Acylcarnitines Workshop October 22th - 2021

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Quantitative analysys of acylcarnitines

- Factors affecting the analysis
- ACS scheme difficulties

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ACS Scheme Scientific Advisor

ACDB scheme difficulties and “weird” DBS acylcarnitines cases

Cristiano Rizzo and Charles Turner

ACDB Scheme Scientific Advisors

INTRODUCTION

As a reminder:

Acylcarnitines (AC) are either involved in the mitochondrial β-oxidation of fatty acids or in branched-chain amino acids catabolism, becoming an important part of the investigation of inherited metabolic diseases in the biochemical genetic laboratories.

The development of tandem mass spectrometry (MS/MS) facilitated the analysis AC, which is used in the newborn screening programs of many countries, using dried blood spots (DBS).

INTRODUCTION

More recently, there had been an increasing demand for satisfactory quality assurance in the biochemical genetic laboratories including external quality control to guarantee comparability of results between different centers (Fowler et al. 2008).

J Inherit Metab Dis (2008) 31:680–689
DOI 10.1007/s10545-008-1025-4

REVIEW

Quality of analytical performance in inherited metabolic disorders: the role of ERNDIM

B. Fowler · A. Burlina · V. Kozich · C. Vianey-Saban

Received: 6 August 2008 / Submitted in revised form: 23 October 2008 / Accepted: 24 October 2008 /
Published online: 21 November 2008

INTRODUCTION

It was also observed that there was a demand for the analysis of AC from a quantitative point of view.

In 2015, a pilot test about analysis in plasma/serum was carried out.

The ERNDIM EQA included the ACS Scheme officially in 2017.

JIMD Reports
DOI 10.1007/8904_2016_533

RESEARCH REPORT

Pilot Experience with an External Quality Assurance Scheme for Acylcarnitines in Plasma/Serum

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QUANTITATIVE ANALYSIS OF ACYLCARNITINES IN PLASMA/SERUM

Before comment some results in ACS Scheme, we are going

to review the methods used in the analysis of AC,

in order to better understand the ACS Scheme results that we will comment on later.

METHODS by MS/MS

Basically, methods are different in :

- **Step of derivatization**
- **Data acquisition mode**
- **Use of chromatography**
- **Use of different stable isotopically labelled standards**

METHODS by MS/MS

Derivatization?

- **Butyl esters of AC:**
  - **Advantages:**
    - **Better Sensitivity in dicarboxylic AC.**
    - **More selectivity, differentiation between some AC with same molecular ion (C3DC/C4OH, C4DC/C5OH).**
    - **Molecular ion is higher, so the effect of low mass interferences is reduced**

- **Underivatized:**
  - **Advantages:**
    - **Simpler preparation.**
    - **Avoid corrosive reagents.**

METHODS by MS/MS

Derivatization?

- **Butyl esters of AC:**
  - **Disadvantages: · Less sentitivity by incomplete butylation or hydrolysis that increases the concentration of free carnitine and decreased of AC**

    · More laborious and time consuming

    · The use of corrosive reagents

- **Underivatized:**
  - **Disadvantages: · Less sensitivity in dicarboxylic**

    · Less selectivity in AC with same M^+ (C3DC/C4OH, C4DC/C5OH)

    · dirtier mass spectra

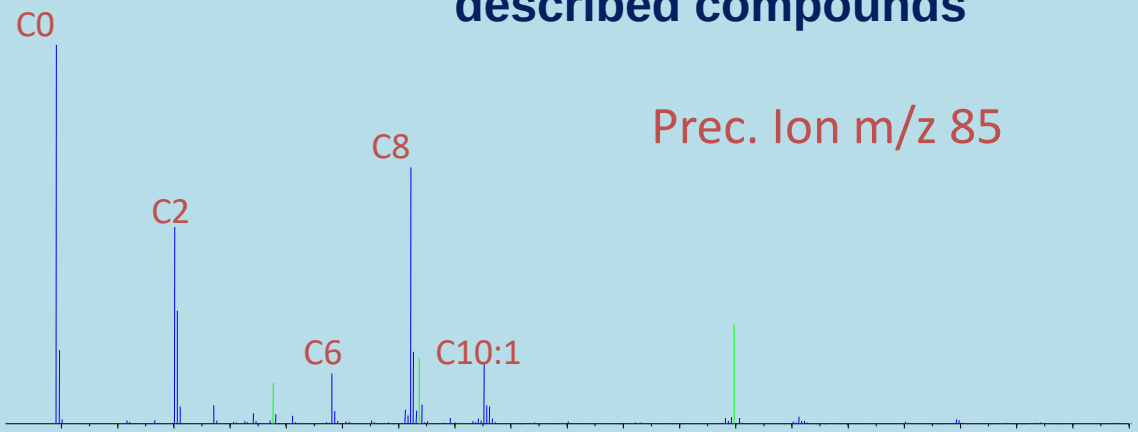
METHODS by MS/MS

Acylcarnitines with same molecular ion, Underivatized or as butyl esters:

Underivatized			Derivatized		
m/z	Notation	AC	*m/z*	Notation	AC
232	C4	Butyryl Isobutyryl	288	C4	Butyryl Isobutyryl
244	C5:1	Tigly l 3-Methylcrotonyl	300	C5:1	Tigly l 3-Methylcrotonyl
246	C5:0	Isovaleryl 2-Methylbutyryl-(D+L) Pivaloyl	302	C5:0	Isovaleryl 2-Methylbutyryl-(D+L) Pivaloyl
248	C3DC C4OH	Malonyl 3-Hydroxybutyryl	360 304	C3DC C4OH	Malonyl 3-Hydroxybutyryl
262	C4DC C5OH	Methylmalonyl-(D + L) Succinyl 3-Hydroxyisovaleryl 2-Methyl-3-hydroxybutyryl	374 318	C4DC C5OH	Methylmalonyl-(D+L) Succinyl 3-Hydroxyisovaleryl 2-Methyl-3-hydroxybutyryl
276	C5DC C6OH	Glutaryl Hydroxyhexanoyl	388	C5DC C10OH	Glutaryl Hydroxydecanoyl
290	C6DC	Methylglutaryl Adipyl	402	C6DC	Methylglutaryl Adipyl

METHODS by MS/MS

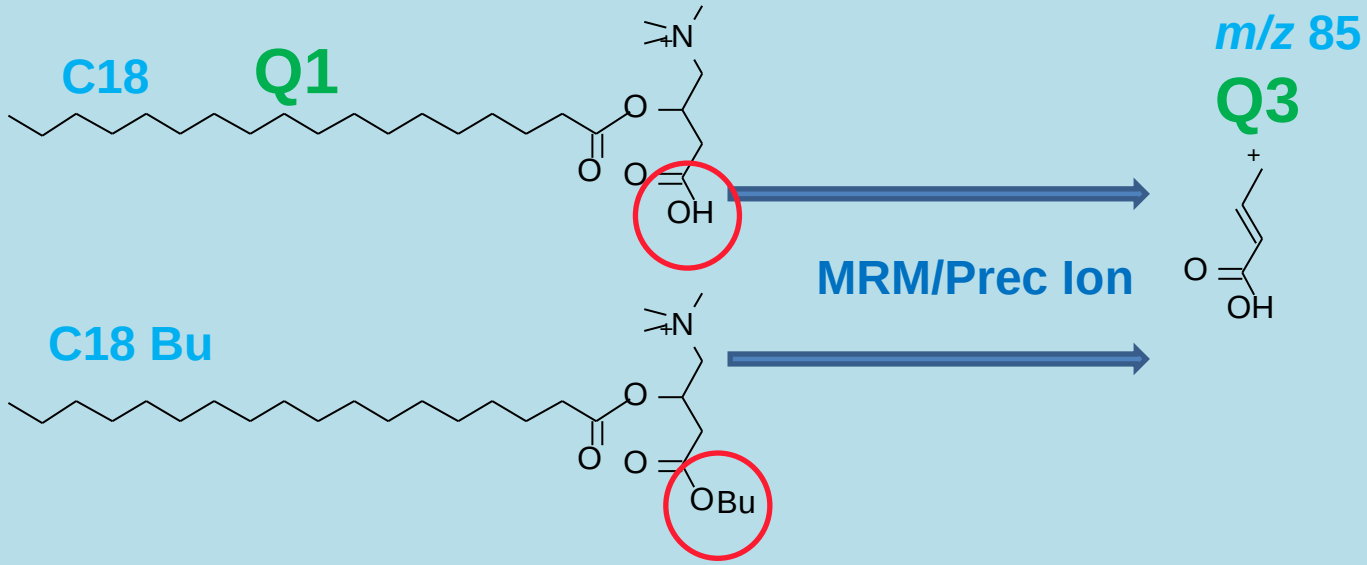
- **Data acquisition mode?**
  - **MRM (multiple reaction monitoring): more sensitivity but only detects the ACs that are indicated from the beginning**
  - **Precursor ion: Mass spectrum is available to check interferences, unexpected or recently described compounds**



MCAD deficiency

METHODS by MS/MS

- **Data acquisition mode:**
- **ion fragment in 3rd quadrupole usually is the same (*m/z* 85) independent of the method:**



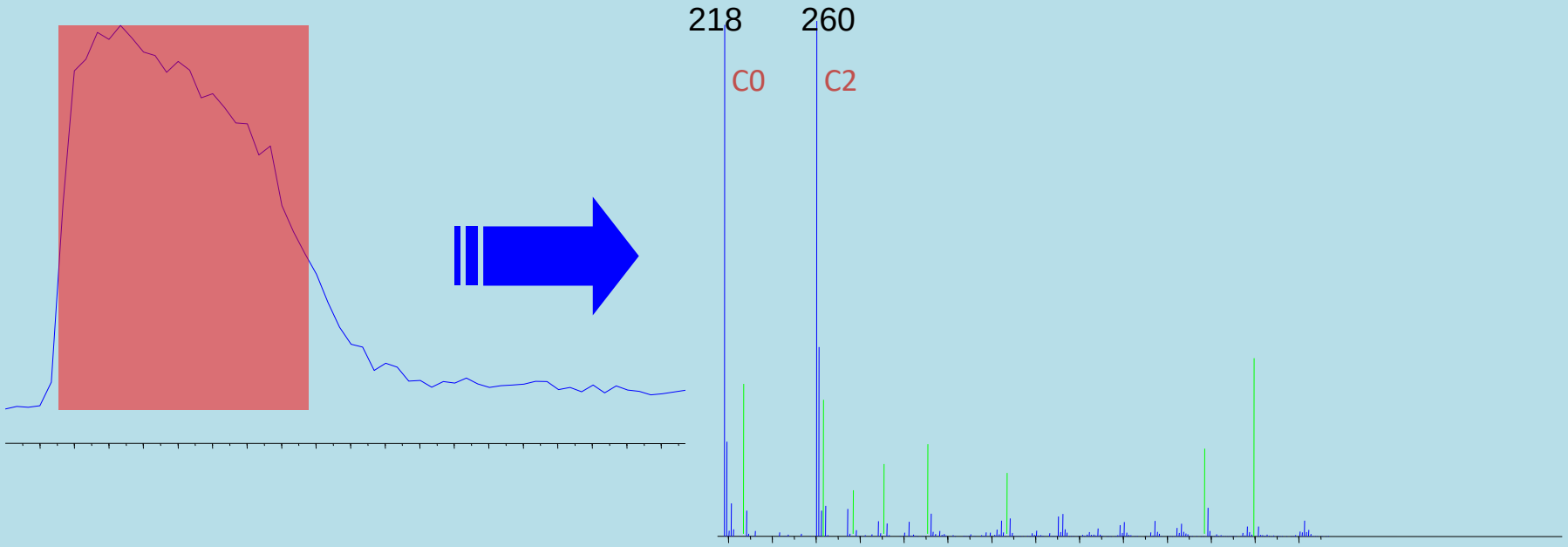
- **However, Precursor Ion of m/z 99 is also used.**

METHODS by MS/MS

- Use of chromatography?
  - NO? samples are injected by Flow injection analysis (FIA)

Butylated AC in plasma by FIA

Precursor Ion m/z 85



METHODS by MS/MS

- **Use of chromatography?**
  - **NO? samples are injected by Flow injection analysis (FIA)**
  - **PROS and CONS:**

AC are quantified by a software used in newborn screening (NBS), based on known concentrations of deuterated internal standards. There is no need of a calibration curve in NBS.

Run time in FIA is short and quantification is fast. However, the quantification is based on one point calibration (the internal standard concentration).

Triple Quadrupole MS/MS response is considered linear in a wide range of concentrations, but an AC concentrations far from the IS could loss accuracy.

Response factor could be known: C8 with d3-C8 will be 1. But is there a commercial IS for…C3DC? How much would it cost? Response factor could be calculated or just supposed = 1. Each laboratory has to consider what is its need and what is not.

METHODS by MS/MS

- **Use of chromatography?**
  - **NO? samples are injected by Flow injection analysis (FIA)**
  - **PROS and CONS:**

AC with same M+ are added in the quantification:

Underivatized			Derivatized		
m/z	Notation	AC	*m/z*	Notation	AC
232	C4	Butyryl Isobutyryl	288	C4	Butyryl Isobutyryl
244	C5:1	Tiglyl 3-Methylcrotonyl	300	C5:1	Tiglyl 3-Methylcrotonyl
246	C5:0	Isovaleryl 2-Methylbutyryl-(D+L) Pivaloyl	302	C5:0	Isovaleryl 2-Methylbutyryl-(D+L) Pivaloyl
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262	C4DC	Methylmalonyl-(D + L) Succinyl	374	C4DC	Methylmalonyl-(D+L) Succinyl
	C5OH	3-Hydroxyisovaleryl 2-Methyl-3-hydroxybutyryl	318	C5OH	3-Hydroxyisovaleryl 2-Methyl-3-hydroxybutyryl
276	C5DC C6OH	Glutaryl Hydroxyhexanoyl	388	C5DC C10OH	Glutaryl Hydroxydecanoyl
290	C6DC	Methylglutaryl Adipyl	402	C6DC	Methylglutaryl Adipyl

METHODS by MS/MS

- **Use of chromatography?**
  - Yes?

