Acylcarnitines –some things to make you think…

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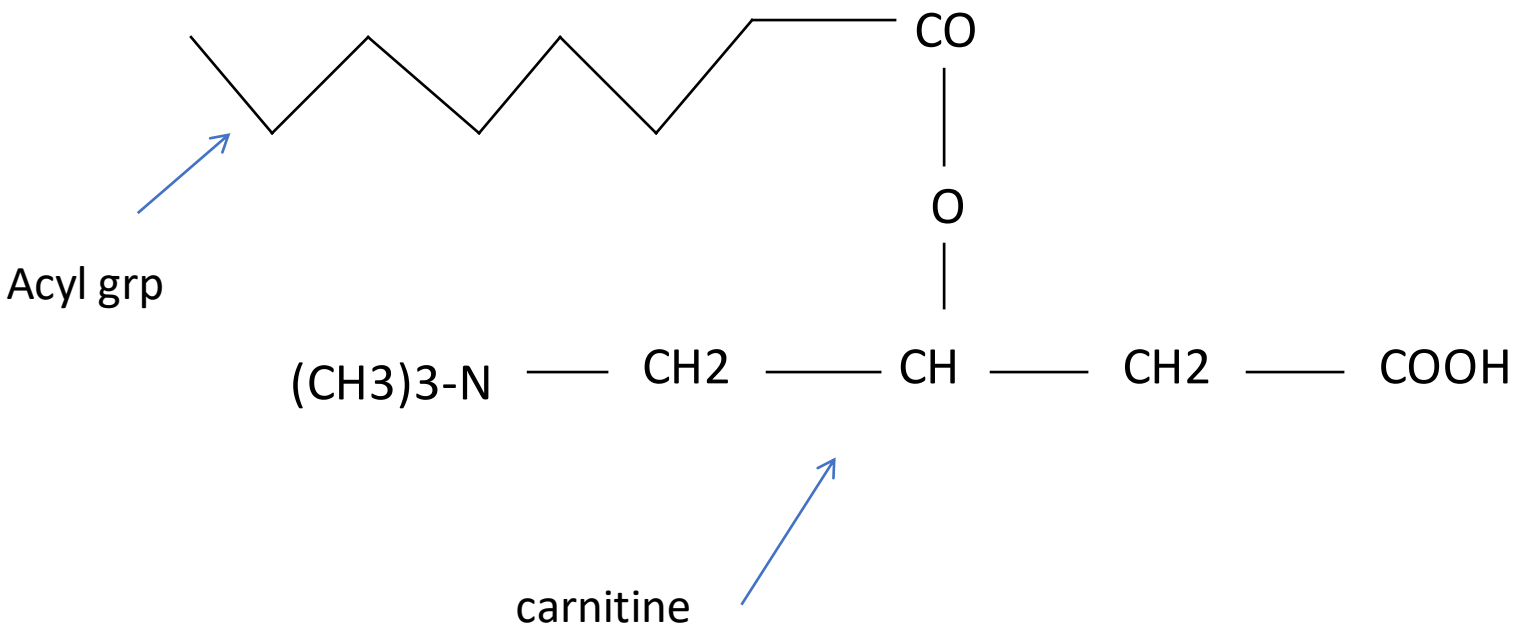
Content

- Firstly some key information on the nature of acylcarnitines and on the method so that we are all on the same page
- Continuing with examples of a number of issues and / or rare diagnoses and problems with interference

What is an Acylcarnitine?

- Acyl = carbon chain containing a carboxyl group
- Carnitine = a simple carrier molecule derived from the amino acid lysine

- A number of products of metabolism form acylcarnitine species therefore we can identify fatty acid oxidation disorders and organic acidaemias



Nomenclature

- We use a particular nomenclature to describe the various acylcarnitine species e.g.

➢ C3 = acyl group with 3 carbon chain

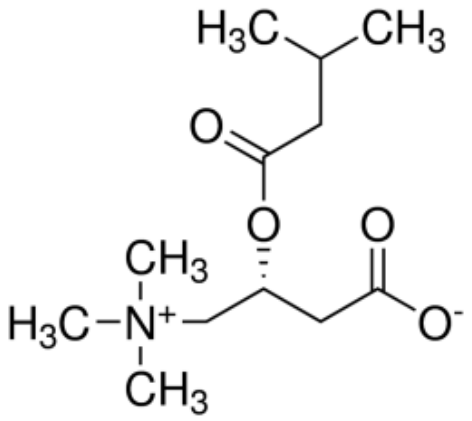
➢ C5DC = acyl group with 5 carbon chain and two carboxylic acid groups (dicarboxylate)

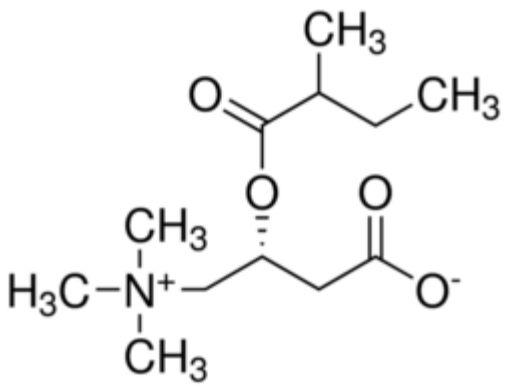
➢ C5-OH = acyl group with 5 carbon chain and a hydroxyl group

➢ C14:1 = acyl group with a 14 carbon chain that contains 1 double bond

Why do we use this nomenclature?

