

Interpretation and follow-up of abnormal newborn screening results from MS/MS

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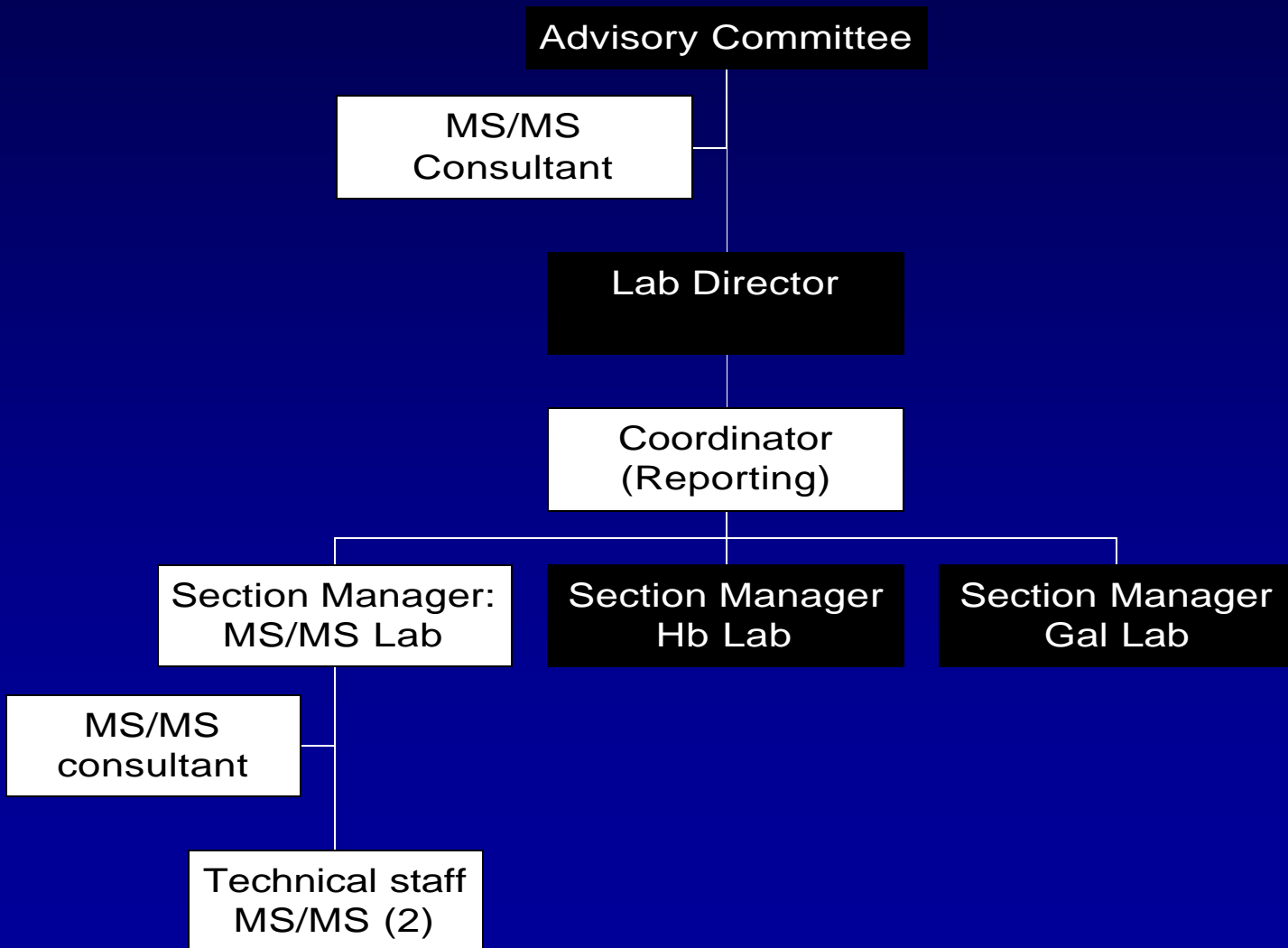
Department of Pediatrics

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Organizational Chart: NBS Program Utilizing MS/MS



Responsibilities of MS/MS Section Manager

- Manages MS/MS lab and personnel
- Reviews all daily MS/MS results
- Monitors daily QA/QC
- Orders repeat analysis of all abnormal results prior to reporting
- Determines action on abnormals based on predetermined criteria (cut-offs)
- Reports abnormal values to Coordinator

Minimum Credentials of MS/MS Section Manager

- Experienced in management of a section of a NBS Lab - “knows the system” - dedicated to NBS
- Background in analytical chemistry/computer applications (esp. spreadsheets)
- Educated in principles and practice of mass spectrometry and MS/MS; has continuous access to expertise (consultant)
- Has attended relevant training courses (e.g. extra-mural; MS/MS instrument manufacturer)
- Active participant in MS/MS workshops, seminars

Responsibilities and credentials of results coordinator

- **Contacts physicians and informs them of test results and required action**
- **Follows through to ensures that action is taken**
- **Records outcome of each case**
- **Must understand the technology (MS/MS), and have sufficient knowledge of biochemical genetics to understand the biochemistry of and required follow-up testing for disorders being screened for**

Diseases Expected to be Found by MS/MS in Expanded Newborn Screening Programs

Acylcarnitine Profile:

MCAD, SCAD, VLCAD, LCHAD, TFP, CPT I, CPTII, CAT, GA-II (ETF, ETFQO), GA-I, Malonic Acidemia, 3-MCC, SKAT, IBDH, 2MBDH, PA, MMA, IVA, MCD, HMG, 3MGA

Amino Acid Profile:

PKU, MSUD, Methioninemia (Homocystinuria), Citrullinemia, Argininosuccinic Aciduria, Tyrosinemias (3 ?), Hyperglycinemia (?) Hyperornithinemia (?), Argininemia (?)

General Approach to Interpretation

- Two-tiered response is recommended depending on magnitude of abnormal result:
 - Result for primary marker exceeds cut-off. Re-analyze; if mean of both values exceeds cut-off, request second NBS specimen.
 - Result for primary marker exceeds alert value – re-analyze same specimen to verify and report immediately.
- If initial result is an alert, or abnormal results are obtained on two different NBS specimens, further testing is recommended to establish diagnosis

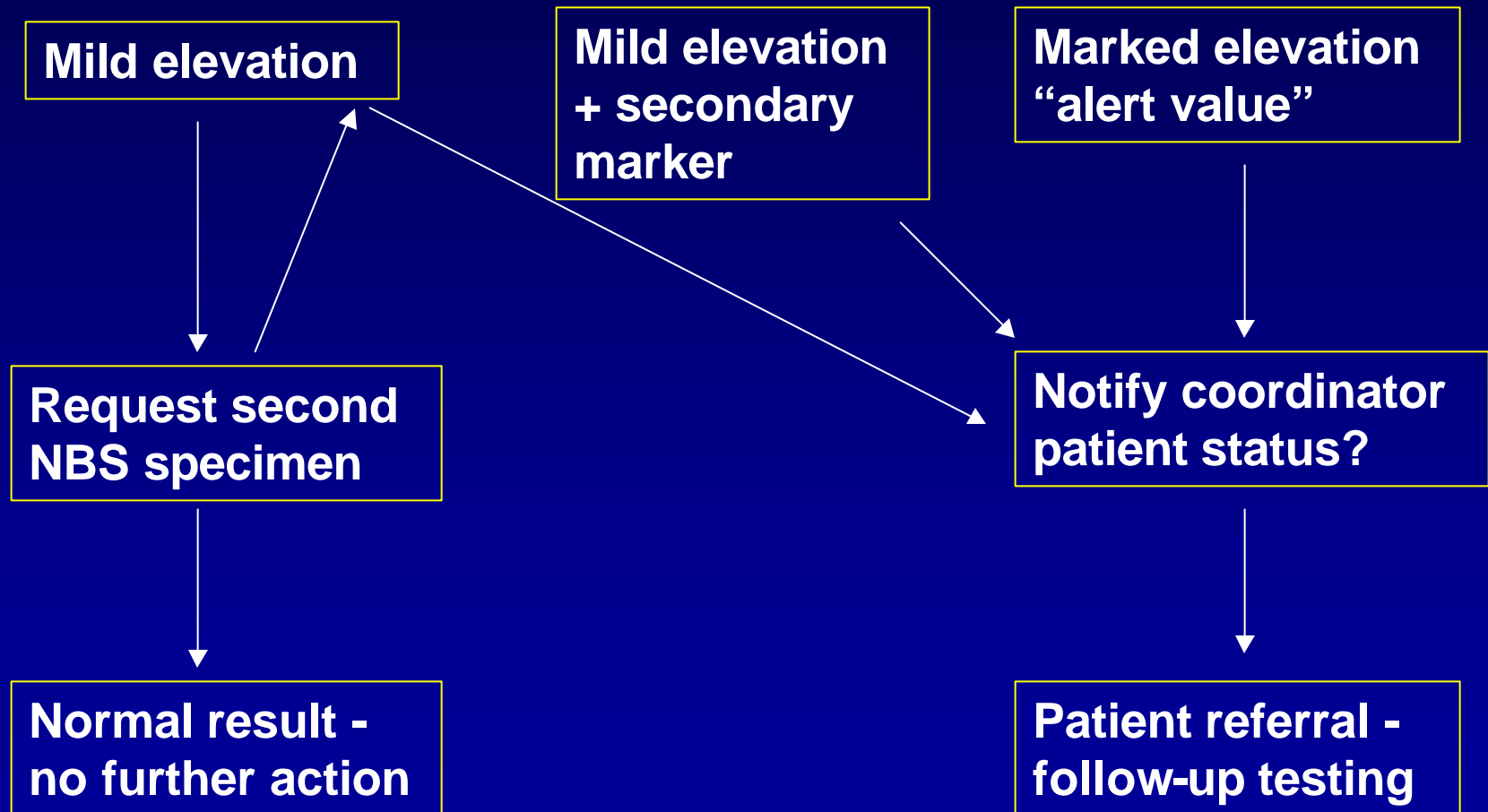
Definition and Use of Primary and Secondary Markers

- Primary analytes – used to establish presumptive positives. A full acylcarnitine profile has up to 13 primary analytes!
- Secondary markers – used in conjunction with primary analyte results to assign risk
- Isolated elevations of secondary markers are considered unimportant

Secondary metabolites and criteria for mild elevation of primary marker

- For PKU: PHE/TYR ratio > 3
- For MSUD: LEU+ILE and VAL
- For PA and MMA: C3/C2 ratio > 0.4
- For MCAD: C8/C10 ratio > 3
- For VLCAD: C14:1/C12:1 ratio > 3
- For LCHAD: C16-OH plus at least one of the following: C18:1-OH, C16, C18:1
- For CPT-II/CAT: C16 and C18:1
- No suitable secondary markers for C3-DC, C4, C5, C5-OH, C5-DC