Allows a calibration curve. Improves and ensures the precision the accuracy. May be suitable for plasma monitoring

But:
The chromatography is time consuming: Long run times. To prepare and calculate the calibration curves.

How many samples can be run in a certain time?

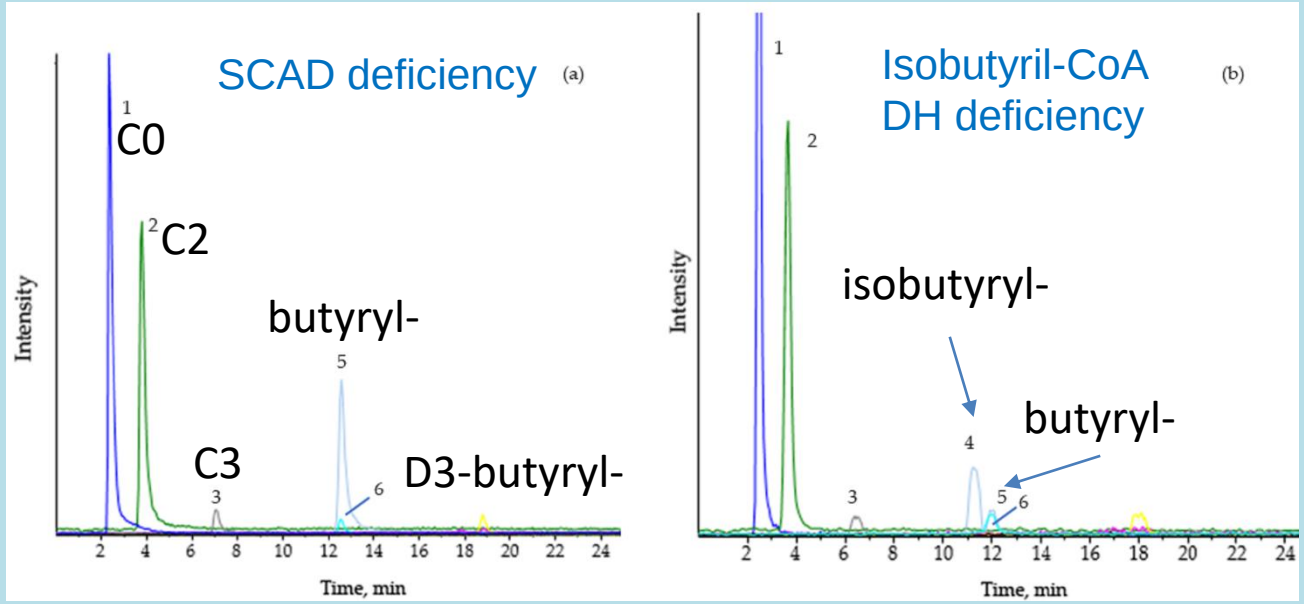
How many calibration curves can be done at the same time?

METHODS by MS/MS

- **Use of chromatography?**
  - Yes?

The HPLC columns separates AC isomers

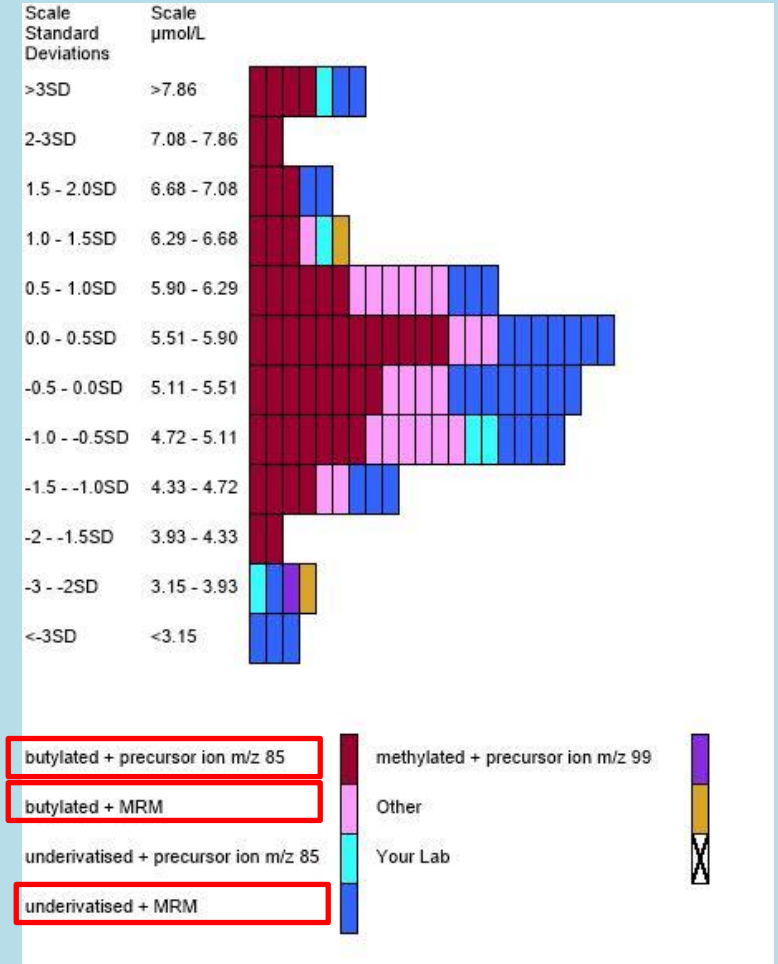
Butyl-AC in plasma by HPLC/MS/MS, MRM Mode:



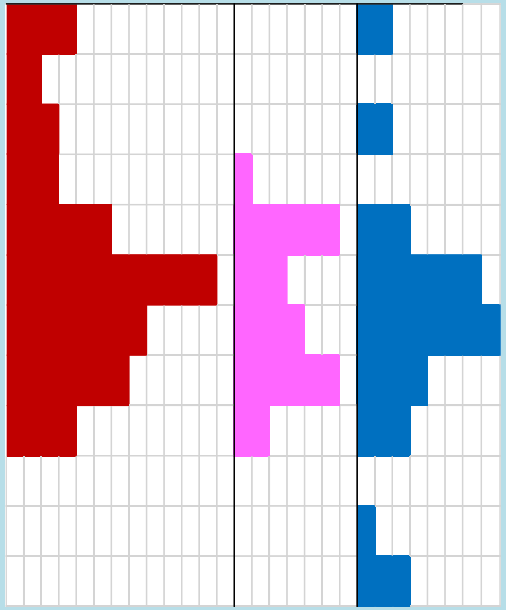
ERNDiM

Let's see some interesting results about the ACS scheme

C8 Octanoylcarnitine
ACS 2021.1



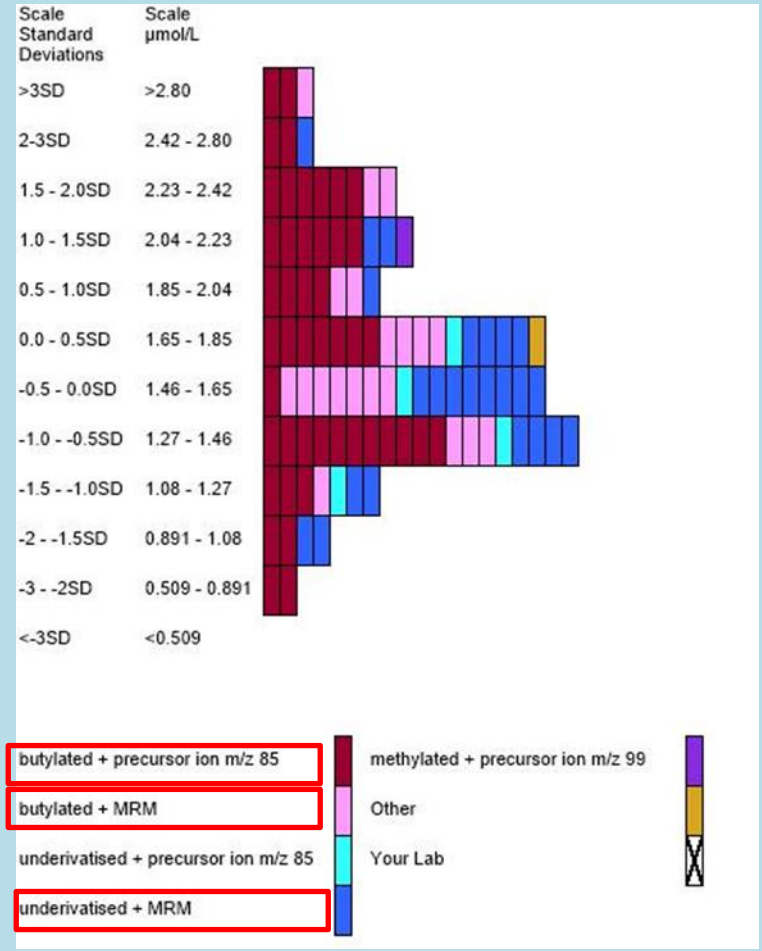
its quantification is quite well established, usually with deuterated-C8.
There is no problem of the commented previously

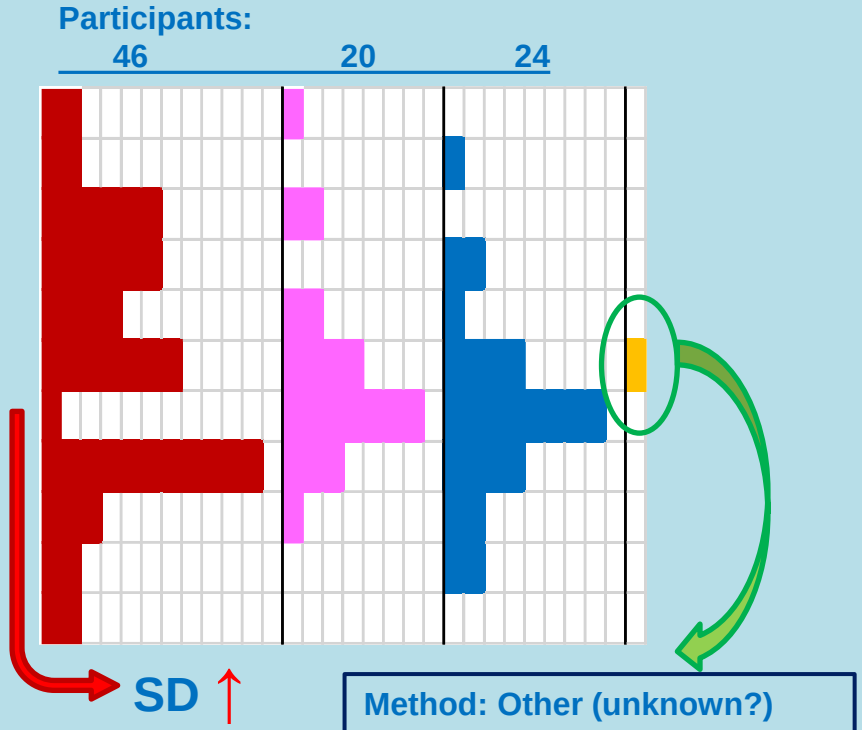


73 participants butylate Octanoylcarnitine
38 participants analyse Free Octanoylcarnitine

(ratio ≈ 2:1)

C5:1 Tyglylcarnitine
ACS 2020.7 (added 2 µmol/L)



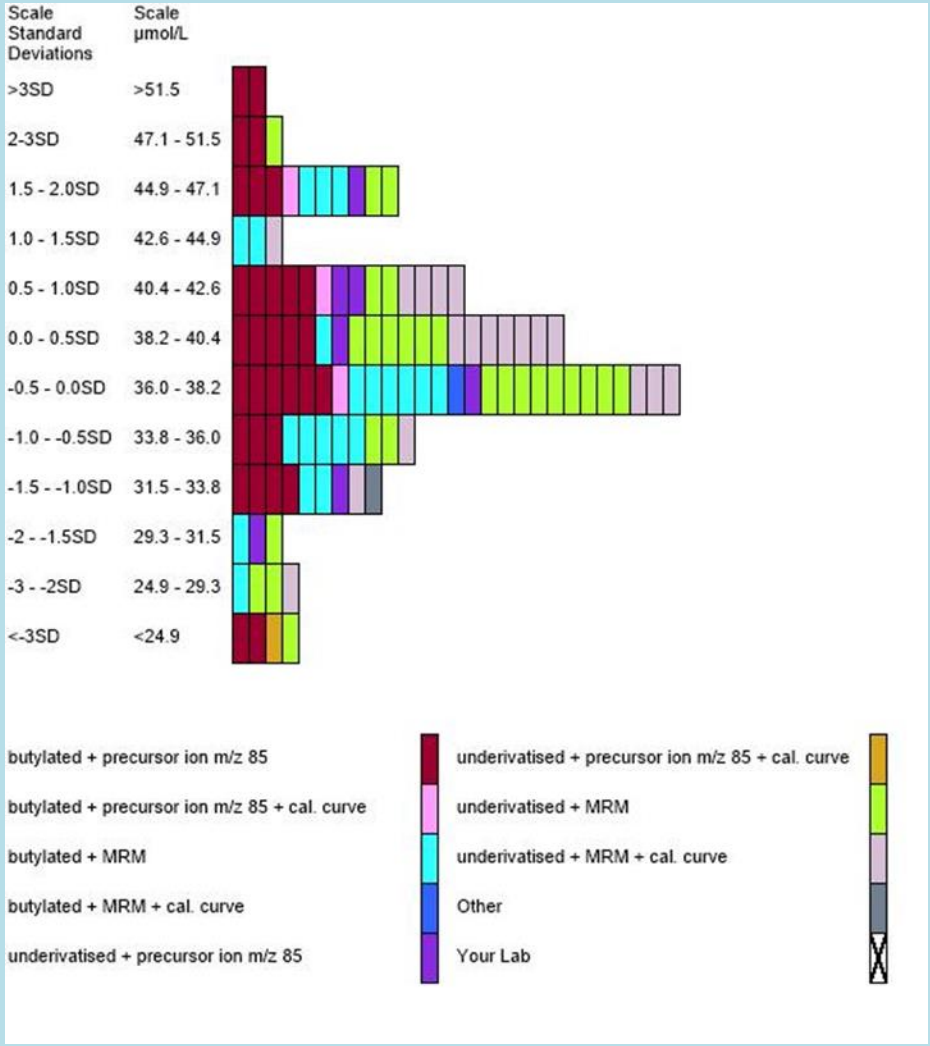


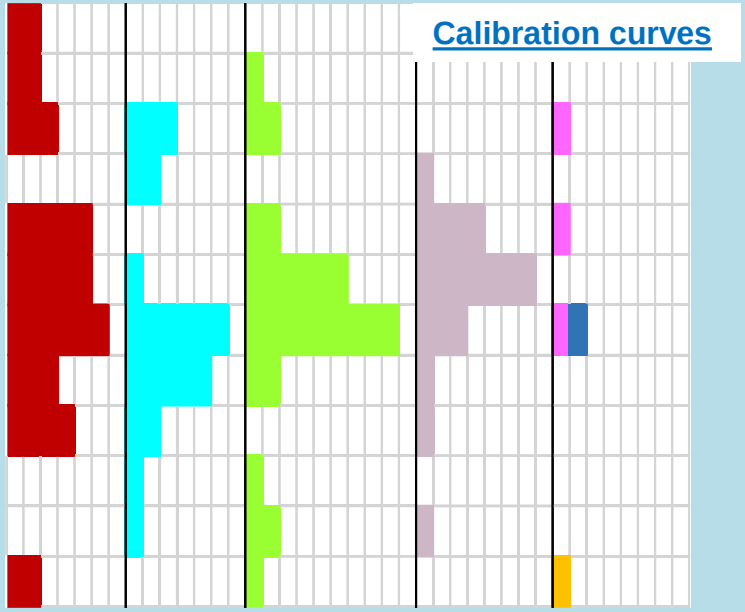
Method: Other (unknown?)

mean+0.5SD Good Results ✓

The standard deviation is high, but there are some aspects unknown. Which is the IS used by each participant?
Which will be the chosen response factor?