- Because we can't distinguish between acylcarnitine species that have the same molecular weight and are structural isomers by the MS/MS method
- e.g. C5 can be isovalerylcarnitine or it can be 2-methylbutyrylcarnitine (or pivaloylcarnitine)





DBS vs Plasma – what's the difference?

- DBS = gives you a more averaged out picture (thus avoiding metabolic noise) but less sensitive for milder disorders
- Plasma = can capture abnormalities that might only be detectable in acute phase / crisis sample e.g. mild CPT2 / VLCADs
- Some acylcarnitines species are naturally higher in DBS than plasma – so reference ranges need to adjust accordingly
- Biggest differences are in C3, C5-OH, C16 and C18:1 – largely due to tendency to stick to red blood cells

Acylcarnitine	DBS ref range	Plasma ref range
C3	<3.6	<1.3
C5-OH	<0.5	<0.06
C16	<5.3	<0.24
C18:1	<3.1	<0.28

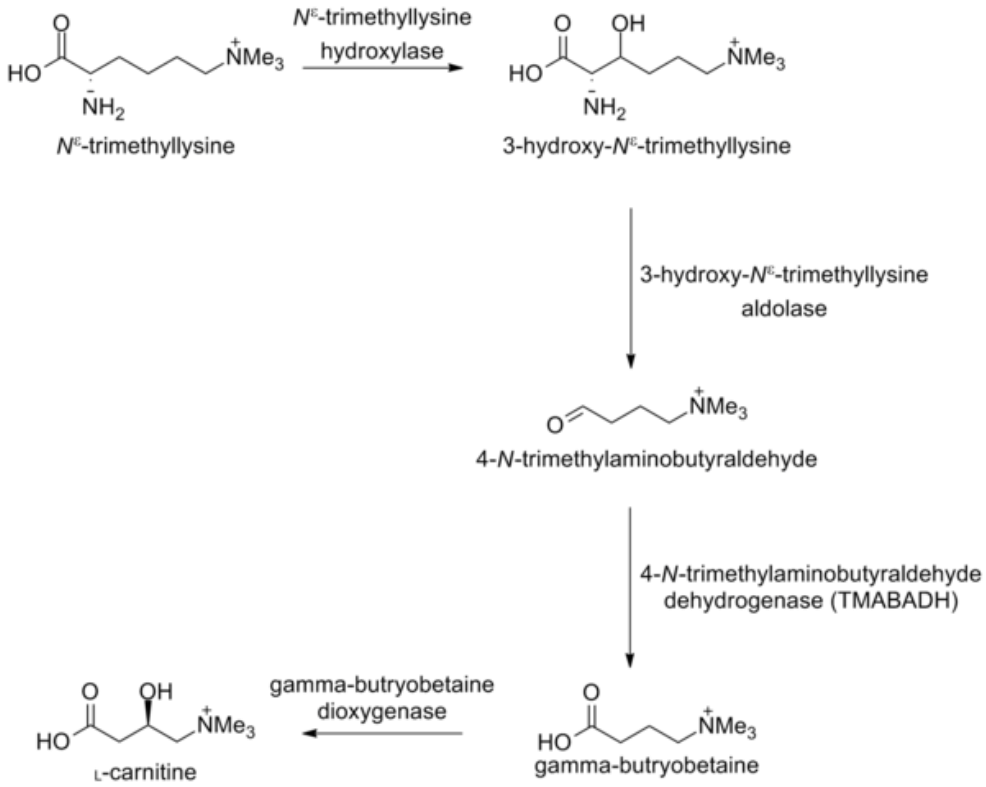
DBS vs Plasma – which is best?

- Plasma avoids the problem of poor or variable blood spot quality (and Guthrie cards that get sent to Newborn screening by mistake…)
- Otherwise depends on what you a trying to detect and what you are used to…
- But typically;
  - DBS is best for diagnosing **CPT1** (high free carnitine and low long chains) – because it is easier to low long chains against a background of typically higher C16 and C18:1
  - Plasma is best for **CPT2** - because easier to spot high C16 and C18:1 when they are typically lower – this is important factor in plasma being more sensitive for mild / late presenting CPT2

Let's Talk about Carnitine

Where does it come from?