Algorithm for Abnormal Screening Results



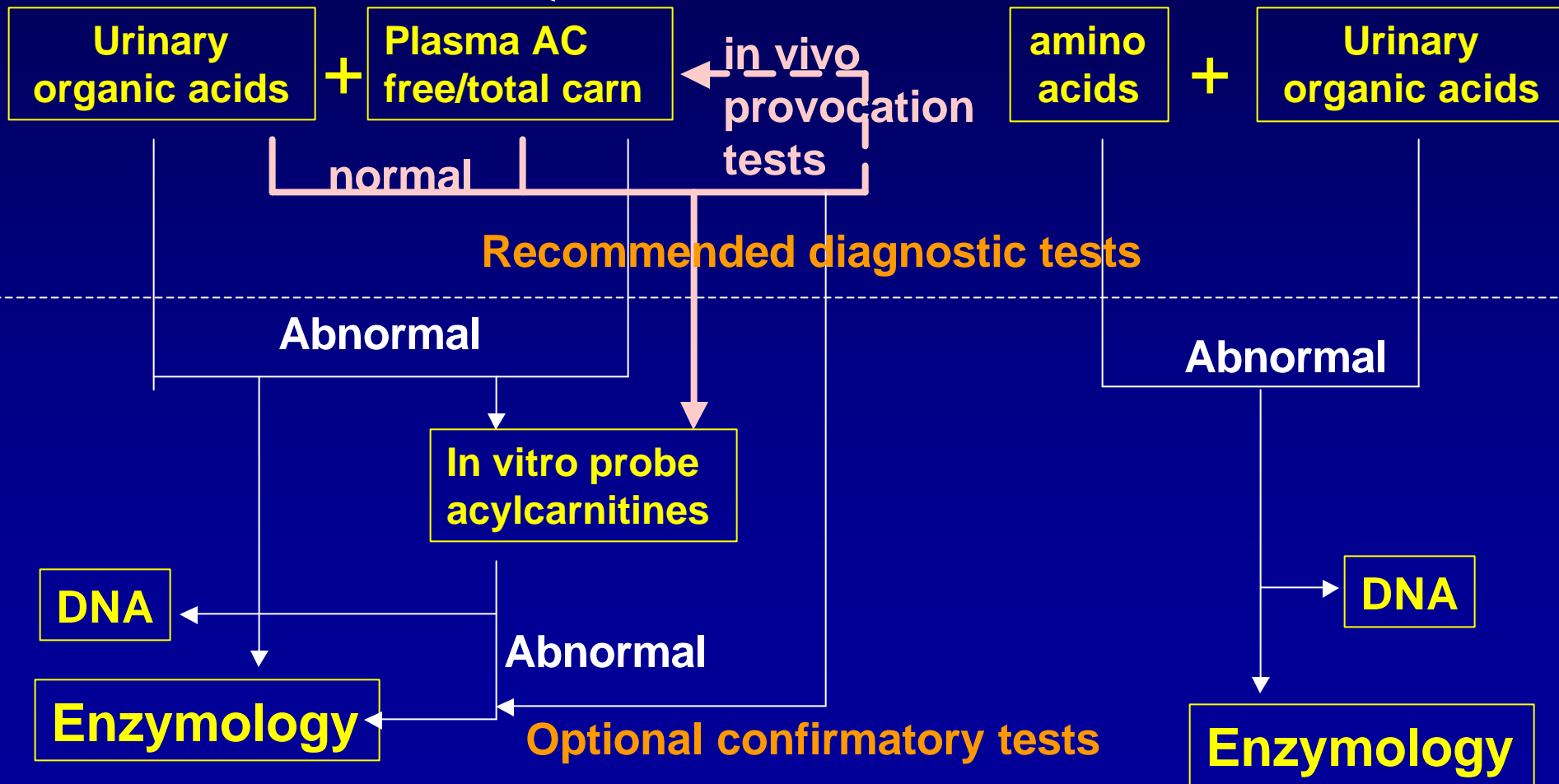
Action taken by co-ordinator

- **Low risk:** contact physician of record, check clinical status of pt., request second blood spot specimen, recommend follow-up testing if symptomatic
- **Moderate risk** (includes positive test on repeat specimen from above and/or presence of secondary markers): request follow-up testing; recommend referral to regional metabolic center if child symptomatic
- **High risk:** recommend immediate referral to metabolic center, follow-up testing and initiate appropriate therapy regardless of clinical status

Abnormal newborn screening acylcarnitine or amino acid result (repeated)

FAO and BCAA

AA



Follow-up testing for elevated glycine

Possible diagnosis: NKH (nonketotic hyperglycinemia)

- CSF amino acids - elevated glycine
- Plasma amino acids - elevated glycine
- Urine organic acids - rules out other metabolic causes for elevated glycine

Confirmation:

- Ratio of CSF: plasma glycine > 0.08
- Reduced activity of the glycine cleavage system (liver)
- *Note: patients usually present with intractable neonatal seizures – prognosis is poor*

Follow-up testing for elevated proline

Possible diagnosis: hyperprolinemia type I or type II

- Plasma amino acids - elevated proline
- Urine organic acids (to rule out lactic acidosis and check for P5C - see below)

Confirmation:

- Type II - P5C dehydrogenase deficiency - by marked elevation of Δ^1 -pyrroline 5-carboxylate (P5C) in urine and plasma
- Type I - proline oxidase deficiency - by exclusion of type II
- *Note: very few known cases in US*

Follow-up testing for elevated valine and leucine + isoleucine

Possible diagnosis: MSUD (maple syrup urine disease)

- Plasma amino acids - elevated BCAA and *allo*-Ile
- Urine organic acids (elevated branched-chain keto-acids)

Confirmation:

- Presence of *allo*-isoleucine in plasma
- Reduced activity of BCKD (branched-chain ketoacid dehydrogenase) activity in fibroblasts or lymphoblasts

Follow-up testing for elevated methionine

Possible diagnosis: homocystinuria or hypermethioninemia

- Plasma amino acids - elevated methionine and/or total plasma homocysteine
- Urine organic acids to check for liver disease

Confirmation:

Cystathionine β -synthase activity in lymphocytes or fibroblasts (if Hcys and Met elevated)

- Methionine adenosyl transferase activity (if Met only elevated) in liver

Follow-up testing for elevated phenylalanine

Possible diagnosis: PKU (phenylketonuria)

- Plasma amino acids - elevated Phe and Phe:Tyr
- Urine organic acids (elevated phenyllactate and phenylpyruvate) and pterins
- BH₄ loading test (rule out BH₄ synthesis defect)

Confirmation:

- DNA testing at PAH locus (> 98%)
- Reduced activity of PAH (phenylalanine hydroxylase) activity in liver (biopsy)
- *Note: other tests required for non-PKU hyper-Phe*

Follow-up testing for elevated tyrosine

Possible diagnosis: tyrosinemia type I, II or III

- Plasma amino acids - elevated Tyr
- Urine organic acids (elevated tyrosine metab; succinylacetone is diagnostic of type I)
- **Confirmation:**
- Clinical history (hepatorenal phenotype - type I; oculocutaneous phenotype - type II)
- Reduced activity of FAH (fumaryacetoacetate hydrolase) activity in lymphocytes - type I
- **Note:** *transient tyrosinemia of the newborn is by far the most common cause of elevated Tyr*

Follow-up testing for elevated citrulline

Possible diagnosis: citrullinemia (ASD); argininosuccinic aciduria (ASLD)

- Plasma amino acids - elevated Cit, also Asa in ASL
- Urine amino acids (grossly elevated arginino-succinic acid (Asa) is diagnostic of ASL def)
- Urine organic acids - orotic acid may be elevated
- **Confirmation:**
- Argininosuccinate synthetase (ASS) activity in liver or cultured fibroblasts
- Argininosuccinate lyase (ASL) deficiency is confidently diagnosed from Asa levels (see above)

Follow-up testing for elevated arginine

Possible diagnosis: arginase deficiency

- Plasma amino acids - marked elevation of Arg
- Urine amino acids - elevated Arg, Lys, Cys Orn
- Urine organic acids - orotate
- **Confirmation:**
- Arginase activity (RBC)

Follow-up testing for elevated ornithine

Possible diagnosis: HHH syndrome; gyrate atrophy

- Plasma amino acids - markedly elevated Orn
- Urine amino acids - elevated Orn, homoCit
- Urine organic acids - orotic acid
- **Confirmation:**
- elevated ammonia in addition to Orn and increased excretion of homocitrulline (homoCit) are diagnostic of HHH syndrome - a mitochondrial membrane transporter defect (ORNT1)
- ornithine aminotransferase activity in lymphocytes (gyrate atrophy)

Summary – Amino Acid Disorders

- MS/MS has been validated only for detection of PKU, MSUD and Homocystinuria thus far
- PKU is by far the most common amino acid defect; MS/MS reduces false positives by allowing use of Phe/Tyr ratios (>2.5)
- The most common abnormality in NBS for amino acids is elevated tyrosine; most cases are NOT tyrosinemia I, II or III (these are very rare)
- The most common urea cycle defect, OTC deficiency, is not currently detectable by MS/MS (possibility of low citrulline?)
- It is not clear that Tyr-I, NKH, HHH, Hyperprolinemia or Arginase deficiency are detectable in the neonate ($< 5d$ of age)

Acylcarnitines in Organic Acidemias: Primary Markers

<u>Acylcarnitine species</u>	<u>Disorder to be considered</u>
C3-	PA, MMA, MCD
C4 -	IBCD, (SCAD, MAD)
C5 -	IVA, 2MBCD (MAD)
C5:1 - (with C5-OH)	SKAT, 3-MCC
C5-OH -	3-MCC, HMGL, SKAT, MCD, 3-methyl- glutaconyl hydratase def
C3-DC -	MA
C5-DC -	GA-I
C6-DC - (with C5-OH)	HMG

Follow-up testing for elevated C-3

Possible diagnosis: propionic acidemia (PA), methylmalonic aciduria (MMA), multiple carboxylase deficiency (MCD)

Plasma acylcarnitine analysis - elevated C3

Urine organic acids analysis - immediate differential diagnosis of PA and MMA; metabolites characteristic of both PA and 3-MCC likely in MCD

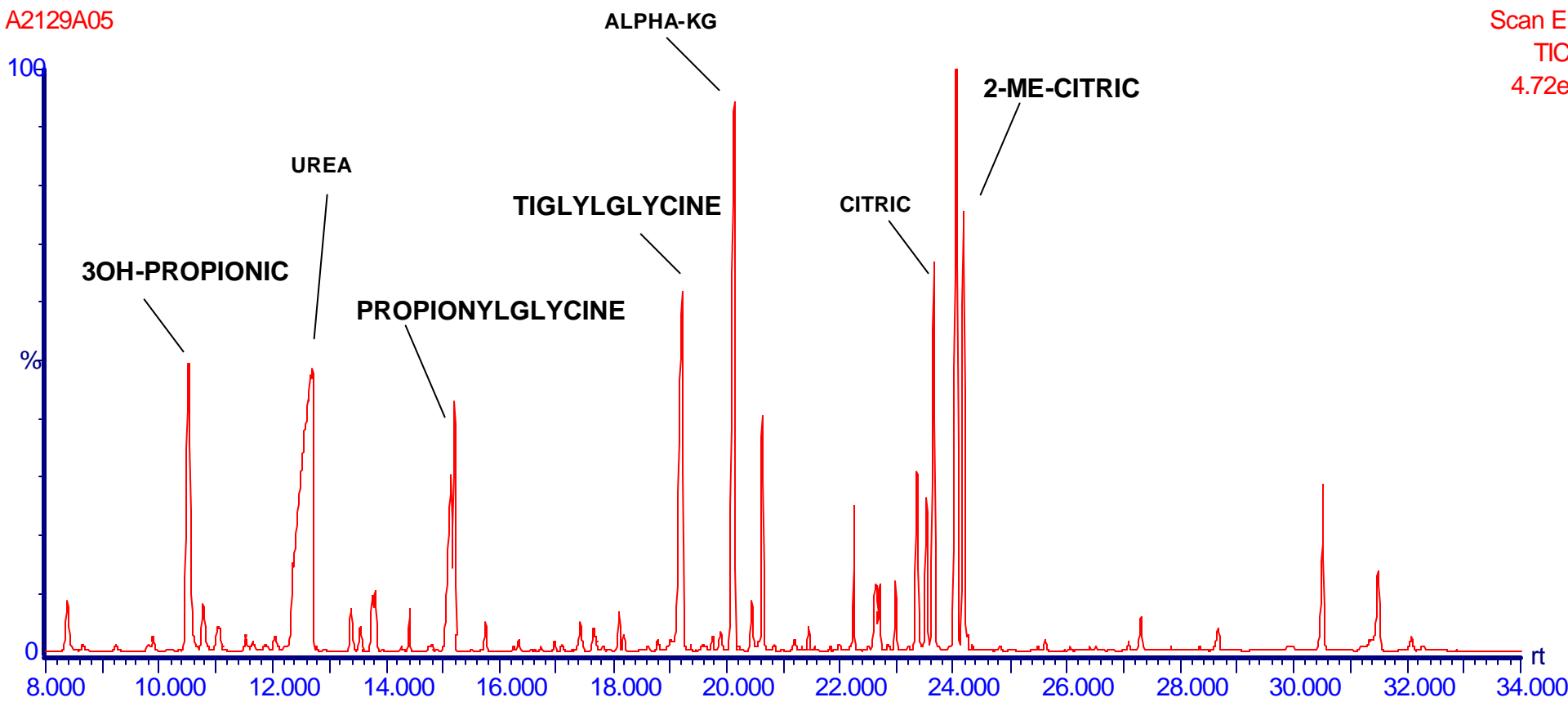
Confirmation:

Propionyl-coA carboxylase assay

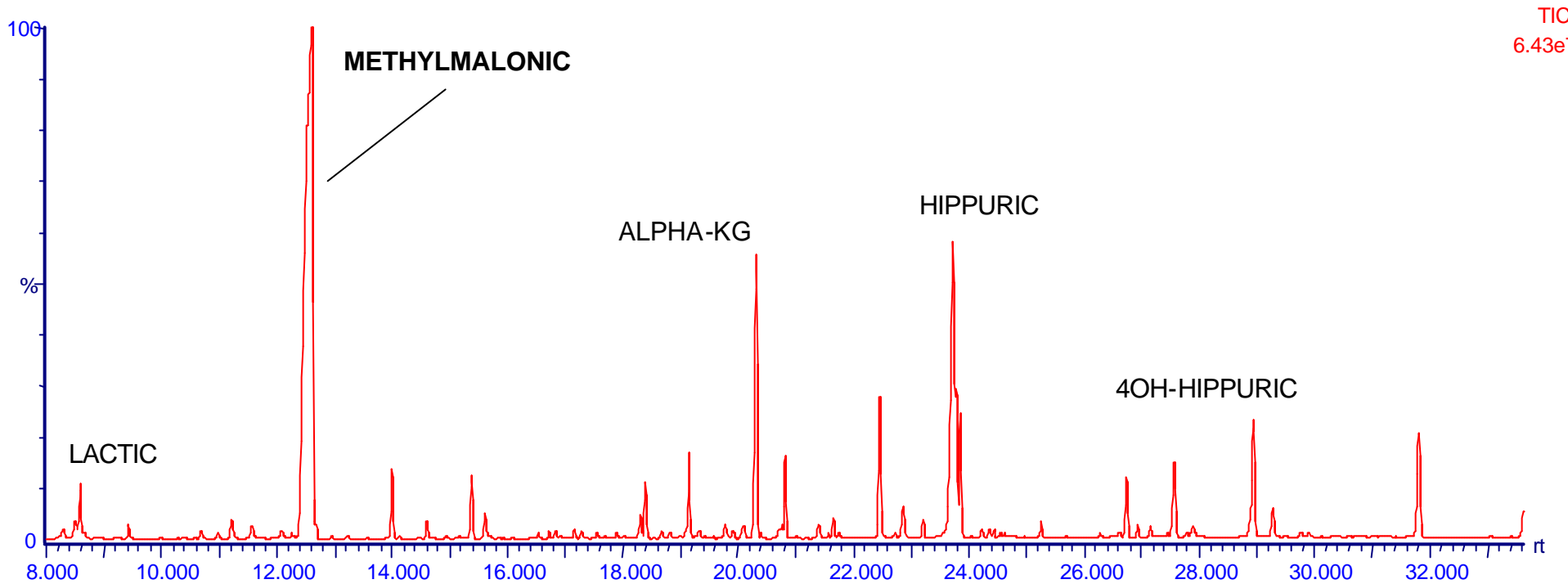
Methylmalonyl-coA mutase (several sub-types)

Holocarboxylase synthase/ biotinidase assay

Urine organic acids analysis - propionic acidemia



Urine organic acids analysis - methylmalonic aciduria



Follow-up testing for elevated C-4

Possible diagnosis: isobutyryl-coA dehydrogenase deficiency(IBCD), (SCAD deficiency, MAD deficiency)

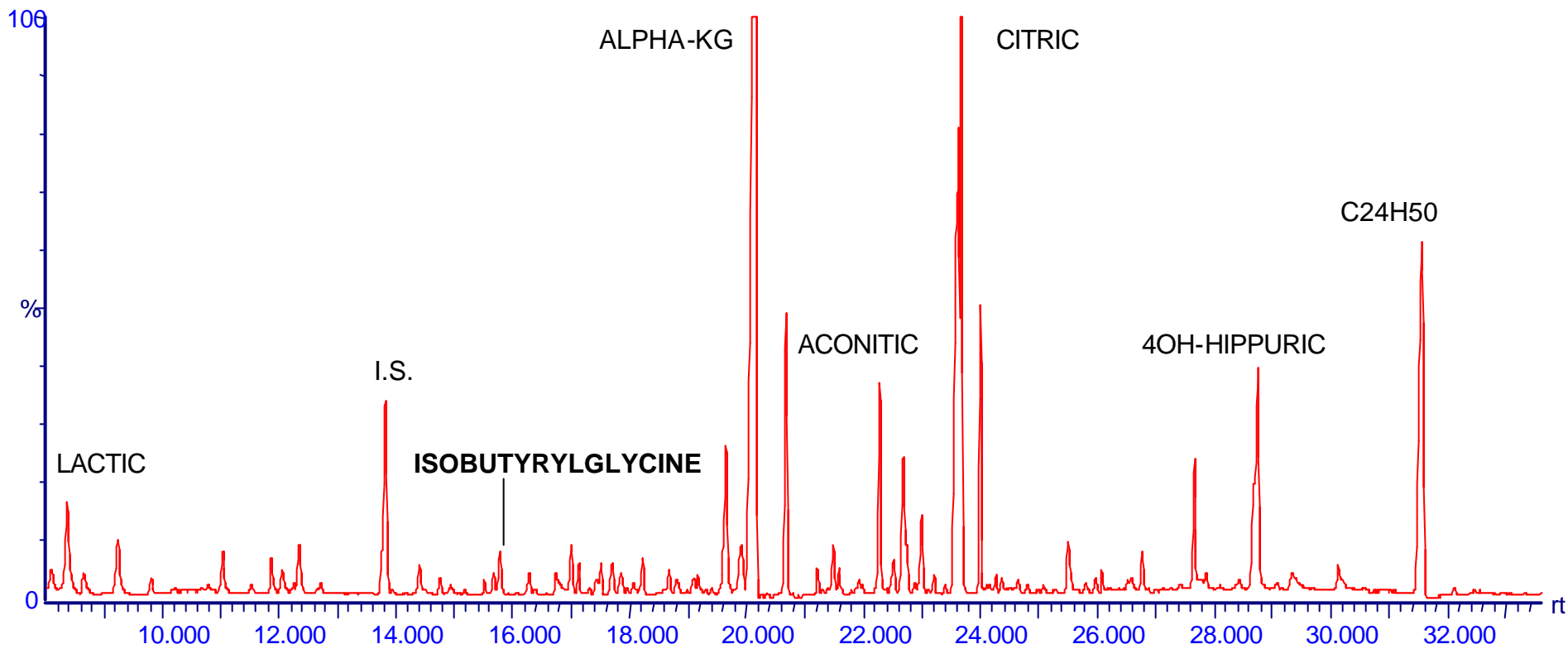
Plasma acylcarnitine analysis - elevated C4 (+ others in MAD deficiency)

Urine organic acids analysis - elevated isobutyryl-glycine (usually not very pronounced)- differentiates IBCD deficiency from the fatty acid oxidation defects

Confirmation:

In vitro testing of valine metabolism in cultured fibroblasts

Urine organic acids analysis - IBCD (isobutyryl-coA dehydrogenase deficiency)



Follow-up testing for elevated C-5

Possible diagnosis: isovaleryl-coA dehydrogenase deficiency, 2-methylbutyryl-coA dehydrogenase deficiency (2-MBCD), multiple acyl-coA dehydrogenase(MAD deficiency)

Plasma acylcarnitine analysis - elevated C5 (+ others in MAD deficiency)

Urine organic acids analysis - marked elevation of isovalerylglycine, or elevated 2-methylbutyryl-glycine (usually not very pronounced)- differentiates the amino acid defects from each other and from MAD

Confirmation:

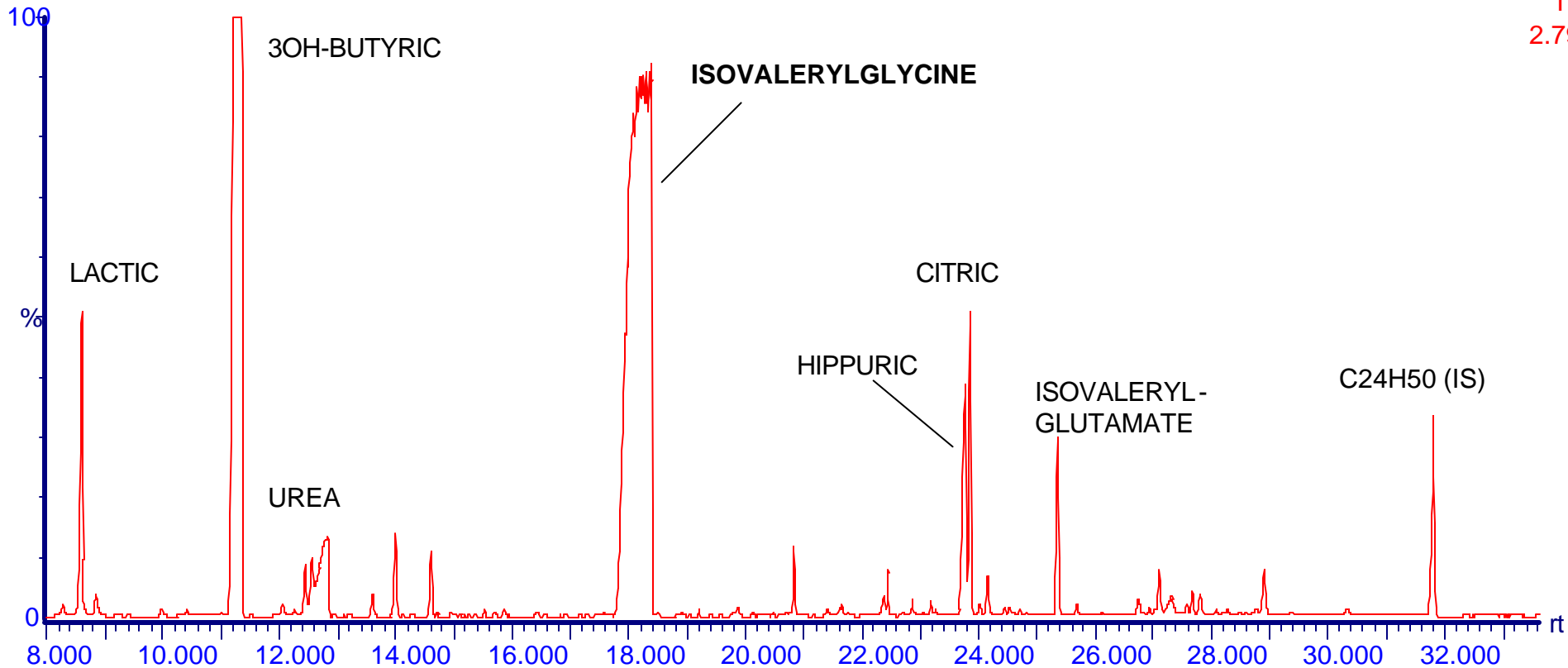
Isovaleryl-coA dehydrogenase activity (fibroblasts)