C0 Free carnitine
ACS 2021.2



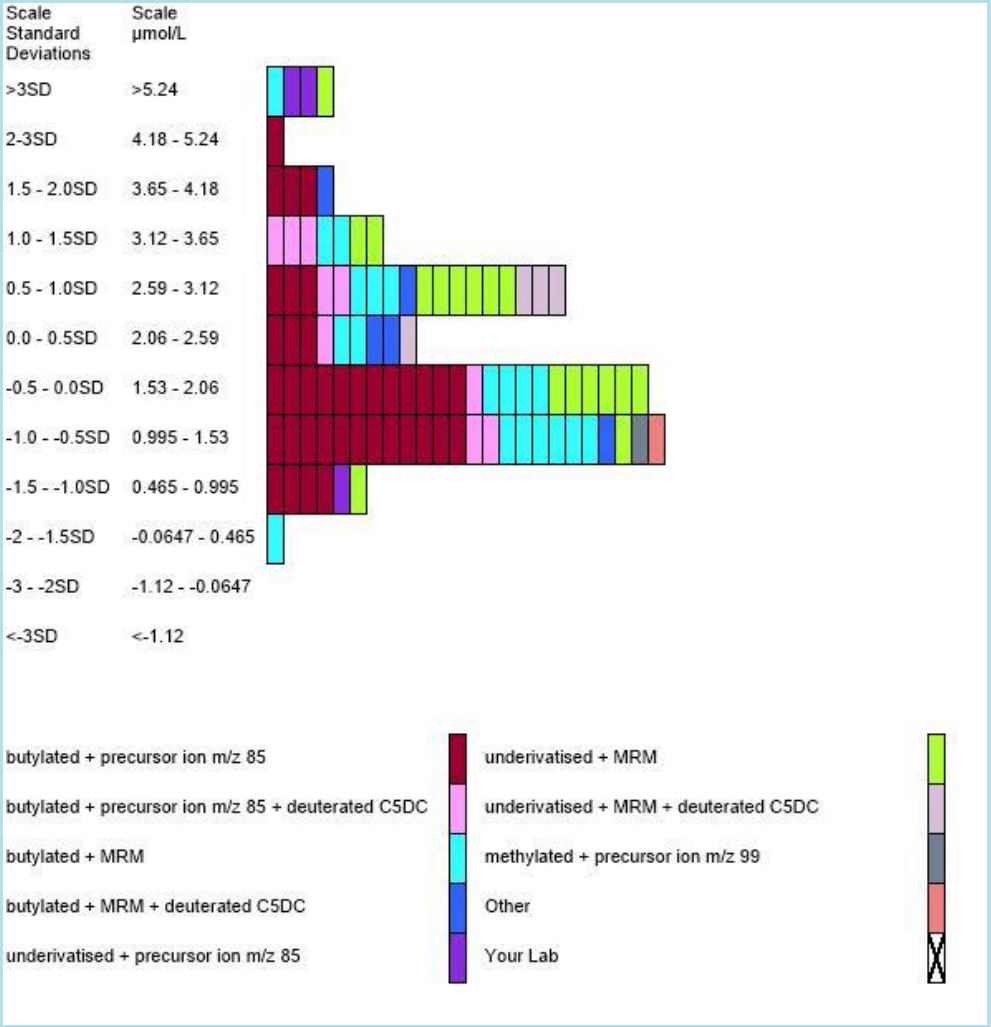


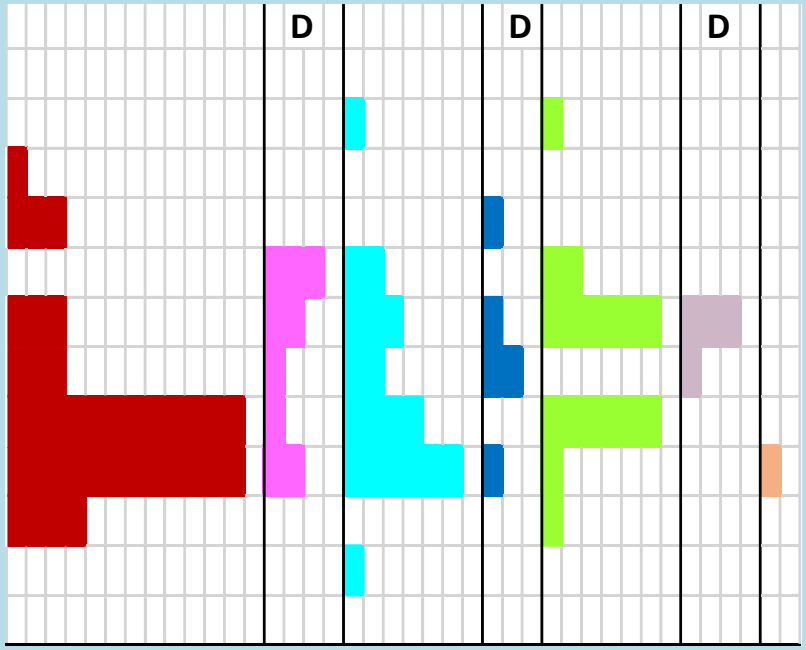
C0 could be adequate to quantify with calibrated.
But most of participant don't use it.

Nevertheless, the trend of the results is quite similar with or without calibration.

C5DC Glutarylcarnitine

D=deuterated C5DC as IS



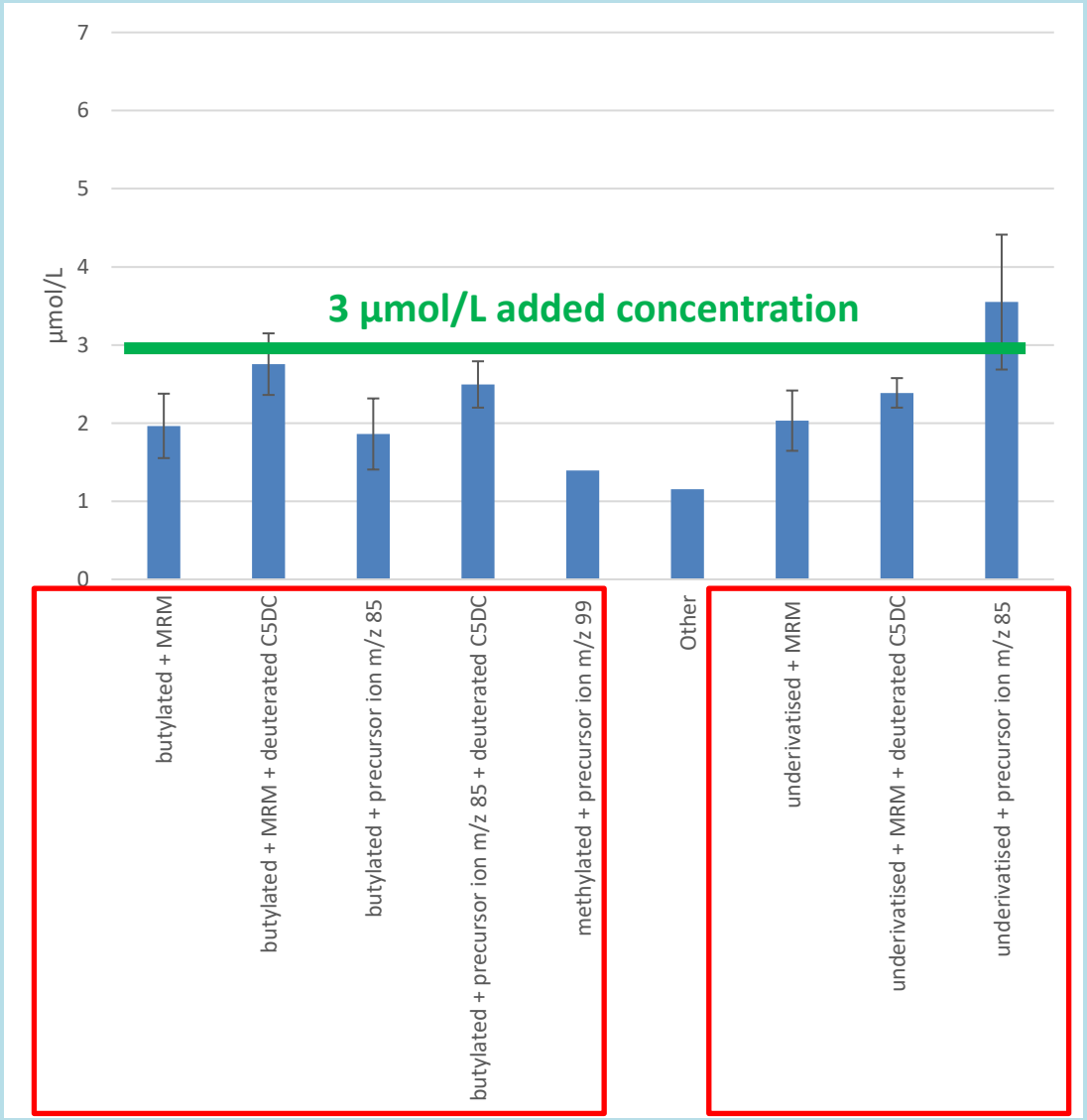


Deuterated C5DC is present in quite a few IS kits, thinking C5DC is considered difficult to detect in low-excretor GA I patients in NBS.

However, less than a half of participants use it.

C5DC Glutarylcarnitine (Pair 3-8)

(ACS 2020. Average and VC of the results given by participants with the same method)



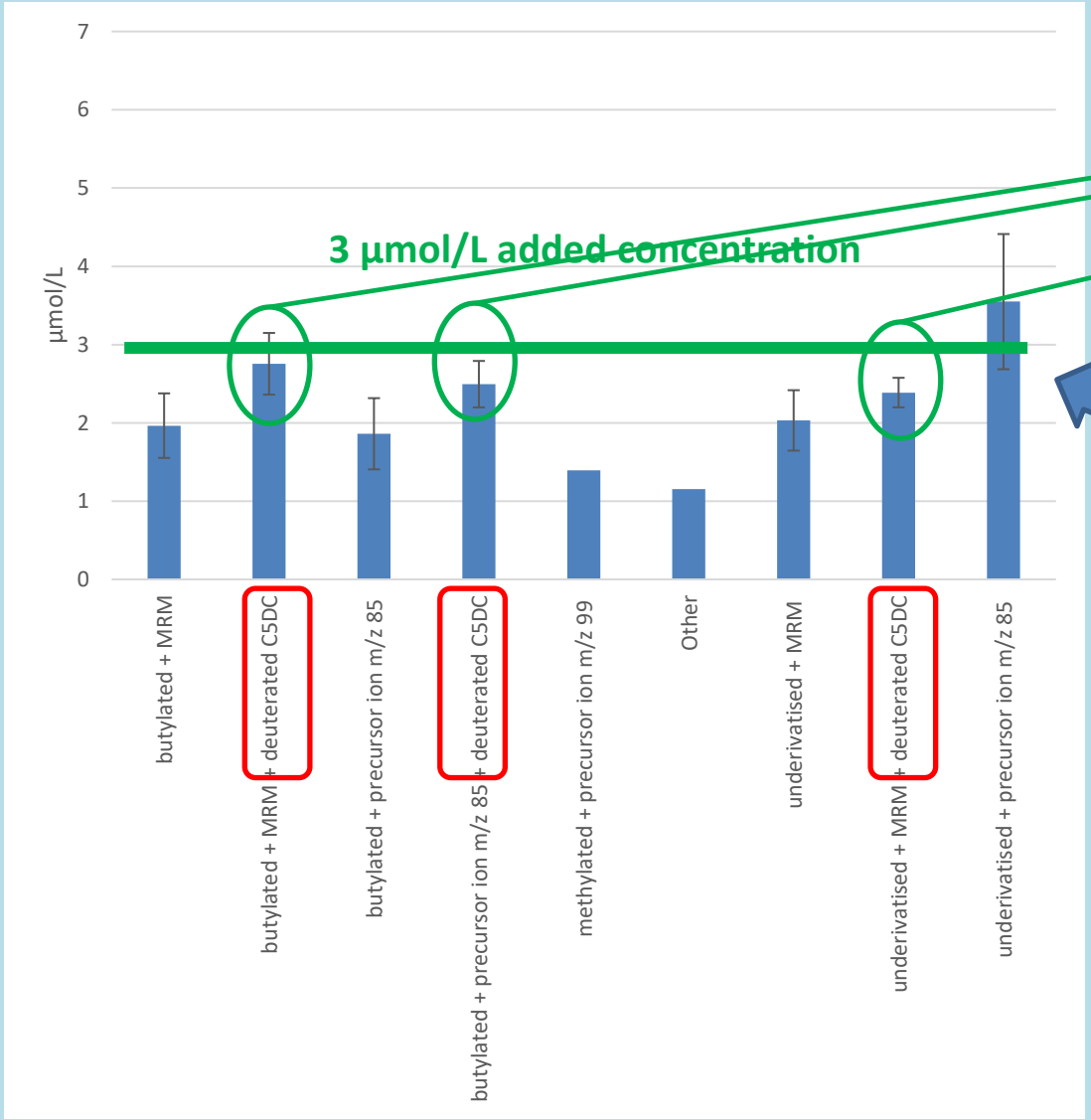
It is been described that the recovery or the accuracy of dicarboxylic acylcarnitines in methods without derivatization are worse than those in methods that include derivatization.