- Diet AND endogenous synthesis
- Found in higher quantities in meat and dairy products so vegan diets are naturally low
- Synthesised in liver from N-trimethyllysine by a 4 enzyme pathway
- So far only deficiency of the 1st and 4th enzymes has been identified in humans
- However, carnitine synthesis disorder patients DO NOT typically have particularly low free carnitine as dietary sources are adequate and renal carnitine reabsorption efficient



Potential causes of low / lowish free carnitine (colour coded for likely severity)

- Neonate – fairly common in first week or two to see free carnitine mildly below reference range. Typically doesn't mean anything.
- Vegan diet – likely to have lower free carnitine on average
- Utilisation for fatty acid oxidation – may see mildly low free carnitine in aftermath of catabolic episode
- TPN – there is no free carnitine in standard TPN preparations. People on TPN are relying on biosynthesis unless supplemented
- Utilisation to form acylcarnitines when there is an underlying FAOD or organic acid disorder
- Carrier status for Primary Carnitine deficiency
- Primary Carnitine deficiency (Carnitine Transporter deficiency) results in profoundly low free carnitine – intracellular as well as in plasma – dietary intake and endogenous production cannot keep up with renal loss

What's the problem with low free carnitine?

- Without free carnitine you can't make acylcarnitines – and you can potentially miss a diagnosis

- E.g.

  19 yr old male, previously completely well, -collapsed / unconscious / encephalopathic

  Plasma free carnitine around 1-2 umol/L (ref 15-53)

  All acylcarnitine species very low / undetectable except C6, C8 and C10:1 which were detectable, C8 was on borderline of reference range

  Fortunately, urine for organic acids was available and confirmed diagnosis of MCAD

Another low free carnitine case…

70 yr old lady with lipid storage myopathy, mostly wheelchair dependent, head drop

- First Acylcarnitine Sample
- Free carnitine = 9 umol/L (15-53)
- Acylcarnitine profile –No significant abnormality
- Second Acylcarnitine sample AFTER carnitine supplementation….

Carnitine / Acylcarnitine	Plasma result	Reference range
C0	38	15-53
C4	0.68	<0.40
C6	0.41	<0.12
C8	1.19	<0.22
C10	2.20	<0.3
C12	0.72	<0.1
C14:1	0.71	<0.18
C16	0.43	<0.24
C18:1	0.47	<0.28

Is this rr MADD or other riboflavin related disorder?

- Repeat acylcarnitines showed same abnormal pattern
- But organic acids completely normal
- Patient given riboflavin…
- After 1 month she no longer had head drop and was walking with a frame! – so a clear **clinical** response to riboflavin
- However -Repeat acylcarnitines as this time showed…….

ABSOLUTELY NO BIOCHEMICAL RESPONSE TO RIBOFLAVIN!

(Genetics are currently awaited!)

Case 1

- 4 yr old boy, previously well
- Presented with hypoglycaemic seizure after episodes of vomiting the day before
- BM initially unrecordable, insulin appropriate, FFA > 3OHBA – therefore ?FAOD
- Dicarboxylic aciduria on organic acids
- Initial acylcarnitine (DBS) analysis showed very low free carnitine
- ? Primary Carnitine Deficiency
- Plasma sample found from same time point as urine – calculated RENAL TUBULAR MAXIMAL REABSORPTION OF FREE CARNITINE (TMAC)

TMAC

- Requires plasma and urine free carnitine and plasma and urine creatinine values (similar to TMP/GFR calculation)
- Relates free carnitine reabsorption to creatinine clearance
- Expressed as a percentage of free carnitine reabsorbed
- Normal is >95%
- Carriers of PCD may have mildly low reabsorption (approx. between 80-85% and 95%)
- Classic PCD patients have reabsorption in 30-40% range
- Most reliable if samples taken BEFORE any carnitine supplementation

Patient Results

- Plasma free carnitine = 1 umo/L
- Urine free carnitine = 6 umol/L
- Tubular reabsorption of free carnitine = 99%

NOT CONSISTENT WITH PCD – But why is free carnitine so low?

- Transpires that whole family including this patient were following a **VEGAN** diet
- Is this the cause? But if veganism alone was sufficient for such a low free carnitine wouldn't we see far more people with profoundly low free carnitine
- What about a CARNITINE SYNTHESIS DISORDER? – samples sent to Amsterdam for analysis

Carnitine Synthesis Intermediate Results

Plasma

- N-6-Trimethyllysine = 2.79 umol/L (0.20-1.20)
- Gamma butyrobetaine = 0.01 umol/L (0.30-1.40)

Urine

- N-6-Trimethyllysine = 47.9 mmol/mol creat (3.4-12)
- Gamma butyrobetaine = 0.00 mmol/L creat (0.00-0.60)

Results consistent with **TMLHE deficiency** (first enzyme in pathway)

Is postulated that the combination of **TMLHE deficiency and veganism** resulted in the profoundly low free carnitine – and caused a PCD / FAOD-like presentation during a catabolic episode. (Sadly, have not yet been able to confirm TMLHE genetically)

TMLHE deficiency

- N-Trimethyllysine hydroxylase deficiency
- First enzyme in carnitine synthesis pathway
- Converts N-trimethyllysine to 3-hydroxy-N-trimethyllysine
- TMLHE is on the X chromosome so is an X-linked disorder
- Has been reported to have an association with autism – a common exon 2 deletion is reported to be much more common in families with multiple affected males
- TMLHE deficiency has been found in 1 in 350 control males!
- Likely low penetrance if it is a risk factor for autism
- Levels of free carnitine reported to be low normal or mildly low

