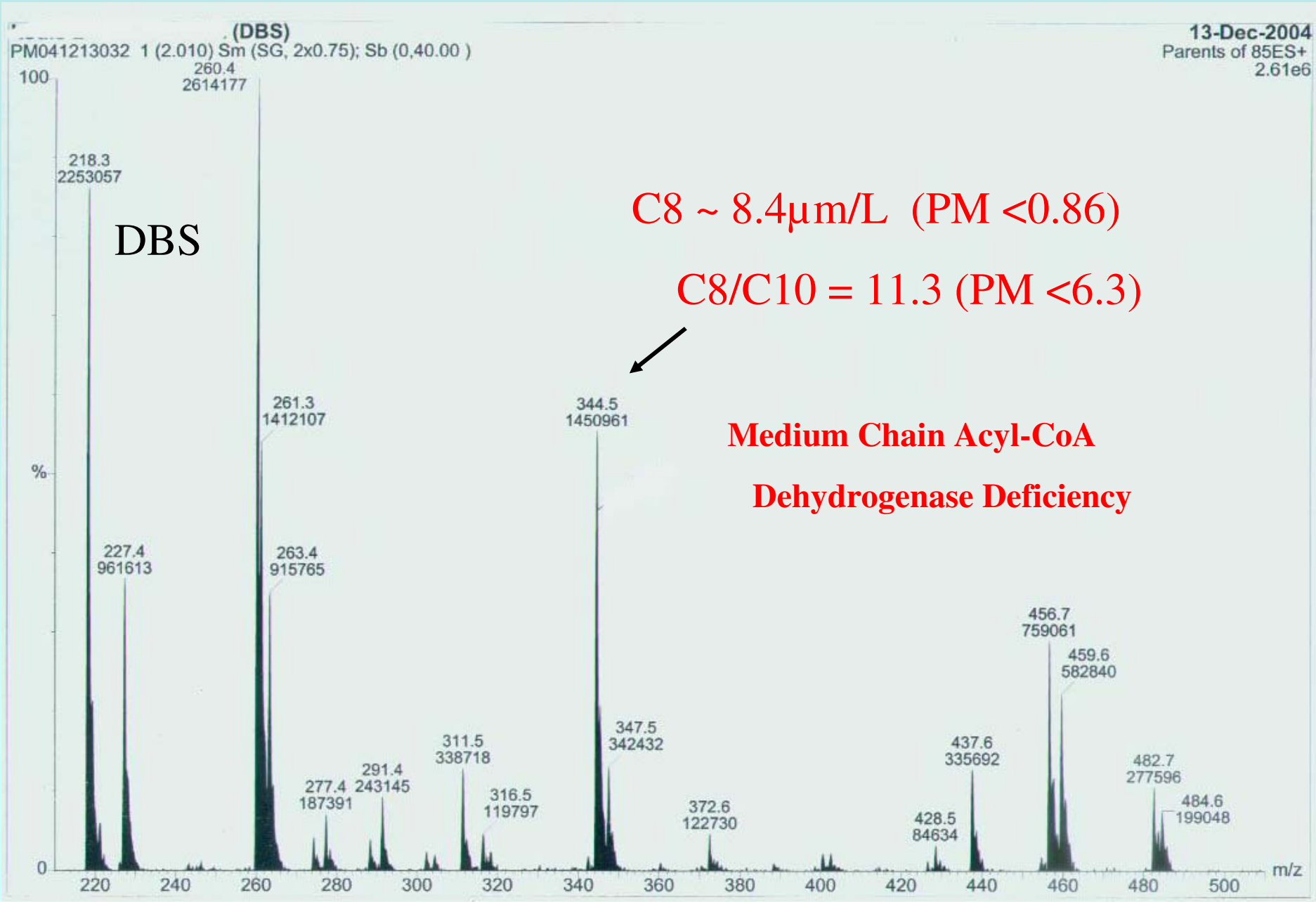
Natural history of this disease

- 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

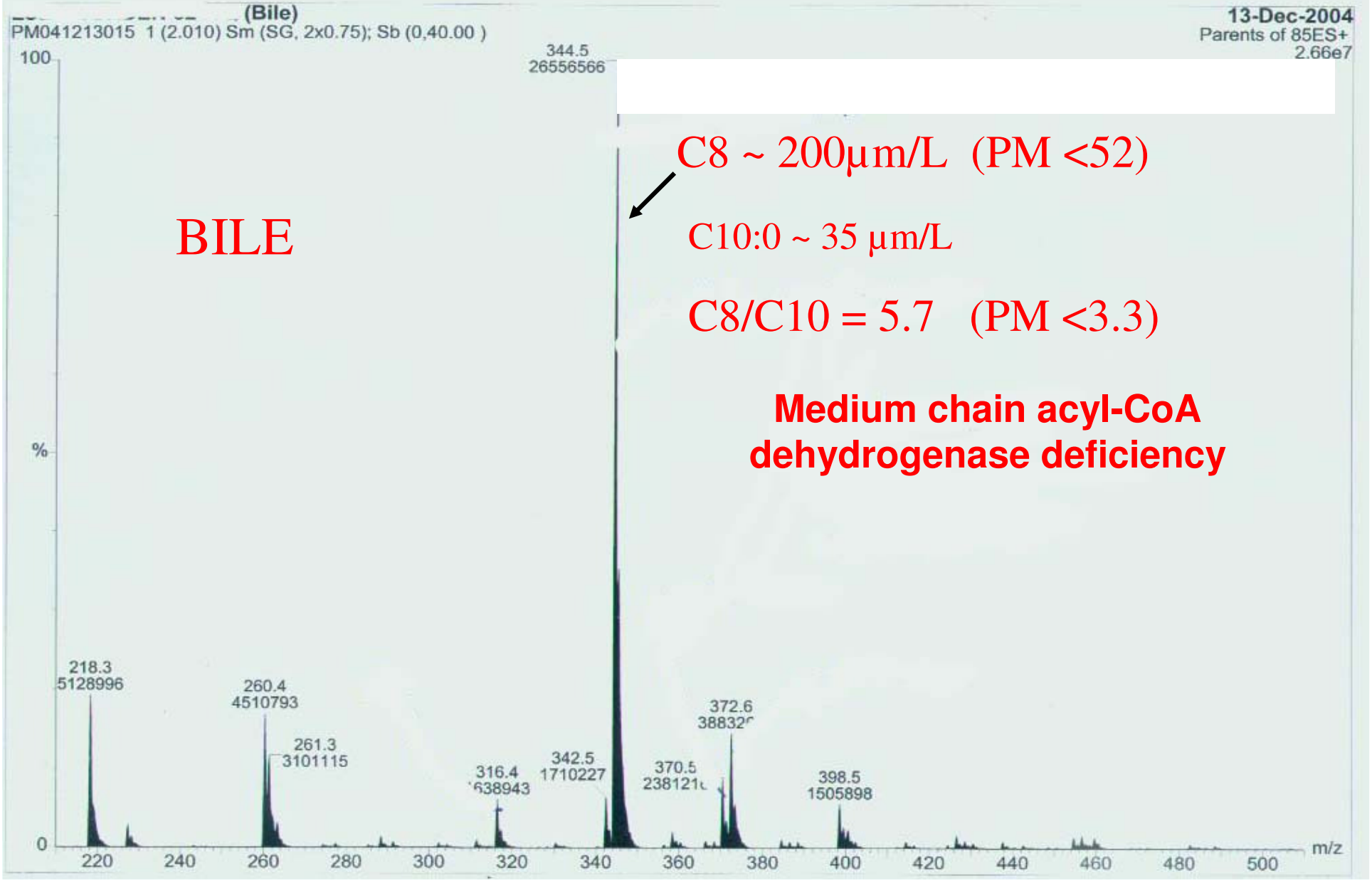
Acylcarnitines in neonatal blood spot in MCAD