But in these results there is not a clear difference in accuray/recovery between these groups of methods

Commercial non-derivatized Kits usually have calibrators to improve the accuracy. Sensitivity is no longer a problem with the latest MS/MS. So, these kits may be more used in the future

C5DC Glutarylcarnitine (Pair 3-8)

(ACS 2020. Average and VC of the results given by participants with the same method)



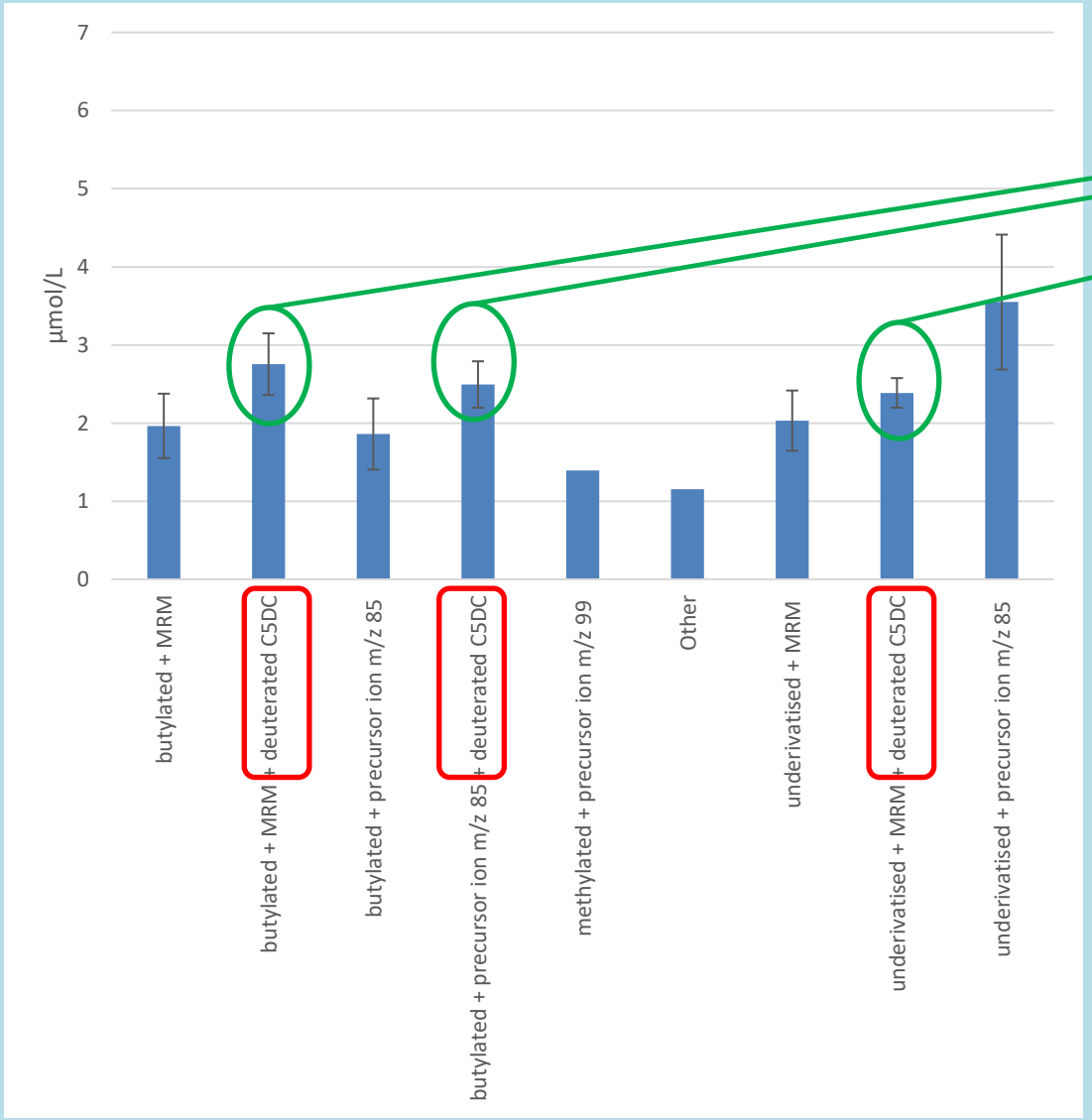
The use of Deuterated-C5DC could improve the accuracy

These results seem to confirm this improvement, since they are better than methods witout C5DC

We will see these results in more detail later

C5DC Glutarylcarnitine (Pair 3-8)

(ACS 2020. Average and VC of the results given by participants with the same method)



The use of Deuterated-C5DC could improve the precision (VC variation coefficient)

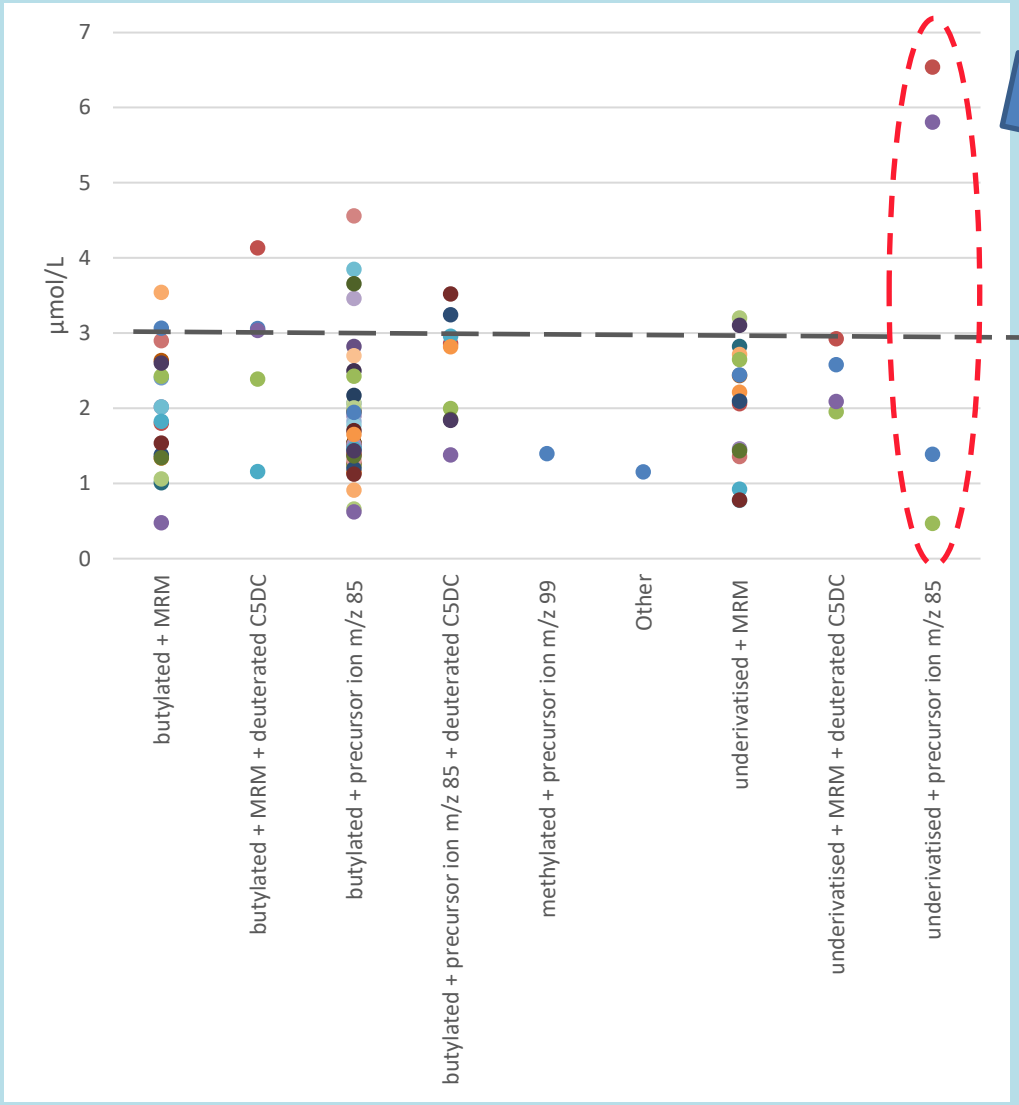
These results seem to confirm this improvement, since VC is lower

C5DC Glutarylcarnitine (Pair 3-8)

(ACS 2020. Average of Pair with highest concentration/ parcitipants)

Every dot is one participant.

These are the before results but without calculatios. Results of every participant is shown isolated.



3 µmol/L adde[illegible]oncentration

The average of these results could seem to be very accurate. However, the precisión is quite poor

Matters about ACS Scheme itself

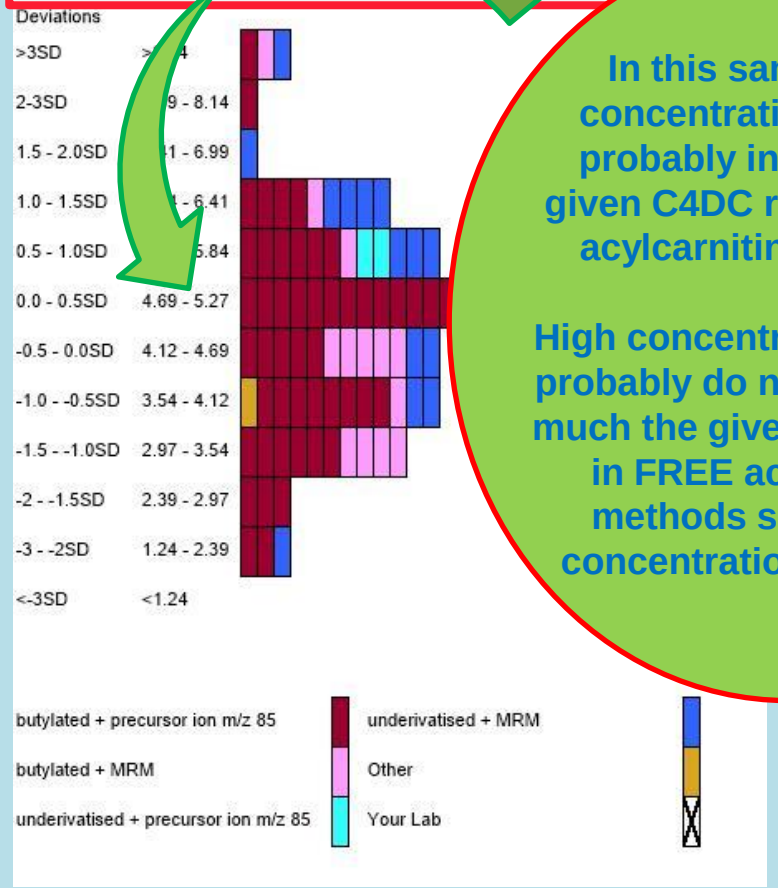
ERNDiM

ACS 2020.6

C5OH
3-OH-isovalerylcarnitine

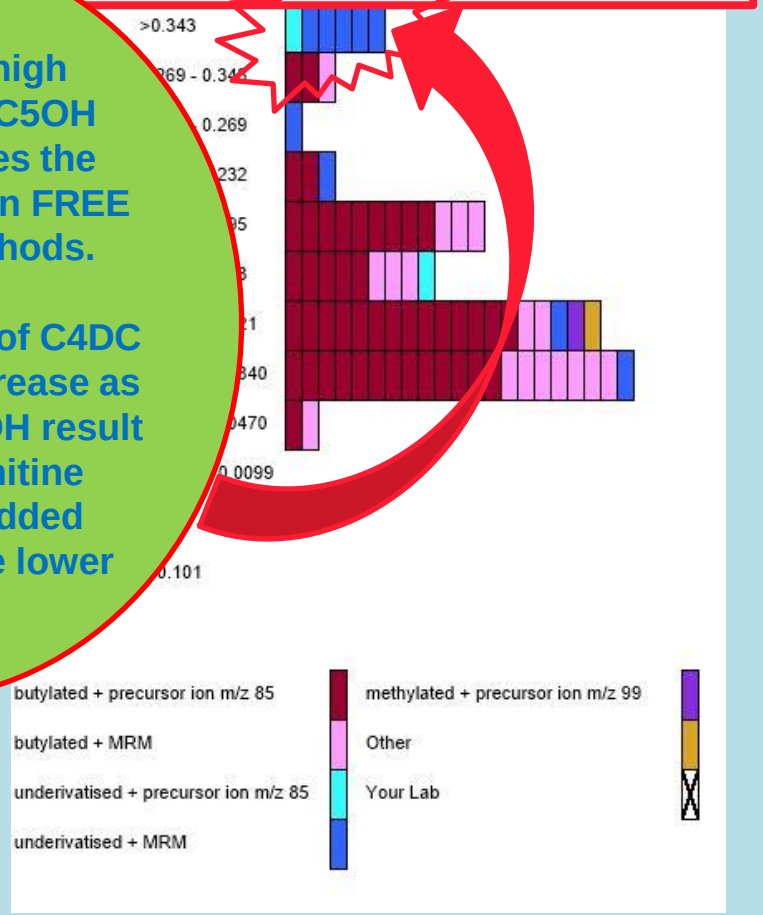
C4DC
methylmalonylcarnitine

Ratio butylated/free ~4 in C5OH and ≈6 in C4DC, probably because many participants who do not derivatise properly not to avoid bad scores.

