Introduction to the biochemical genetics laboratory

Tim Wood

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- I have no conflicts of interest to report

- I consider myself to be proficient at the techniques/assays but I continue to learn and evolve. The concepts and ideas I will share have been taught to me by many bright and dedicated individuals over the years for which I want to say "Thanks".

- Thanks to the BGL laboratory at CHCO and the faculty and staff in the Section of Genetics and Metabolism.

- Thanks to Dr. Pete Baker for organizing this lecture series

SIMD laboratory workgroup

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- Amino acids
- Organic acids
- Fatty acid oxidation
- Purine / Pyrimidines
- Bile acids
- Cholesterol / Lipids
- Steroid synthesis
- Porphyrias
- Energy metabolism (mitochondrial)
- Creatine synthesis and transport
- Simple carbohydrates
- Peroxisomal disorders
- Lysosomal disorders
- Vitamin disorders
- Copper / Zinc / Iron
- Leukotrienes

- Many assays separate classes or groups of compounds.

- Historically, screening for IEMs was done using common urine chemical reactions (ferric chloride, nitroprusside, etc.), followed by more specific tests (2nd tier)

- Evolution toward more precise/diagnostic testing as 1st tier testing occurred in the 1950s–1990s
  - Amino acid analysis
  - Urine organic acid analysis
  - Acylcarnitine analysis
  - Enzyme testing

- With the advent of NGS testing, biochemical genetic testing continues to evolve

What to look for…

Elevated

Substrate

~~Enzyme~~

Product

Decreased

Qualitative tests

- For some assays/tests, just identifying the compound or a pattern of compounds is enough to make the diagnosis.

- Based on experience we can make an estimate of whether the sample is normal or abnormal.

- Most biochemical tests started this way. As technology has improved we have been able to put numbers / values with results.

- Common **qualitative** tests include urinary organic acids, urinary oligosaccharides, and TLC analysis of glycosaminoglycans

Quantitative tests – give you a number

- Reporting an analytical value for an analyte is essential for determining treatment efficacy. So many tests that involve treatment have been advanced to quantitative tests

    - Plasma amino acids
    - Single analytes such as total and free carnitine

- Because most analytes are present in normal individuals, we need to develop a normal range for each compound.

- Values outside this range are considered abnormal....but are they pathogenic/disease related?

How do we decide if it is abnormal?

- **Development of a normal range**
  - **Mean +/- 2/3 standard deviations**
  - **z score**
  - **May be age related**
  - **Are often sample type specific**
  - **Need to run normals (100?)**
  - **Need to know if the normal range and affected/pathogenic range differ…..if there is overlap**

Factors that can influence values

- Medications (valproic acid)
- Vitamins/supplements (MCT)
- Time since last meal
- Health (liver dysfunction)
- Time since blood draw
- Sample collection tube
- Gut microbes

- Metabolites of uncertain significance (MOUS)!

What we will discuss……

- Amino acid analysis
- Organic acid analysis
- Acylcarnitine analysis
- Enzyme analysis
- Macromolecules

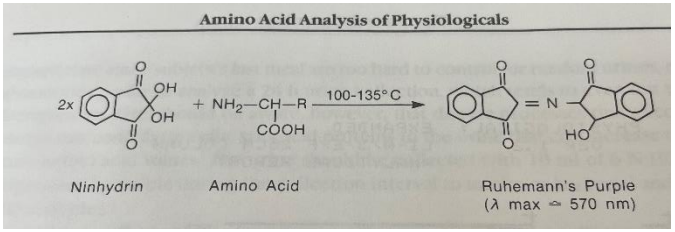
Amino acid (AA) analysis

Rationale for analysis

- Blood levels are fairly constant in blood after meal effects have subsided
- Renal tubules have transporters to reabsorb various AAs from the urine
- Plasma/serum/blood spots — best for *pathway* defects (i.e., non-transporter disorders):
  - Example, aminoacidopathies
- Urine — optimal for disorders of AA *transport:*
  - Cystinuria
  - Lysinuric protein intolerance
  - Hartnup disease
  - Renal Fanconi syndrome (many causes — e.g., mitochondrial disorders, cystinosis)
- CSF — used for *cerebral* aminoacidopathies:
  - Glycine encephalopathy (a.k.a. non-ketotic hyperglycinemia)
    - Often collect blood for CSF/plasma glycine ratio
  - Serine biosynthesis defects
  - Asparagine and glutamine synthetase deficiencies

Adapted from NAMA 2020

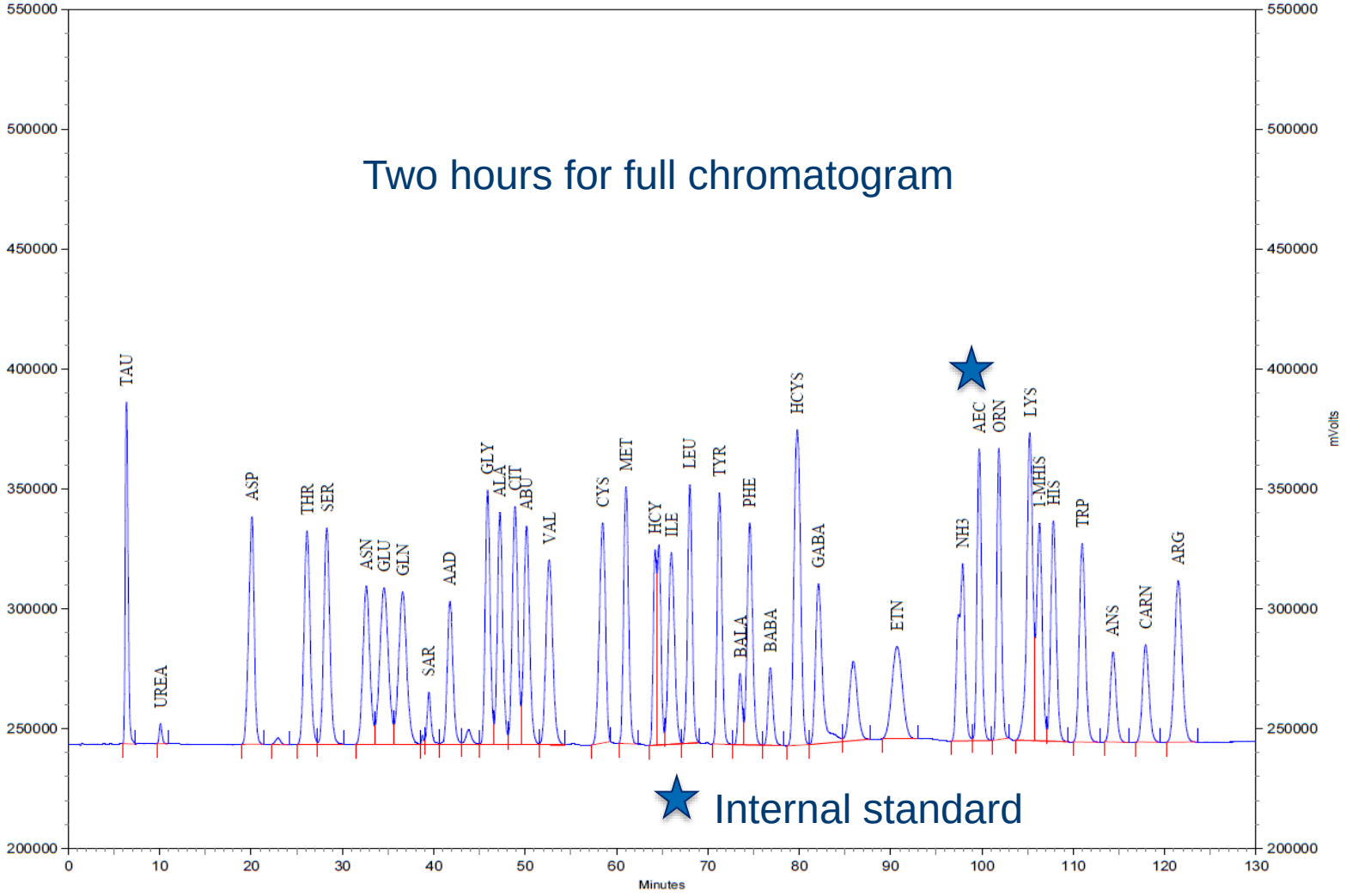
Method of analysis



- Plasma is separated and deproteinized, and an internal standard is added
- Analysis most commonly done by ion-exchange columns or UHPLC chromatography
- For ion exchange chromatography, separation is based upon pH and salt concentration of eluent; temperature is also used.
- **Retention time is main factor for identification.**
  - **Important for compounds like methionine and homocitrulline that co-elute in most systems**
- For IEC, two wavelengths (570nm) may be used for detection (440 for imino acids)
- Quantity is assessed by peak in relation to the internal standard and a standard (calibrating) run of all quantitated AAs

Adapted from NAMA 2020

Amino acid standard



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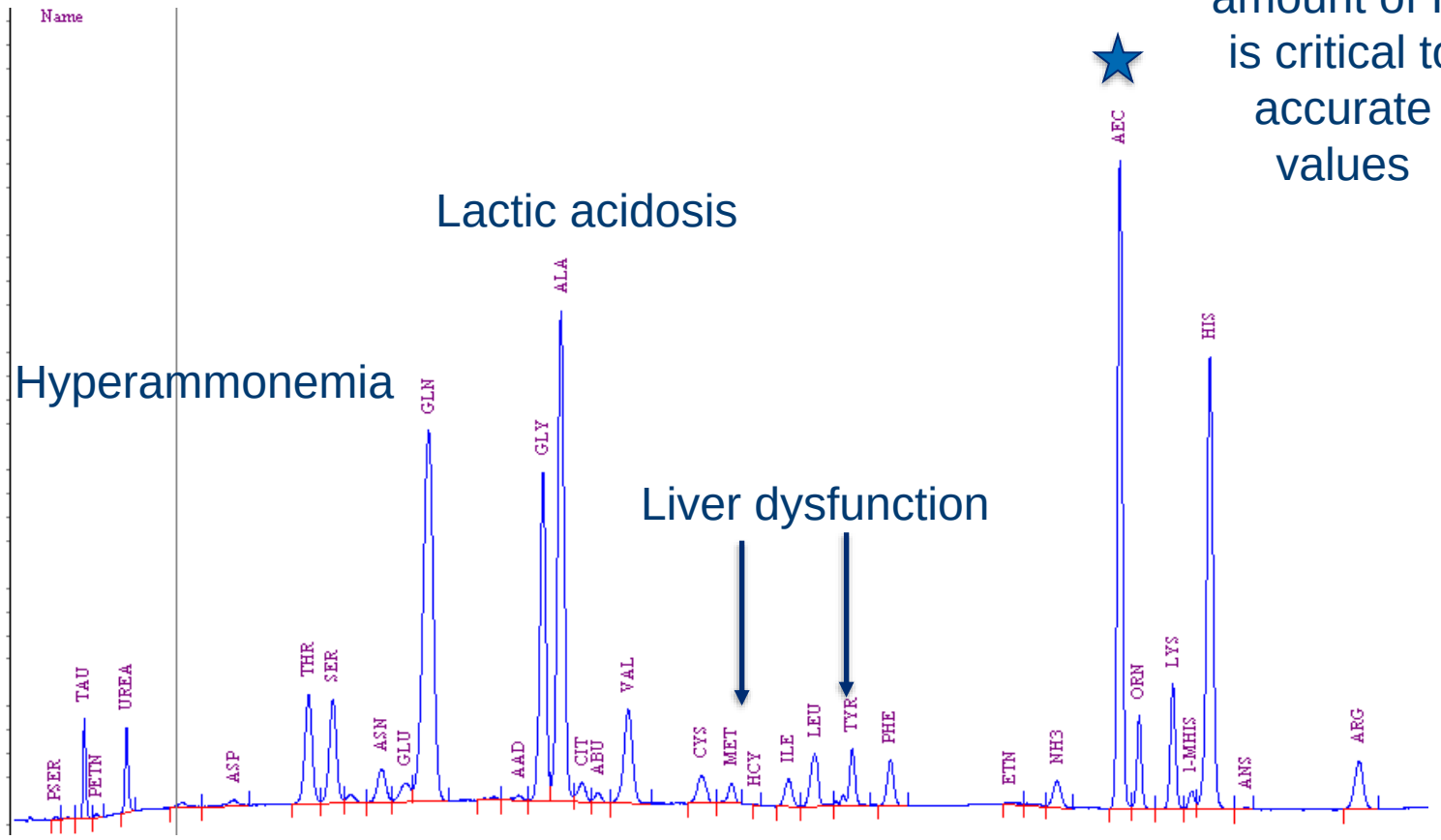
Thanks to Dr. Laura Pollard