In vitro testing of isoleucine metabolism in cultured fibroblasts for 2-MBCD

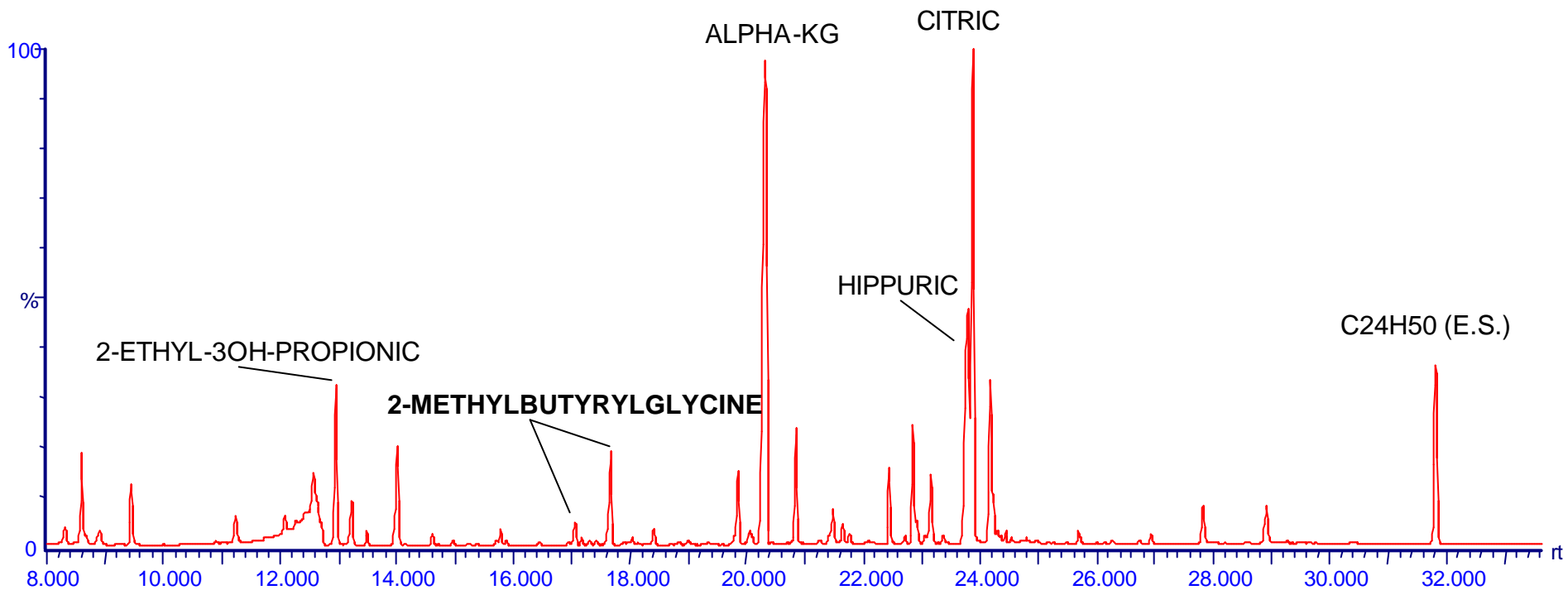
Urine organic acids analysis - isovaleric acidemia

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Urine organic acids analysis - 2-MBCD deficiency (2-methylbutyryl-coA dehydrogenase deficiency)



Follow-up testing for elevated C-5OH

Possible diagnosis: 3-methylcrotonyl-coA carboxylase deficiency (3-MCC); β -ketothiolase deficiency (SKAT); 3-methyl-3-OH-glutaryl-coA lyase deficiency (HMG); multiple carboxylase or holocarboxylase synthetase deficiency (MCD); 3-methylglutaconyl-coA hydratase deficiency (glutaconic aciduria type I)?

Plasma acylcarnitine analysis - elevated C5-OH; also with C5:1 in 3-MCC and SKAT, or with C6DC in HMG; or with C3 in MCD (holocarboxylase synthetase def).

Urine organic acids analysis - moderate or marked elevation of 3OH-isovalerate, with 3-methylcrotonylglycine (3-MCC); or with 3-methylglutaconic and 3Me-3OH-glutaric acids (HMG); or with 3-methylglutaconic acid (glutaconic aciduria type I); or with metabolites of propionic acidemia in MCD.

Follow-up testing for elevated C-5OH (contd.)

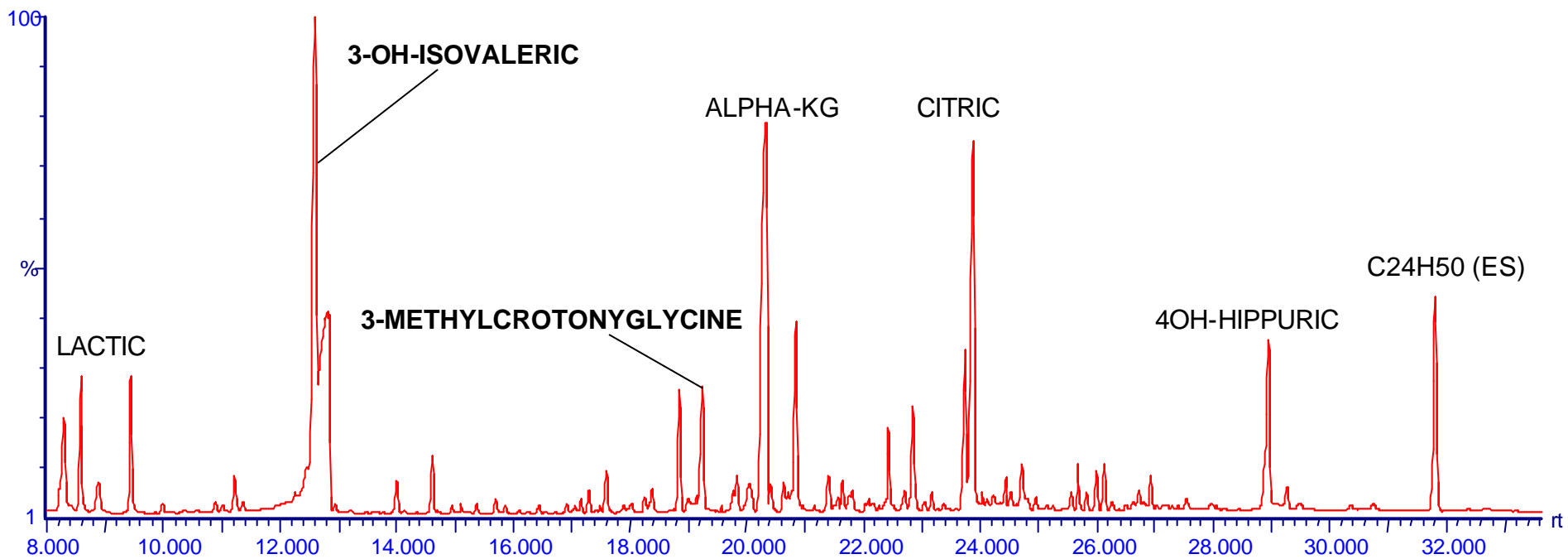
In β -ketothioase deficiency (SKAT), there is marked elevation of 2-methyl-3-OH-butyric and 2-methylacetoacetic acids, with tiglylglycine.

Confirmation:

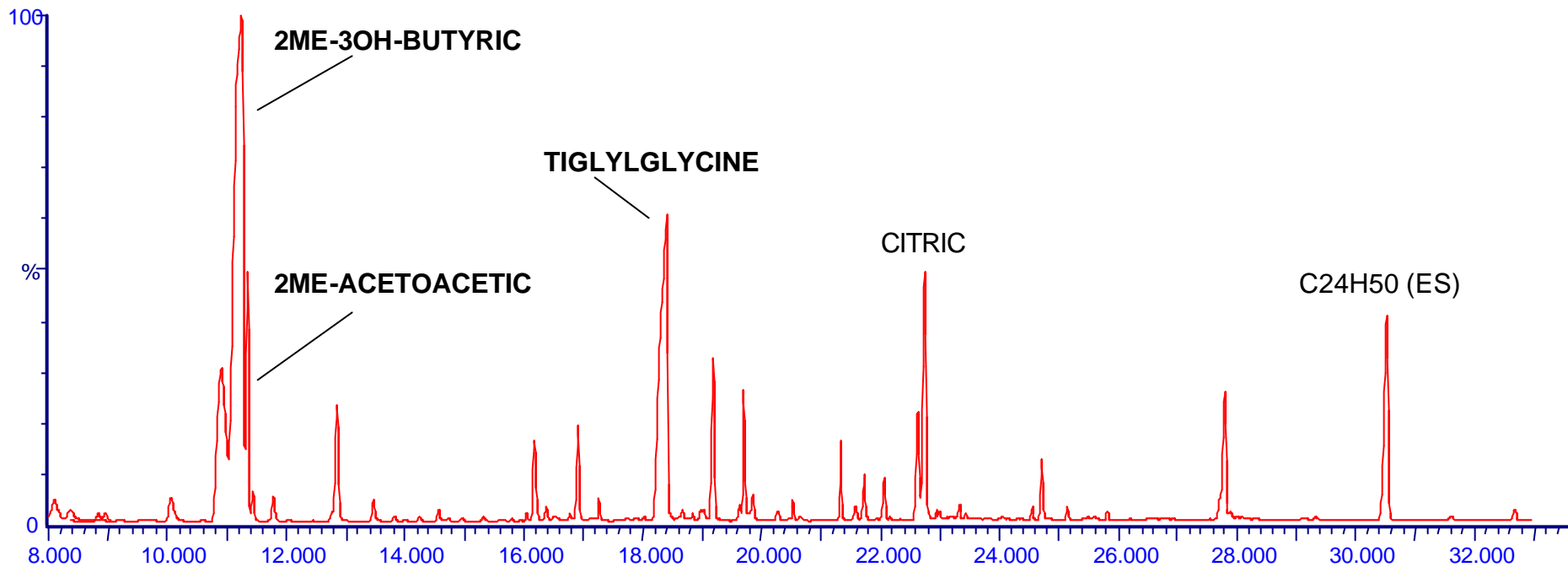
Not usually necessary for HMG or SKAT

Enzyme activity of 3-MCC and PCC is assayed in cultured fibroblasts

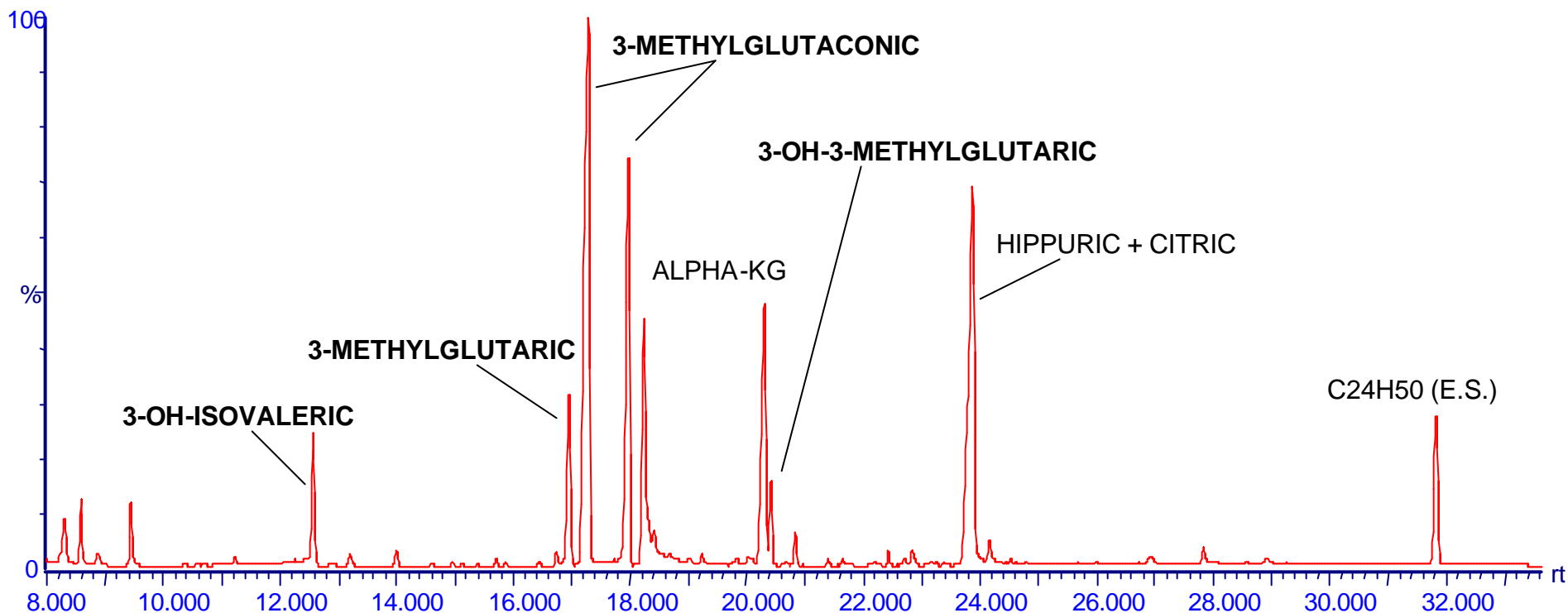
Urine organic acids analysis - 3-MCC deficiency