Case 2 – 6 month old boy, presenting with hypoglycaemia

Carnitine / Acylcarnitine	Plasma result / umol/L	Reference range
Free Carnitine	26	15-53
C2	69.7	5.5-27
C3	1.37	<1.30
C4	0.72	<0.40
C4-OH	0.1	<0.07
C12	0.22	<0.10
C14	0.69	<0.20
C16:1	0.51	<0.08
C16	2.23	<0.24
C18:1	2.25	<0.28
C18	0.30	<0.10
(C16+C18:1)/C2	0.065	(<0.05, Diagnostic range = >0.08)

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What would you suggest is the diagnosis?

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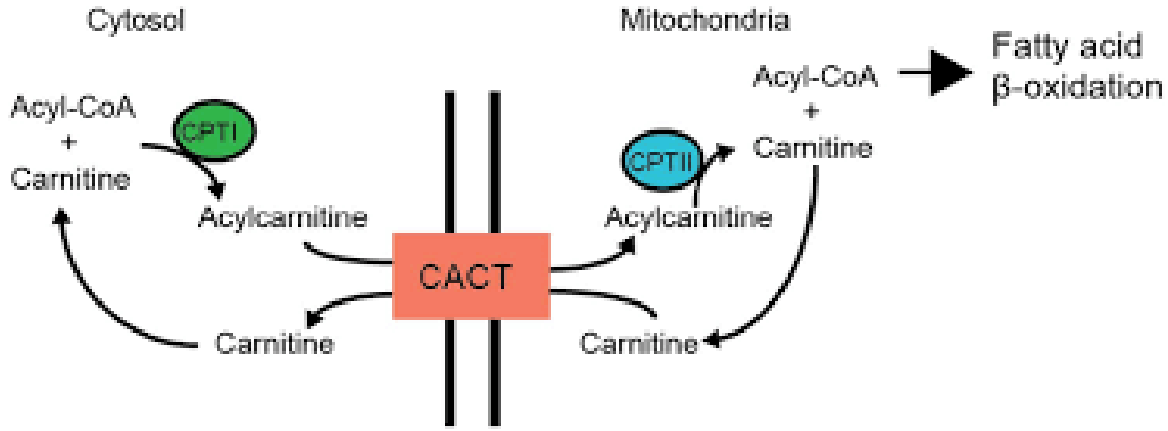
Case 2 – second sample a few weeks later

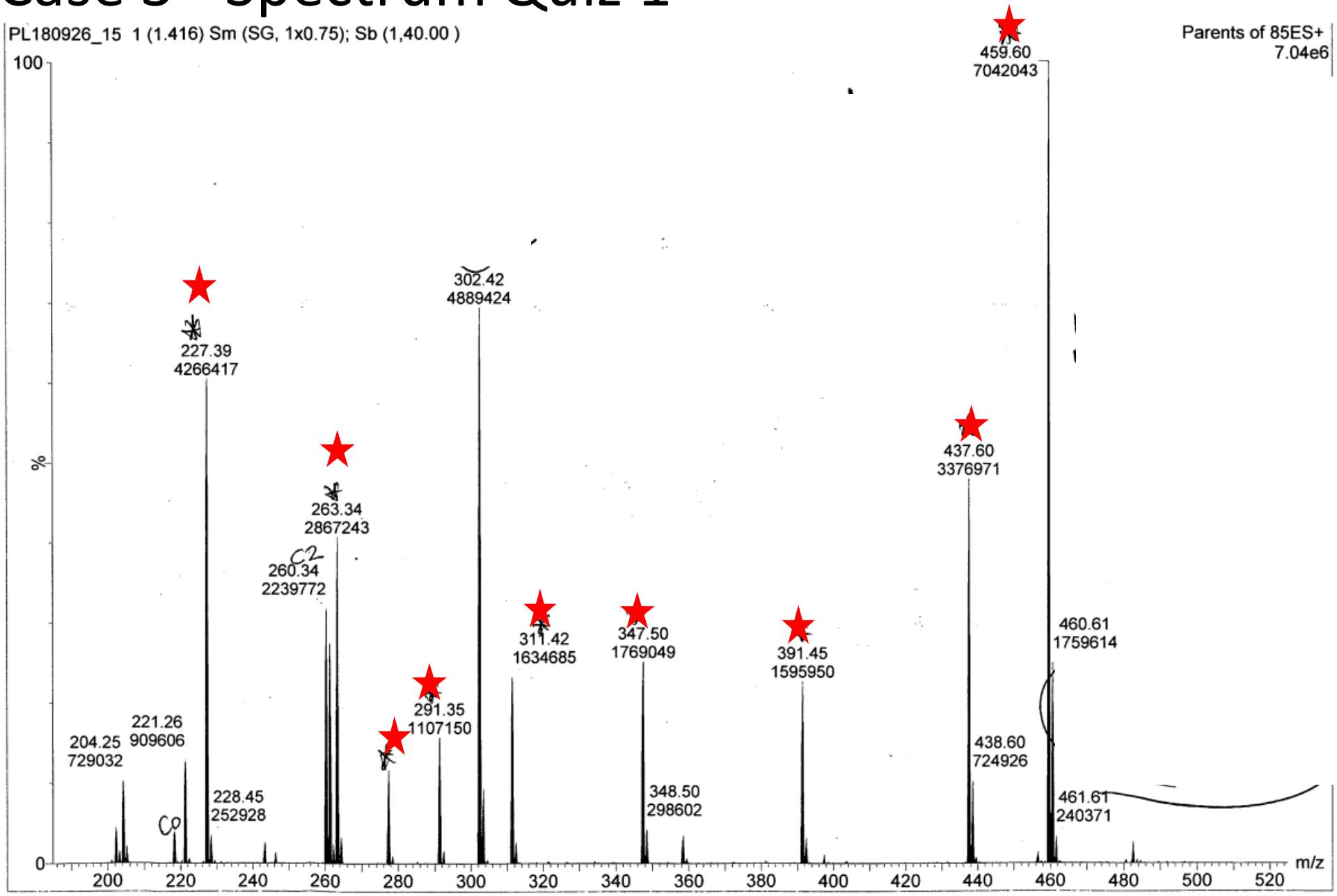
Carnitine / Acylcarnitine	Plasma result / umol/L	Reference range
Free Carnitine	9	15-53
C2	21.8	5.5-27
C3	-	<1.30
C4	0.42	<0.40
C4-OH	-	<0.07
C12	0.19	<0.10
C14	0.67	<0.20
C16:1	0.34	<0.08
C16	1.78	<0.24
C18:1	1.62	<0.28
C18	0.28	<0.10
(C16+C18:1)/C2	0.156	(<0.05, Diagnostic range = >0.08)