Amino acid chromatogram - Normal



Consistent amount of IS is critical to accurate values

Lactic acidosis

Hyperammonemia

Liver dysfunction

Internal standard

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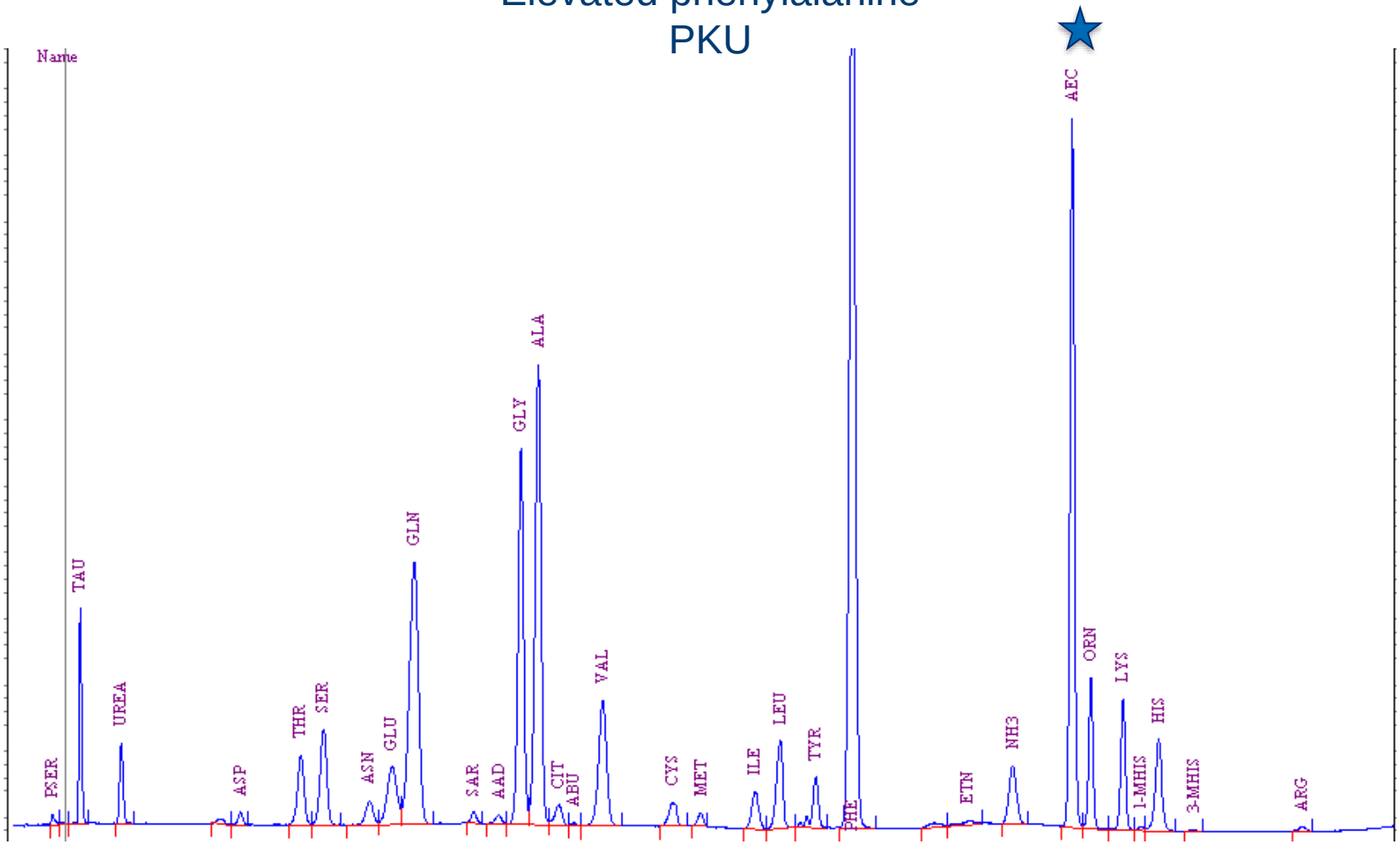
Elevated phenylalanine
PKU



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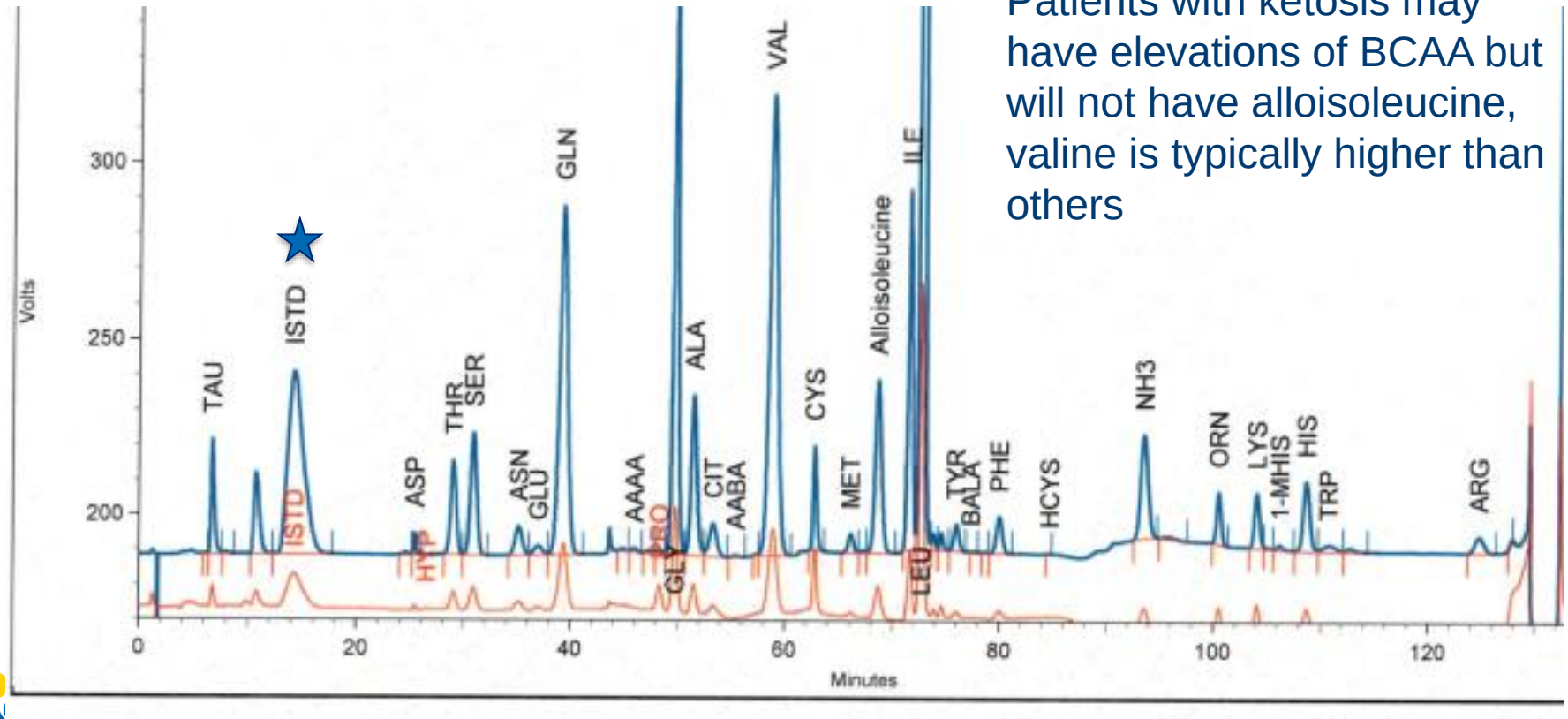
Elevations of leucine, isoleucine and valine, including alloisoleucine
Maple syrup urine disease



Patients with ketosis may have elevations of BCAA but will not have alloisoleucine, valine is typically higher than others

★ Internal standard

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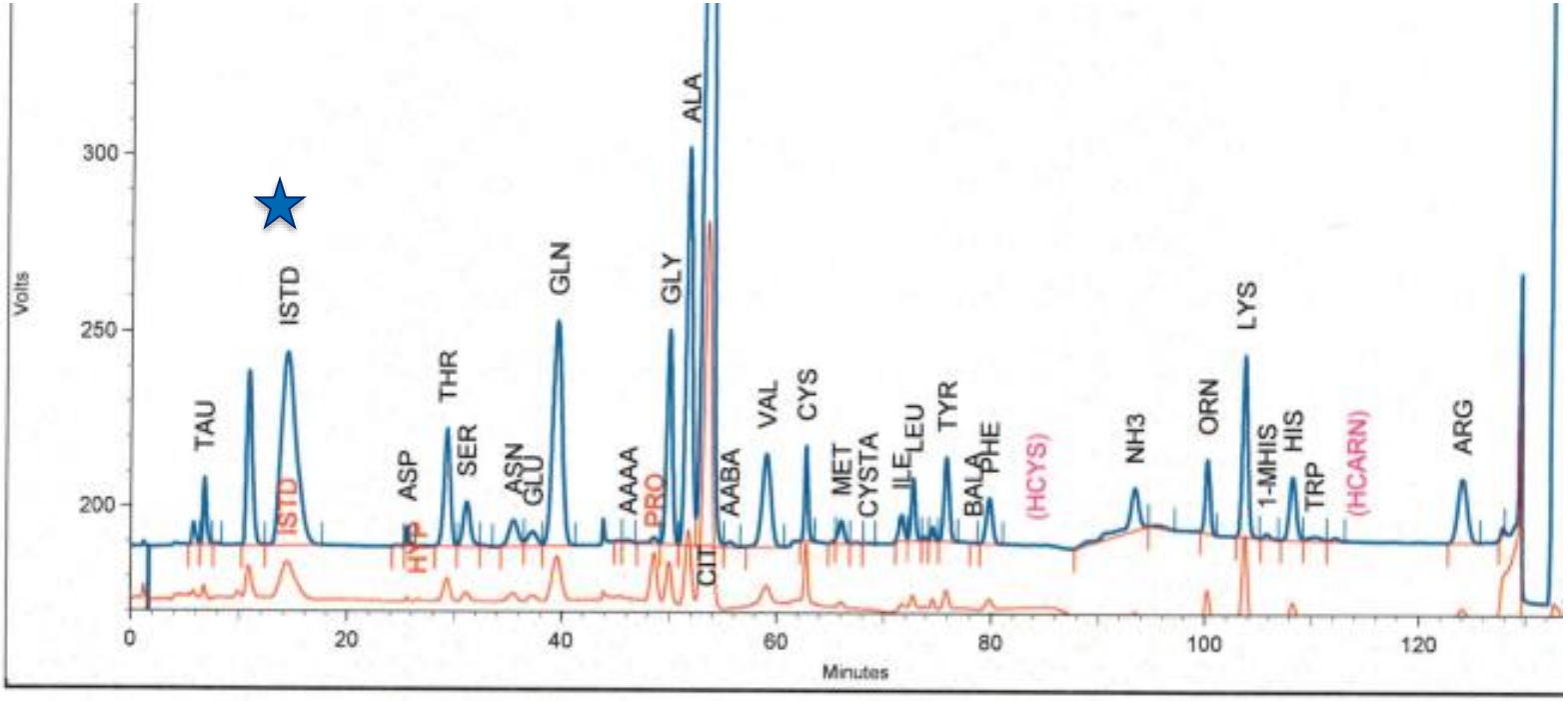
Elevated Citrulline
Citrullinemia