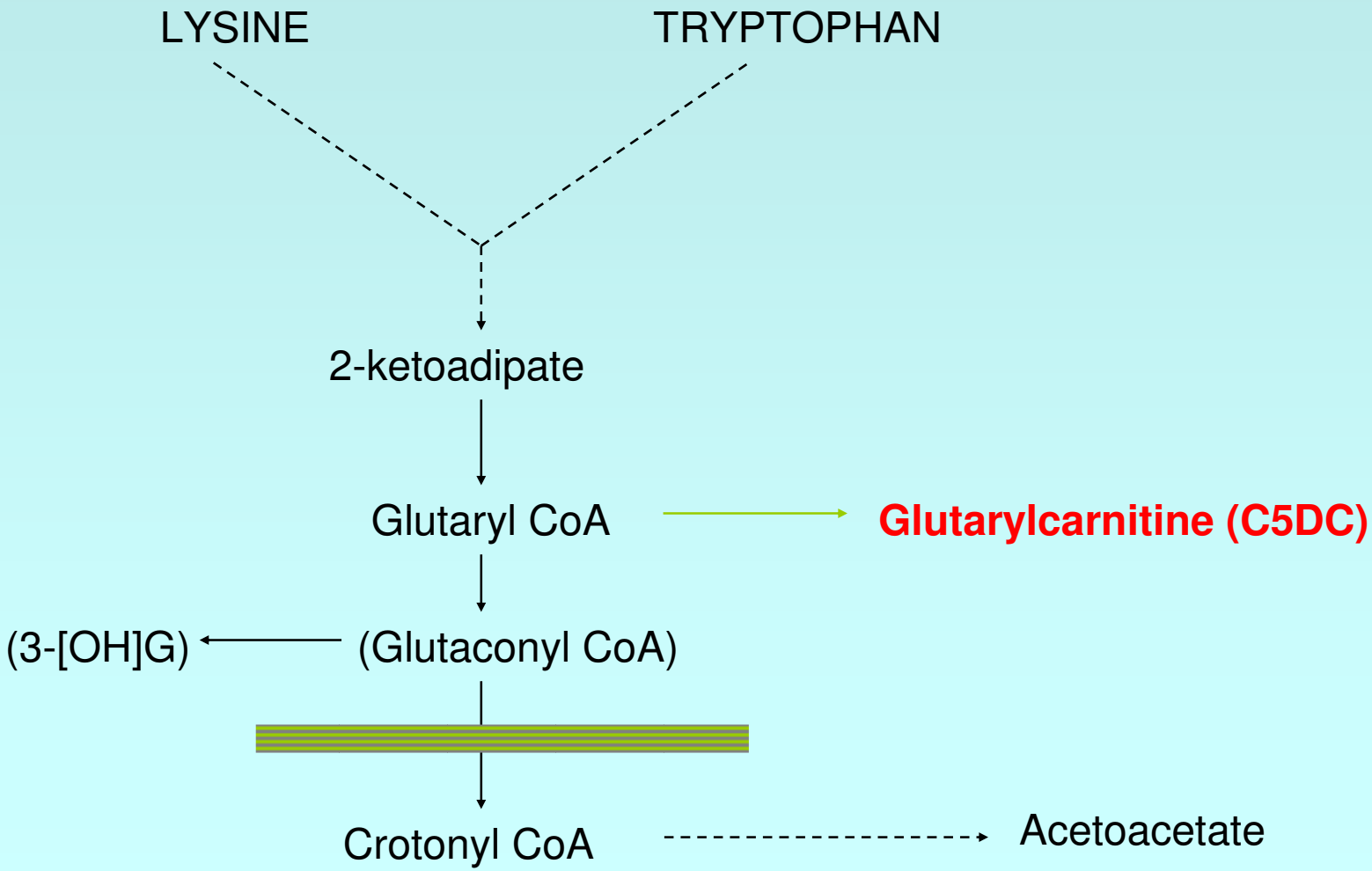
Post mortem sample



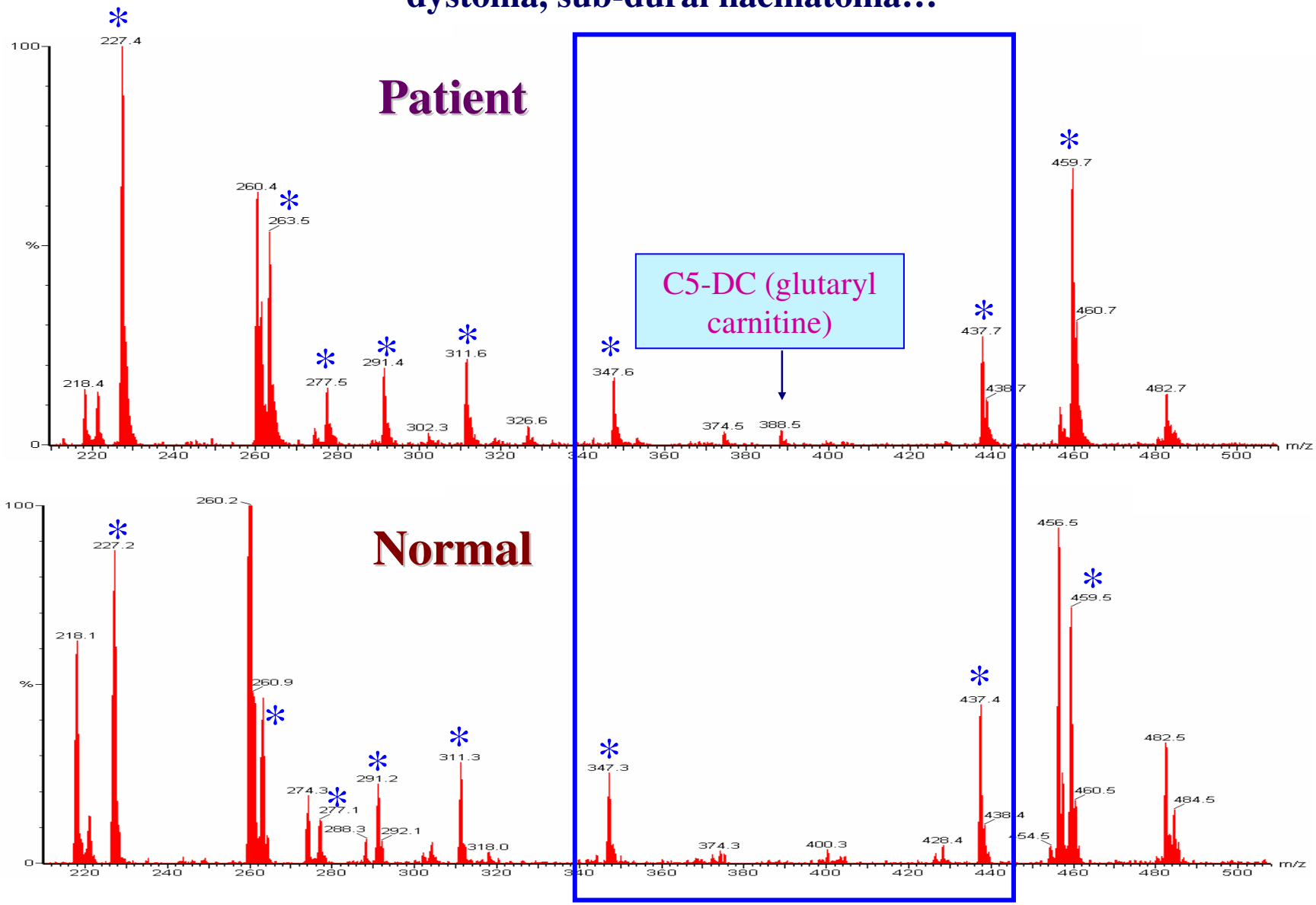
Post mortem sample



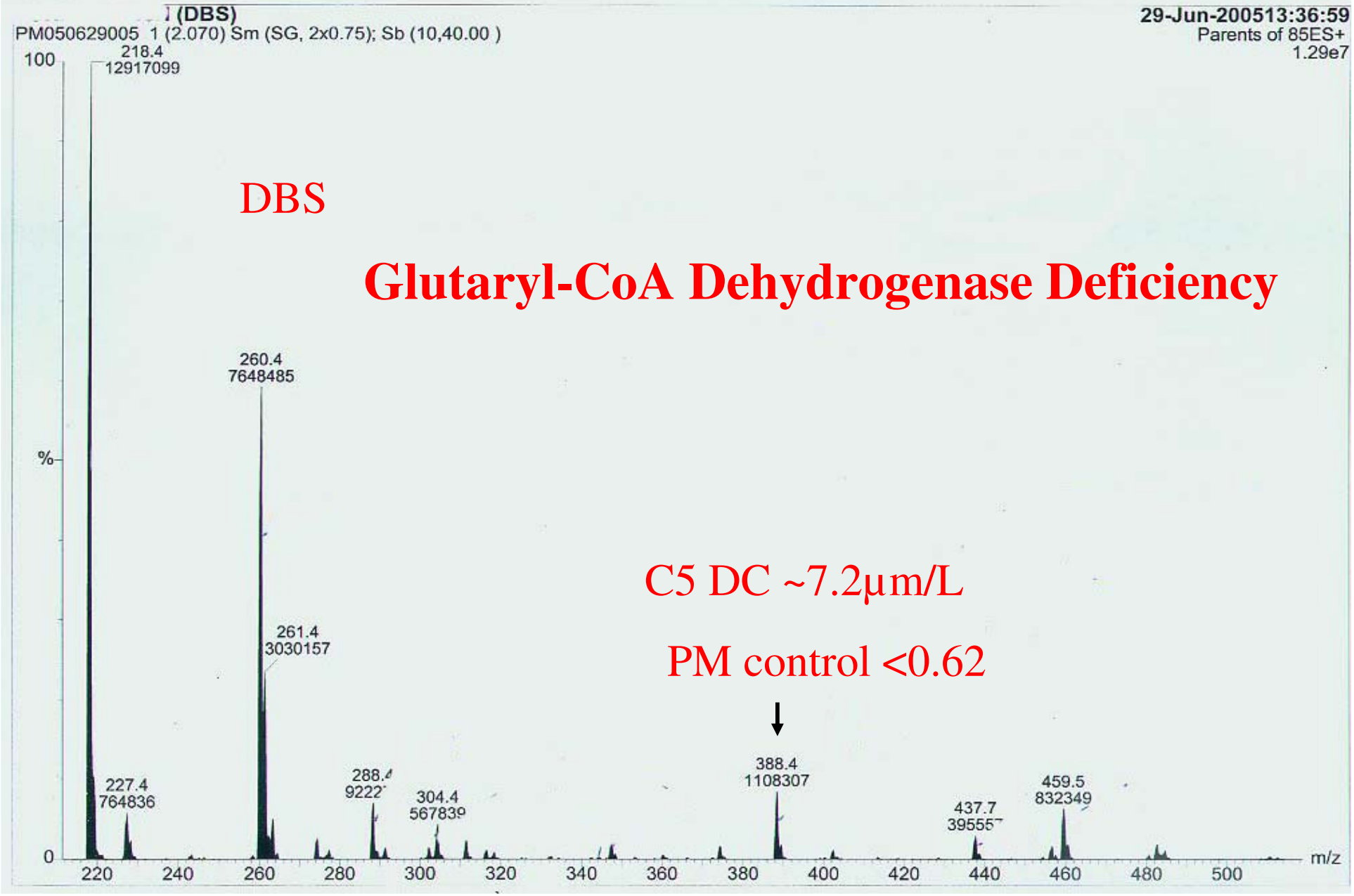
Glutaryl CoA dehydrogenase deficiency (GA1)