Urine organic acids analysis - β -ketothiolase (short-chain ketoacylthiolase - SKAT) def



Organic acids analysis - HMG-CoA Lyase deficiency (3OH-3-methylglutaryl-coA dehydrogenase deficiency)



Follow-up testing for elevated C5-DC

*Possible diagnosis: Glutaryl-coA dehydrogenase deficiency
(GA-I)*

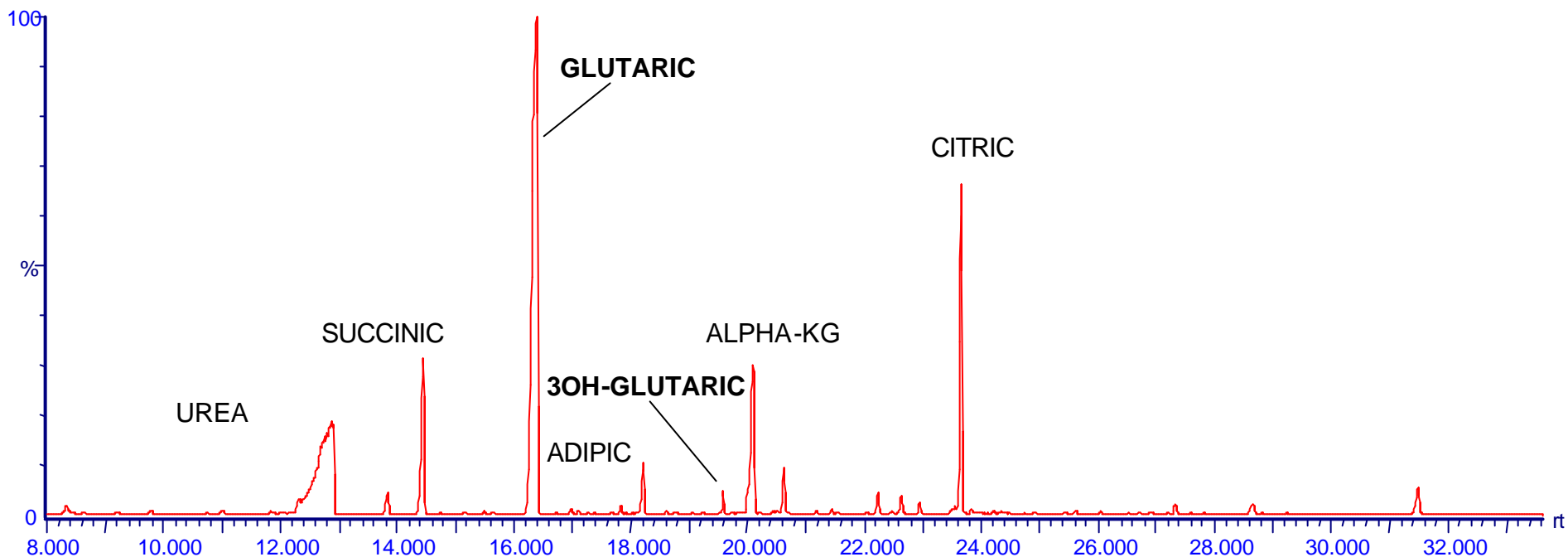
Plasma acylcarnitine analysis - elevated C5-DC, in some cases mild

Urine organic acids analysis - marked - 3-OH-glutarate, with or without elevated glutarate

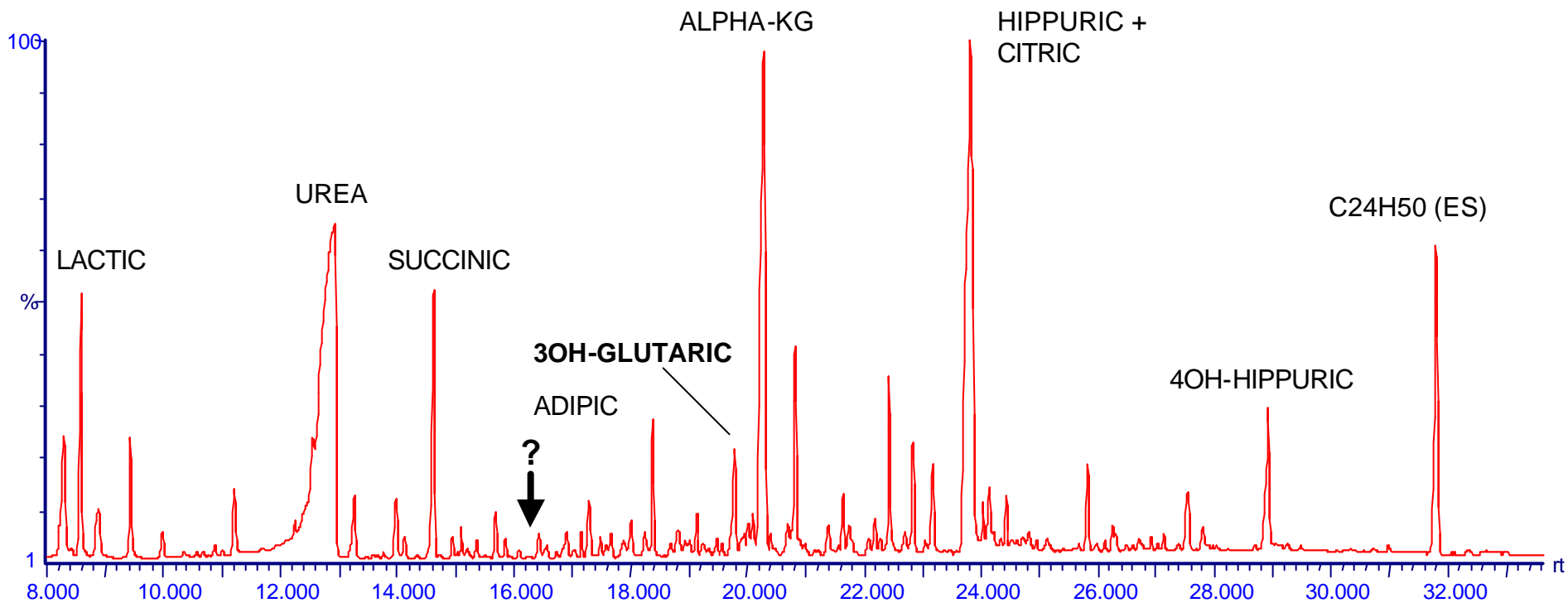
Confirmation:

Glutaryl-coA dehydrogenase activity (where available)

Urine organic acids analysis - glutaric acidemia “classical”



Urine organic acids analysis - glutaric acidemia “low excretor” - glutaric acid not observed!



Acylcarnitines in FAO defects: Summary

Acylcarnitine species	Disorder to be considered
C0 -	Transporter defect
C4 -	SCAD, MAD
C5 - (with C4)	MAD
C6 - (with C8; C10:1)	MCAD
C8 -	MCAD
C10 - (with C8, C10:1)	MCAD
C10:1 - (with C8)	MCAD
C14:1 -	VLCAD

Acylcarnitines in FAO defects: Summary

Acylcarnitine species	Disorder to be considered
C14:1-OH - (with C16-OH)	LCHAD/TFP
C16 - (usually with C18:1)	CPT-II, CAT
C18:1 - (with C16)	CPT-II, CAT
C16-OH -	LCHAD, TFP
C18:1-OH - (with C16-OH)	LCHAD, TFP
C16 ⁻ (with C18:1 ⁻)	CPT-I
C18:1 ⁻ (with C16 ⁻)	CPT-I

Follow-up testing for low C-0

Possible diagnosis: carnitine plasma membrane transporter deficiency

Plasma acylcarnitine analysis - low C0 (usually <10 μ M); low acylcarnitine signals generally

Urine organic acids analysis - non-specific findings; absence of dicarboxylic acids.

Confirmation (required):

Enzyme activity in patient cells - lymphocytes, fibroblasts

Follow-up testing for elevated C-4

Possible diagnosis: short-chain acyl-coA dehydrogenase (SCAD) deficiency, multiple acyl-coA dehydrogenase (MAD) deficiency

Plasma acylcarnitine analysis - elevated C4 (+ others in MAD deficiency, including C5, with or without various C6-C16 saturated species and C18:1)

Urine organic acids analysis - marked elevation of ethylmalonic and 2-methylsuccinic acids, butyrylglycine (“classical” SCAD); modest elevation of ethylmalonic (“mild variant” SCAD); one or more of the following modestly elevated: ethylmalonic acid, adipic acid, glutaric acid, butyrylglycine, isobutyrylglycine, isovalerylglycine, hexanoylglycine, suberylglycine (MAD)

Follow-up testing for elevated C-4 (contd.)

Possible diagnoses: short-chain acyl-coA dehydrogenase (SCAD) deficiency, “variant” SCAD deficiency, multiple acyl-coA dehydrogenase (MAD) deficiency

Confirmation (not easy):

Enzyme activity fibroblasts, muscle (SCAD)

MAD can result from riboflavin deficiency, electron-transferring flavoprotein (ETF) or ETF-DH deficiency

In vitro testing of palmitic acid metabolism in cultured fibroblasts

DNA testing/sequencing for inactivating mutations and polymorphisms in exons 5 & 6

Follow-up testing for elevated C8

Possible diagnosis: medium-chain acyl-coA dehydrogenase (MCAD) deficiency; “mild variant” MCAD

Plasma acylcarnitine analysis - marked elevation of C8, usually with elevations of C6, C10, C10:1. The C8:C10 ratio is always elevated in MCAD (>3:1), except in the mild variants.

Urine organic acids analysis - elevated hexanoylglycine and suberylglycine, often with 5-OH-hexanoic acid, also with dicarboxylic acids when fasting. Variants can be normal.