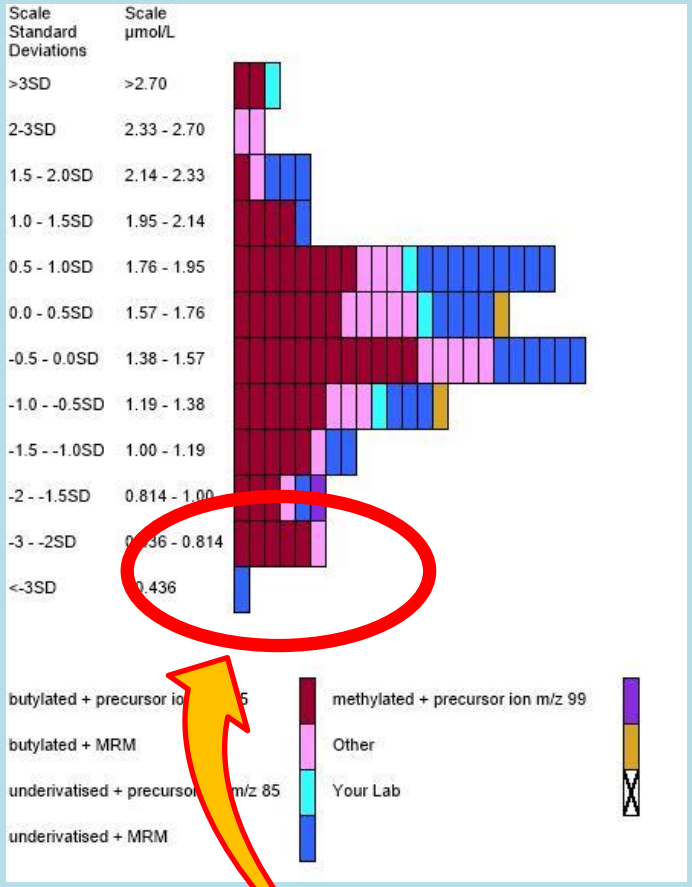


In this sample, high concentration of C5OH probably increases the given C4DC result in FREE acylcarnitine methods.

High concentration of C4DC probably do not increase as much the given C5OH result in FREE acylcarnitine methods since added concentrations are lower

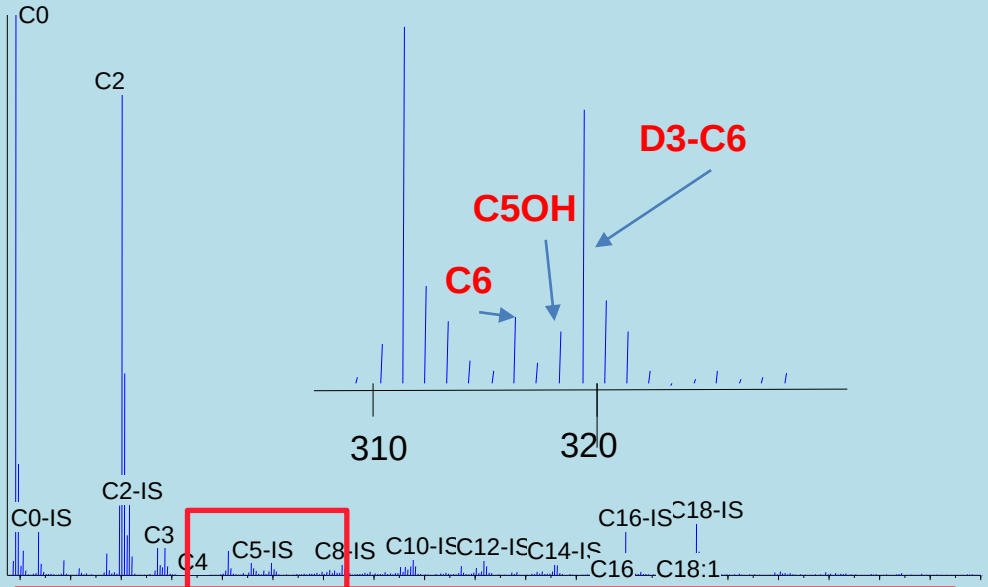


C6 Hexanoylcarnitine ACS2021.1



m/z in Quadrupole 1

BUTYL	BUTYL	BUTYL	FREE	FREE	FREE
C6	C5OH	d3-C6	C6	C5OH	d3-C6
316	318	319	259	261	262



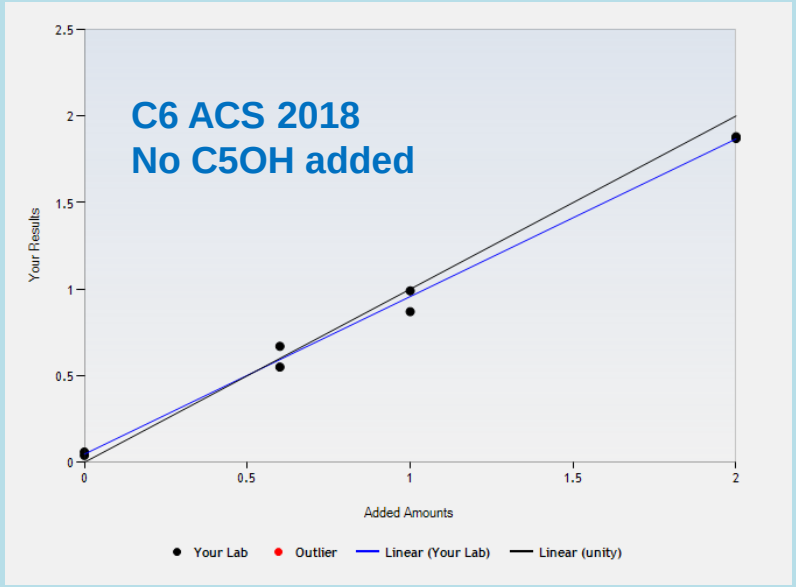
The naturally occuring isotope distribution of C5OH contributes with m/z 318+1=319, distorting in excess the intensity of d3-C6.

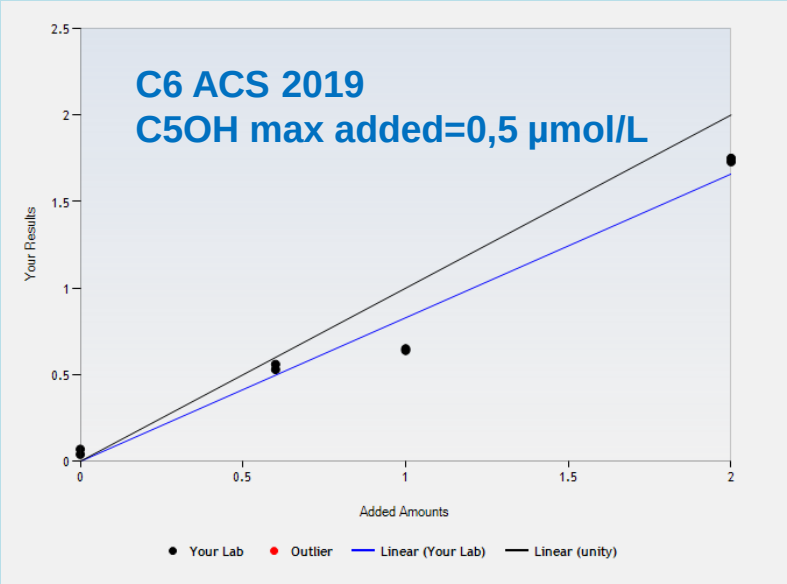
The higher added concentration of C5OH, the lower concentrations of C6 (wrongly found).

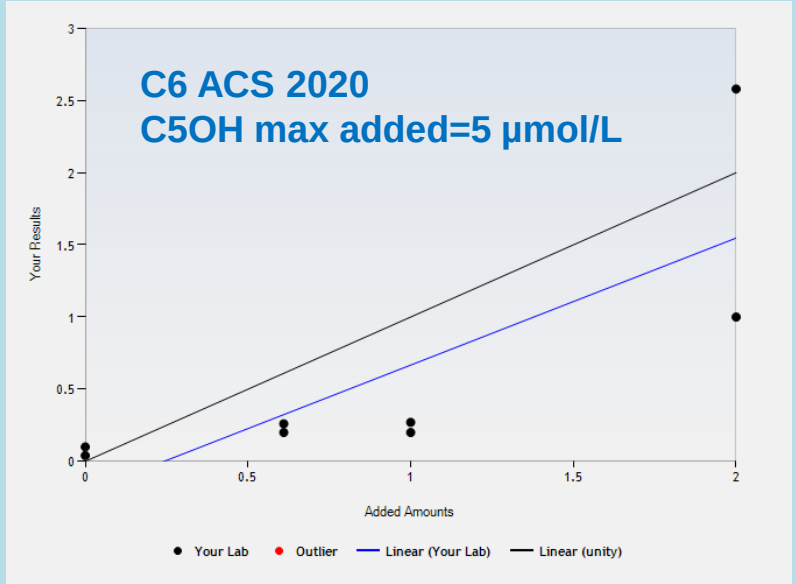
C6 Hexanoylcarnitine ACS

In 2020 ACS Scheme, the increase of the added concentrations of C5OH revealed even more the worsening of the results during 2019.

This situation, in which C6 and C5OH could be bioamarkers of a disease, is not seen in real samples.
However, at least for the participation in ACS a decision has to be made to change these results. Possibly changing the method with the addition of an HPLC column, but the duration is lengthened, in comparison with the Flow injection analysis.







Acylcarnitine measurement in dried blood spots
ERNDIM Qualitative QA scheme

Blood spots from real clinical cases: Education & Interpretation

2003-2009 Began with 45 registered participants

London, current scientific advisor: Charles Turner

2010 split between two centres, 60+ participants in each centre

Heidelberg, current scientific advisor: Joachim Janda

2017 Third centre added, 40+ participants in each centre

Zurich now Rome, current scientific advisor: Cristiano Rizzo

ERNDIM Qualitative Acylcarnitine dbs QA scheme (2020: 131 participants from 42 countries)

Country (s)	Participants
FRANCE	16
UNITED KINGDOM	15
ITALY	13
GERMANY	9
SPAIN	8
USA	6
BELGIUM, NETHERLANDS	5
AUSTRALIA, CANADA	4
ARGENTINA, MALAYSIA, PORTUGAL, TURKEY	3
CHINA. CZECH REPUBLIC, ISRAEL, SLOVAKIA, SWITZERLAND, TAIWAN	2
AUSTRIA, BRAZIL, BULGARIA, CHILE, CROATIA, ESTONIA, IRELAND, JAPAN, KINGDOM of SAUDI ARABIA, KUWAIT, LEBANON, LITHUANIA, LUXEMBOURG, MOROCCO, NEW ZEALAND, POLAND, QATAR, REPUBLIC OF SINGAPORE, RUSSIA, SLOVENIA, SULTANATE OF OMAN	1

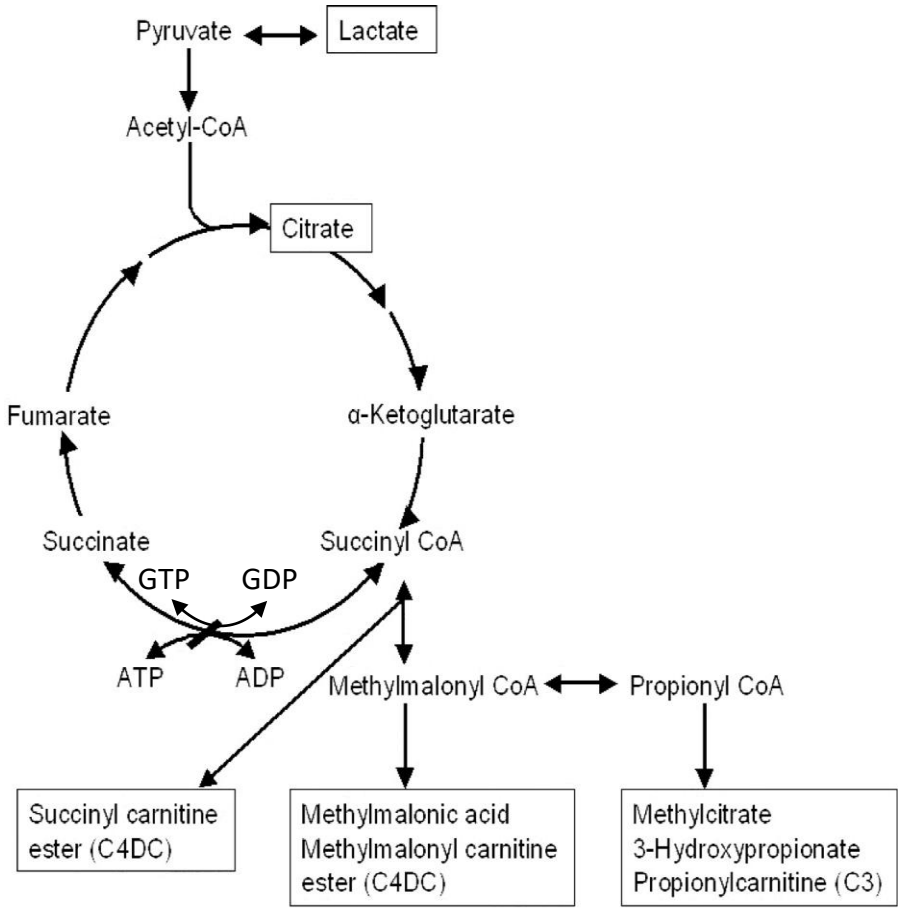
ERNDiM

Sample ACDB-IR-2020 F

- ✓ 15 year old male. Patient admitted at the age of 7 months for encephalopathy, psychomotor retardation and hypotonia. In treatment
- ✓ Significant elevation of C4DC acylcarnitines, (succinylcarnitine)
- ✓ This sample was from a patient with **Succinyl-CoA ligase subunit beta** (SUCLA2)
- ✓ Educational sample

Sample ACDB-IR-2020 F (SUCLA2)

Relevant metabolic pathways illustrating the metabolic effects of ADP-forming succinyl-CoA synthetase deficiency.



SUCLA2 mutations are associated with mild methylmalonic aciduria, Leigh-like encephalomyopathy, dystonia, and deafness. Carrozzo,et al. Brain 130: 862-874, 2007.