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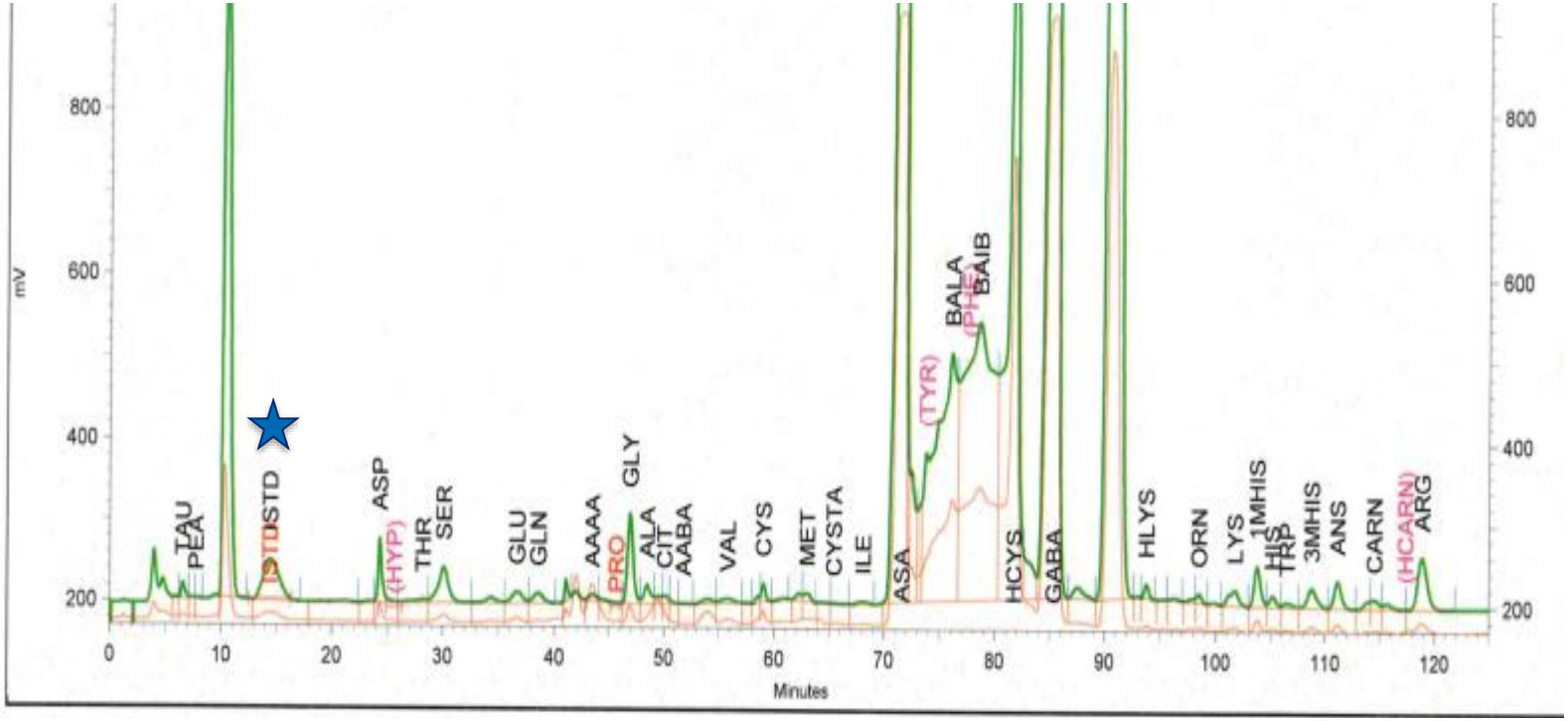
★ Internal standard

Urine sample

Argininosuccinic acid



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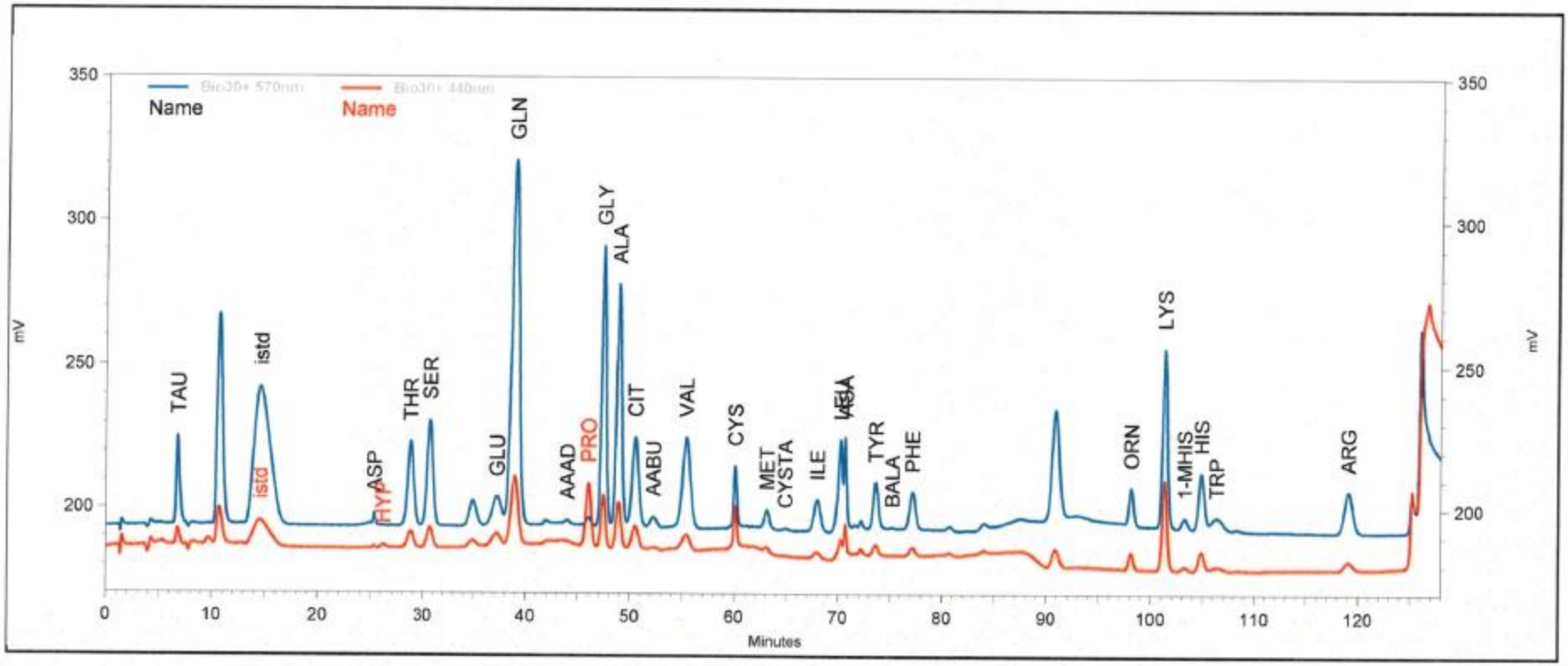
Argininosuccinic acidemia- mild



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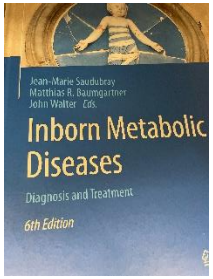
Current trends in AA separation

- Ultra performance liquid chromatography (UPLC)
  - Shorter run time 30 minutes
- Tandem mass spectrometry
  - MSMS has shorter run time
  - MSMS can separate compounds that were co-eluting on ion exchange.
  - MSMS is more expensive and a dedicated instrument to amino acid analysis may be necessary
  - Increased sensitivity of MSMS can lead to changes in what is "normal" – presence of alloisoleucine
  - IEC allows for analysis of full chromatogram where MSMS is typically targeted
  - Needed for dried blood spot analysis

Thanks to Marzia Pasquali

Plasma Amino Acid	Variation	Other Plasma Amino Acids	Investigations in other fluids	Diagnoses
Alanine	↑	See Gln, Pro, Gly		Hyperlactacidemia
Arginine	↑	Gln ± ↑, Cit ± ↓, Orn ↓	U: ± ↑	Arginase deficiency
	↓	Gln ± ↑, Pro ↓, Cit ↓, Orn± ↓		P5CS deficiency
		Orn ↓, Lys ↓	U: ↑++, Orn ↑, Lys ↑ UOA: Orotic ↑	LPI
Argininosuccinic acid	±↑	Gln ± ↑, Cit ± ↑	U: ASA ↑	ASLD late-onset form
	↑	Gln ↑, Cit ↑	U: ASA ↑++	ASLD neonatal form
Asparagine	↓	All normal	CSF ↓	Asn synthetase deficiency
Branched chain AA	↑	no Alle, other AA ± ↓		Starvation
		no Alle, other AA ± ↑		Fed state
		Alle +++, Ala ↓	U: ↑	MSUD
		Alle± ↑, Ala ↑, Gln ↑	UOA: Lac ↑, 2KG ↑	E3 deficiency
	↓	All normal	CSF ↓	BCKAD kinase deficiency
		Met ↑, Tyr ↑		Hepatic failure
		Cit ↑, Cys2 ↑, 3Mhis ↑		Renal failure



ACMG TECHNICAL STANDARD | Genetics inMedicine

Laboratory analysis of amino acids, 2018 revision: a technical standard of the American College of Medical Genetics and Genomics (ACMG)

J. Daniel Sharer, PhD[1], Irene De Biase, MD, PhD[2], Dietrich Matern, MD, PhD[3], Sarah Young, PhD[4], Michael J. Bennett, PhD[5] and Adviye A. Tolun, PhD[6];
on behalf of the ACMG Laboratory Quality Assurance Committee

PMID: 30459394

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Urine organic acid (OA) analysis

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Rationale for analysis

- Intermediates in the degradation of AAs, carbohydrates, and lipids
- Formation produces a proton and hence the potential to cause acidosis
- *Not* reabsorbed by the kidney, therefore urine is the ideal specimen for analysis; plasma is generally not used.
- Quantitative vs. qualitative analysis
  - For diagnosis, qualitative is usually sufficient
  - Quantitation is difficult (need for internal and external standards, values may differ between labs
  - There is limited evidence supporting the use of precise quantitation in diagnosis and disease monitoring

Adapted from NAMA 2020

Sample preparation

- Determination of creatinine concentration; and addition of internal standards.
- Urine specimens are normalized to a fixed amount of creatinine (0.25mg)
- Urine specimen is acidified to pH of 1, where OAs are uncharged
- Organic solvents (e.g., diethylether & ethylacetate) are used to extract the OAs
- Ether is removed with nitrogen gas stream
- OAs are derivatized to their trimethylsilyl esters for detection
- Oximation preserves ketoacids (pyruvic, alpha ketoglutarate, BCKAs), otherwise get converted to 2-hydroxyacids and not be derivatized

Method of analysis

- Gas chromatography/mass spectrometry
- Derivatized/extracted organic acids are first separated via gas chromatography
- The eluted OA subjected to an electron beam that fragments the parent compound into daughter ions
- The collection of daughter ions by the mass spectrometer is like a fingerprint that can identify the parent compound
- The better the separation (longer the run?) and the easier it is to identify compounds.

Concept 1: Mass Spectrometer

Mass Spectrometrist Definition:

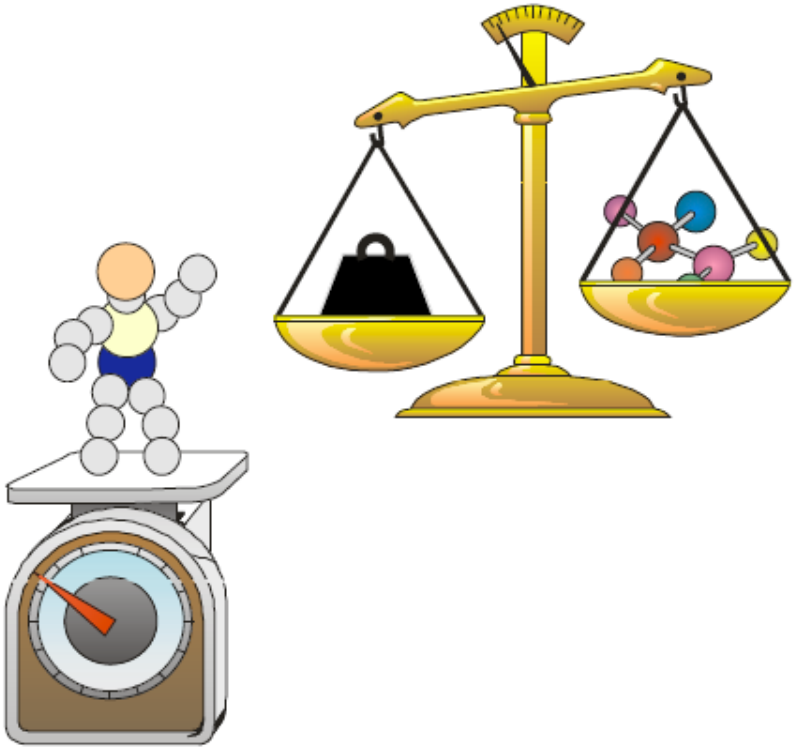
A mass spectrometer is an instrument that measures the masses of individual molecules that have been converted to ions; i.e., molecules that have been electrically charged.