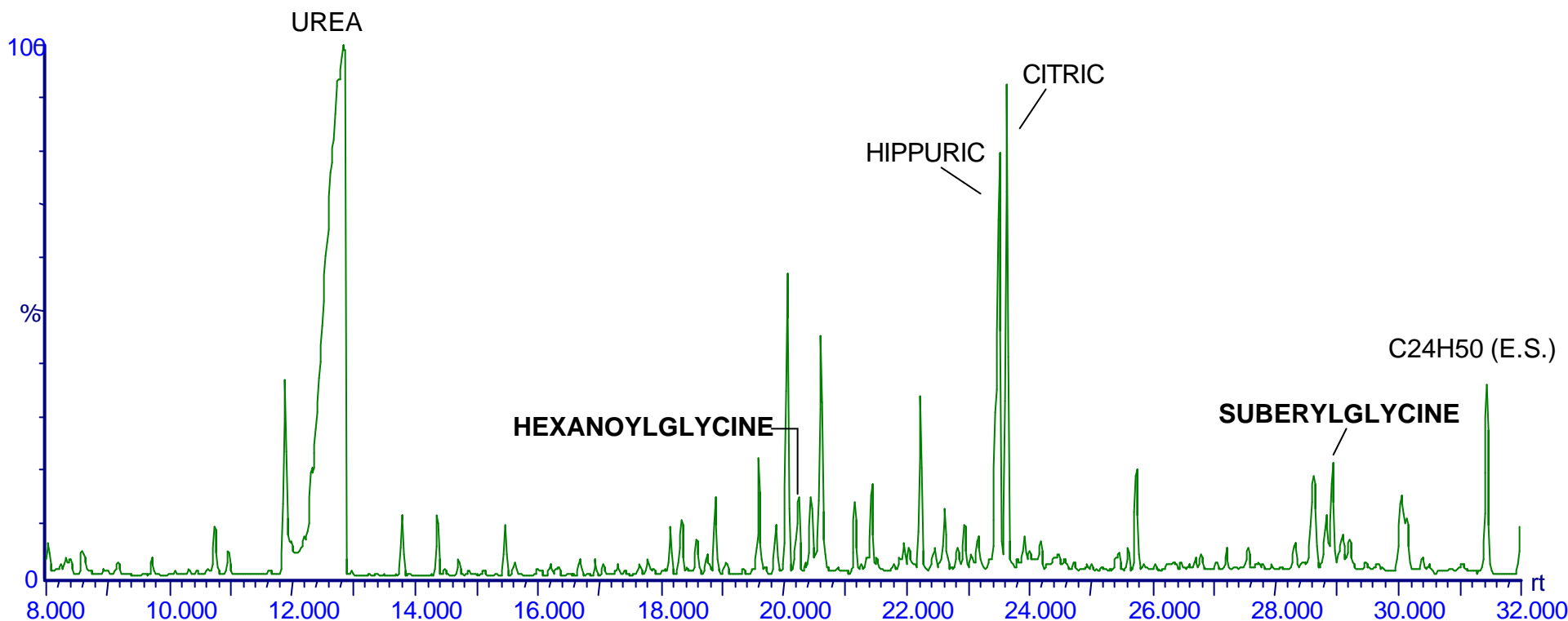
Confirmation:

Not necessary for classical form

DNA testing for A985G mutation usually done

DNA testing available for mild variants (exon 11 sequencing)

Urine organic acids analysis - MCAD deficiency



Follow-up testing for elevated C14:1

Possible diagnosis: very long-chain acyl-coA dehydrogenase (VLCAD) deficiency

Plasma acylcarnitine analysis - modest to marked elevation of C14:1, often with elevations of C14:2, C14, C16, C18:1. The C14:1:C12:1 ratio is (always?) elevated in VLCAD (>3:1)

Urine organic acids analysis - either normal, or showing dicarboxylic aciduria with reduced ketones when fasting. No specific markers for VLCAD

Confirmation:

Enzyme assay or in vitro test using C16 as substrate (fibroblasts)

DNA testing available

Follow-up testing for elevated C16-OH

Possible diagnosis: long-chain 3-OH-acyl-coA dehydrogenase deficiency, trifunctional protein deficiency

Plasma acylcarnitine analysis - mild to marked elevation of C16-OH, often with elevations of C18:OH, C14:1, C16, C18:1.

Urine organic acids analysis - either normal, or showing dicarboxylic aciduria and 3-hydroxydicarboxylic aciduria with reduced ketones when fasting. No specific diagnostic metabolites for LCHAD; 3-OH-monocarboxylic acids might accumulate in TFP deficiency

Confirmation:

Enzyme assay or in vitro test using C16 as substrate (fibroblasts). Difficult to distinguish LCHAD from TFP

DNA testing available - G1528C mutation common in LCHAD

Follow-up testing for elevated C16 & C18:1

Possible diagnosis: carnitine palmitoyl transferase -II (CPT-II) def., carnitine-acylcarnitine translocase def.)

Plasma acylcarnitine analysis - mild to marked elevation of C16 and C18:1, often with elevation of C14 (not C14:1).

Urine organic acids analysis - either normal, or showing dicarboxylic aciduria and 3-hydroxydicarboxylic aciduria with reduced ketones when fasting. No specific diagnostic metabolites.

Confirmation:

Enzyme assay (fibroblasts or lymphocytes).

in vitro test using C16 as substrate (fibroblasts). Difficult to distinguish CPT-II from CAT.

Follow-up testing for low C16 & C18:1

*Possible diagnosis: carnitine palmitoyl transferase-I
(CPT-I) deficiency*

Plasma acylcarnitine analysis - normal; plasma carnitine levels elevated. Blood acylcarnitines still show low C16 and C18:1 with normal or elevated C2

Urine organic acids analysis - unremarkable. No specific diagnostic metabolites.

Confirmation:

Enzyme assay (fibroblasts or leukocytes)

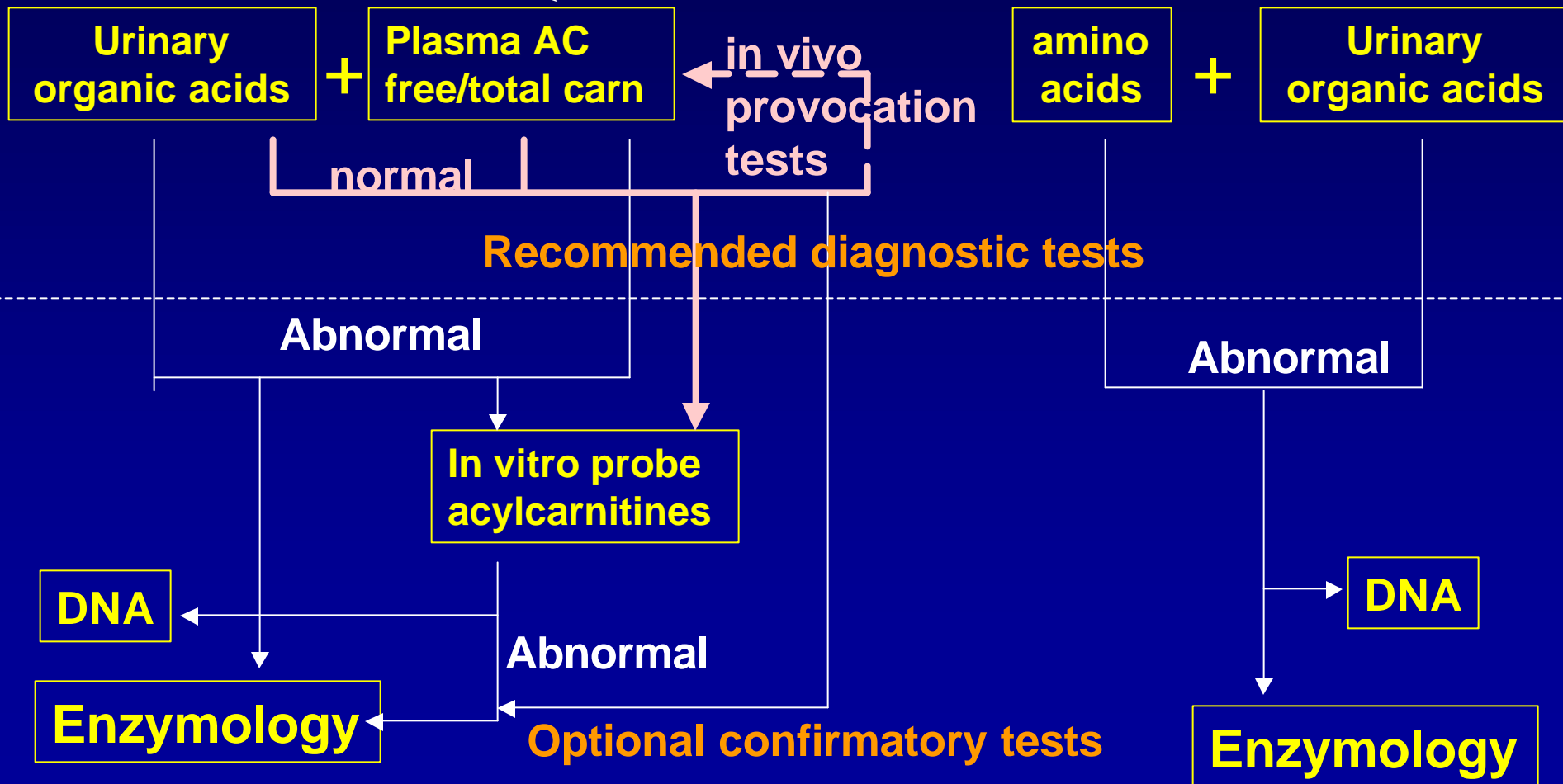
First-tier Tests: Summary

Analysis of plasma amino acids, plasma carnitine/acylcarnitines and urine organic acids constitute the main tier of follow-up diagnostic testing for abnormal results from MS/MS NBS. In order to achieve optimal results, these tests must be entrusted to a specialty biochemical genetics laboratory that has an established reputation for high quality diagnostic work and a director or in-house consultant that can discuss results intelligently with the referring physician

Abnormal newborn screening acylcarnitine or amino acid result (repeated)