Succinyl-CoA synthetase (SCS), also called succinate ligase, is a Krebs cycle enzyme that not only converts succinyl-CoA to succinate and free Coenzyme A, but also converts ADP to ATP and GDP to GTP.

The substrate specificity for ADP and GDP is determined by the β-subunits, whereas the α subunit is shared.

The α-subunit is coded by the gene *SUCLG1*, whereas the β-subunit is encoded by *SUCLA2* for the ADP specificity, and by *SUCLG2* for the GDP specificity.

Patients SCS-related defects *SUCLG1* or *SUCLA2* mutation present hypotonia, muscle weakness, hypoacusis, Leigh disease, lactic acidosis, polyneuropathy, mild methylmalonic aciduria and mild elevation of C4DC-Carnitine

ERNDiM

Sample ACDB-IR-2020 F (SUCLA2)

27/38 **(71%)** respondents reported an increase of C4DC (succinyl/methylmalonilcarnitine or C5OH)

	median	range	interquantile range	median URL	range URL	interquartile range URL	# respondents (URL)
C4DC/C5OH	1.2	0.39-3.8	0.80-1.72	0.64	0.37-2.6	0.50-0.73	27 (26)

19/38 (50%) respondents considered Succinate-CoA ligase deficiency (SUCLA2 or SUCLAG1)

- ✓ 11 respondents considered a normal acylcanitines profile

- ✓ 2 respondents suggested primary carnitine deficiency
- ✓ 2 respondents suggested methylmalonic acidemia
- ✓ 2 respondents suggested 3-methycrotonyl-CoA carboxylase deficiency
- ✓ 1 suggested multiple carboxylase deficiency
- ✓ 1 suggest a valproate therapy profile

The alternative differential diagnosis suggested by respondents included:

- Methylmalonic acidemia (n=5)
- 3-hydroxy-3-methylglutaryl-CoA lyase deficiency (HMG) (n=3)
- Beta-ketothiolase deficiency (n=2)
- 2-methyl 3-hydroxybutyryl-CoA dehydrogenase deficiency (2M3HBA) (n=4),
- 3-methylglutaconic aciduria (3MGA) (n=6)
- Biotine deficiency (n=3)
- 3-methylcrotonyl-CoA carboxylase (3MCC) deficiency (n=7)
- Mitochondrial DNA depletion (n=2)
- Valproate treatment (n=1)

Sample ACDB-IR-2020 F (SUCLA2)

27/38 **(71%)** respondents reported an increase of C4DC/C5OH.

16 derivatized 13 suggest SCS-related defects 81%
11 underivatized 6 suggest SCS-related defects 54%

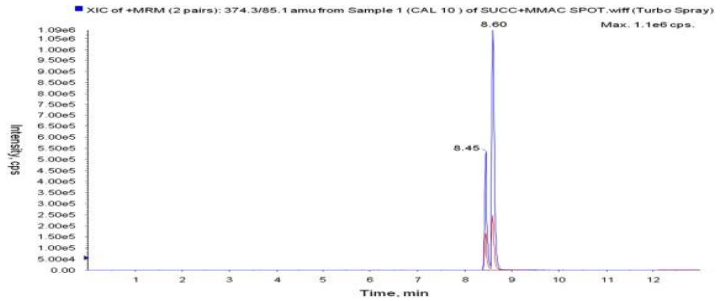
Derivatized method
C5OH 318→85
C4DC 374→85

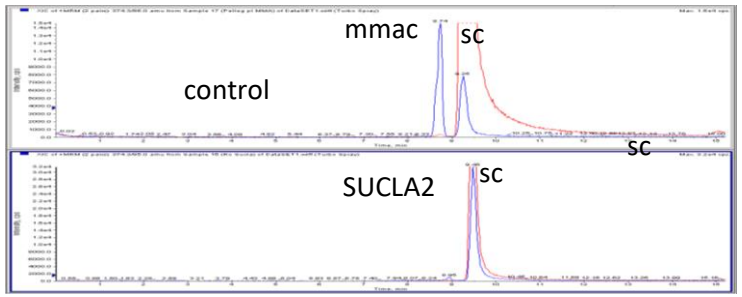
Underivatized method
C5OH 262→85
C4DC 262→85

Clinica Chimica Acta
Volume 429, 15 February 2014, Pages 30-33

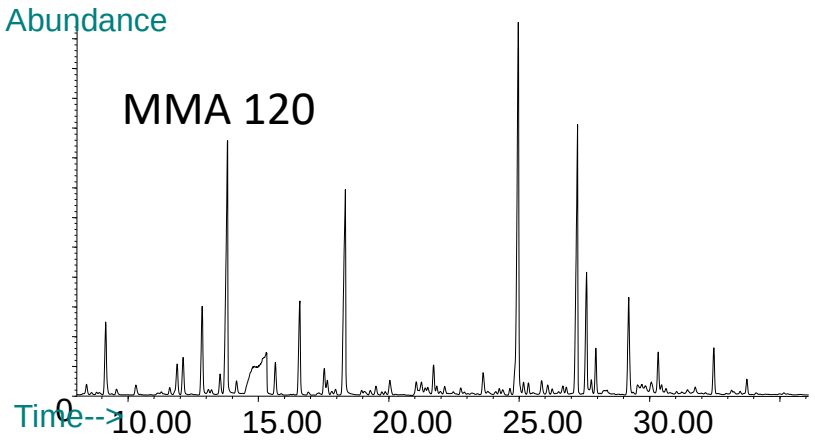
Measurement of succinyl-carnitine and methylmalonyl-carnitine on dried blood spot by liquid chromatography-tandem mass spectrometry

Cristiano Rizzo [a, 1], Sara Boenzi [b, 1], Rita Inglese [a], Giancarlo la Marca [c, d], Maurizio Muraca [a], Tegra Barreiro Martinez [e], David W. Johnson [f], Eleonora Zelli [a], Carlo Dionisi-Vici [b]





Organic acids analysis



ERNDiM

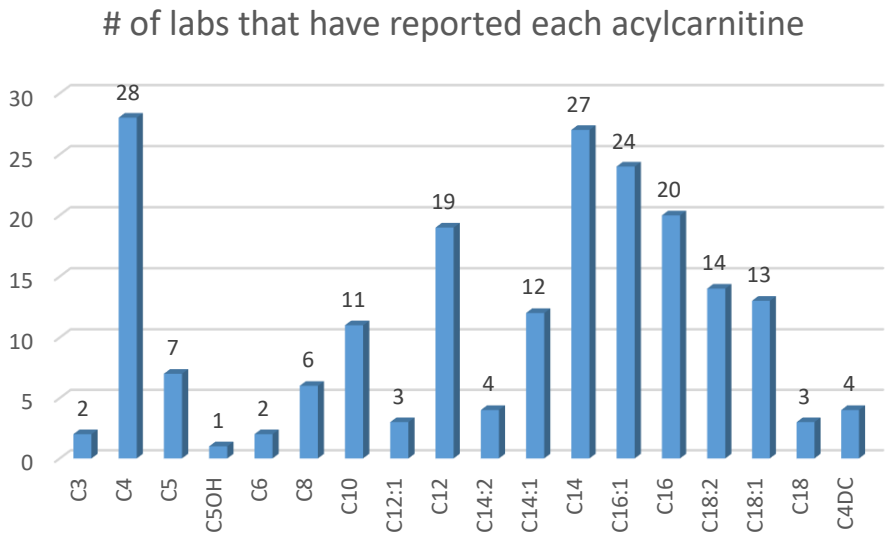
Sample ACDB-IR-2020 A

- ✓ 19 year old male.
- ✓ Patient admitted at the age of 3 days for vomit, hypoglycemia, hyperammonemia and hepatic dysfunction.
- ✓ In treatment with MCT
- ✓ Most likely Carnitine Acylcarnitine translocase deficiency or Carnitine Palmitoyltransferase II deficency.
- ✓ This sample was from a patient with **Carnitine Acylcarnitine translocase deficiency (CACT – OMIM 212138)**

Sample ACDB-IR-2020 A (CACT)

Significant increase was found in long chain acylcarnitines (from C14 to C18), medium chain acylcanitines and C4-carnitine.

	median	range	interquartile range	median URL	range URL	interquartile range URL	# respondents (URL)
C4	1.24	0.79-1.66	1.10-1.35	0.59	0.20-1.80	0.42-0.74	29 (28)
C8	0.15	0.11-0.21	0.14-0.16	0.1	0.07-0.16	0.10-0.11	5 (5)
C10	0.3	0.20-0.41	0.23-0.32	0.2	0.12-0.30	0.15-0.23	10 (10)
C12	0.32	0.19-0.77	0.23-0.38	0.16	0.08-0.41	0.10-0.31	13 (13)
C14:1	0.27	0.16-0.47	0.21-0.38	0.17	0.08-0.40	0.10-0.31	13 (13)
C14	0.51	0.40-0.86	0.45-0.61	0.3	0.11-0.55	0.21-0.43	28 (28)
C16:1	0.73	0.45-1.31	0.58-0.83	0.27	0.09-0.81	0.15-0.47	23 (22)
C16	3.56	2.79-7.43	3.2-4.21	2	1.32-5.72	1.71-2.34	21 (21)
C18:2	0.97	0.60-1.40	0.80-1.05	0.58	0.39-1.33	0.48-0.70	14 (14)
C18:1	2.3	0.88-4.73	2.09-2.54	1.78	1.33-378	1.78-2.20	15 (15)
C18	1.05	0.11-0.21	0.14-0.16	1	0.07-0.16	0.10-0.11	5 (5)
(C16+C18:1)/C2	0.45	0.43-0.70	0.43-0.51	0.28	0.20-0.30	0.27-0.30	5 (5)



Elevation of C4-carnitine and medium chain acylcarnitine found in this sample are the result of MCT treatment of this patient.

Sample ACDB-IR-2020 A (CACT)

9/41 (**22%**) respondents considered CACT o CPT2 deficiency as the most likely diagnosis

38/41 (**92%**) respondents considered BOX defects as the most likely diagnosis

Fig 2. 1st suggested diagnosis

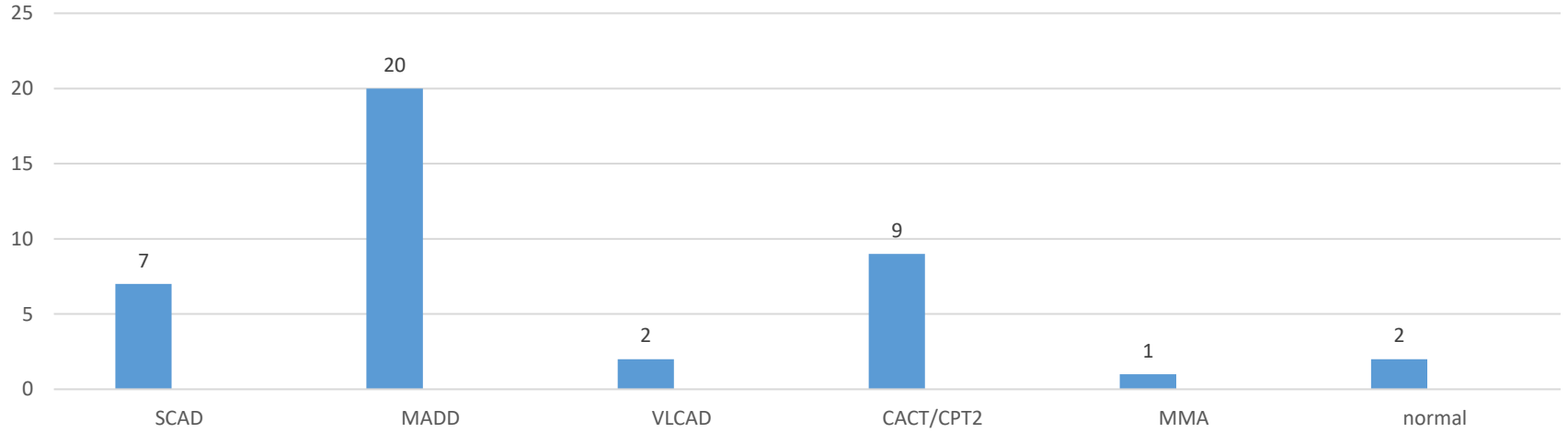
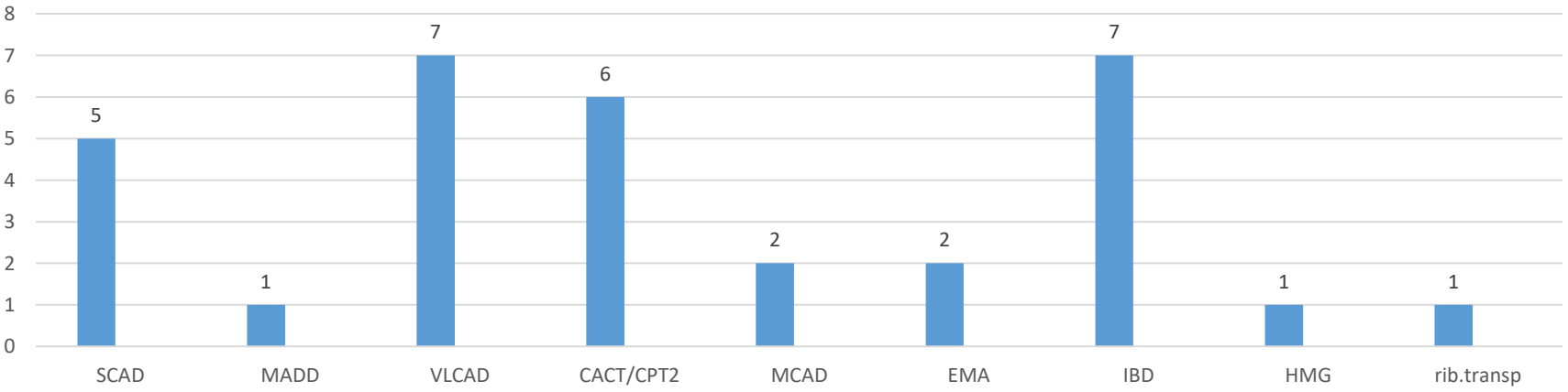


Fig 3. alternative suggested diagnosis



ERNDiM

ACDB-UL-2020-A (CACT)