Layperson Understanding:

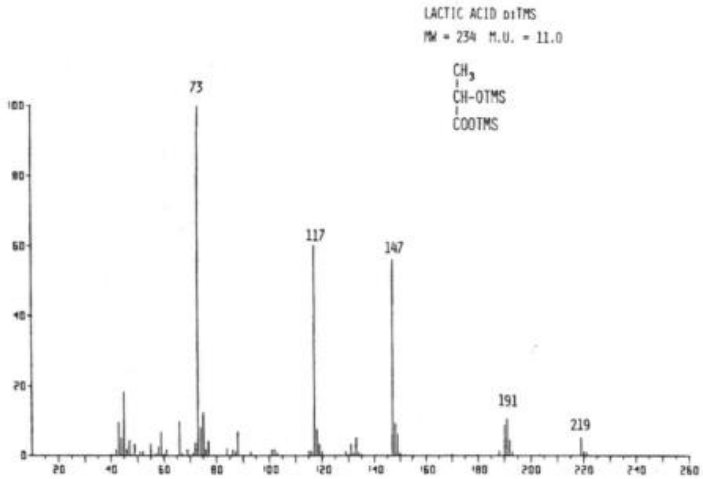
The terms "masses" and "ions" may not be understood

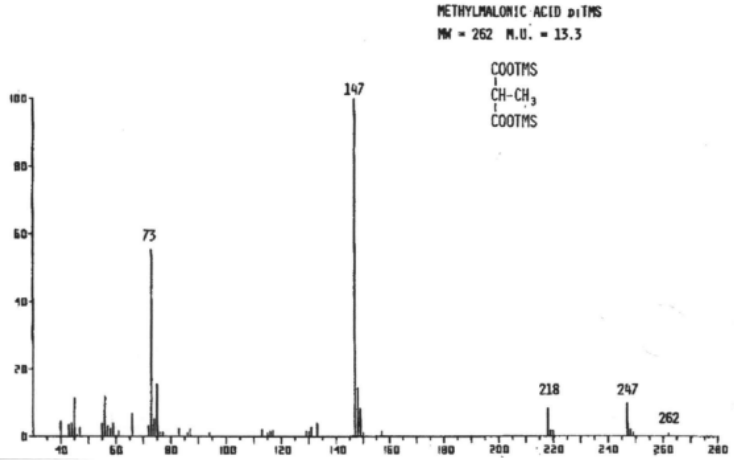
Simple Definition:

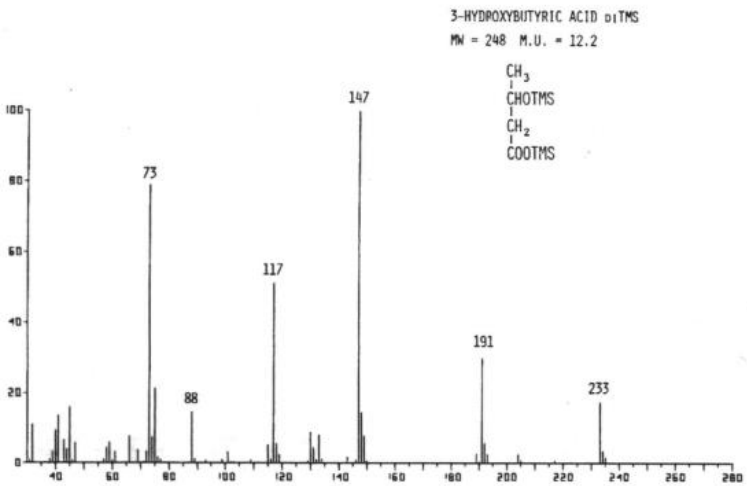
A machine used to weigh molecules.
A molecular scale.