FAO and BCAA

AA



In vitro analysis of acylcarnitine intermediates

Fibroblasts/lymphocytes in culture

3-5 days

**16:0, [U-¹³C]16:0, BCAAs
excess L-carnitine**

Harvest medium

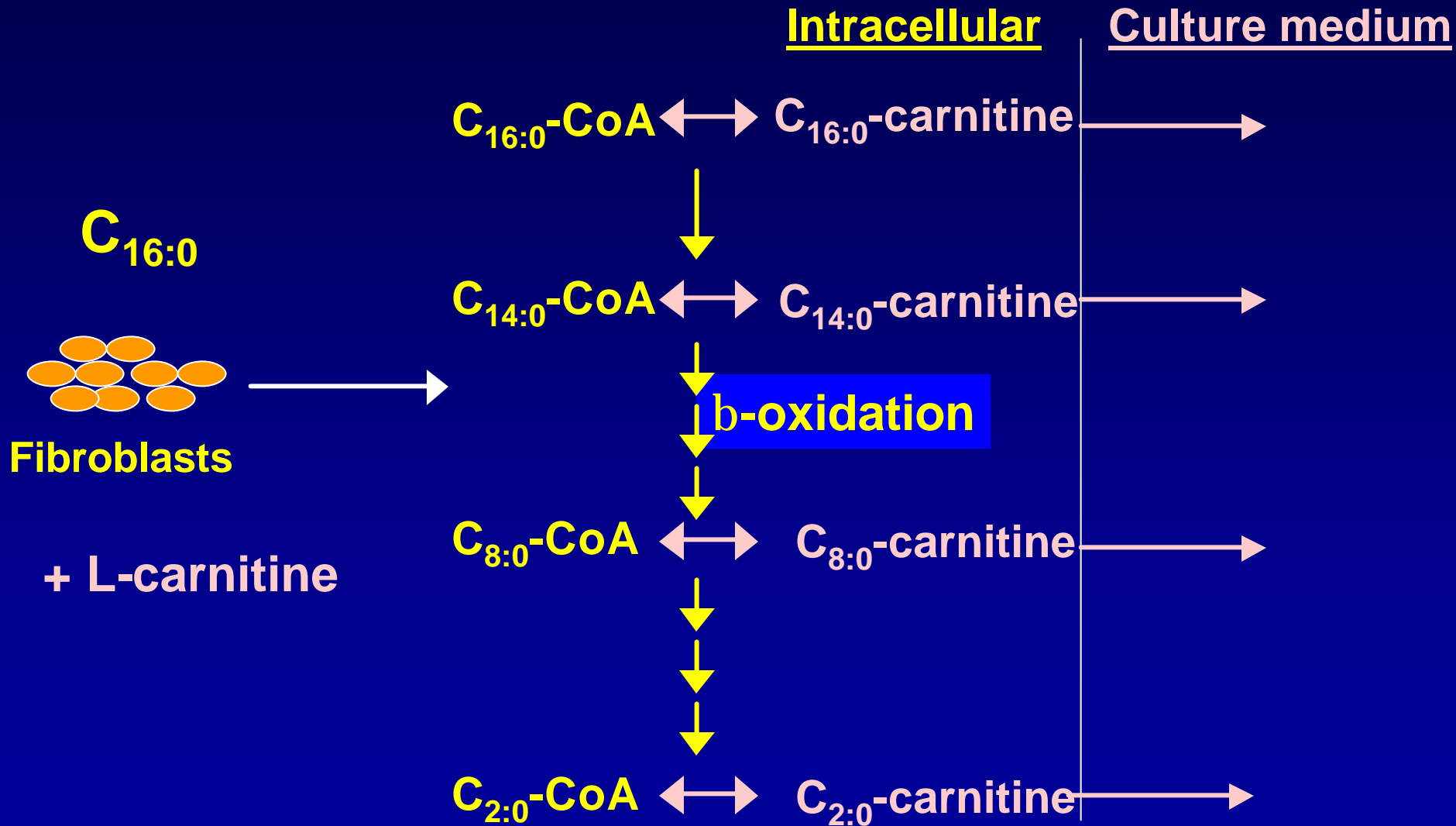
Add internal standards

Spot onto cotton fiber filter paper

Dry, solvent extract, derivatize

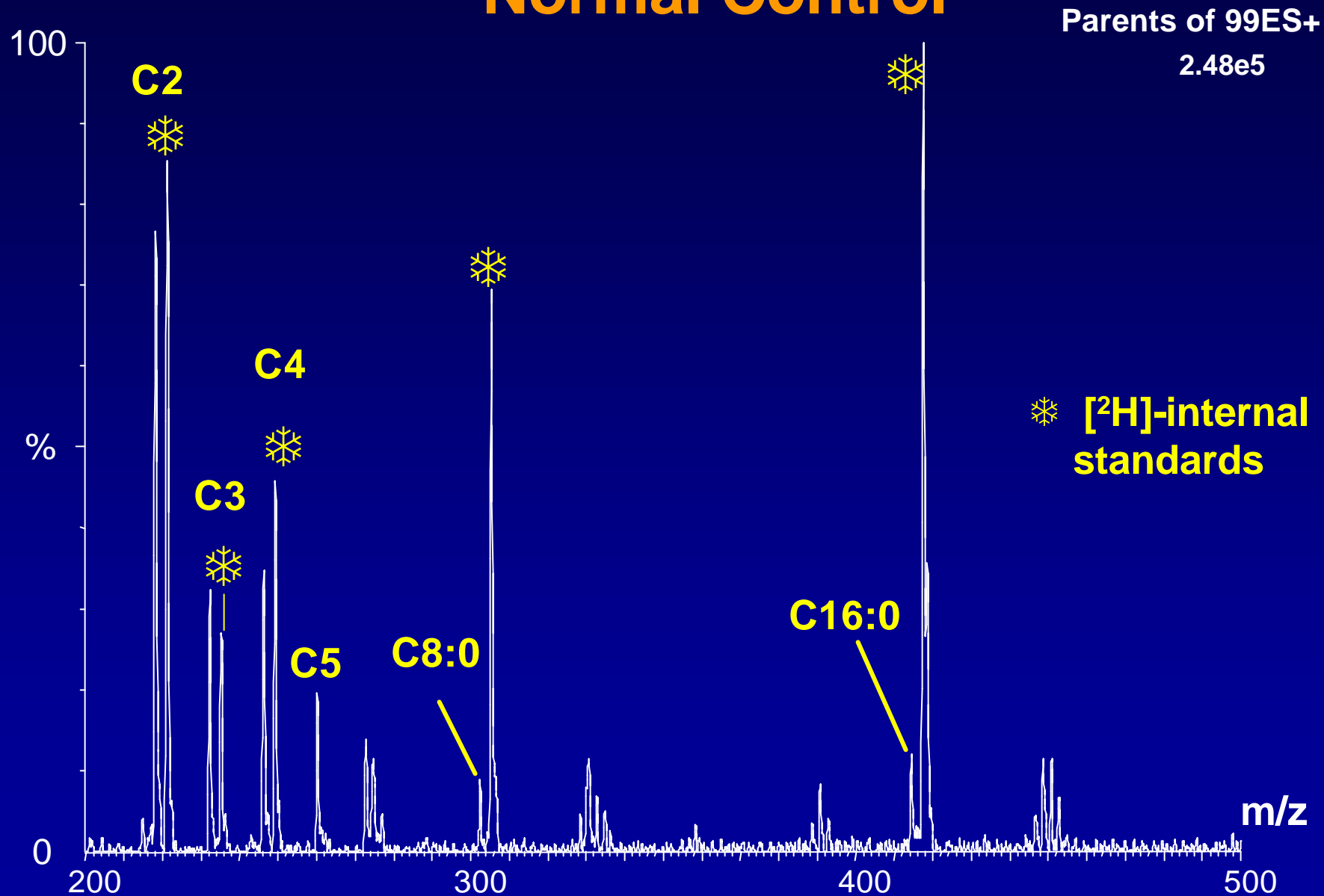
ESI-MS/MS analysis

In vitro probe analysis - FAO defects

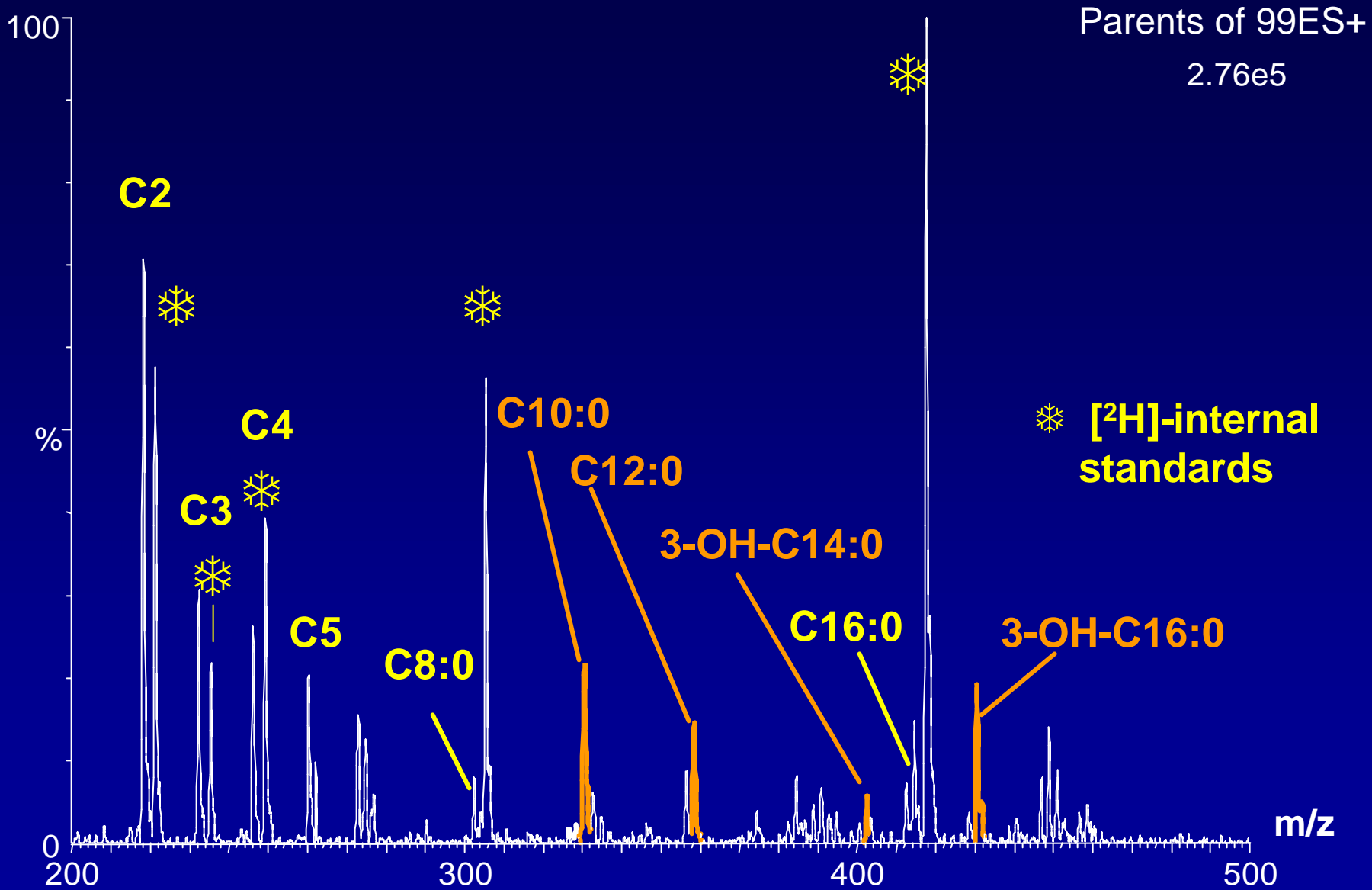


In Vitro Probe – C16:0 (palmitate)