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



Post mortem sample



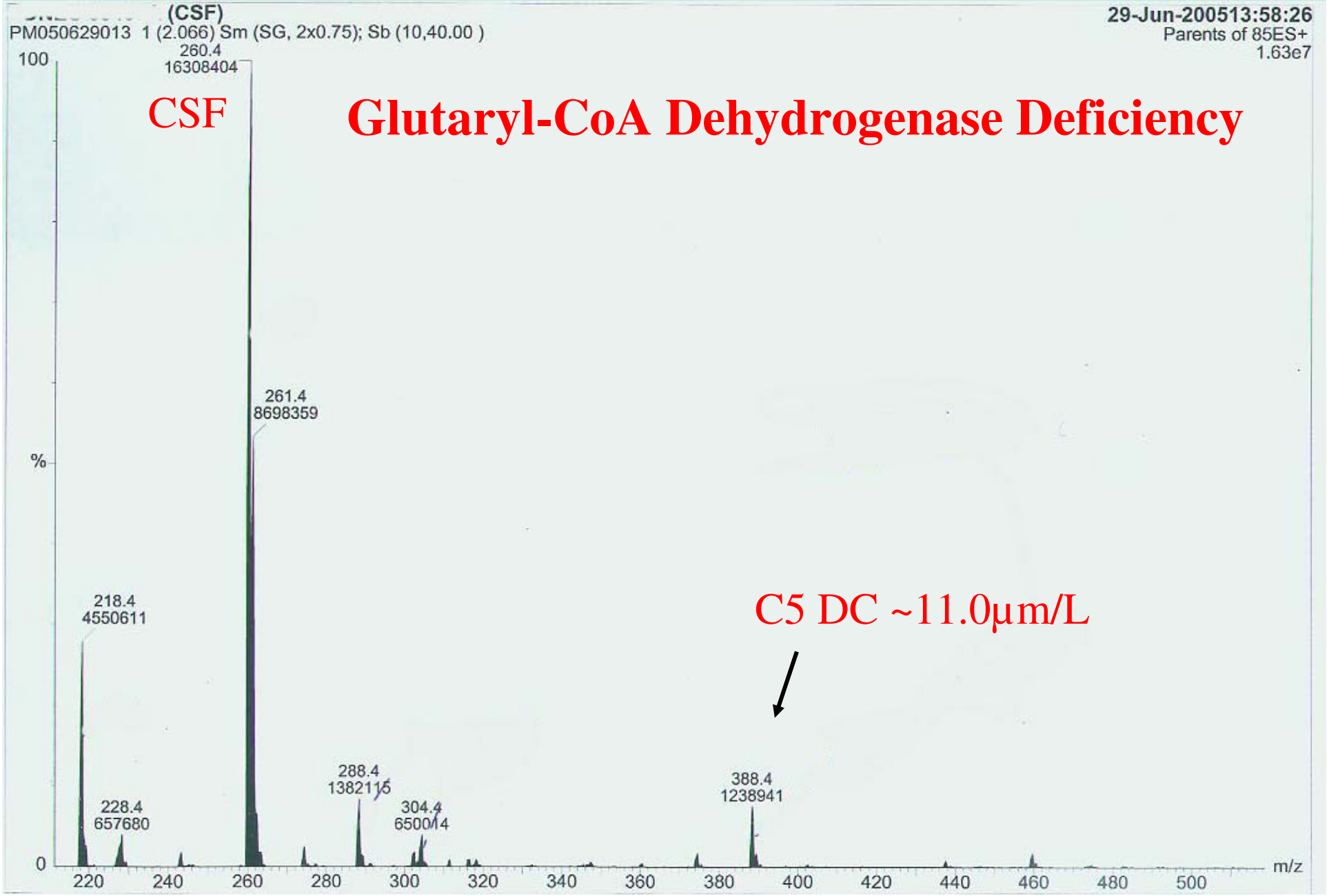
DBS

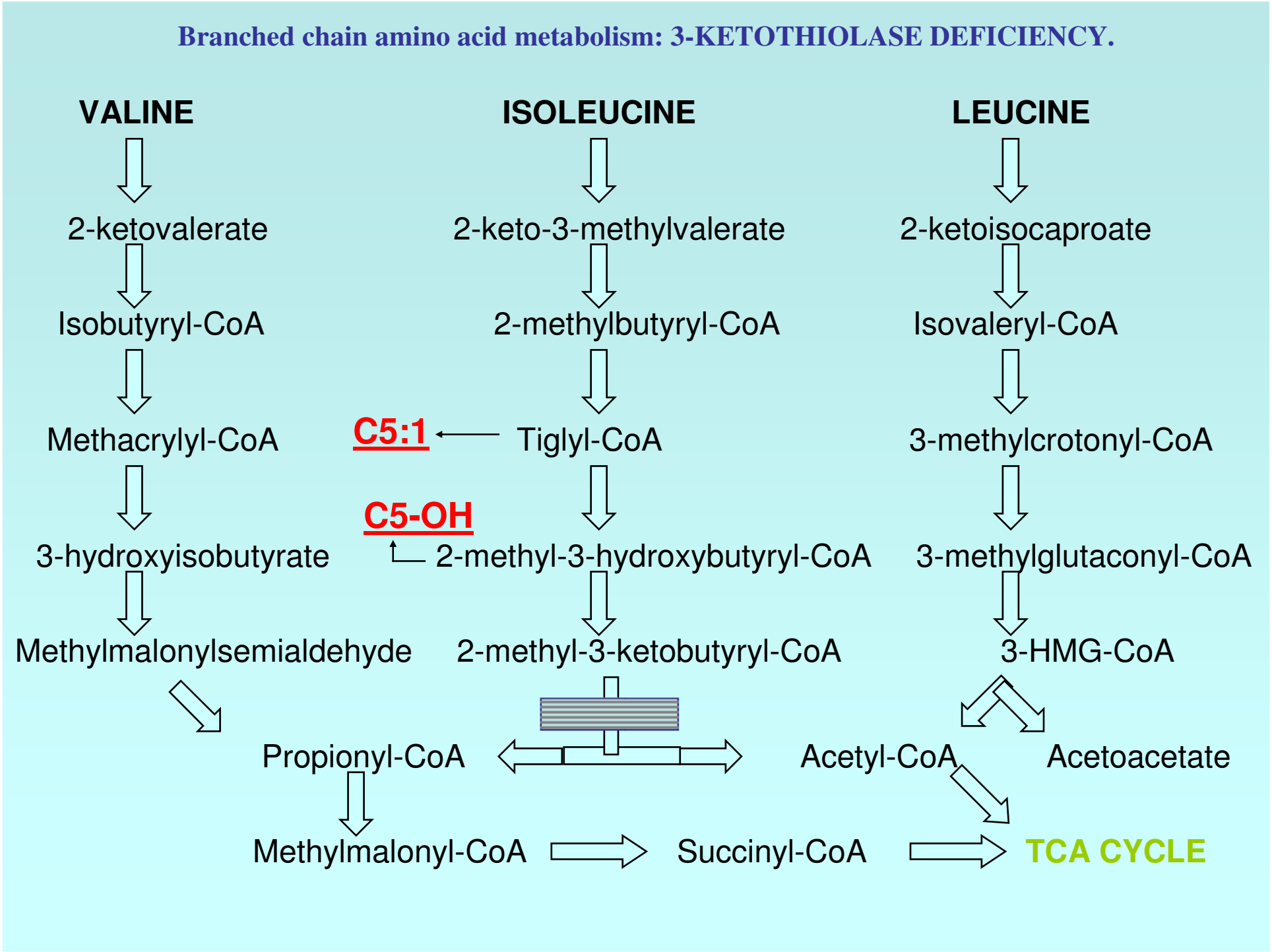
Glutaryl-CoA Dehydrogenase Deficiency

C5 DC ~7.2µm/L

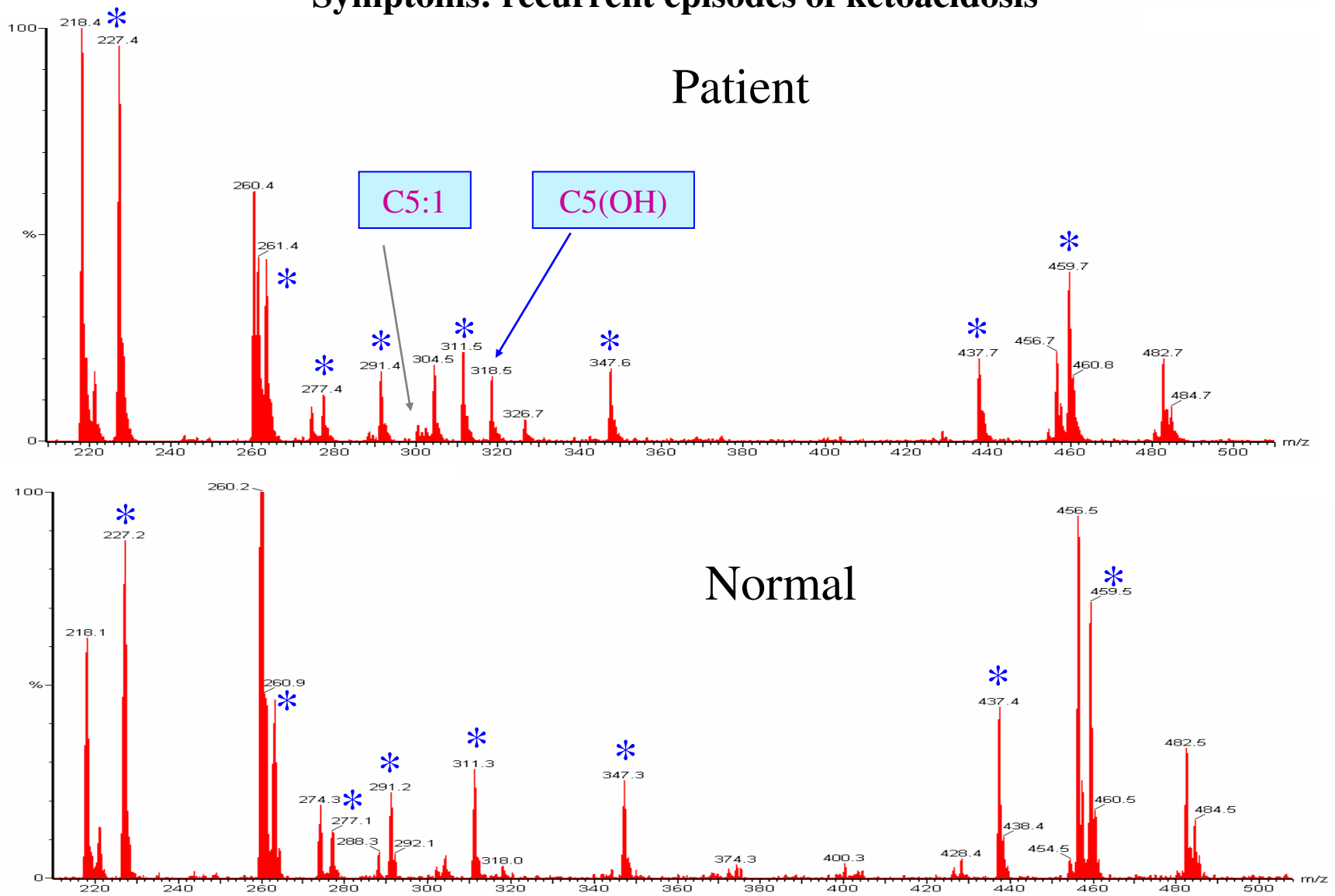
PM control <0.62

Post mortem sample

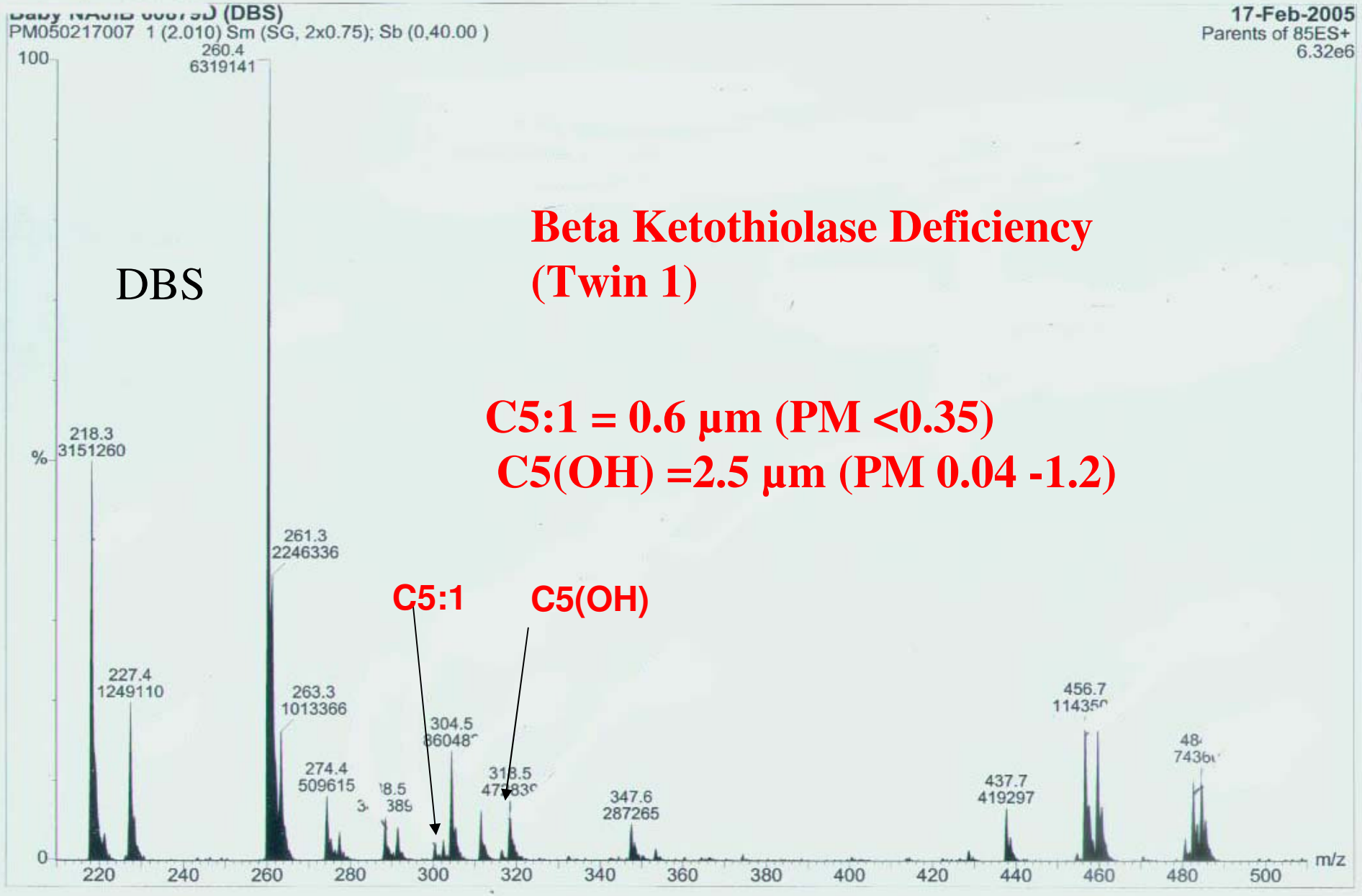



Branched chain amino acid metabolism: 3-KETOTHIOLASE DEFICIENCY.
VALINE
ISOLEUCINE
LEUCINE
2-ketovalerate