Case 2 cont…

- Second sample reported as likely CPT 2 deficiency – please refer to metabolic consultant and treat as affected until confirmed
- Genetics report 6 months later;
- Homozygous for c.82G>T p.(Gly28Cys) variant in SLC25A20 -consistent with **CACT (carnitine acylcarnitine transporter) deficiency**
- This particular mutation is known to be associated with attenuated disease that is later onset in presentation and associated with long term survival
- For unclear reasons these milder cases often have mildly elevated C4 acylcarnitine
- CACT is otherwise very difficult to distinguish from CPT2



Case 3 -Spectrum Quiz 1



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What is interesting about this spectra?

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Patient Info

- C0 = 1.5 umol/L (15-53)
- C5 = 2.8 umol/L (<0.5)
- All other acylcarnitines normal / low

- Patient is 68 yr old man, no clinical details, no previous samples

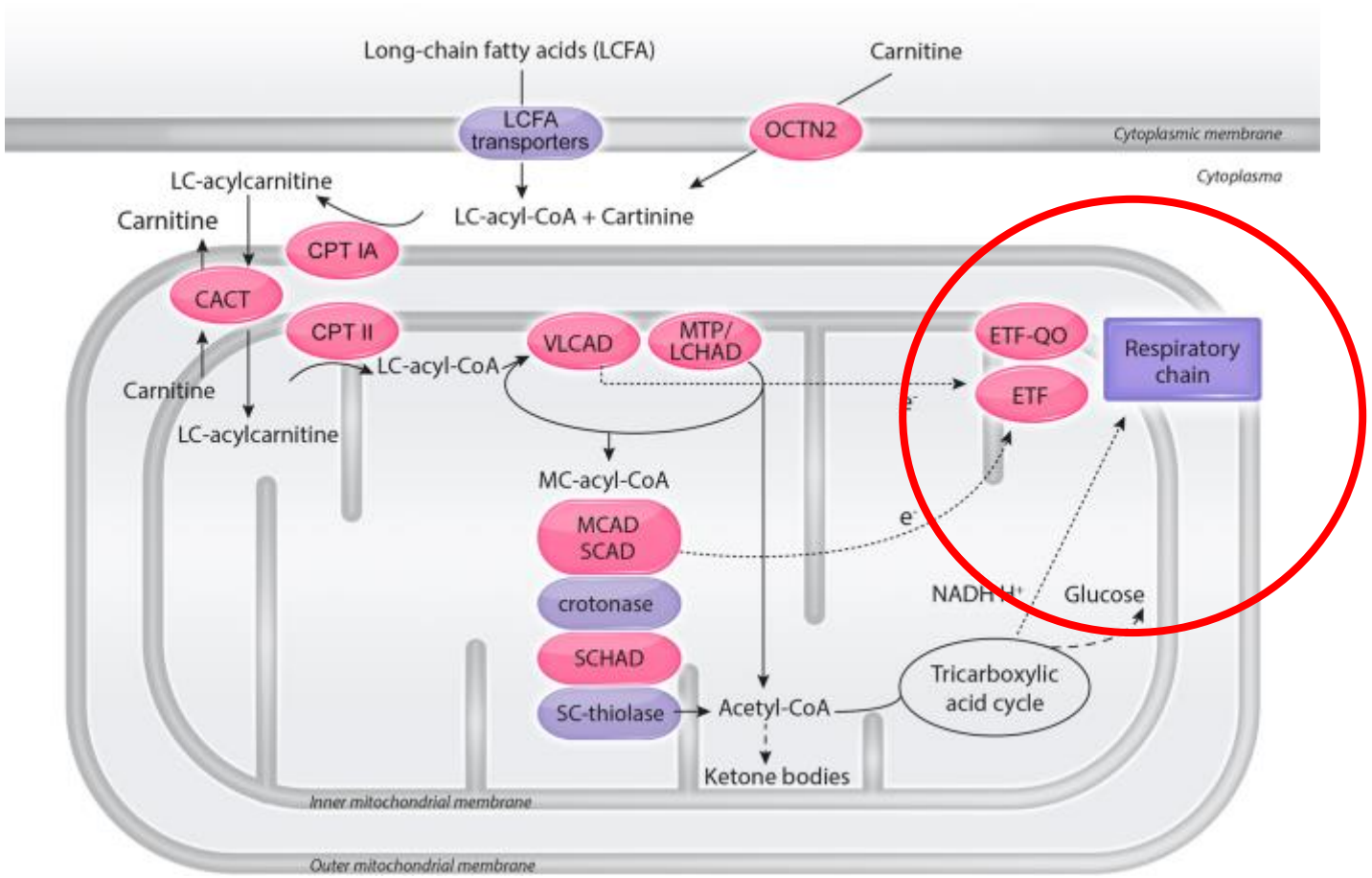
- What do you do next? What questions do you ask?