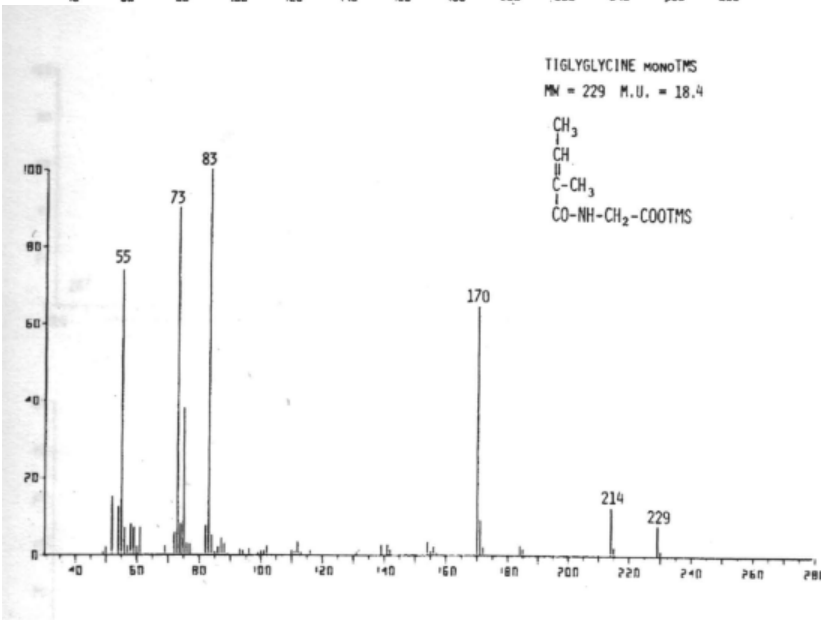


Spectrums for various compounds

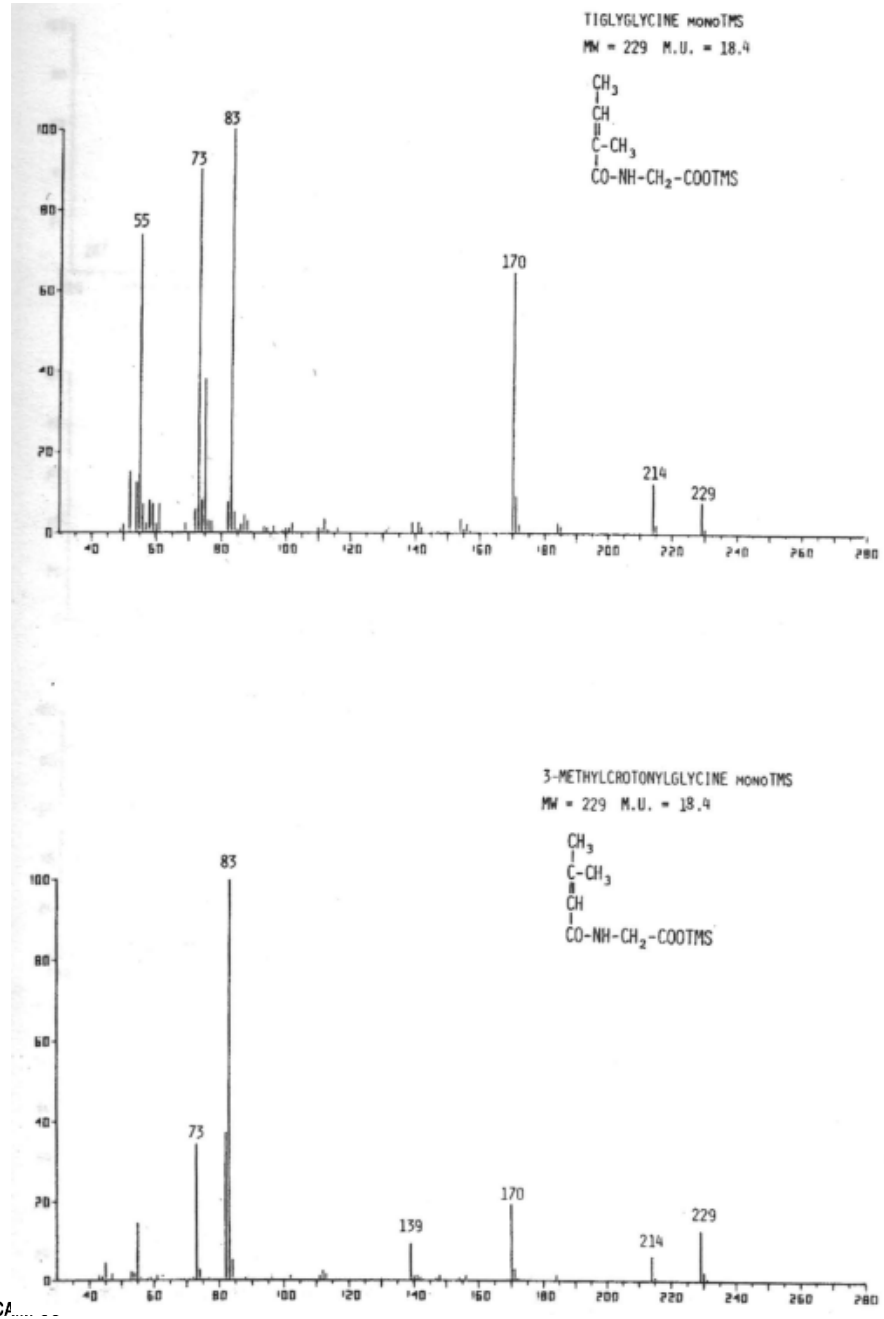




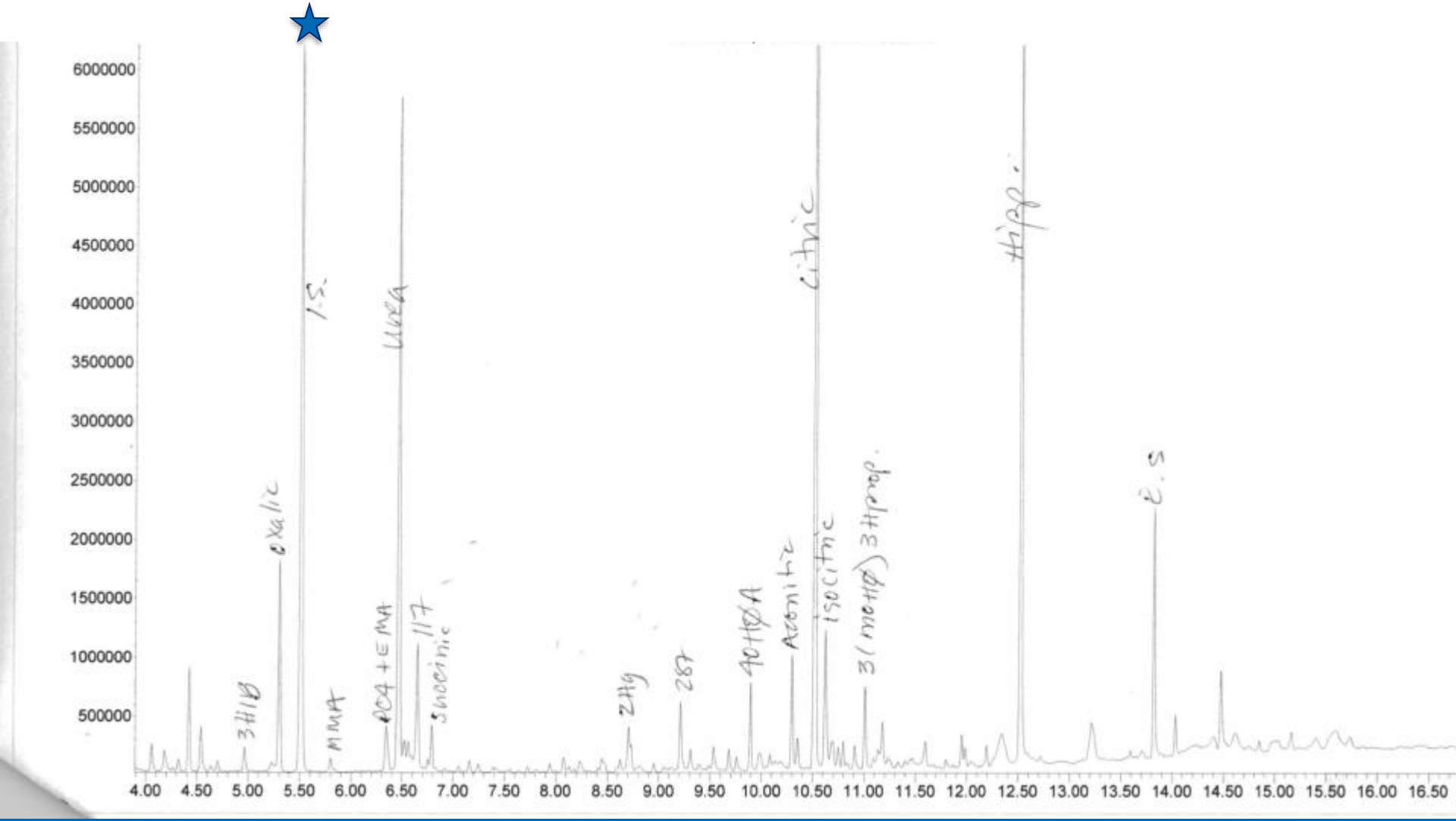




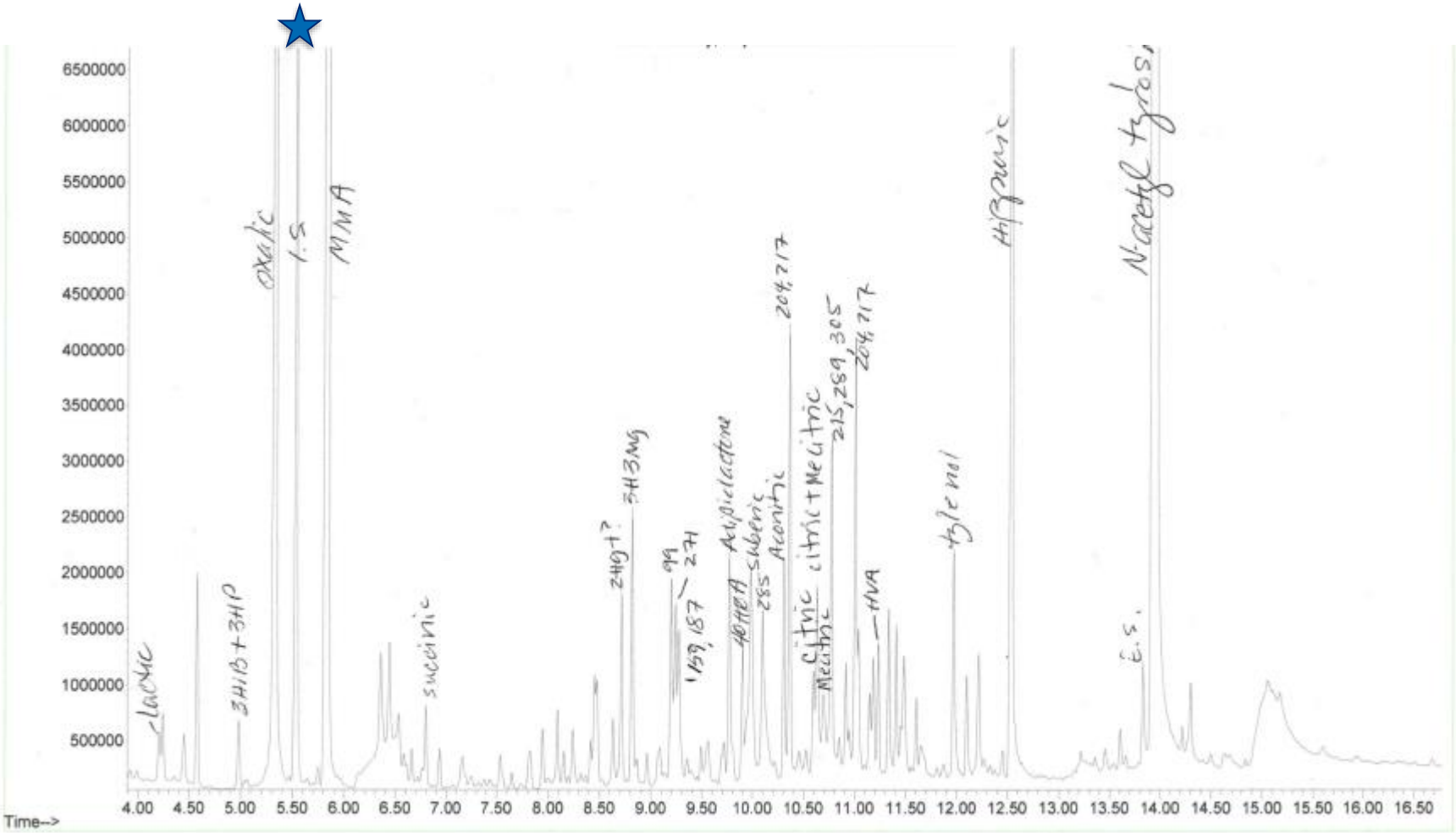
Similar compounds may have similar fragmentation patterns



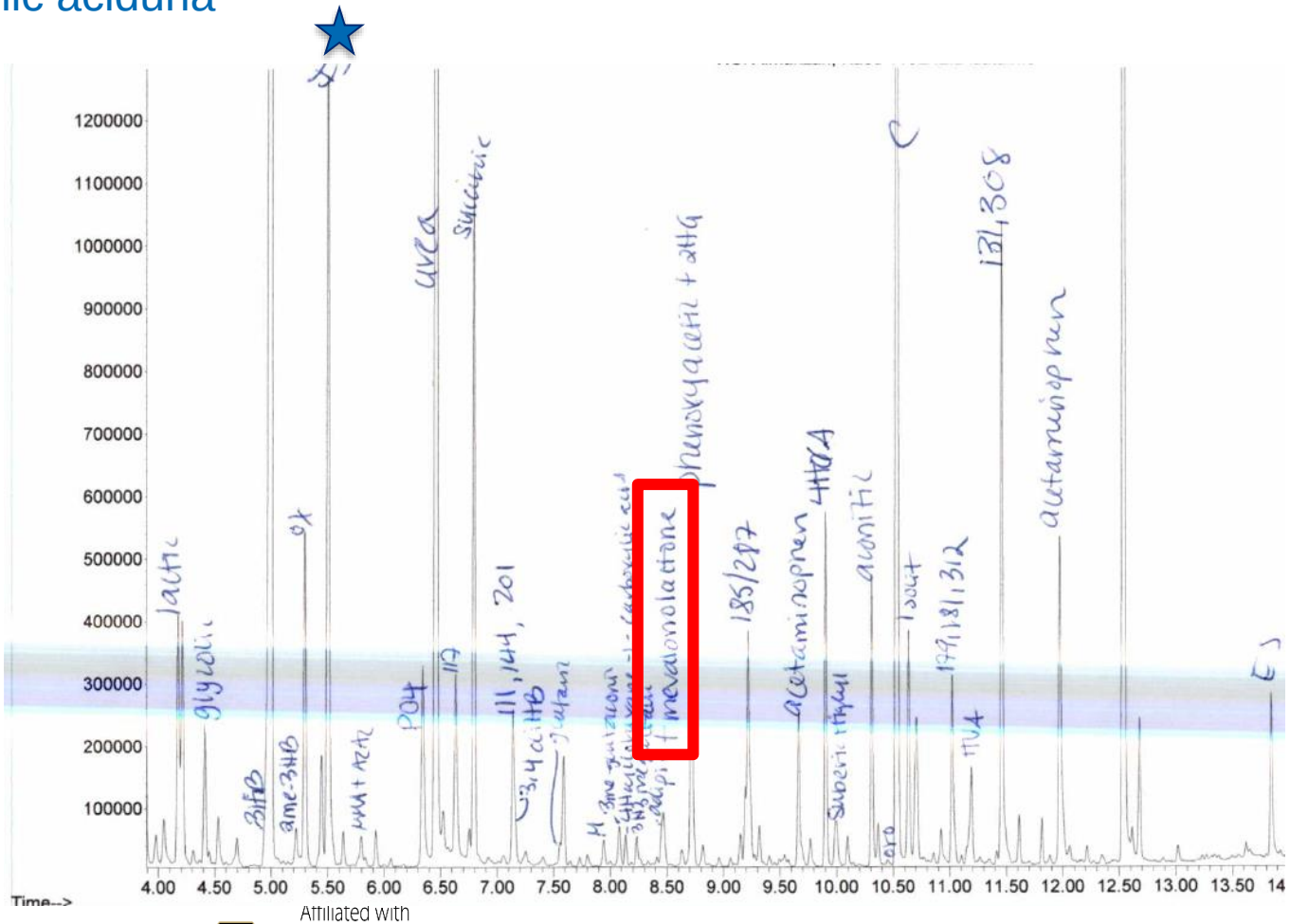
Normal organic acid



Elevated MMA and methylcitric



Mevalonic aciduria



Compound	Non-IEM	IEM
Methylmalonic	B12 deficiency, intestinal bacteria	MMA, Cobalamin disorders, SUCLA2, CMAMMA
Methylcitric	Malnutrition	PA and MMA
3-OH propionic	Intestinal bacteria	PA
Tiglylglyine	Seizure meds (?)	PA, several inborn errors, mito(?)
Isovalerylglycine	Seizure meds (?)	IVA, mito(?)
N-acetylaspartic	Premature infants(?)	Canavan

Reference

Clinical Chemistry 48:5
708–717 (2002)

Review

Metabolic, Nutritional, Iatrogenic, and Artifactual Sources of Urinary Organic Acids: A Comprehensive Table

Alain Kumps, Pierre Duez, and Yves Mardens*

PMID: 11978597

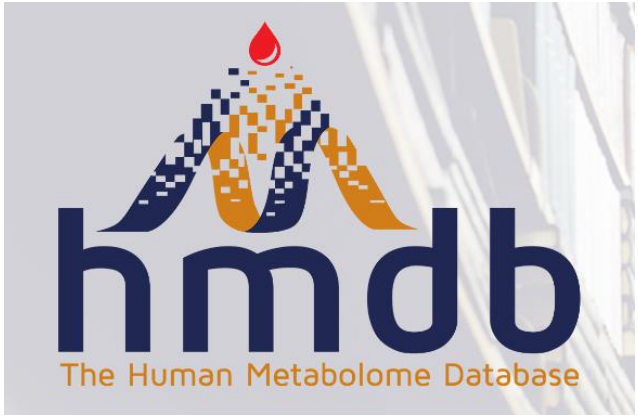
Table 1. Possible origins of abnormal excretion patterns of urinary organic acids.

Acid/Metabolite	Non-IEM *(4, 12, 15, 16, 22)*	IEM
Aromatic amino acid metabolism *(23)*		
2-Hydroxyphenylacetate	Uremia	PKU; BH4[a] deficiency
4-Hydroxyphenylacetate *(24, 25)*	Bacterial gut metabolism and bacterial contamination (from tyrosine); short bowel syndrome; liver diseases	Tyrosinemia; PKU; hawkinsinuria
4-Hydroxyphenyllactate *(24–27)*	Bacterial gut metabolism; short bowel syndrome; liver diseases (e.g., secondary to PA, galactosemia, fructosemia); scurvy; lactic acidosis	Tyrosinemia; PKU; Zellweger; hawkinsinuria; lactic acidosis
4-Hydroxyphenylpyruvate	VPA; liver diseases (e.g., secondary to PA, galactosemia, fructosemia)	Tyrosinemia; hawkinsinuria
Homogentisate		Alcaptonuria
Mandelate *(28)*	Preservative in albumin solution for intravenous perfusion; methenamine mandelale; gastrointestinal malabsorption diseases	PKU
N-Acetyltyrosine	Some parenteral solutions	Tyrosinemia
Phenylacetate	Intestinal bacterial origin (from phenylalanine)	PKU; BH4 deficiency
Phenylacetylglutamine	Bacterial metabolism (from phenylacetate); hyperammonemia treated with phenylbutyrate or phenylacetate; uremia	PKU
Phenyllactate *(29)*	Bacterial gut metabolism (D-form); liver diseases	PKU; tyrosinemia (L-form); BH4 deficiency
Phenylpyruvate	Bacterial gut metabolism; liver diseases	PKU; BH4 deficiency
Succinylacetoacetate		Tyrosinemia type I
Succinylacetone		Tyrosinemia type I

Laboratory analysis of organic acids, 2018 update: a technical standard of the American College of Medical Genetics and Genomics (ACMG)