2-keto-3-methylvalerate
2-ketoisocaproate
Isobutyryl-CoA
2-methylbutyryl-CoA
Isovaleryl-CoA
Methacrylyl-CoA
C5:1
Tiglyl-CoA
3-methylcrotonyl-CoA
C5-OH
3-hydroxyisobutyrate
2-methyl-3-hydroxybutyryl-CoA
3-methylglutaconyl-CoA
Methylmalonylsemialdehyde
2-methyl-3-ketobutyryl-CoA
3-HMG-CoA
Propionyl-CoA
Acetyl-CoA
Acetoacetate
Methylmalonyl-CoA
Succinyl-CoA
TCA CYCLE

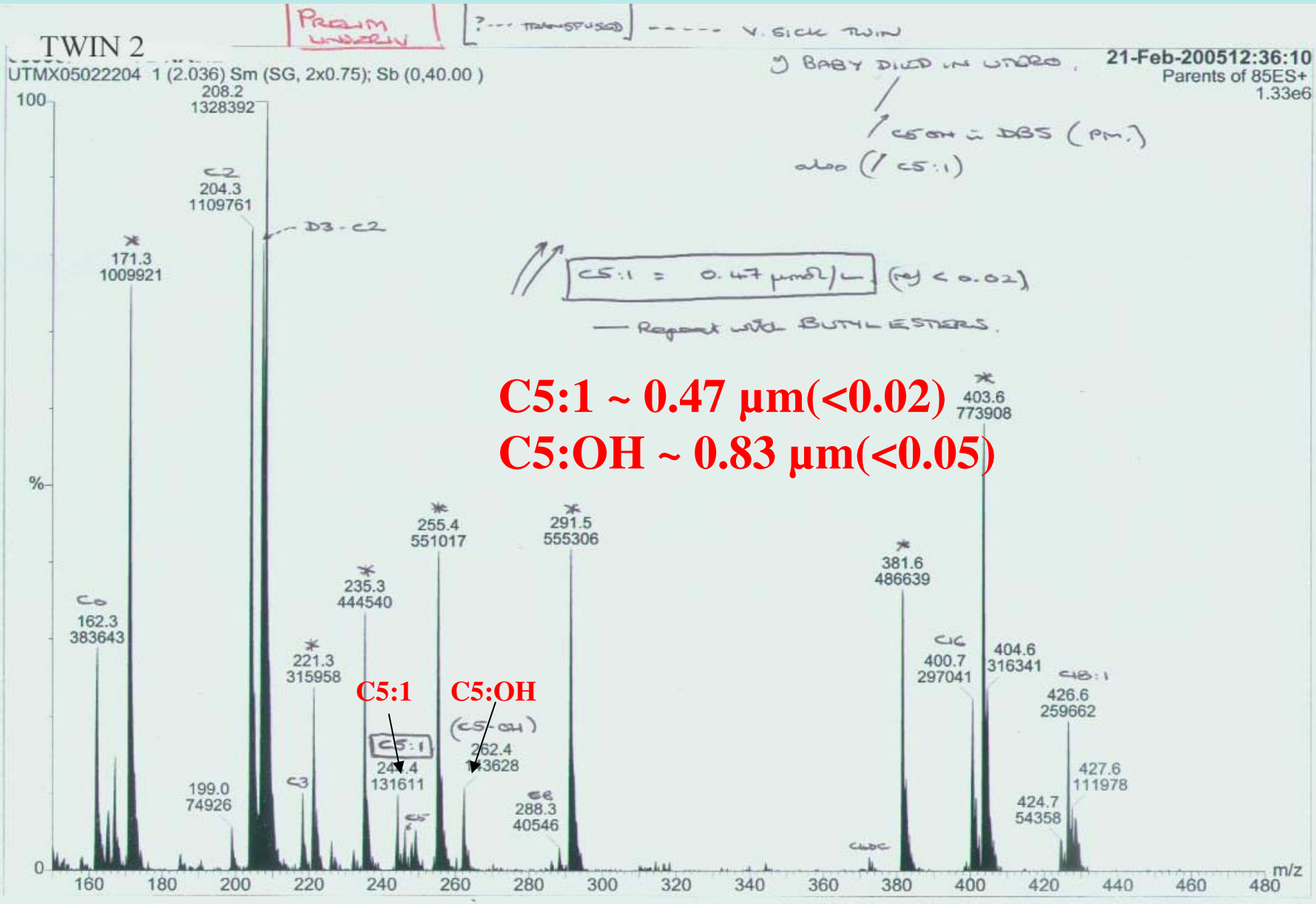
Symptoms: recurrent episodes of ketoacidosis