- ✓ 1 year old female.
- ✓ Admitted acutely unwell with Cardiomyopathy, hypoglycaemia
- ✓ Not on specifc treatment at time of sampling
- ✓ Most likely Carnitine Acylcarnitine translocase deficiency or Carnitine Palmitoyltransferase II deficency.
- ✓ This sample was from a patient with **Carnitine Acylcarnitine translocase deficiency (CACT – OMIM 212138)**

Sample ACDB-UL-2020 A (CACT)

Acylcarnitine species	Number of respondents
^C8	42
^C6	36
Low C0	29
^C16	23
^C10	22
^C4	19
^C14	16
^C16:1	15
^C8/C10	13
^C18	13
^C12	12
C4DC	8
^C18:1	8
C10:1>	7
^C14:1	6
^C8/C2	4
Succinyl carnitine	1
^(C16+C18:1) /C2	1

Acylcarnitine	C8	C6	C0	C10	C16	C16:1
Median	1.21	0.49	7.8	0.30	5.13	0.82
Range	0.65-1.72	0.31-0.82	3.2-24.0	0.21-0.49	0.96-6.20	0.54-5.76
Interquartile Range	1.05-1.32	0.44-0.53	5.6-9.7	0.26-0.36	4.58-5.47	0.73-0.97
URL (LRL) Median	0.20	0.15	(9.6)	0.24	1.90	0.27
URL (LRL) Range	0.06-0.45	0.05-0.40	(3.2-20.2)	0.10-0.42	0.80-6.70	0.13-1.04
URL (LRL) Interquartile range	0.16-0.30	0.11-0.20	(8.7-11.8)	0.20-0.29	1.64-3.44	0.20-0.40
Number of respondents (URL)	40 (37)	34 (31)	31 (28)	28 (25)	23 (21)	17(16)

15/41 suggested CACT/CPT2 as the most likely diagnosis
20/41 suggested MADD
6/41 suggested MCADD

All recognized a fatty acid oxidation defect

Sample ACDB-RM-2019 F

- ✓ 5 months old asymptomatic male.
- ✓ Acylcarnitines alteration was found by NBS.
- ✓ This sample was from a patient with **3-methyl-crotonyl-CoA carboxylase deficiency (3MCC**) Mutation in MCCC1

	median	range	interquartile range	median URL	range URL	interquartile range URL	# of repondents (URL)
C5OH	0.81	0.51-3.56	0.68-1.08	0.43	0.20-1.80	0.32-0.51	32 (31)
C5OH/C8	13.47	10.83-23.5	11.08-15.9	10	6-17.30	9-13	8 (7)

32/42 respondents (76%) reported an increase of **C5-Hydroxy-carnitine (C5OH)**

30/42 (71%) respondents considered 3-methyl-crotonyl-CoA carboxylase deficiency (3MCC) as the most likely diagnosis; 2 respondents considered glutaryl-CoA dehydrogenase deficiency

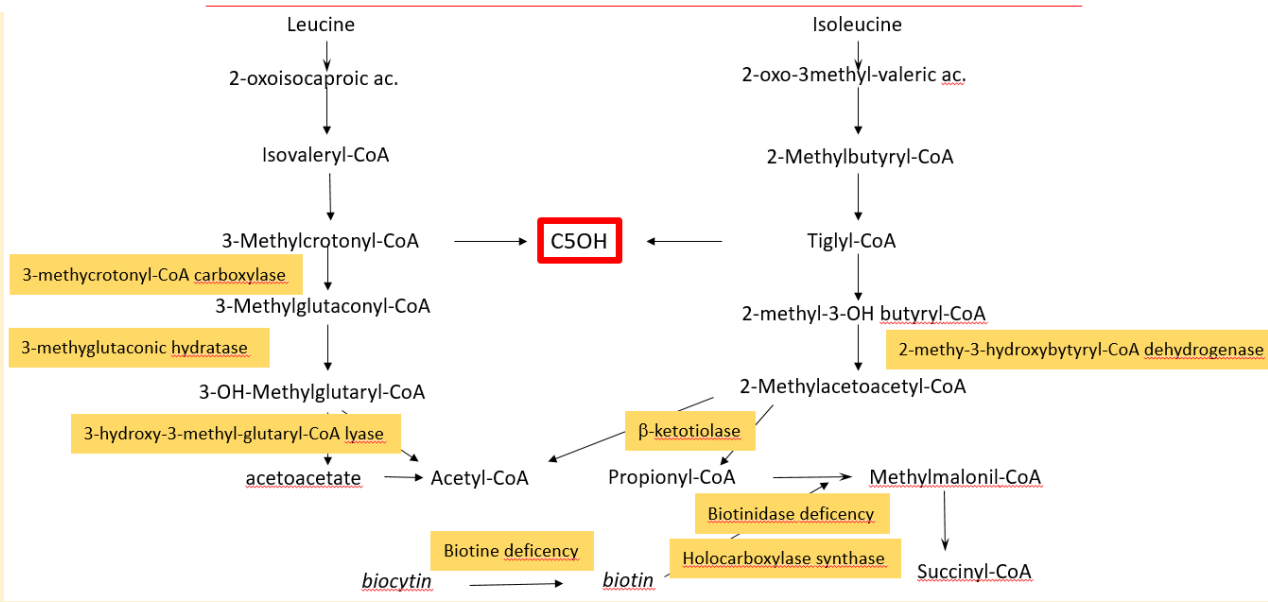
32 respondents suggested **gene mutation analysis guided by the results of the urinary organic acids analysis** to confirm the diagnosis.

Sample ACDB-RM-2019 F (3MCC)

Causes of elevated C5OH-carnitine include:

- ✓ 3-hydroxy-3-methylglutaryl-CoA lyase
- ✓ Beta-ketothiolase
- ✓ 2-methyl 3-hydroxybutyryl-CoA dehydrogenase
- ✓ Biotine deficiency
- ✓ Biotinidase
- ✓ Holocarboxylase synthetase
- ✓ Maternal 3-methylcrotonyl-CoA carboxylase (3MCC)
- ✓ 3- methylglutaconic acidurias (3MGAs)
- ✓ Valproate therapy

Main causes of C5OH elevation in leucine and isoleucine pathway



- ✓ **MT-ATP6**

- ➢ C5OH ↑
- ➢ C3 ↑
- ➢ citrulline↓

Mitochondrion
Volume 44, January 2019, Pages 58-64

Biochemical signatures mimicking multiple carboxylase deficiency in children with mutations in *MT-ATP6*

Austin A. Larson [a, b], Shanti Balasubramaniam [c, d], John Christodoulou [e, f], Lindsay C. Burrage [g, h], Ronit Marom [g, h], Brett H. Graham [g, h], George A. Diaz [i], Emma Glamuzina [j], Natalie Hauser [k], Bryce Heese [l], Gabriella Horvath [m], Andre Mattman [m], Clara van Karnebeek [m, n], S. Lane Rutledge [o], Amy Williamson [i], Lissette Estrella [i], Johan K.L. Van Hove [a, b], James D. Weisfeld-Adams [a, b]

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Prospective diagnosis of *MT-ATP6*-related mitochondrial disease by newborn screening

Ryan H. Peretz [a], Nicholas Ah Mew [b], Hilary J. Vernon [c], Rebecca D. Ganetzky [d,e,*]

Rome-Case 1

Newborn screening

	day3	day9	cutoff
C4	1.29	1.19	0.87
EMA	11	6.8	2.34

clinical signs: cyanosis, hypotonia , vomiting and metabolic acidosis

	DBS					plasma		
	day10	day 11	cutoff			day 10	day 11	cutoff
C0	9.09	10.9	>9 -56		C0	8.76	15.6	27
C4	1.11	1.78	0.69		C4	1.95	3.48	1.05
C5	0.65	0.29	0.33		C5	1.08		0.62
C6		0.27	0.2		C6	0.26	0.63	0.22
C8		0.56	0.31		C8		1.76	0.44
C10		0.55	0.3		C10		1.48	0.9
C12:1		0.76	0.35		C5DC	0.25	0.21	0.09
C12		0.87	0.35		C12:1		1.43	0.36
C14:1		1.16	0.3		C12		1.4	0.34
C14		0.63	0.35		C14:1		2.9	0.34
C16:1		0.4	0.35		C14		1.46	0.14
					C16:1		1.2	0.2

urinary organic acids			
EMA	170	170	<15
glutarate	80	2	<3
ESG	↑	↑	
2MBG	↑		
IVG	↑	↑	

EMA/ETHE1 or MADD???

ERNDiM

Case 1

Pannel for GAII (ETFA, ETFB, ETFDH, FLAD1, SLC25A32, SLC52A1, SLC52A2, SLC52A3) were all normal

ETHE1: **single variant in heterozygosity in ETHE1**, no deletions

Mater riboflavin dosage (25/03): **113** mcg /L (137-370 mcg /L)

Mater riboflavin dosage (10/05): 199 mcg /L (137-370 mcg /L) in supplementation 50 mg / day

CASE REPORT | Open Access

Abnormal VLCADD newborn screening resembling MADD in four neonates with decreased riboflavin levels and VLCAD activity

Marne C. Hagemeijer, Esmee Oussoren, George J. G. Ruijter, Willem Onkenhout, Hidde H. Huidekoper, Merel S. Ebberink, Hans R. Waterham, Sacha Ferdinandusse, Maaike C. de Vries, Marleen C. D. G. Huigen, Leo A. J. Kluijtmans, Karlien L. M. Coene, Henk J Blom, ... See fewer authors

«This report demonstrates that a secondary (alimentary) maternal riboflavin deficiency in combination with reduced VLCAD activity in the newborns can result in an abnormal VLCADD/MADD acylcarnitine profile and can cause false-positive NBS. We hypothesize that maternal riboflavin deficiency contributed to the false-positive VLCADD neonatal screening results.»

Rome-Case 2

Born at 38 weeks of gestational age

Consanguineous parents (first cousins)

Normal NBS. Underivatized method

Hospitalised with these clinical symptoms:

- axial hypotonus
- facial dysmorphisms (hypertelorism, mild retrognathia) congenital heart disease
- cryptorchidism
- hyper echogenic spots in the liver

ERNDiM

Rome-Case 2

Clinicians request the following tests (patient's age 2 months)

- DBS acylcarnitine (derivatized method)
- Plasma acylcarnitines (derivatized method)
- Urinary organic acids

DBS AC
C16DC **0.04** µmol/l (NV<0.03)
C18DC **0.05** µmol/l (NV<0.03)
C24-C 0.05 µmol/l (NV<0.02)

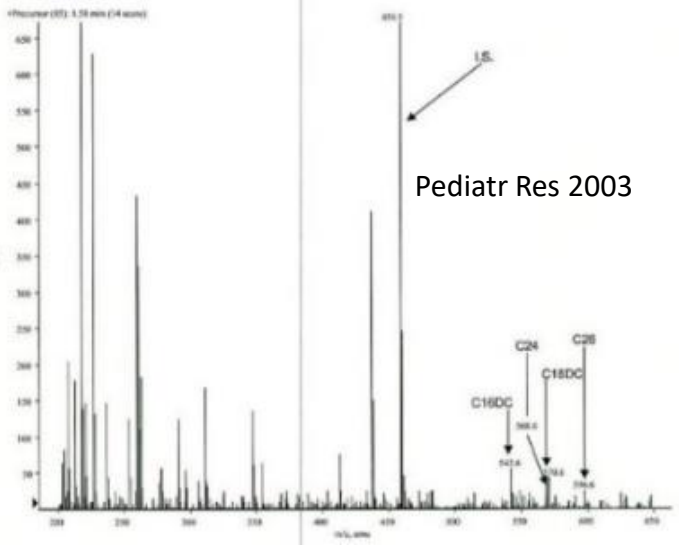
Plasma AC
C16DC **0.17** µmol/l (NV<0.03)
C18DC **0.18** µmol/l (NV<0.03)

Organic acids
Pimelic ↑ ; Azelaic ↑
2-OH-sebacic ↑
C14 Epoxydicarboxylic ↑

C26:0-lyso-PC **404** µmol/l (NV<25)

Characteristic Acylcarnitine Profiles in Inherited Defects of Peroxisome Biogenesis: A Novel Tool for Screening Diagnosis Using Tandem Mass Spectrometry

CRISTIANO RIZZO, SARA BOENZI, RONALD J.A. WANDERS, MARINUS DURAN, UBALDO CARUSO, AND CARLO DIONISI-VICI



Pediatr Res 2003

Metabolites analysis

VLCFA	↑	C26 8.02 µmol/l (NV<0.9)
Phytanic a.	↑	4.79 µmol/l (NV<3)
Pipecolic a.	↑	35.8 µmol/l (NV<3.5)
DHCA	↑	16.6 µmol/l (NV<0.35)
THCA	↑	6.67 µmol/l (NV<0.44)
Plasmalogens	↓	

Genetic analysis

microdeletion in chromosomal region 11p11.2 involving exons 1 and 2 of the **PEX16** gene in homozygous condition

ERNDIM QA Scheme London Glutaric Aciduria Type 1

2003 2a 11month old male, collapse during intercurrent illness, large head with frontal bossing

GlutarylCoA dehydrogenase deficiency (type 1 glutaric aciduria) – enzyme confirmed

0/32 ^C5-dicarboxyl-carnitine

Blood spot/plasma acylcarnitines consistently normal

Urine organic acids - only once was 3OH-glutarate suspicious

2005 5c 3y old male, intercurrent infection, altered consciousness, hypoglycaemia

52/53 ^C5-dicarboxyl-carnitine and suggested a diagnosis of GA1

201 15a 4y old, movement disorder subsequent to acute illness

39/44 ^C5-dicarboxyl-carnitine and suggested a diagnosis of GA1

2012 20c 3 year old female, epilepsy, basal ganglia changes on MRI

50/52 ^C5-dicarboxyl-carnitine, 7/52 ^C5DC/C8 48/52 suggested a diagnosis of GA1

2016e 23y old male, movement disorder following intercurrent illness in childhood

56/56 ^C5-dicarboxyl-carnitine, 11/56 ^C5DC/C8. 56/56 suggested a diagnosis of GA1

A normal acylcarnitine result does not exclude type 1 glutaric aciduria

High awareness of GA1, unlikely to be missed if metabolite elevated

Increasing use of ratios

No apparent difficulty for underivatised analysis

ERNDIM QA Scheme Glutaric Aciduria Type 1

Year Sample	correct	Median	Range	n	URL Median	URL Range	URL n
2005 5c	52/53	1.2	0.39-3.10	40	0.15	0.03-0.5	34
2010 15a	39/44	0.38	0.13-1.02	34	0.15	0.03-0.57	25
2012 20c	48/52	0.77	0.3-2.6	39	0.14	0.02-0.4	25
2016 e	56/56	4.38	1.47-12.1	46	0.17	0.03-0.51	34

Problems with quantitation and reference ranges

Some correlation between concentration & diagnostic accuracy

Rome-Case 3

-One year old girl hospitalized for suspected mitochondrial encephalopathy.