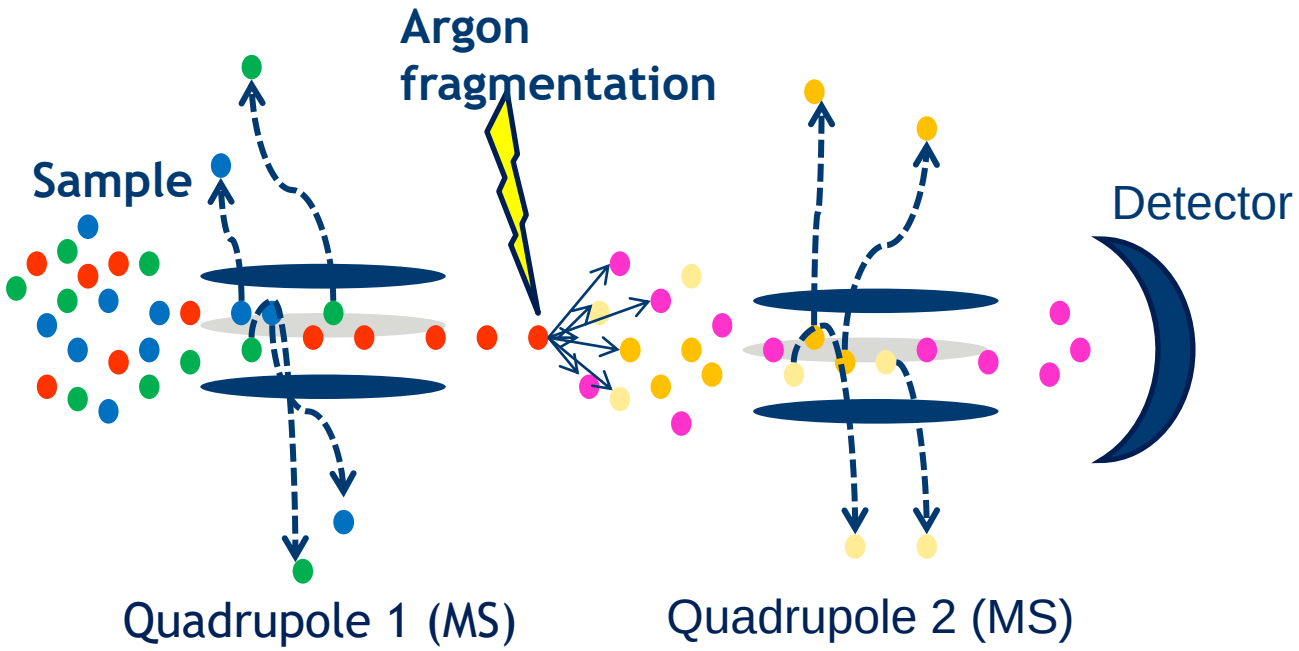
Renata C. Gallagher MD, PhD[1], Laura Pollard, PhD[2], Anna I. Scott, PhD[3,4], Suzette Huguenin, PhD[5], Stephen Goodman, MD[6], Qin Sun, PhD[7]; on behalf of the ACMG Biochemical Genetics Subcommittee of the Laboratory Quality Assurance Committee

PMID: 29543224



Acylcarnitine (AC) analysis

Tandem mass spectrometer

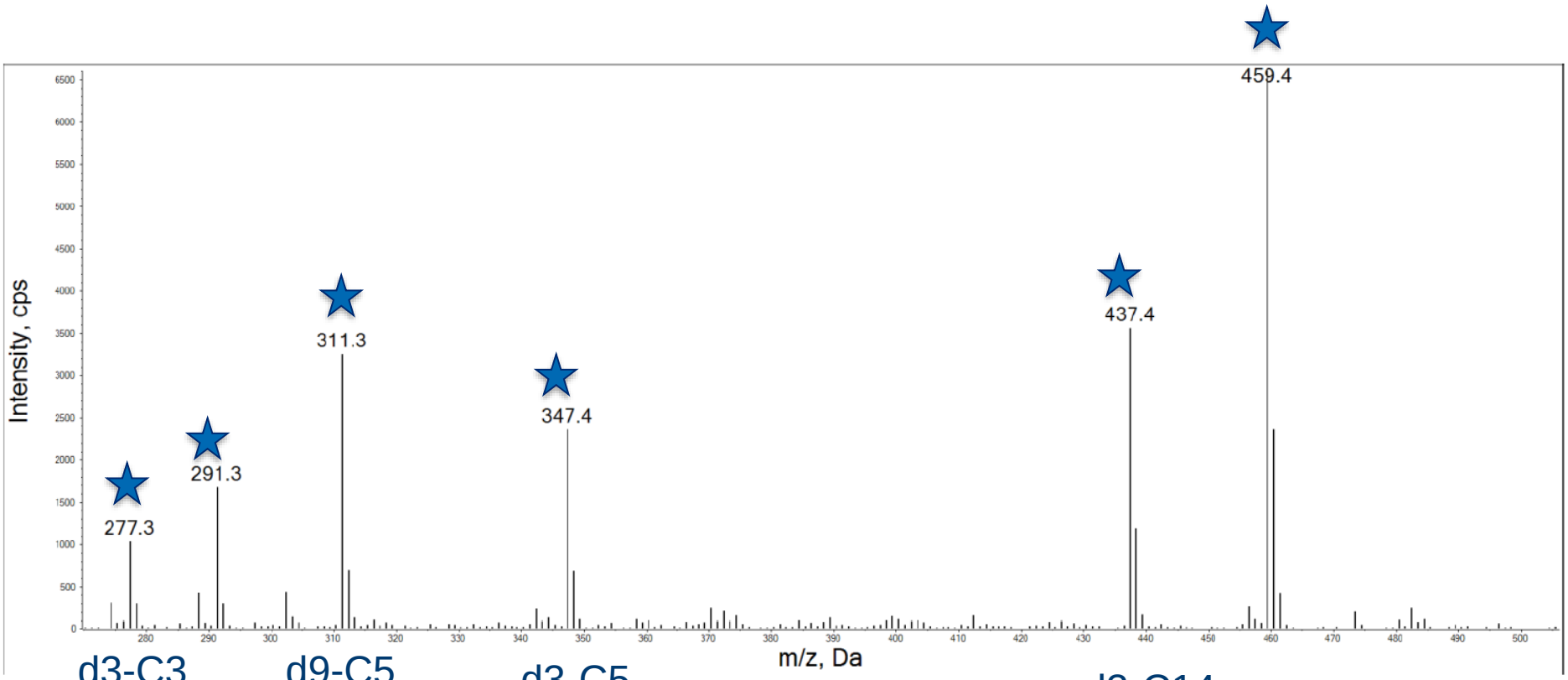


Rationale for analysis

- 1980s: Discovery of carnitine deficiency in a patient with propionic acidemia; Roe, Millington, and Bohan suspected propionylcarnitine as a detoxification mechanism, and identified this compound.
- Used tandem mass spectrometry (MS/MS) method — single analytic method for all analytes of interest
- Any pathway where a –CoA compound might accumulation
- Single test allows for the detection of fatty acid oxidation disorders but also several organic acid disorders
- Method has been applied to dried blood spot analysis allowing the addition of several FAOD and organic acidemias to NBS
- Initial analyses incorporated derivatization (butyl or methyl esters) but many analyses are currently performed without derivatization. (Parent of 85 scan)

Normal acylcarnitine profile – butylester derivatives
Length of carbon chain (C3-C18)
Subset of stable label isotope internal standards are added for quantitation

C3 → C18



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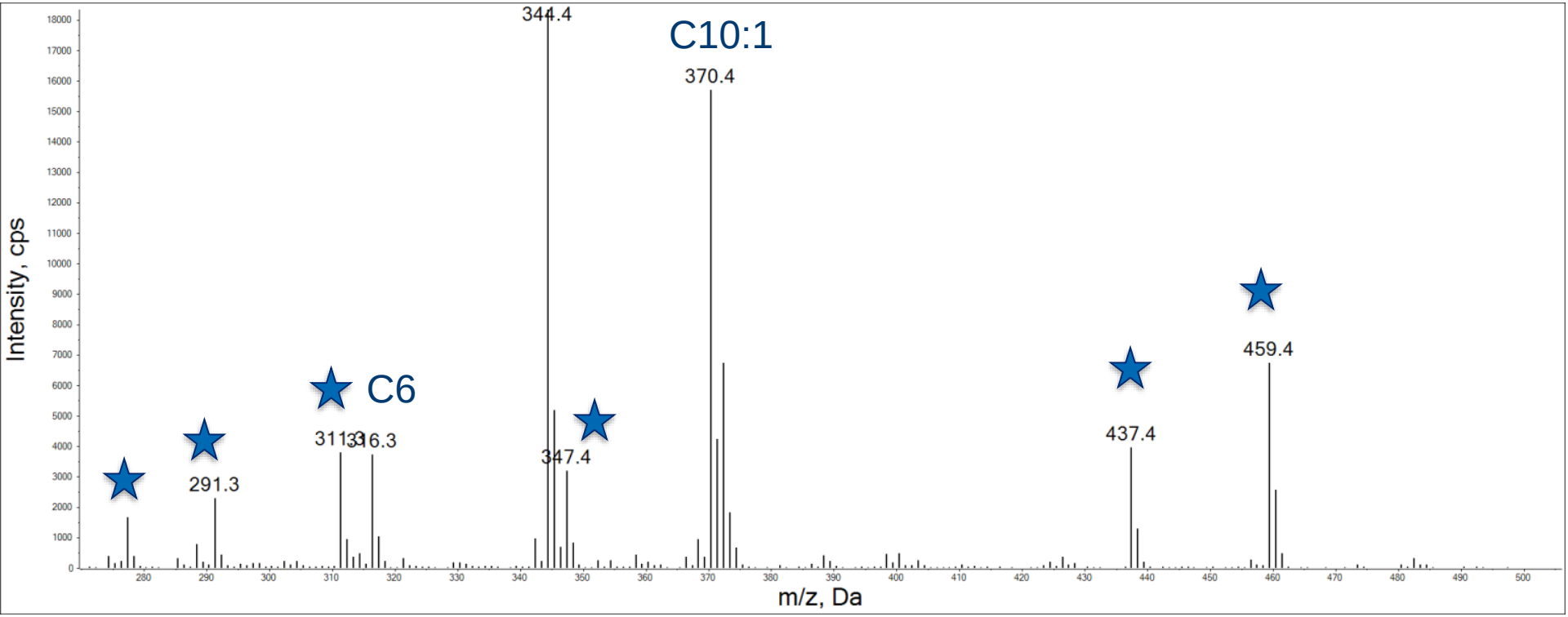
MCAD deficiency
Abnormal C8/C10 ratio (normal < 3)