- Phone call to referral hospital uncovered that patient was being treated with **pivampicillin** (he was a retired doctor who had read that pivampicillin could result in carnitine depletion and ask his GP to send sample)
- Pivaloyl containing antibiotics can cause really marked carnitine depletion, and it's not just seen in the context of NBS false positives for IVA

Late onset riboflavin responsive-MADD

- Patients most likely to have raised medium chains – plus or minus increases in shorter (C4, C5) or longer (C14:1, C16, C18:1 etc) chain acylcarnitines, or C5DC, that look like partial MADD profiles
- Some patients may have organic acid abnormalities –(most likely increase in EMA, 2-hydroxyglutarate or small amounts of acylglycines)
- Common clinical details – myopathy, progressive limb weakness that is typically proximal, weakness of neck muscles / head drop- however some patients will present in a ketoacidotic crisis
- Muscle biopsy may show lipid storage myopathy (lipid droplets in muscle fibres)
- Unfortunately, it can be VERY difficult to confirm the diagnosis
- If mutations are found they are typically in ETFDH (rather than ETFA or ETFB) but very common that despite all the hallmarks no or only one mutation is found
- Can’t rule in or out with fatty acid oxidation studies in fibroblasts because you can’t grow the fibroblasts in a riboflavin deficient medium

Reminder of underlying defect in MADD



To transfer electrons from acylCoA-dehydrogenases to respiratory chain requires **ETF** (electron transport flavoprotein) which has two subunits **ETFA** and **ETFB**, and **ETF-QO** (ETF coenzyme Q oxidoreductase) which is the product of **ETFDH** gene

Also affects some dehydrogenases involved in metabolism of some amino acids, in particular lysine (hence the glutarate)

Case 4 - ?rr-MADD

- 17 year old girl, - “Myopathy - ? Metabolic”

Plasma acylcarnitine profile:
Free carnitine = 16 µmol/L -(ref. 15 - 53).
C8 = 0.86 µmol/L (<0.22)
C10 = 1.35 µmol/L (<0.3)
C5DC = 0.16 µmol/L (<0.06)
C14:1 = 0.30 µmol/L (<0.18)
C18:1 = 0.44 µmol/L (<0.28)

Organic Acids: Increased ethylmalonic acid and mildly increased 2-hydroxyglutaric acid

- Responded well clinically to riboflavin and found to be heterozygous for mutation in ETFDH gene (no second mutation found)

Case 5 and 6 - ? rr-MADD

- 25 yr old woman, “worsening limb weakness”
- C8 = 0.79 umol/L (<.022)
- C10 = 1.4 umol/L (<0.30)

- Organic acids = NSA

- One mutation in ETFDH found
- Response to riboflavin

- 55 yr old father of above, - presented 3 yrs later with “new onset proximal weakness”

  C4 = 0.53 (<0.4)
  C5 = 0.82 (<0.5)
  C8 = 0.32 (<0.22)
  C10 = 0.65 (<0.3)
  C5DC = 0.39 (<0.1)
  C14:1 = 0.82 (<0.18)
  C16 = 1.9 (<0.24)
  C18:1 = 2.18 (<0.28)

- Organic Acids - small increase in 2-hydroxyglutarate
- Same ETFDH mutation as daughter