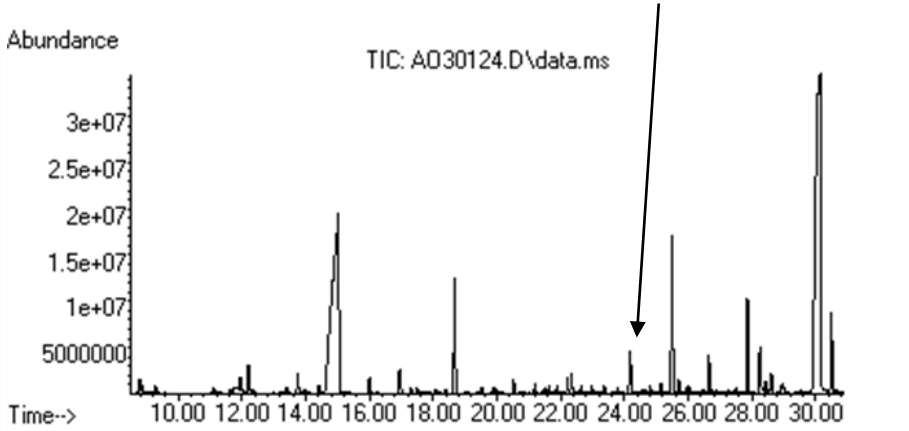
- **NBS was normal**.
-Blood Acylcarnitine normal
- 2 urinary organic acids samples were normal

She presented with motor regression, drowsiness and head rotation movements with intra-rotation of the right limb.

Brain MRI showed hyperintensity at the level of the caudate and bilateral lenticular nuclei

Rome-Case 3

- third sample of organic acids .... slight increase of **3-hydroxy-Glutaric acid**

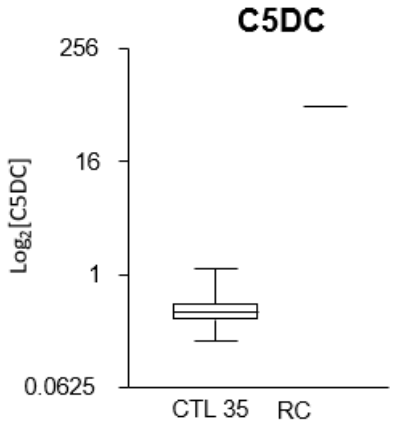


- DBS Acilcarnitines

-NBS C5DC 0.17 μmol (Cutoff <0.20)

- **7 normal DBS acylcarnitine samples**. (C5DC average 0.12 μmol; range 0.08-0.14)

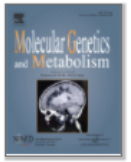
- **Urinary Acilcarnitines** **C5DC 165** μmol (nv<5)



- Genetic analysis confirm **glutaric aciduria type I**

  c.286A>G ;p(Ile96Val); c.1157G>A p(Arg386Val)

Molecular Genetics and Metabolism
Volume 84, Issue 2, February 2005, Pages 137-143

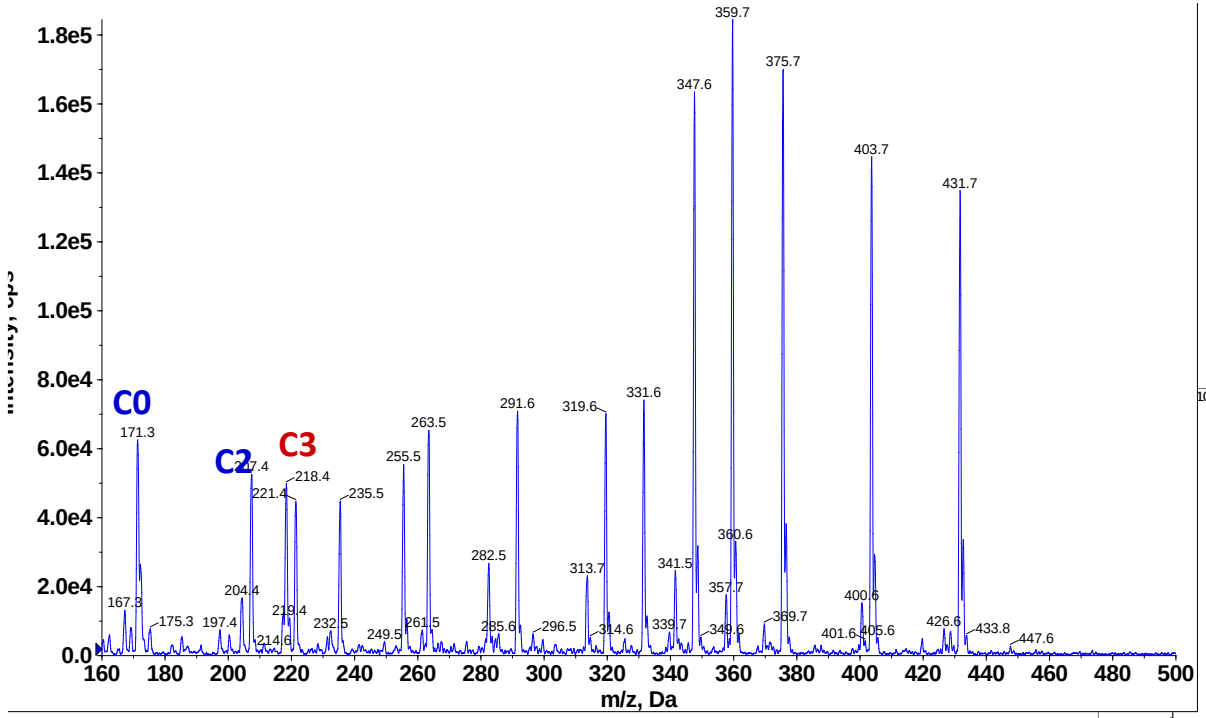


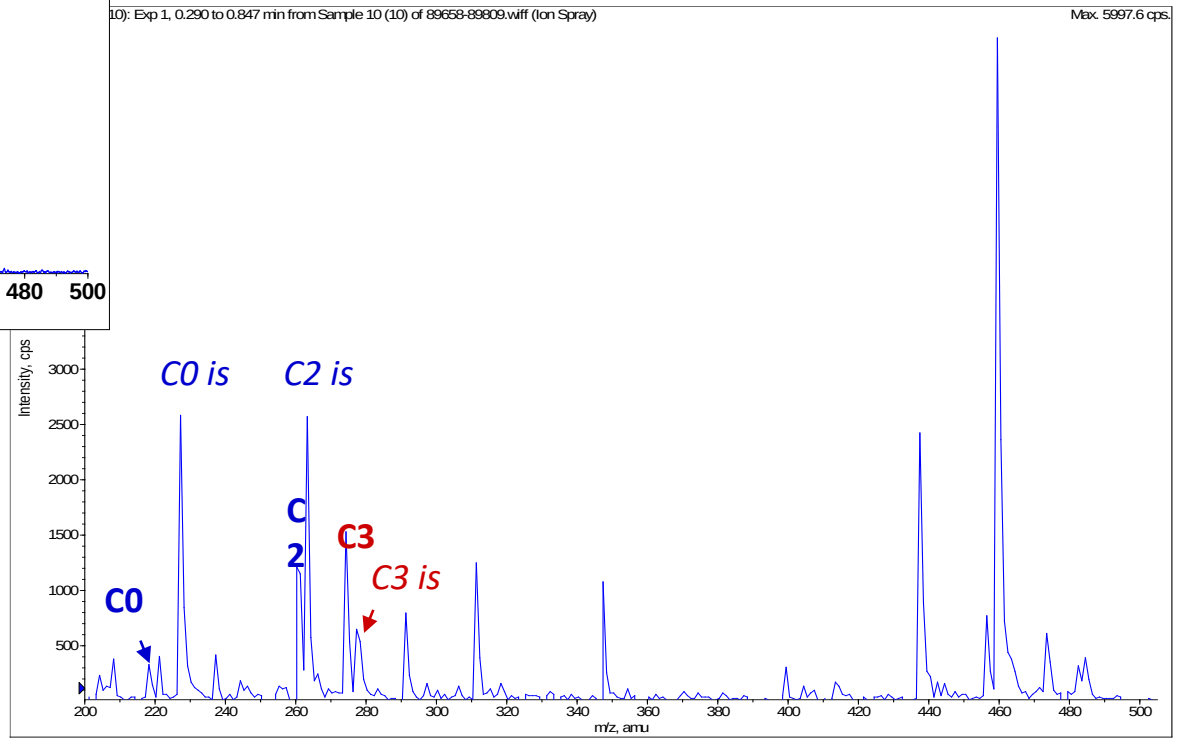
The urinary excretion of glutarylcarnitine is an informative tool in the biochemical diagnosis of glutaric acidemia type I

S. Tortorelli [a], S.H. Hahn [a], T.M. Cowan [b], T.G. Brewster [c], P. Rinaldo [a], D. Matern [a]

"The urinary excretion of glutarylcarnitine is a specific biochemical marker of GA-1 which could be particularly useful in the work up of patients with suggestive clinical manifestations but **without glutaric aciduria and with normal plasma acylcarnitine profiles**".

Sample 13a 8 day old male, hyperammonaemia, coma





Propionic acidaemia Sample 13a

2009 13a 55/73

8 day old male, hyperammonaemia, coma

44 C3 carnitine outside their age appropriate reference limit

13 C3 abnormal by ratios (C3/C2, C3/C0, C3/C16).

C3 concentration: median 3.4μmol/l, range 2.20-9.39, interquartile range 3.00-3.98 n=49.
Upper limit: median 3.14μmol/l, range 1.10-10.70, IQ range 1.80-4.93 n=32

Free carnitine concentration: median 4.2μmol/l, range 2.0-15.0, IQ range 3.36-5.45 n=58.
lower limit: median 9.62μmol/l, range 4.5-25.0, IQ range 7.00-14.00

16 carnitine transporter, 1 fatox, 1 urea cycle defect, 1 no diagnosis

Need for vigilance when carnitine is depleted

Huge variation in quantitative values & reference ranges

Overdependence on quantitative values in some laboratories

3/73 laboratories measured MMA & methylcitrate on the dbs

Critical error?

Case of secondary carnitine depletion

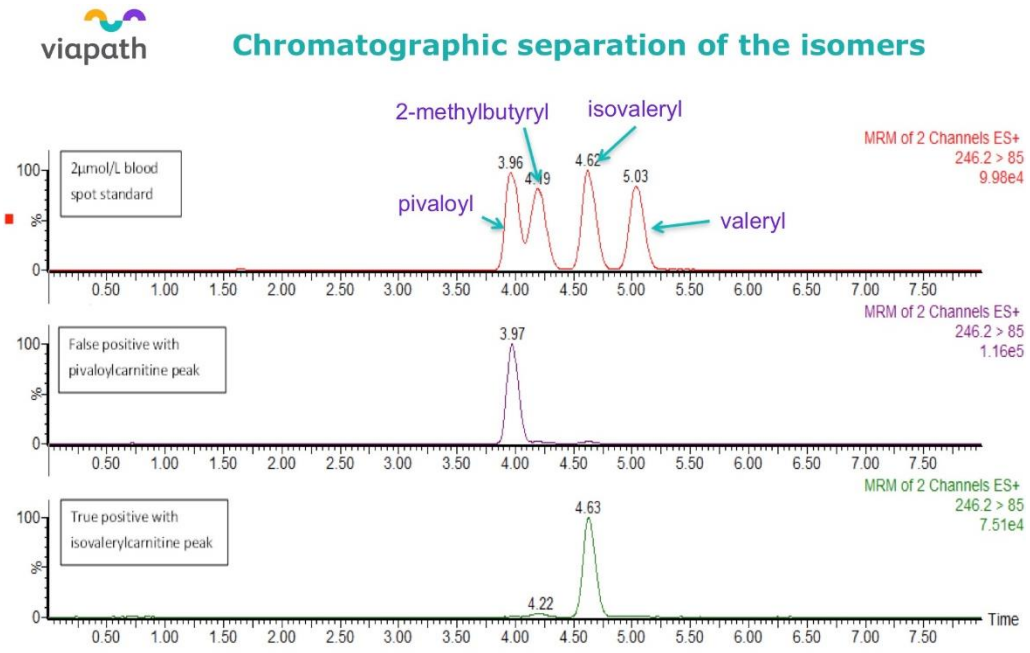
with thanks to colleagues at Viapath: Deborah Burden, Ben McDonald, Rachel Carling

- Positive newborn screen for C5 isovaleryl carnitine
  - C5 2.3µmol/L, C0 <2µmol/L, scan showed all acylcarnitines low except C5
  - Second line chromatographic separation of C5 isobars: 99% pivaloyl carnitine
  - Mother had been prescribed multiple courses of pivalic acid containing antibiotic drugs for recurrent UTI during pregnancy
  - Mother: C5 (pivaloyl) 0.8µmol/L, C0 3µmol/L

Extreme secondary carnitine depletion

May mask other disorders detectable by acylcarnitine profiling

Affects fasting tolerance and exercise capacity



Holme, E., et al. (1992). "Effects of pivalic acid-containing prodrugs on carnitine homeostasis and on response to fasting in children." Scand J Clin Lab Invest **52**(5): 361-372

Abrahamsson, K., et al. (1996). "Pivalic acid-induced carnitine deficiency and physical exercise in humans." Metabolism **45**(12): 1501-1507.

Thank you

We continue to try to improve the ACS and ACDB Schemes

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