C8



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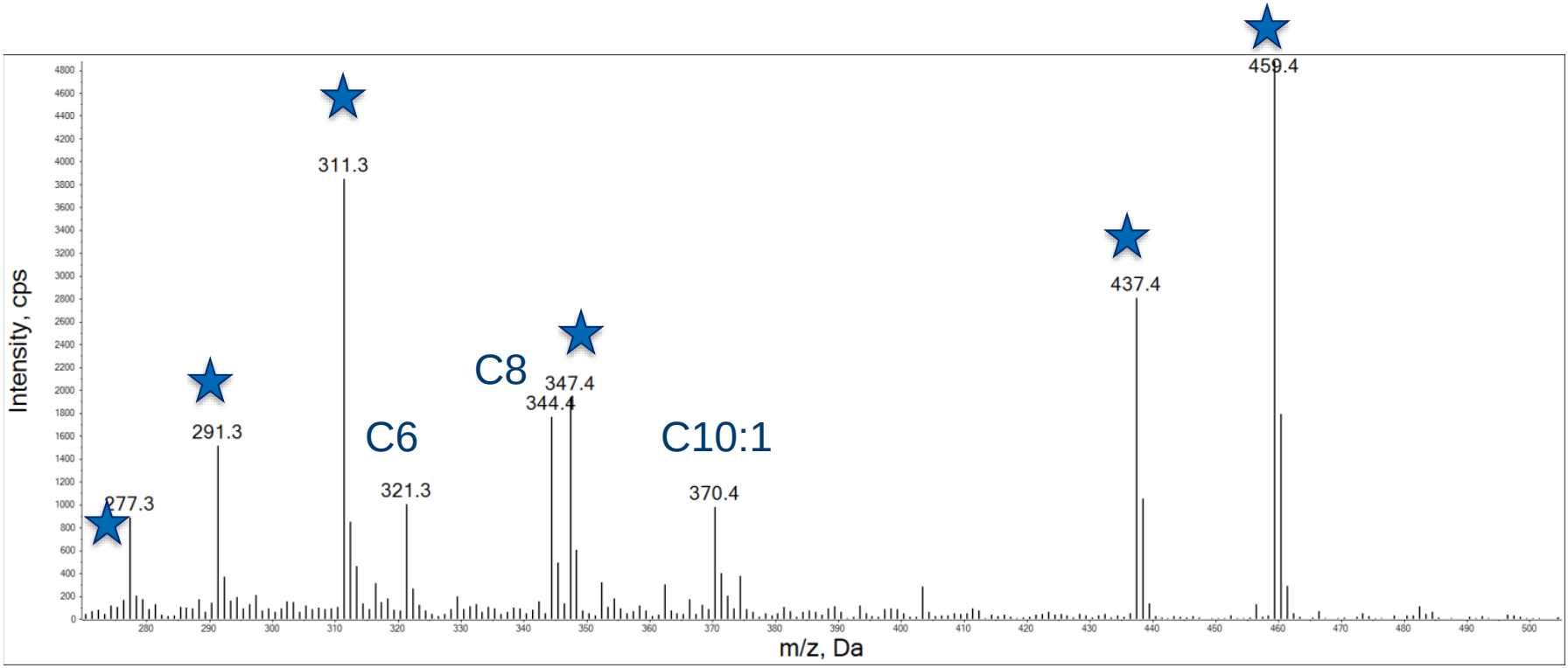
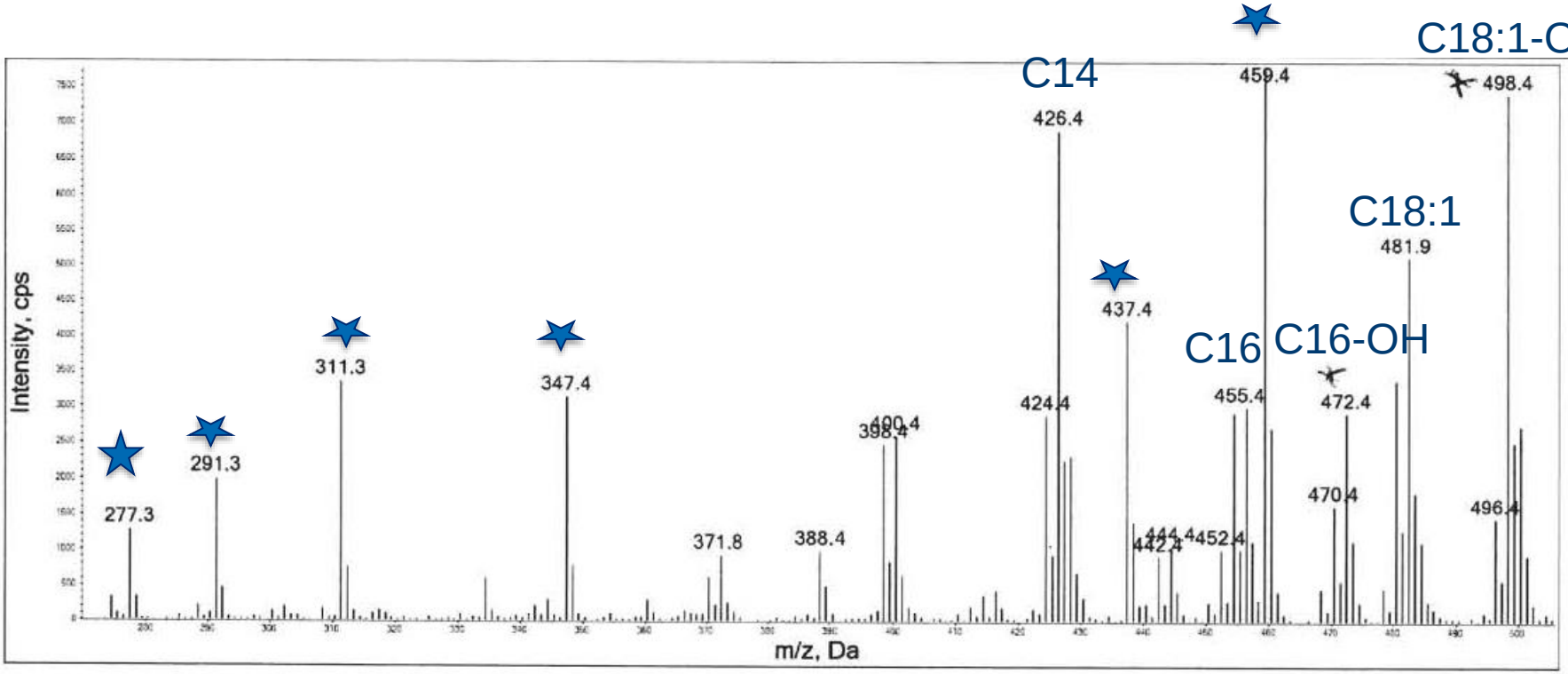
MCAD patient with low carnitine

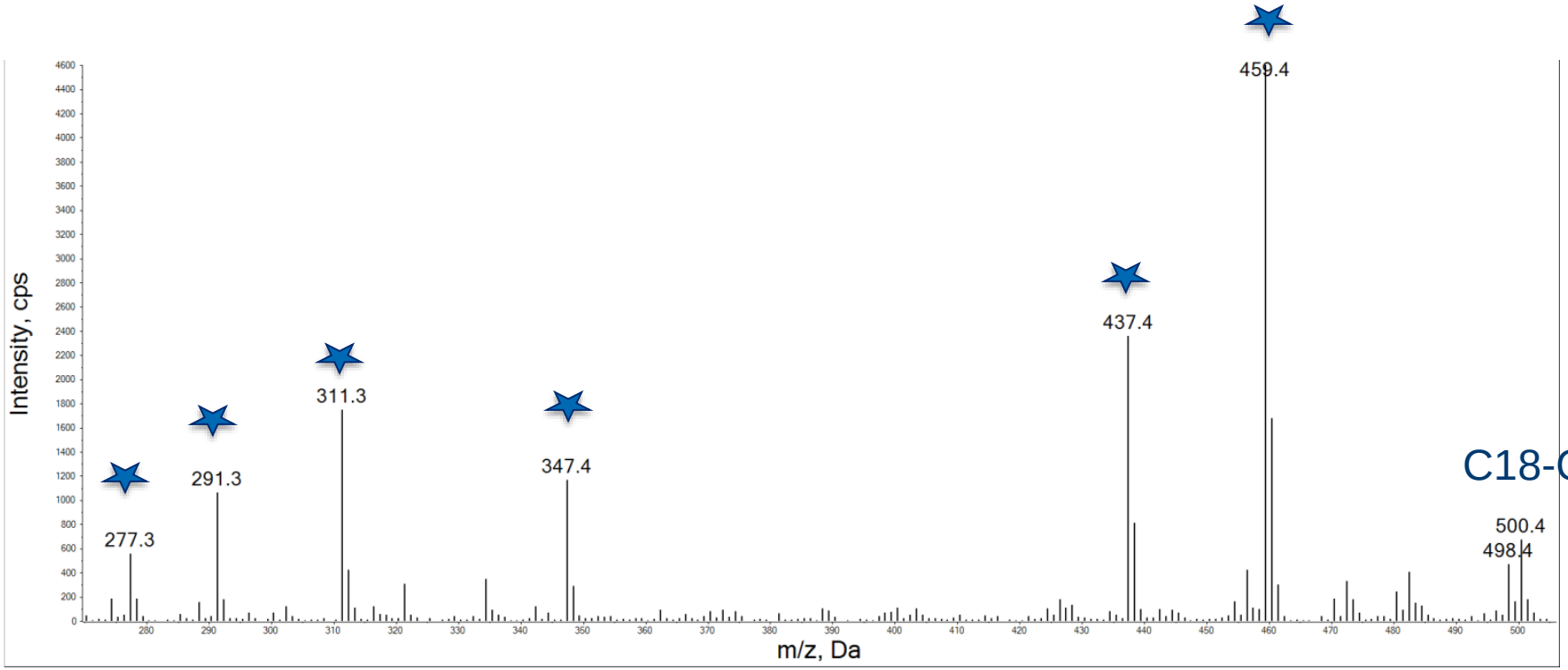


C0 -4 (normal < 10), C2 -1.8 (normal<2)

LCHAD deficiency

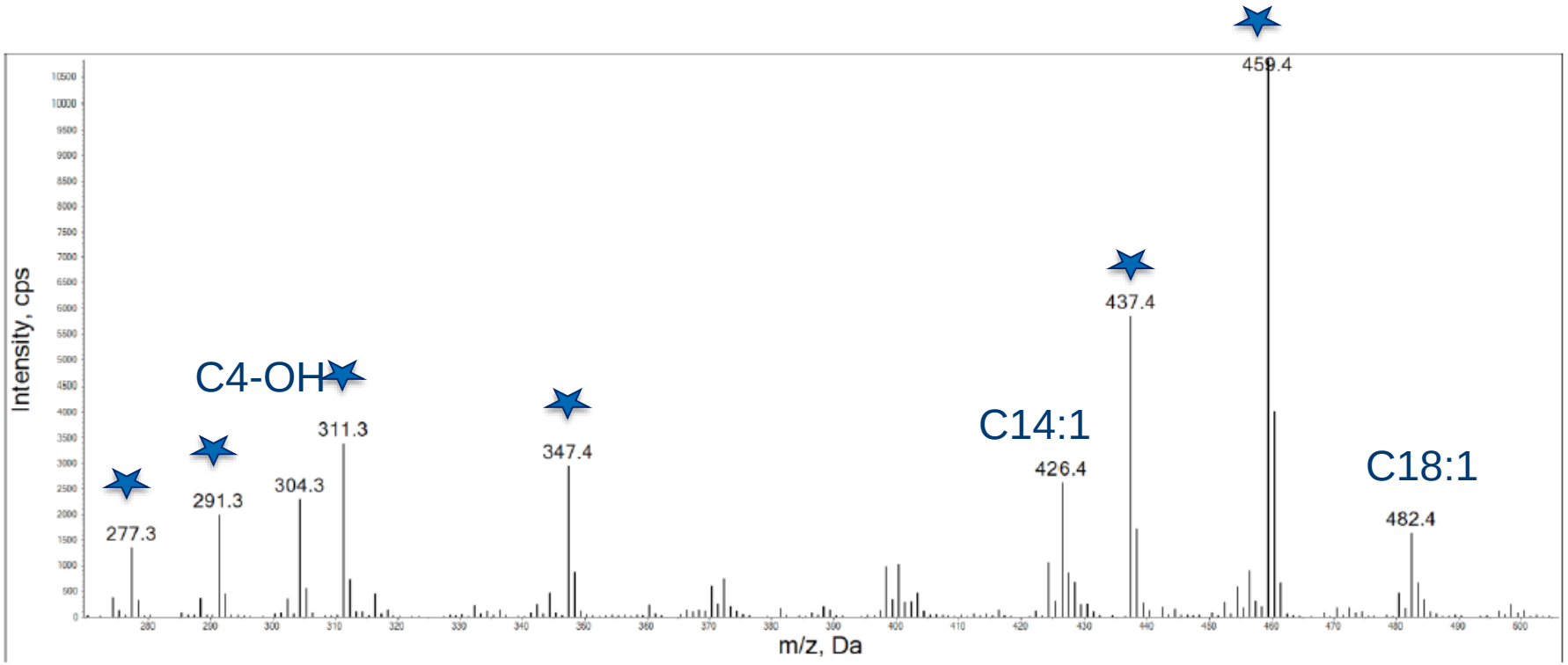


LCHAD at baseline



C18-OH

Ketosis
May also see elevations of C2
C14:1/C12:1 ratio is normal <3



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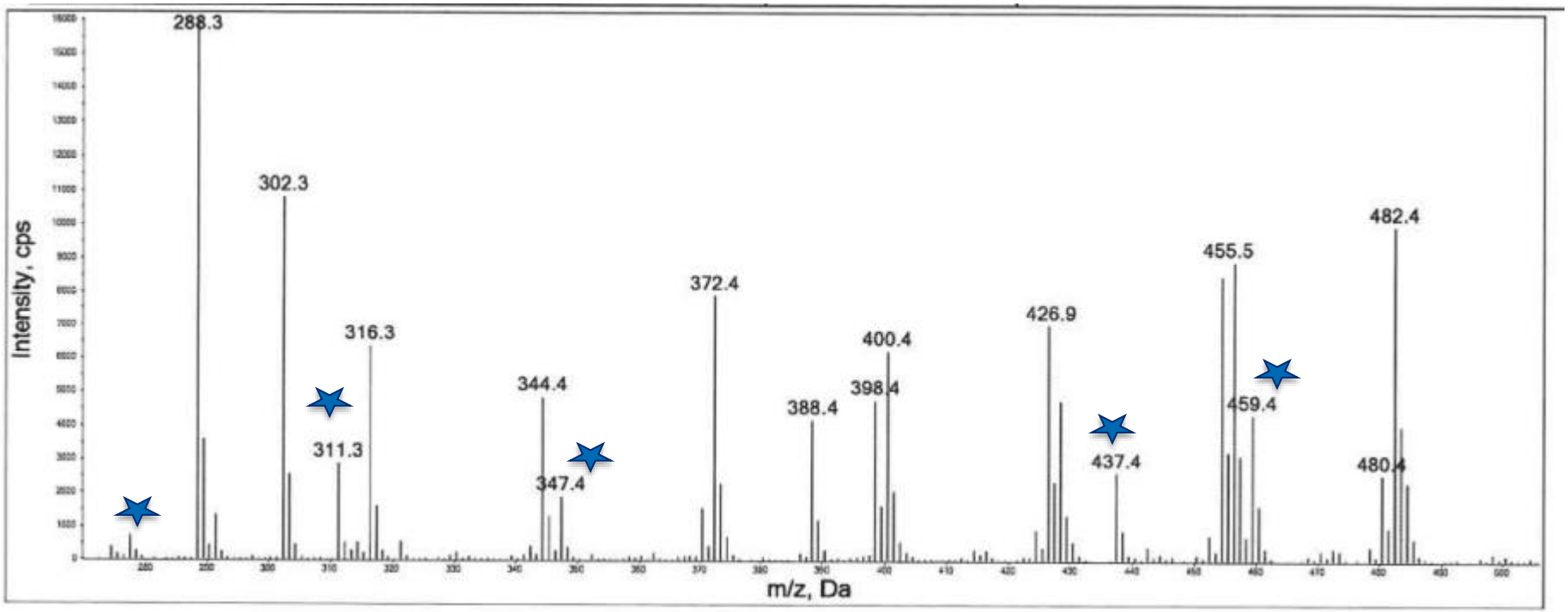
MAD deficiency / glutaric aciduria type II