Post mortem sample

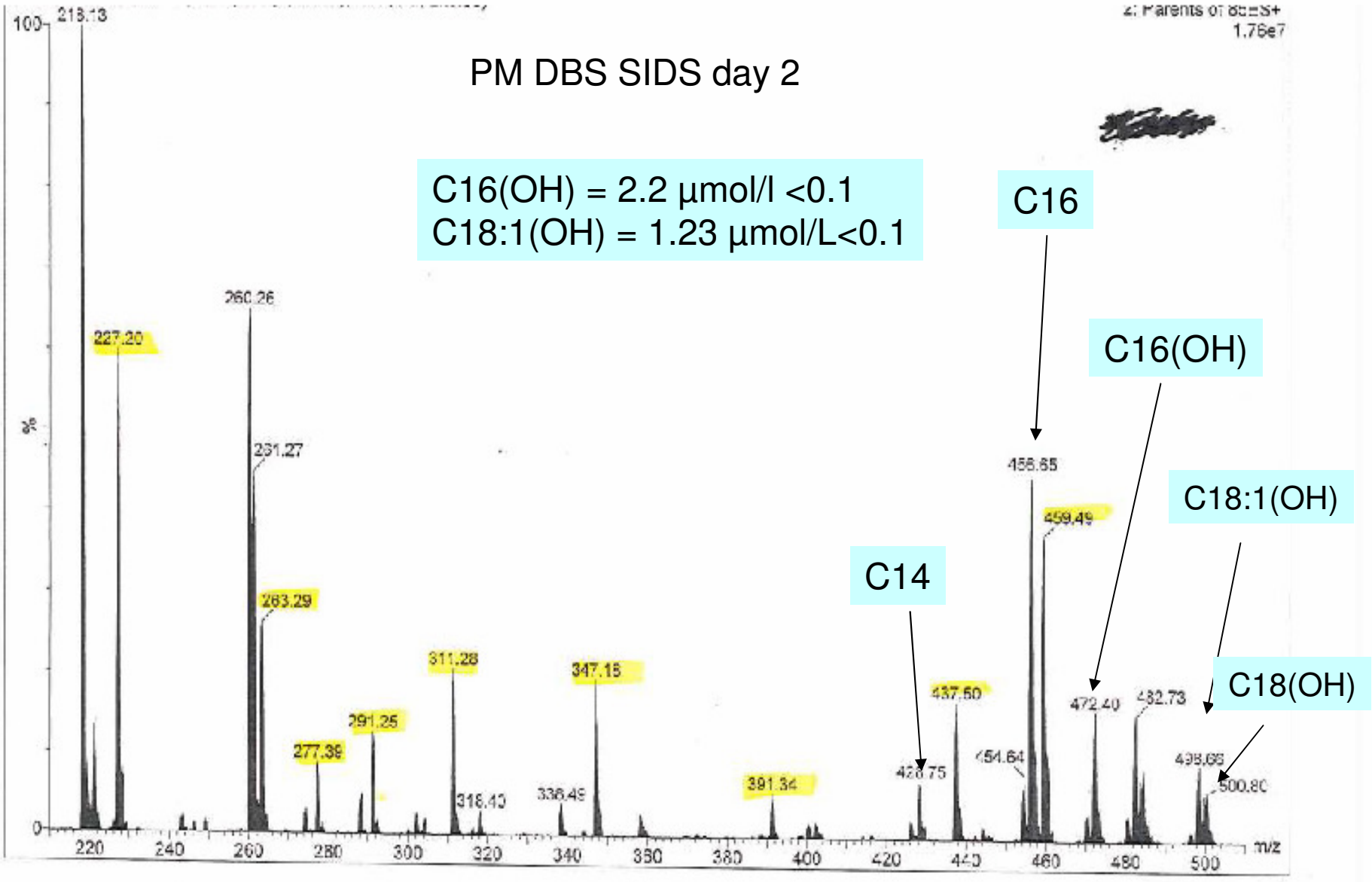


Sample from surviving Twin



PM DBS SIDS day 2

C16(OH) = 2.2 µmol/l <0.1
C18:1(OH) = 1.23 µmol/L<0.1



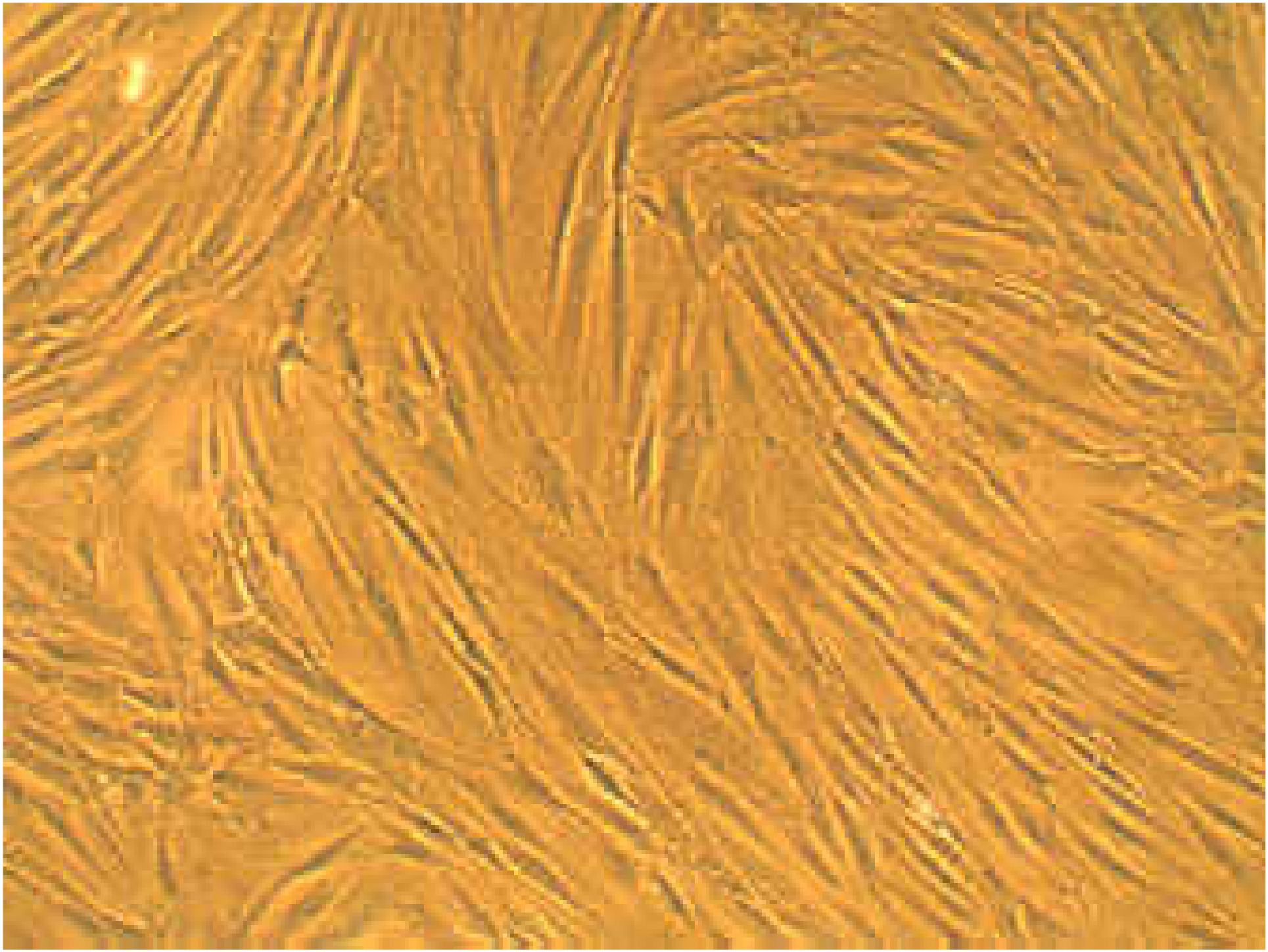
- Fatty acid oxidation
- Myristate = 42%
- Palmitate = 27%
- Oleate = 16%
- Common G1528C LCHAD mutation – not found
- LCHAD – 36 (34-114) nmol/mg/min
- LC thiolase – 2 (58-110)

- HADHB gene c.1292T.C plus c.1301C>T

- Mitochondrial Trifunctional Protein deficiency

POSTMORTEM DIAGNOSES
1989-2012 at SCH on fibroblasts

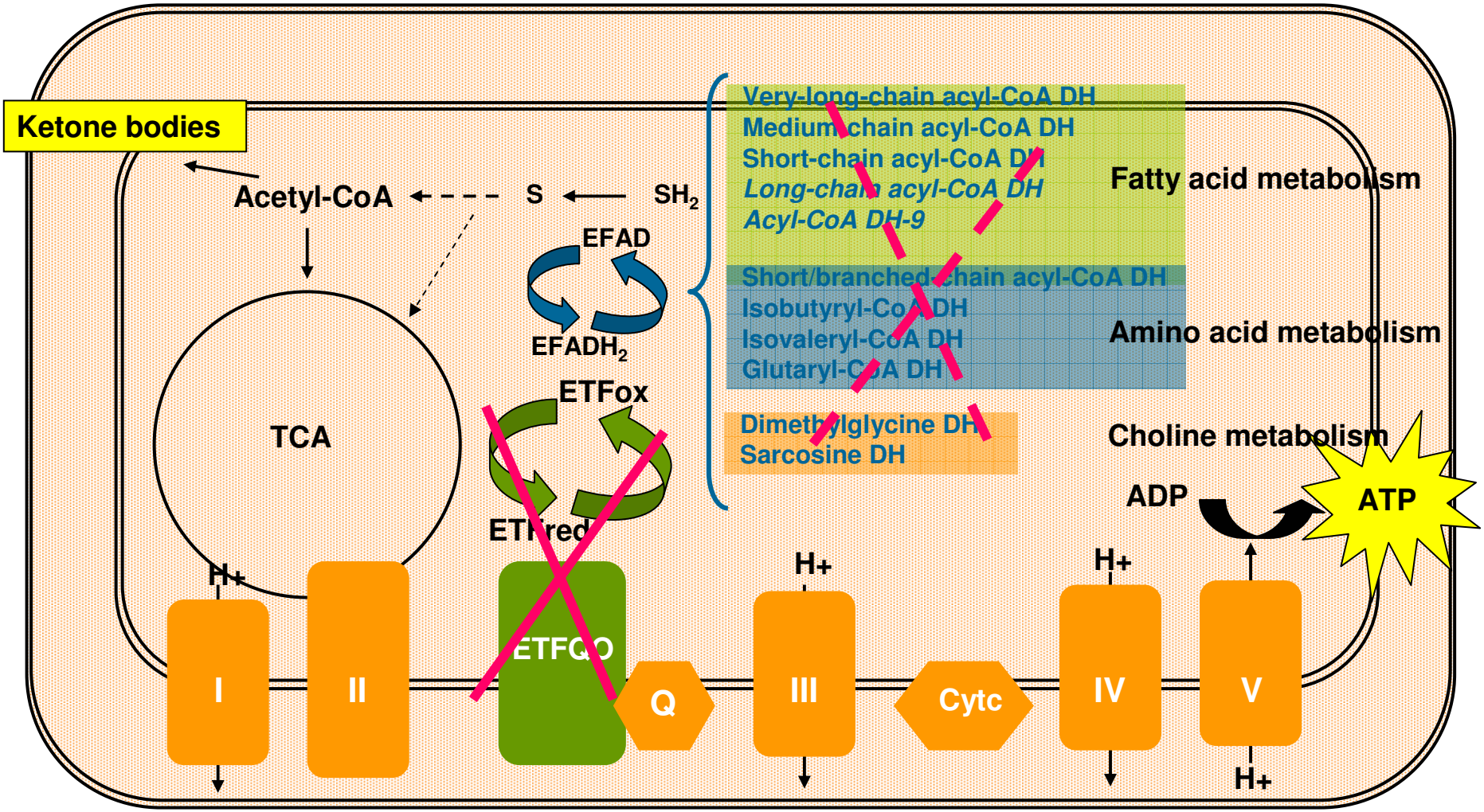
	Number
Respiratory chain defect	15
Multiple acyl-CoA dehydrogenase defect (severe)	12
Medium-chain acyl-CoA dehydrogenase defect	10
Carnitine palmitoyltransferase deficiency Type II	8
Very long-chain acyl-CoA dehydrogenase defect	7
Long-chain 3-hydroxyacyl-CoA dehydrogenase deficiency	7
Carnitine-acylcarnitine translocase	4
Mitochondrial trifunctional protein deficiency	4
Fumarate hydratase deficiency	2
Methylmalonic aciduria	2
Zellweger spectrum	2
Argininosuccinic aciduria	1
Carnitine palmitoyltransferase deficiency type I	1
Glutaric aciduria type I	1
Glutathione synthase deficiency	1
GSD IV	1
Isovaleric acidaemia	1
Congenital disorder of glycosylation type 1	1
Primary carnitine deficiency	1
Pyruvate dehydrogenase deficiency	1
X-linked adrenoleucodystrophy	1
Total diagnoses	**83**
Total number of post mortem cell lines	**1211**