Other causes of mildly raised medium chain acylcarnitines (C8 and C10 in particular, with C8 < C10)

- Theoretically possible to be stressed MCAD carrier (would be unusual to see in adult or older child)
- MCT feeds
- Valproate
- Mitochondrial dysfunction
- NAFLD / NASH
- Sertraline
- Dietary riboflavin deficiency
- Disorders of riboflavin metabolism / transport

Disorders of Riboflavin Transport

- **Disorders of Plasma Riboflavin transporters**:
  - SLC52A2 = Brown Vialetto-Van Laere type 1 = progressive neurologic disease with bulbar palsy, respiratory insufficiency and sensorineural deafness (also Fazio-Londe syndrome, same gene similar features but no deafness)
  - SLC52A3 = BVVL type 2
  - BVVL type patients may or may not show biochemical features of MADD and may have some response to riboflavin but depends on severity and age of diagnosis
  - Predominantly a severe disorder of childhood but some patients present in adulthood with milder / more nebulous symptoms

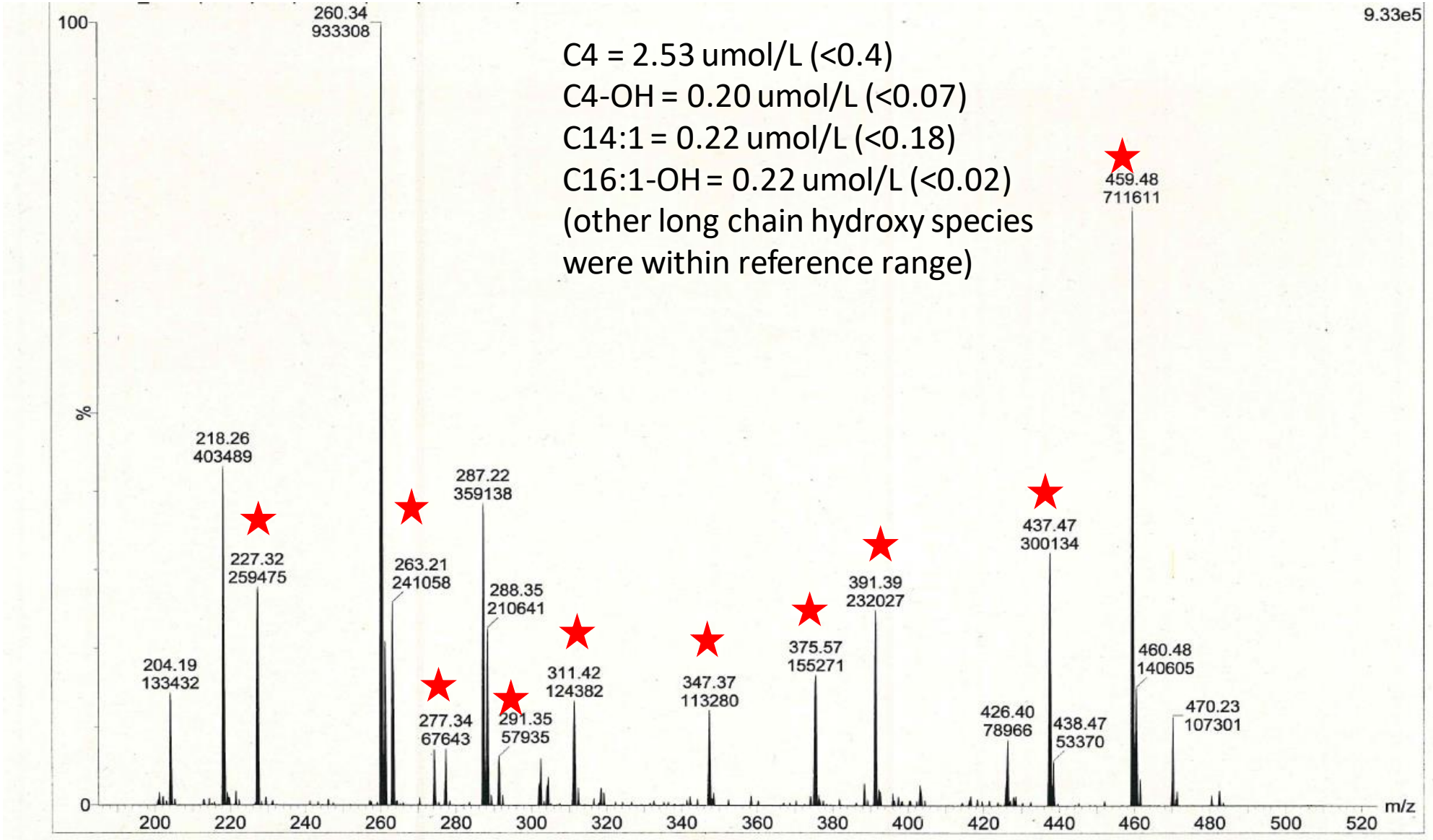
  - SLC52A1 - 2 infants heterozygote for SLC52A1 mutations and mothers with dietary riboflavin deficiency in pregnancy identified after being born with hallmarks of MADD that rapidly corrected on treatment with riboflavin