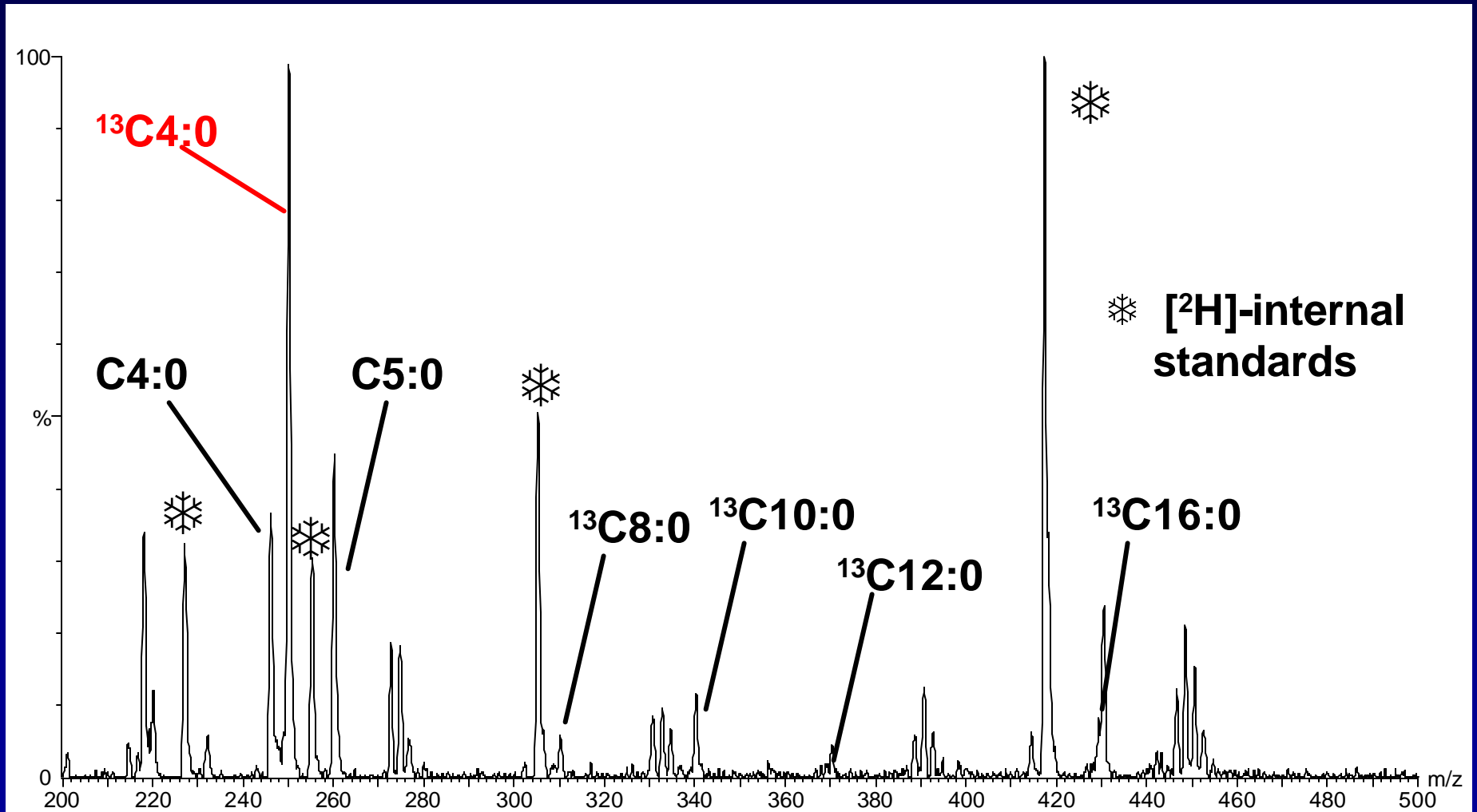
Normal Control



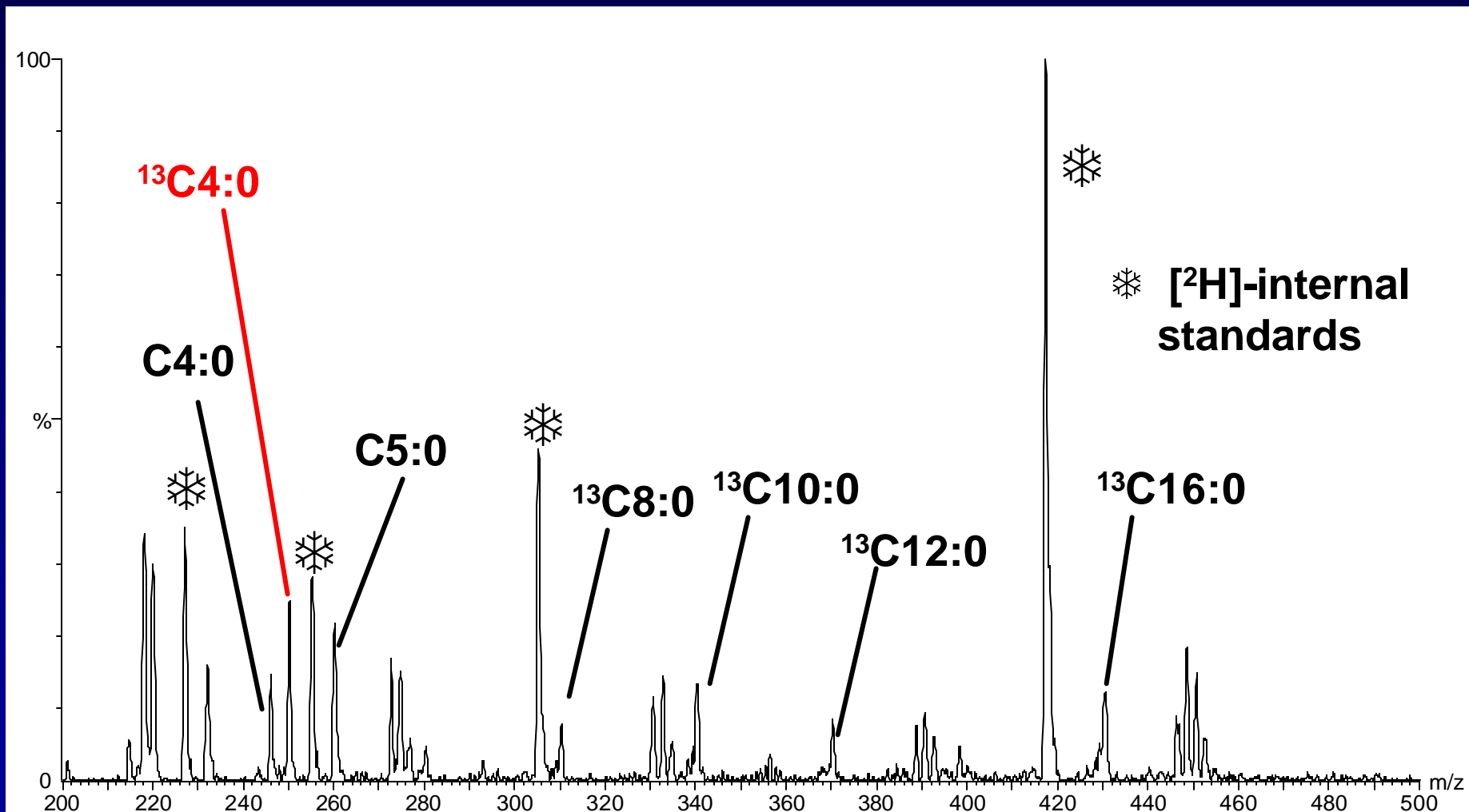
In Vitro Probe - LCHAD or MTP deficiency



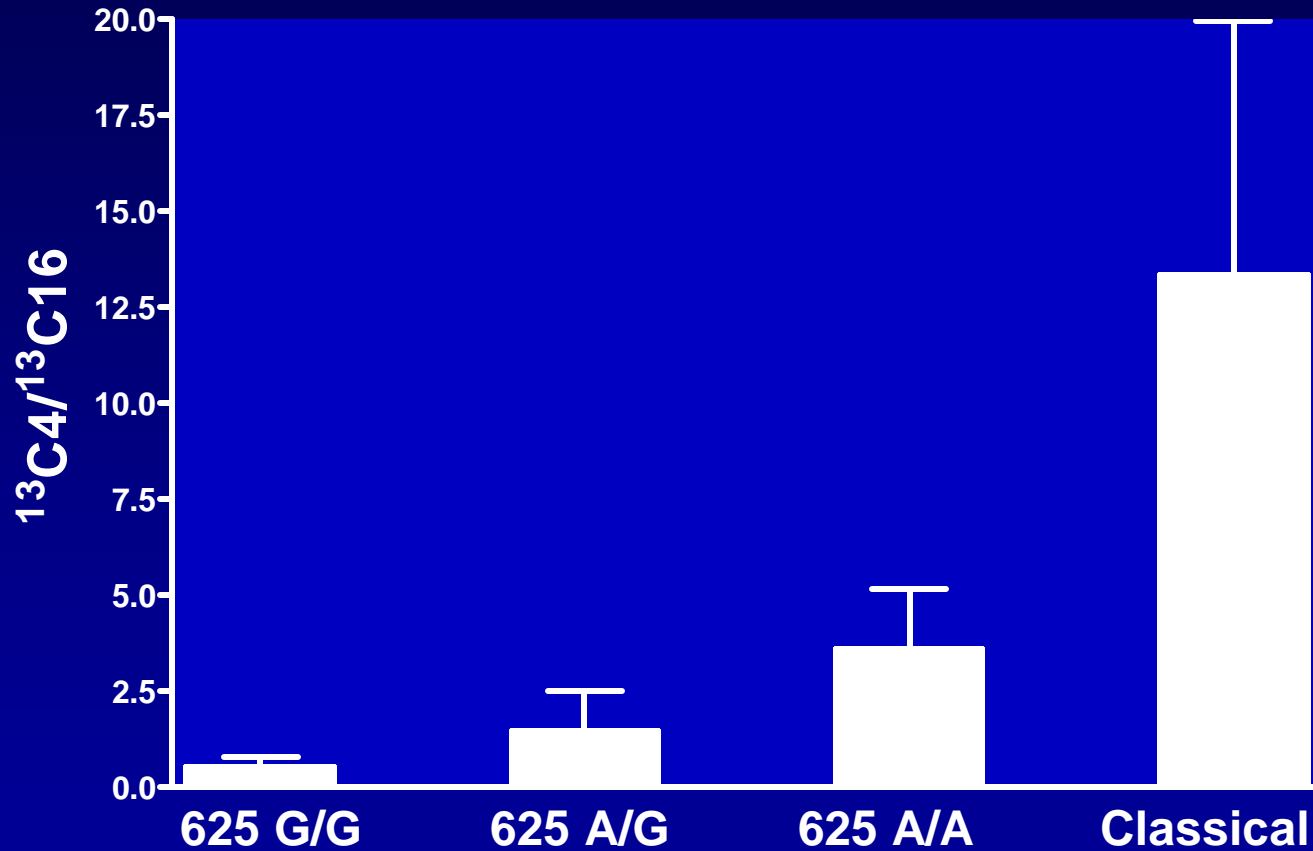
[¹³C]16:0 probe - Classical SCAD



[¹³C]16:0 probe -SCAD variant (625A/625A)



C4-carnitine accumulation in SCAD



In vitro probe analysis - BCAAs

BCAA	Carnitine intermediates	Enzyme deficiency
Isoleucine	C₅ (methylbutyryl) C₃ (Ratio C₅/C₃)	2-Methylbutyryl-CoA DH
Leucine	C₅ (isovaleryl) 3-OH- C₅ (3-OH isovaleryl) C₆DC (3-methylglutaryl)	Isovaleryl-CoA DH 3-methylcrotonyl-CoA carboxylase or 3-HMG-CoA lyase 3-HMG-CoA lyase
Valine	C₄ (isobutyryl)	Isobutyryl-CoA DH

Summary

- **Global (flux) enzyme studies and in vitro profiling methods are useful for confirming defects in fatty acid oxidation and BCAA metabolism (in vitro probe only). In vitro profiling is more informative. Especially useful when blood and urine biochemical indicators are not persistently elevated.**
- **Definitive diagnosis can be made through specific enzyme activity measurements and/or molecular studies.**

MS/MS Newborn Screening in NC: Overall 5 yr Summary

Total screened = 635,168 (7/28/97 to 12/31/02)

Confirmed diagnoses after abnormal screen:

- Fatty acid oxidation disorders 62
 MCADD (48)
- Organic acidemias 37
 3-methylcrotonylglycinuria (13)
- Amino acid disorders 43
 PKU/hyperphenylalaninemia (30) —

Total = 142

Overall Incidence: 1:4,473 (D. Frazier, UNC)

References

- Strategies for the diagnosis of mitochondrial fatty acid b-oxidation disorders Sim et al Clin Chim Acta (2002) 323:37-58
- Wanders RJA et al Disorders of mitochondrial fatty acyl-CoA b-oxidation J.Inher.Metab.Dis (1999) 22:442-487
- Koeberl DD, Young SP, et al. Rare disorders of metabolism with elevated butyryl and isobutyryl carnitine detected by tandem mass spectrometry newborn screening. Ped. Res. 54:219-223 (2003).
- Young SP, Matern D., et al. A comparison of in vitro acylcarnitine profiling methods for the diagnosis of classical and variant short chain acyl-coA dehydrogenase deficiency. Clin. Chim. Acta. 337:103-113 (2003).

Resources

1. <http://biochemgen.ucsd.edu>
2. <http://www.genetests.org>