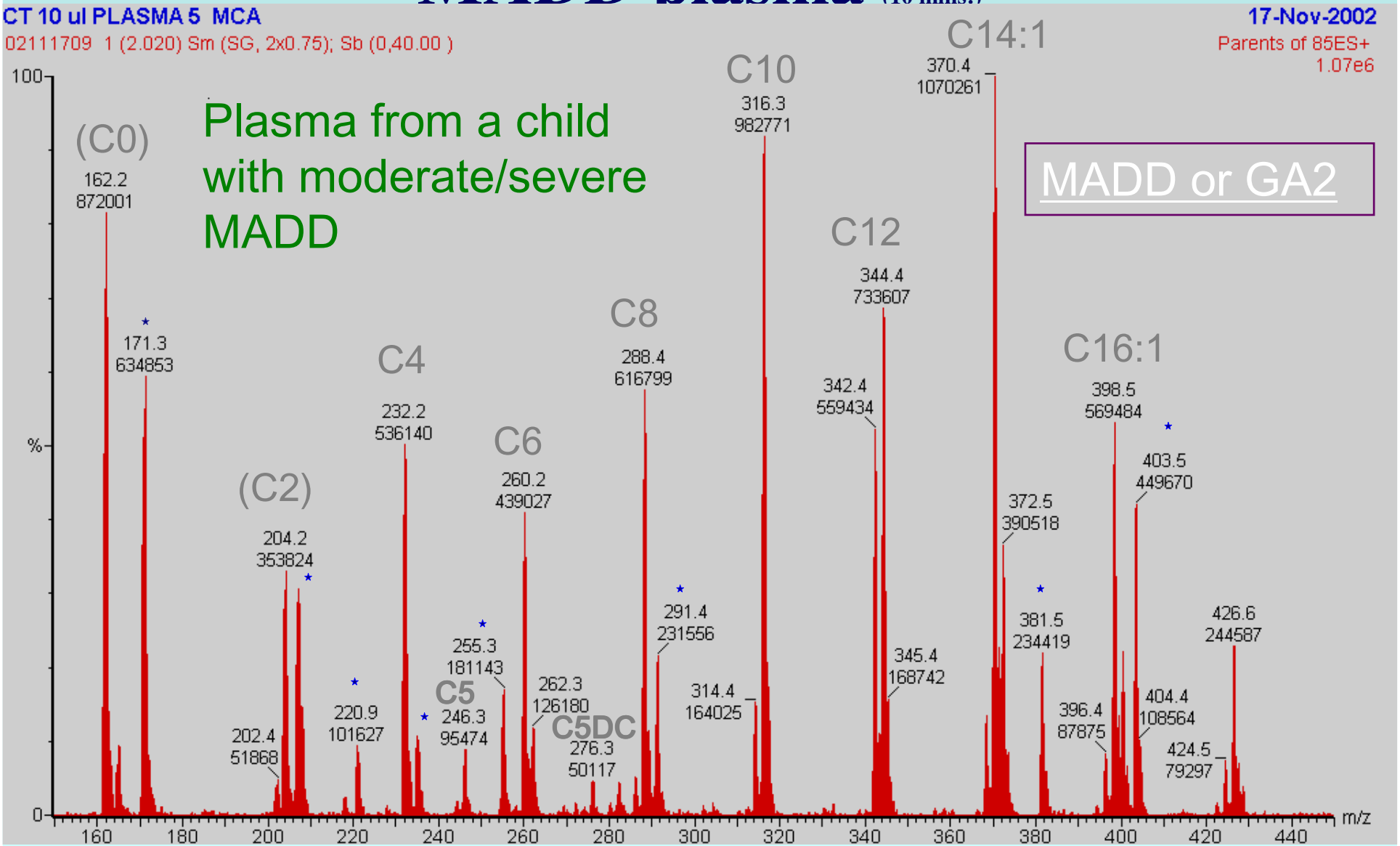
Multiple acyl-CoA dehydrogenase deficiency MADD

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

The Biochemical defect in MADD



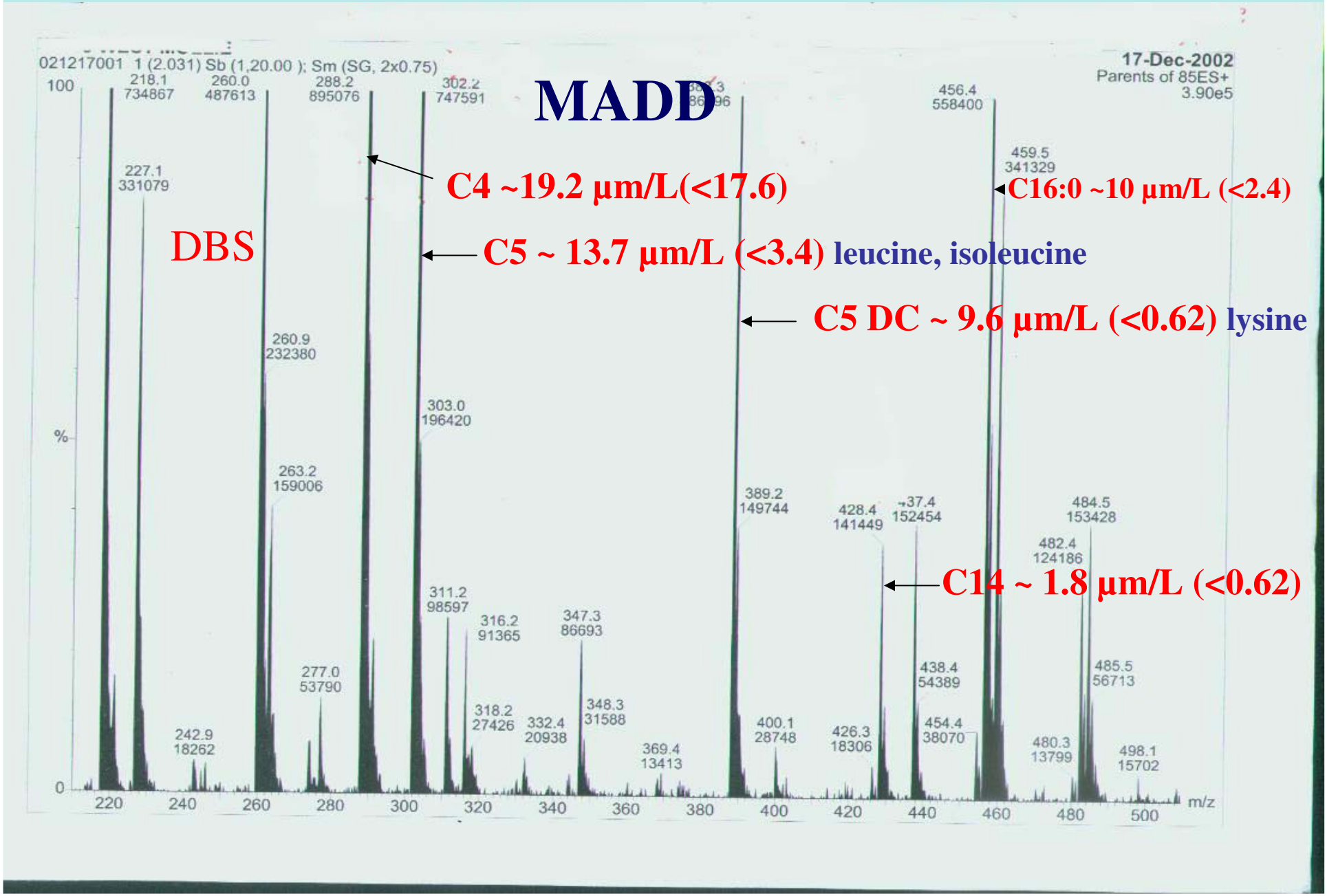
MADD plasma (10 mins!)



M W 19/12/02

- Appeared normal at birth
- Sent home on day 1
- Died during car journey home

Post mortem sample



Tritium release from labelled [9,10-^{3}H] substrates

[9,10-^{3}H]Myristic acid (C14:0)

$CH_3\ CH_2\ CH_2\ CH_2\ CH_2{*}\ CH_2{*}\ CH_2\ CH_2\ CH_2\ CH_2\ CH_2\ CH_2\ CH_2\ COOH$

[9,10-^{3}H]Palmitic acid (C16:0)

$CH_3\ CH_2\ (CH_2)_3\ CH_2\ CH_2{*}\ CH_2{*}\ CH_2\ CH_2\ CH_2\ CH_2\ CH_2\ CH_2\ CH_2\ COOH$

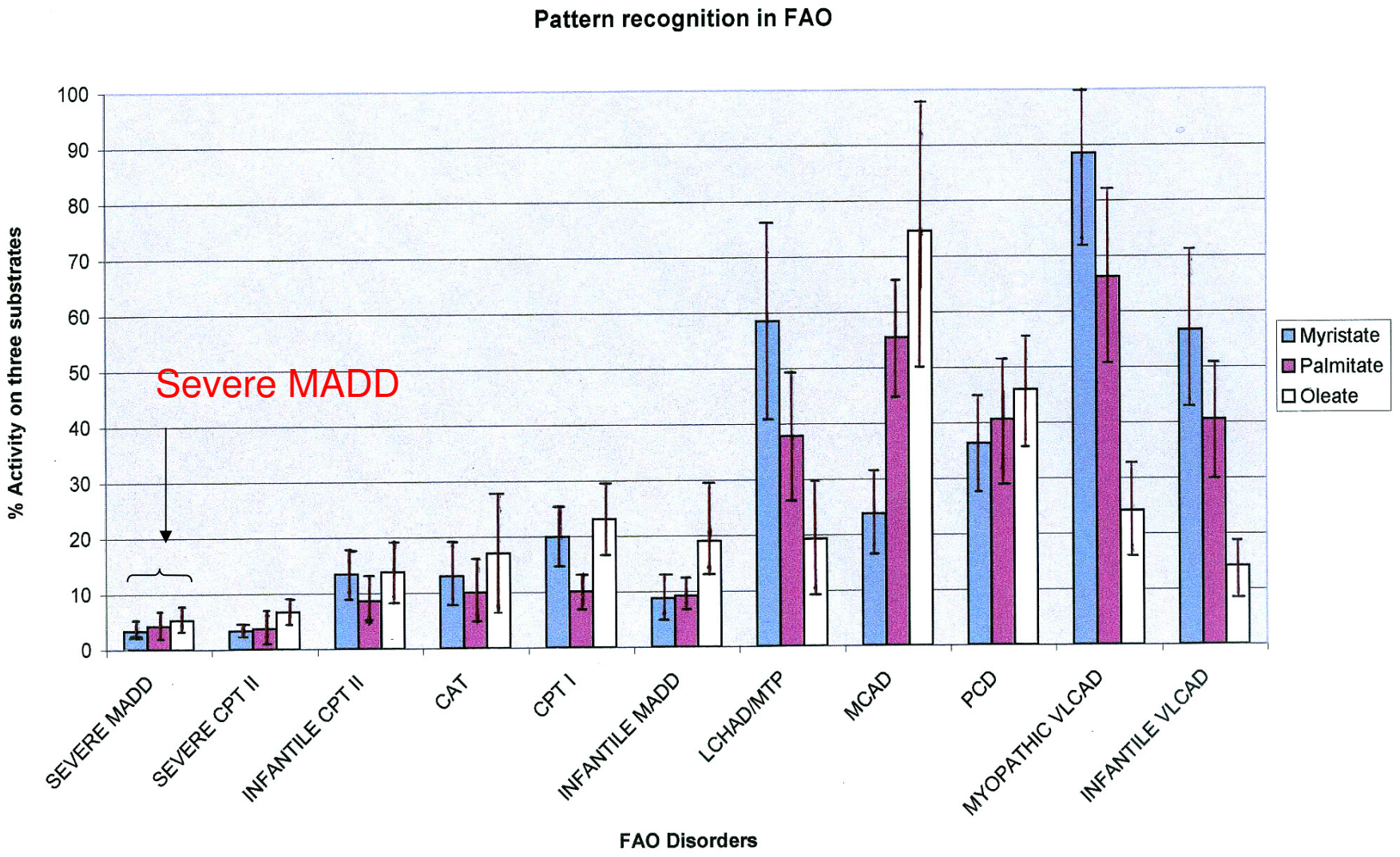
[9,10-^{3}H]Oleic acid (C18:1)