- **Disorders of Mitochondrial Riboflavin transporters**:
  - SLC25A32 – 2 patients with mostly muscle / exercise intolerance type symptoms and lipid storage myopathy on muscle biopsy presented with biochemical features of MADD, - rapid clinical improvement with riboflavin

Disorders of Riboflavin Metabolism (riboflavin to FAD/ FMN)

- FAD (flavin adenine dinucleotide) synthase deficiency (FLAD1 gene) is only known disorder in humans
  - Predominantly presents in infancy with severe neuromuscular problems and lipid storage myopathy, - typically noted to have multiple resp chain deficiency in muscle biopsy
  - Some of these patients have responded well to riboflavin if treated rapidly, untreated survival is poor
  - A few patients have present in adulthood with exercise intolerance and muscle weakness
  - Biochemistry can be typical for classic MADD but may be partial (even in early presenting patients)

Case 7 –Spectrum Quiz 2



C4 = 2.53 umol/L (<0.4)
C4-OH = 0.20 umol/L (<0.07)
C14:1 = 0.22 umol/L (<0.18)
C16:1-OH = 0.22 umol/L (<0.02)
(other long chain hydroxy species were within reference range)

★ = IS

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What do you think about the quantitative results in view of the spectra?

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What's going on?

- C16:1-OH in ISOLATION (might have slight C16-OH as well but C18:1-OH and C18-OH completely normal) indicates CEFOTAXIME interference
- C14:1 is often also elevated at the same time (higher the C16:1-OH the more likely this is the case)

They aren't real results but they do stop you from knowing what the true result is (would they be normal without the interference?)

- Interference from peak at m/z 287 stops you from being able to reliably measure C4 (how much is due to isotopic abundance and how much is truly C4?)

- m/z 287 can be due to **drug interference** (typically acutely unwell ICU patient) or **Formiminoglutamate** (Formiminoglutamic aciduria – probably benign biochemical disorder) – can confirm this by running Organic acids and looking for **hydantoin-5-propionate**

Some other Interferences / Complicating Factors

- In derivatised samples can get interference in m/z 260 (C2) from glutamate
- Benzoylcarnitine can be seen in patients on benzoate therapy = m/z 322
- Liver and / renal disease can cause general increase in the dicarboxylics e.g. C3DC, C5DC, C6DC, C8DC (m/z 430)
- Excess blood on a DBS card will tend increase C14 in particular
- If derivatisation is incomplete you will tend to see peaks at m/z 381 (d3-C14) and 403 (d3-C16)

Case 8

- 61 yr old man, recent history of recurrent rhabdomyolysis
- Initial plasma acylcarnitines results (all other acyls normal)
  - Free carnitine = 38 umol/L (15-53)
  - C16:1 = 0.11 (<0.08)
  - C16= 0.77 (<0.24)
  - C18:1 = 0.55 (<0.28)
  - C18 = 0.25 (<0.25)
  - (C16+C18:1)/ C2 ratio = 0.08 (ref = <0.05, “Diagnostic” = > 0.08)
- Mild raised long chains and ratio = ?? CPT2
- Repeat sample received, C16 and C18:1 a little higher this time and ratio = 0.14
- Sample received for fibroblast fatty acid oxidation flux studies
- Normal results at 37°C but reduced FAO at 41 °C
- Specific assay of CPT2 gave result of 23% of activity of simultaneous controls
- Consistent with a mild CPT2

Case 9

- 11 yr old boy, presented with peripheral neuropathy and myopathy, went on to have rhabdomyolysis type episodes, - ?FAOD – in particular, is it mild CPT2 or VLCAD?
- First DBS acylcarnitine reported as NSA
- Second DBS sample “trace long chain hydroxy acylcarnitines with C16-OH predominating, but only just above the upper limit of normal”
- FAO flux studies – initial results mildly abnormal but unclear
- Repeated with incubation at 41°C – results much more clearly abnormal and suggestive of a long chain defect
- Specific CPT2 assay normal
- LCHADD and long chain thiolase activity assayed – both moderately low - consistent with **mild MTP (mitochondrial trifunctional protein) deficiency**
- MTP is an octamer comprising 4 alpha subunits (HADHA) and 4 beta subunits (HADHB) – and has dehydrogenase (LCHAD), hydratase and thiolase activity
- Isolated LCHADD is caused by HADHA mutations
- MTP deficiency can be caused by HADHA **or** HADHB mutations

Summary

- Watch out for patients with low free carnitines – two questions to ask yourself;
  - Why is the free carnitine low?
  - What is it hiding? (? repeat after carnitine supplementation)

- rr-MADD patients are tricky! (and probably more common than you think) – but you also need to consider other possible IEM and non-IEM causes of the increased medium chains in particular
- Are you measuring what you think you are? Look out for interferences
- Patients with clinically mild versions of all FAOD can have minimally abnormal results particularly when well – need to have a suspicious mind