$CH_3\ (CH_2)_5\ CH_2\ \ CH_2\ \ CH{*}\ CH{*}\ CH_2\ CH_2\ CH_2\ CH_2\ CH_2\ CH_2\ CH_2\ COOH$

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

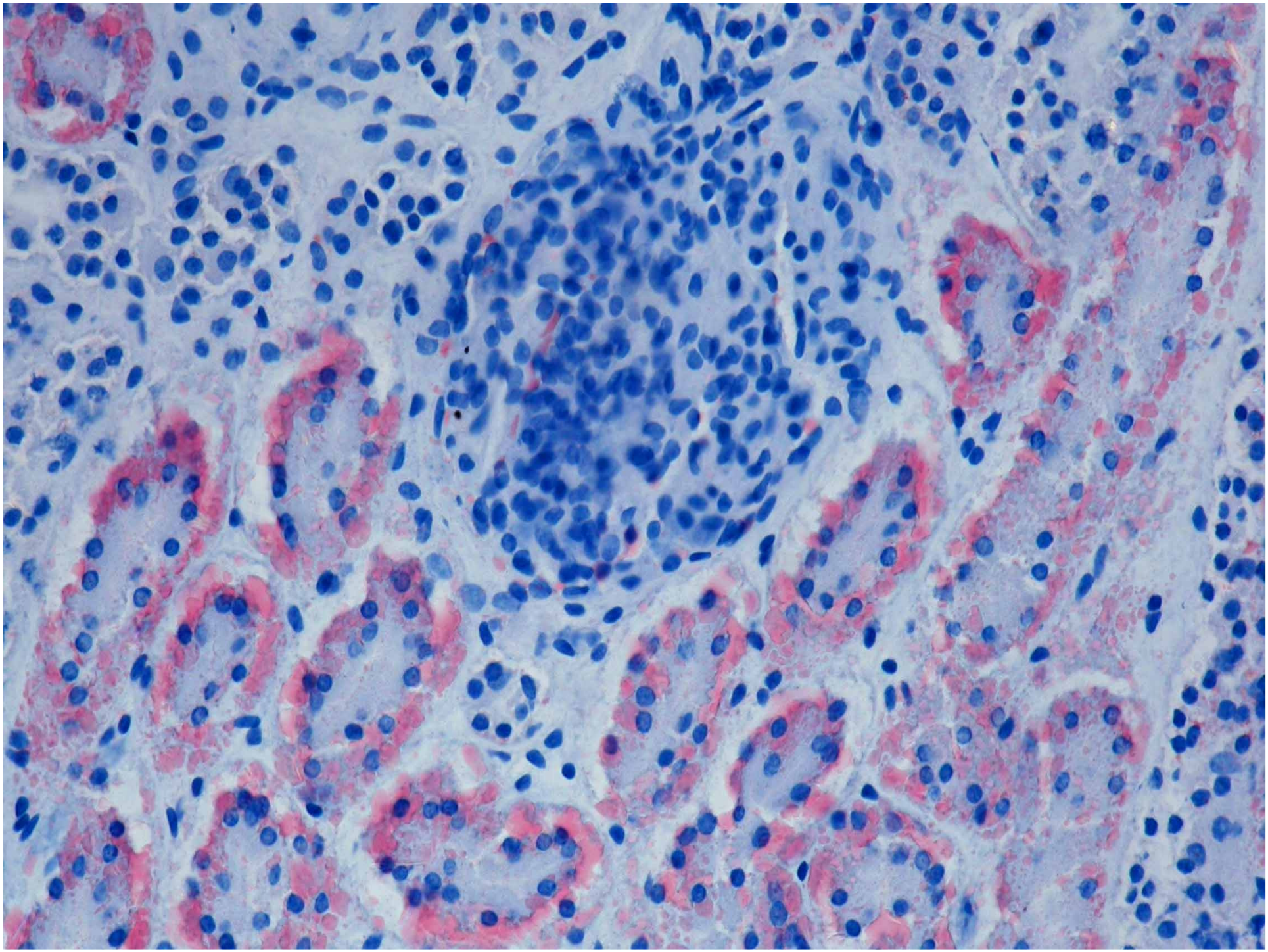


M W 19/12/02

- Two subsequent prenatals on amniotic fluid / cultured amniocytes
  - 1 affected
  - 1 unaffected

MK 14/12/04

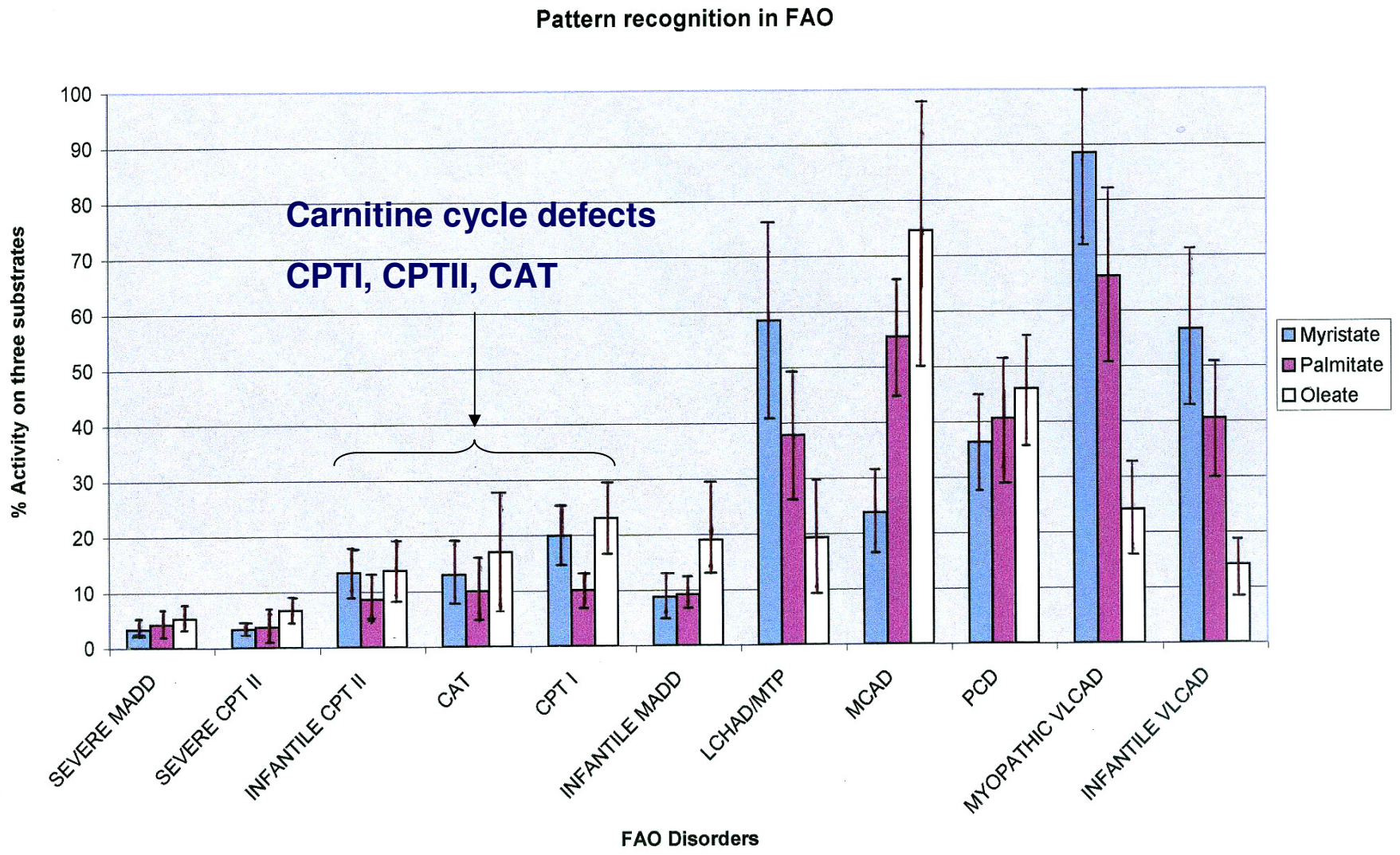
- Sudden death at 3 days
- PM findings
  - gross deposition of fat in liver
  - fat deposition in renal tubules



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



Fibroblast Acylcarnitine Profiling

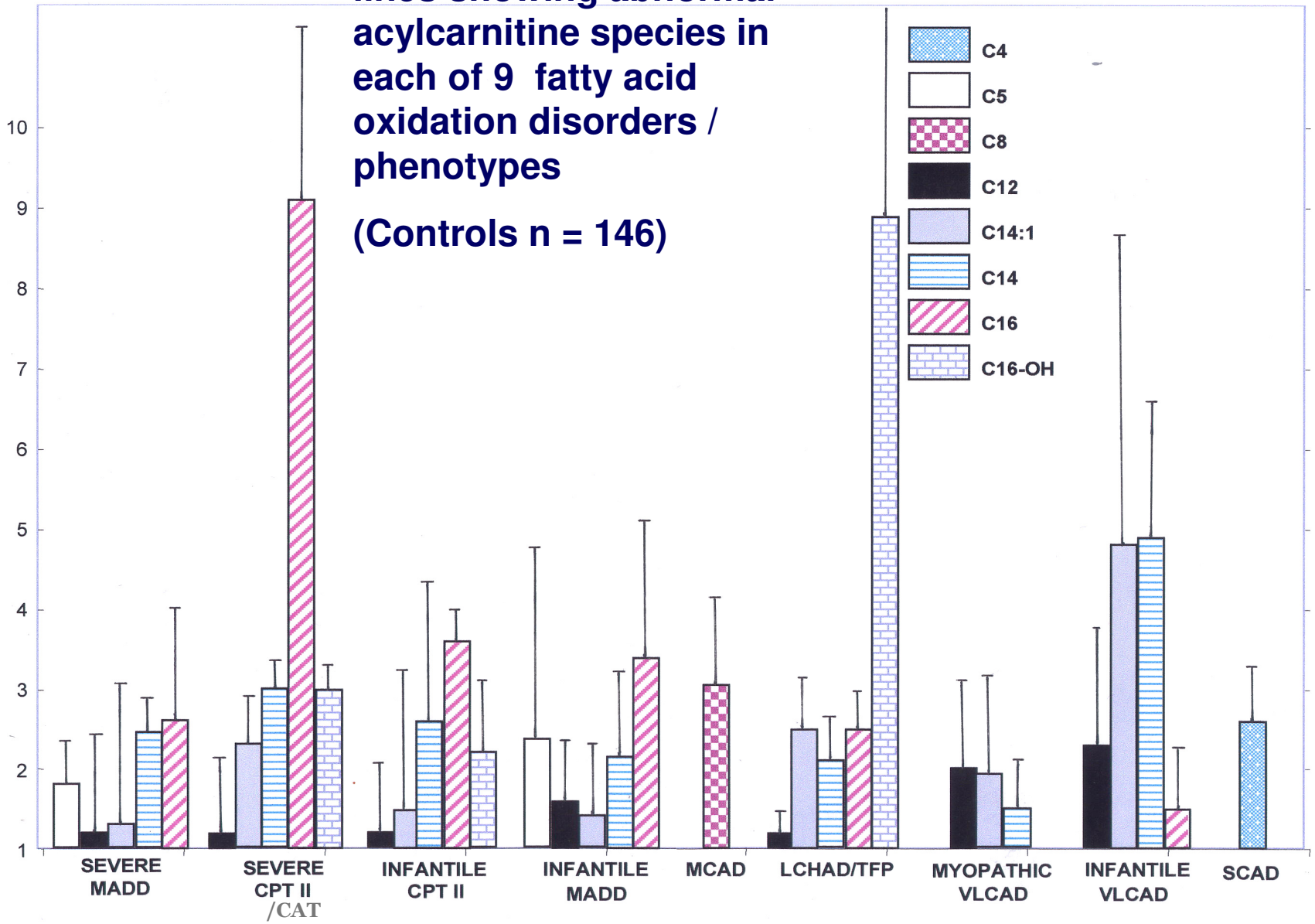
- Non-radioactive methodology
- Easier analysis of end product (MS/MS)
- Improved specificity

Principle of method

- Plate fibroblasts into multi-well plates
- Settle overnight
- Add substrate
  - Fatty acid plus carnitine
    - e.g. 200μm/L palmitate, 400 μm/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)

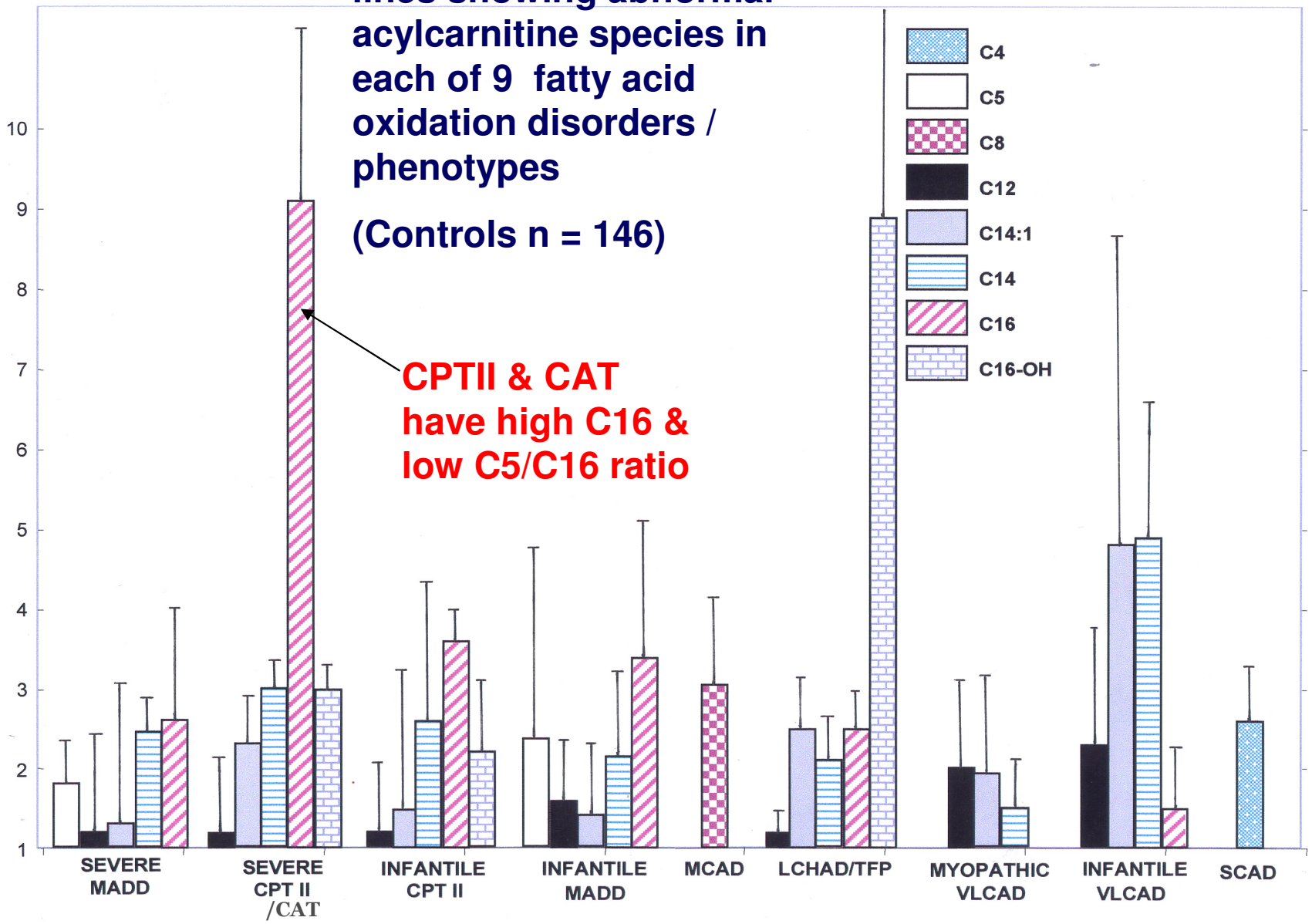


Acylcarnitine profiling in fibroblasts from MK

- 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

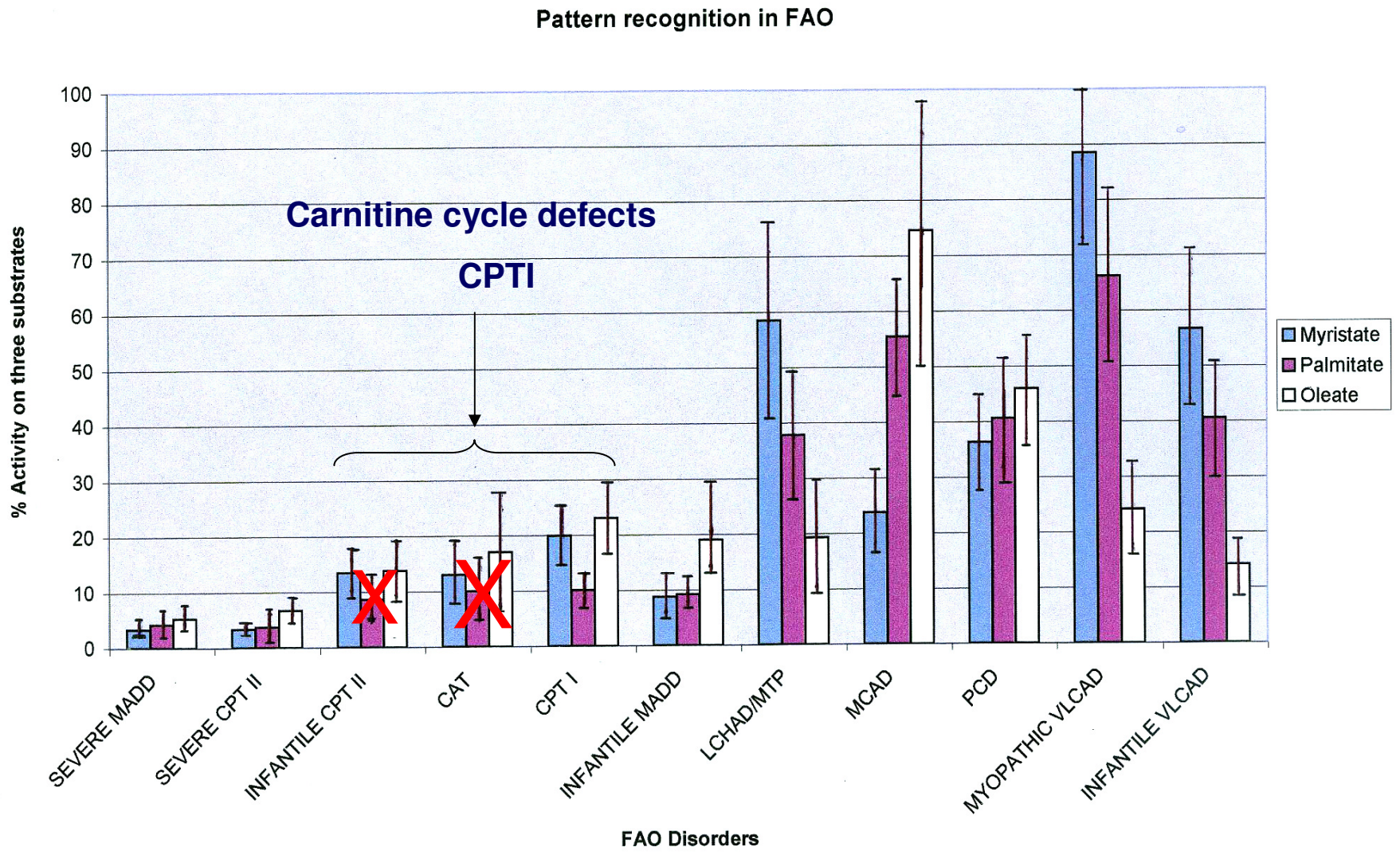
Data from 45 patient cell lines showing abnormal acylcarnitine species in each of 9 fatty acid oxidation disorders / phenotypes

(Controls n = 146)



CPTII & CAT have high C16 & low C5/C16 ratio

% residual activity for M/P/O for various FAOD's



MK has low C16 & high C5/C16 ratio in fibroblasts = CPTI

Acylcarnitine	Patient MK	Controls (n=70) Mean ± 2 SD	Positive CPTI controls N=5
C16	0.06	0.15 - 1.25	0.07; 0.15; 0.08; 0.16
C5/C16 ratio	15.5	0.13 – 1.01	6.1; 3.6; 5.1; 2.3

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

Acknowledgements

Clinical Chemistry

Shirley Clark
Farzana Ghoni
Helen Hind
Nigel Manning
Jenny Watkinson
Kate John
Ed Smith
Camilla Scott
Melanie Downing
Joanne Croft
Roy Talbot
Claire Hart
Jim Bonham

Histopathologists

Marta Cohen
David O'Neill

Sheffield Children's NHS
NHS Foundation Trust