OR Riboflavin deficiency/metabolism disorders



Other acylcarnitine considerations

- C6, C8 + C10, [and especially with the corresponding dicarboxylic acids C6DC (adipic), C8DC (suberic), and C10DC (sebacic) in the urine organic acids]
  - Dietary formulas with medium-chain triglycerides
  - C10:1 is elevated in MCAD deficiency, but should NOT be significantly elevated by MCT oil
  - Fasting
- C8 — Valproate (may be accompanied by carnitine deficiency, esp. in young children)
- C5
  - Pivalic acid is 2,2-dimethylpropanoic acid
  - Originally found in some antibiotics. Also in some creams for nipple sensitivity in nursing mothers and other drugs

Elevations of PA due to more than antibiotics

Original Article

Elevation of pivaloylcarnitine by sivelestat sodium in two children

Kenji Yamada [a,*], Hironori Kobayashi [a], Ryosuke Bo [a,b], Tomoo Takahashi [a], Yuki Hasegawa [a], Makoto Nakamura [c], Nobuyuki Ishige [d], Seiji Yamaguchi [a]

[a] Department of Pediatrics, Shimane University Faculty of Medicine, 89-1 En-ya-cho, Izumo, Shimane 693-8501, Japan
[b] Department of Pediatrics, Kobe University Graduate School of Medicine, 7-5-1 Kusunoki-cho, Chuo-ku, Kobe 650-0017, Japan
[c] Department of Neonatology, Okayama Medical Center, 1711-1 Tamasu, Kita-ku, Okayama 701-1192, Japan
[d] Tokyo Health Service Association, 1-2 Ichigayasadoharacho, Shinjuku-ku, Tokyo 162-8402, Japan

Molecular Genetics and Metabolism 116 (2015) 192–194

Short Communication

Surprising causes of C5-carnitine false positive results in newborn screening

François Boemer [a,*], Roland Schoos [a], Virginie de Halleux [b], Masendu Kalenga [b], François-Guillaume Debray [c]

[a] Biochemical Genetics Laboratory, Human Genetics, CHU Liege, University of Liege, Belgium
[b] Neonatal Intensive Care Unit, University of Liege, Centre Hospitalier Régional de la Citadelle, Liege, Belgium
[c] Metabolic Unit, Human Genetics, CHU Liege, University of Liege, Belgium

Molecular Genetics and Metabolism 111 (2014) 52–54

Article

Raising Awareness of False Positive Newborn Screening Results Arising from Pivalate-Containing Creams and Antibiotics in Europe When Screening for Isovaleric Acidaemia

James R. Bonham [1,*], Rachel S. Carling [2], Martin Lindner [3], Leifur Franzson [4], Rolf Zetterstrom [5], Francois Boemer [6], Roberto Cerone [7], Francois Eyskens [8], Laura Vilarinho [9], David M. Hougaard [10] and Peter C.J.I. Schielen [11]

Int. J. Neonatal Screen. **2018**, 4, 8;

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References

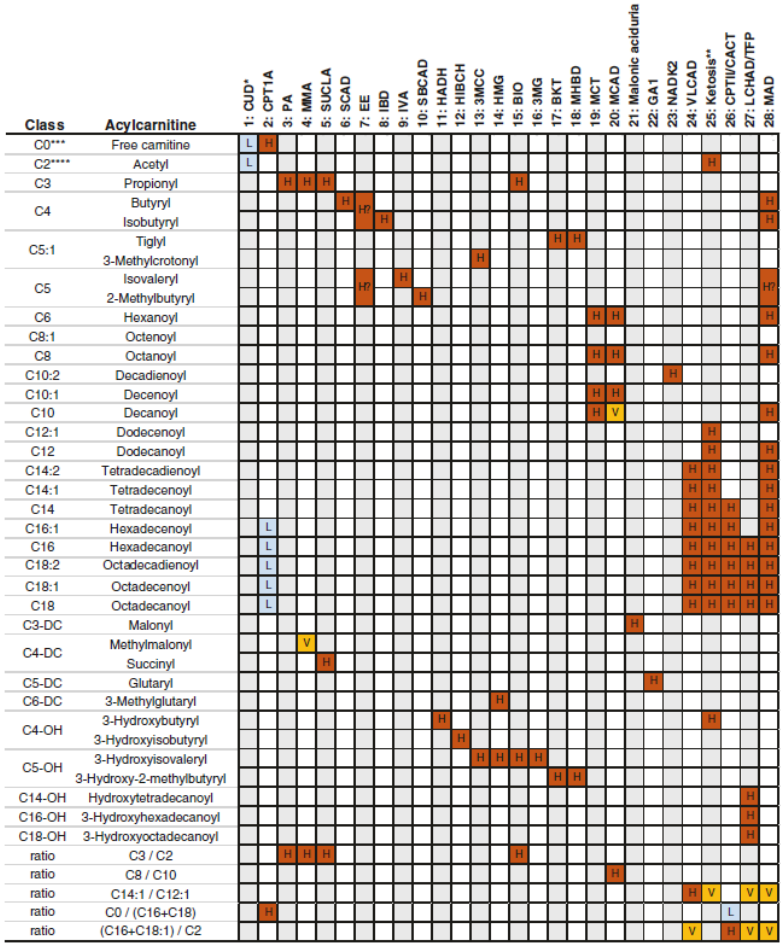

Fig. 1 Common acylcarnitine patterns associated with various disease states. *H* high, *H?* high but specific isomeric species are not clearly defined for

ACMG TECHNICAL STANDARD | Genetics inMedicine

Laboratory analysis of acylcarnitines, 2020 update: a technical standard of the American College of Medical Genetics and Genomics (ACMG)

Marcus J. Miller, PhD[1], Kristina Cusmano-Ozog, MD[2], Devin Oglesbee, PhD[3] and Sarah Young, PhD[4]; ACMG Laboratory Quality Assurance Committee[5]

PMID: 33071282

Supplemental table very helpful!

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Other uses of LCMSMS

Acylglycine Analysis by Ultra-Performance Liquid Chromatography-Tandem Mass Spectrometry (UPLC-MS/MS)

Judith A. Hobert,[1,2,3] Aiping Liu,[3] and Marzia Pasquali[1,2,3]

[1]Department of Pathology, University of Utah School of Medicine, Salt Lake City, Utah
[2]ARUP Laboratories, Salt Lake City, Utah
[3]ARUP Institute for Clinical and Experimental Pathology, University of Utah, Salt Lake City, Utah

Newborn screening for mucopolysaccharidoses: Measurement of glycosaminoglycans by LC-MS/MS

Molly Stapleton[a,b], Francyne Kubaski[c], Robert W. Mason[a,b], Haruo Shintaku[d], Hironori Kobayashi[e], Seiji Yamaguchi[e], Takeshi Taketani[e], Yasuyuki Suzuki[f], Kenji Orii[g], Tadao Orii[g], Toshiyuki Fukao[g], Shunji Tomatsu[a,b,e,g,h,*]

[a] *Nemours/Alfred I. duPont Hospital for Children, Wilmington, DE, United States of America*
[b] *Department of Biological Sciences, University of Delaware, Newark, DE, United States of America*
[c] *Medical Genetics Service, HCPA, Department of Genetics and Molecular Biology-PPGBM, UFRGS, INAGEMP, Porto Alegre, Brazil*
[d] *Department of Pediatrics, Osaka City University Graduate School of Medicine, Osaka, Japan*
[e] *Department of Pediatrics, Shimane University Faculty of Medicine, Shimane, Japan*
[f] *Medical Education Development Center, Gifu University, Japan*
[g] *Department of Pediatrics, Graduate School of Medicine, Gifu University, Gifu, Japan*
[h] *Department of Pediatrics, Thomas Jefferson University, Philadelphia, PA, United States of America*

Research Article

Comparison of C26:0-carnitine and C26:0-lysophosphatidylcholine as diagnostic markers in dried blood spots from newborns and patients with adrenoleukodystrophy

Irene C. Huffnagel[c,d,1], Malu-Clair van de Beek[a,b,1], Amanda L. Showers[e], Joseph J. Orsini[e], Femke C.C. Klouwer[a,b,c,d], Inge M.E. Dijkstra[a,b], Peter C. Schielen[f], Henk van Lenthe[a,b], Ronald J.A. Wanders[a,b], Frédéric M. Vaz[a,b], Mark A. Morrissey[e], Marc Engelen[c,d], Stephan Kemp[a,b,c,d,*]

Development of a rapid UPLC–MS/MS determination of urine sulfocysteine for diagnosis of sulfocysteinuria and molybdenum co-factor deficiencies

Yi Jiang[1], Brandon Mistretta[1], Sarah H Elsea[1,2] & Qin Sun*[,1,2]
[1]Division of Biochemical Genetics, Baylor Genetics, Houston, TX 77021, USA
[2]Department of Molecular & Human Genetics, Baylor College of Medicine, Houston, TX 77030, USA
*Author for correspondence: Tel.: +1 713 798 6032; qsun@bcm.edu.

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Enzyme analysis

Enzyme Analysis

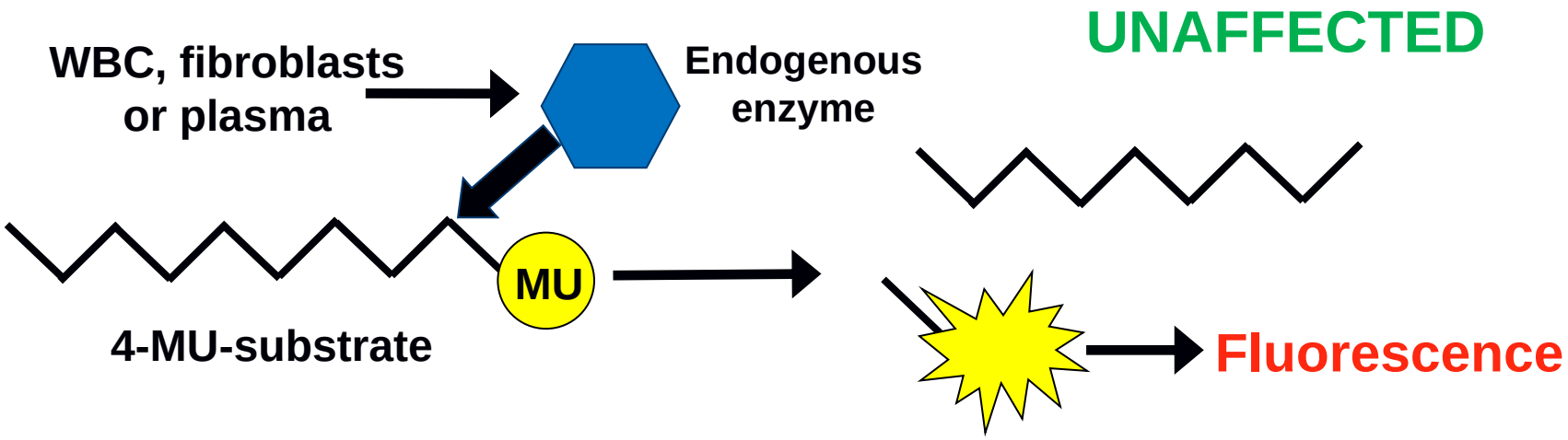
- Enzyme analysis is commonly performed using either blood or cultured fibroblasts.
  - Most enzyme assays are performed in white blood cells (leukocytes)- requires isolation from whole blood (~2 hour process)
  - Protein concentration must be determined for leukocytes and fibroblasts to calculate enzyme activity (~1 hour process)
  - Some assays require two steps requiring 24-48 hours to complete

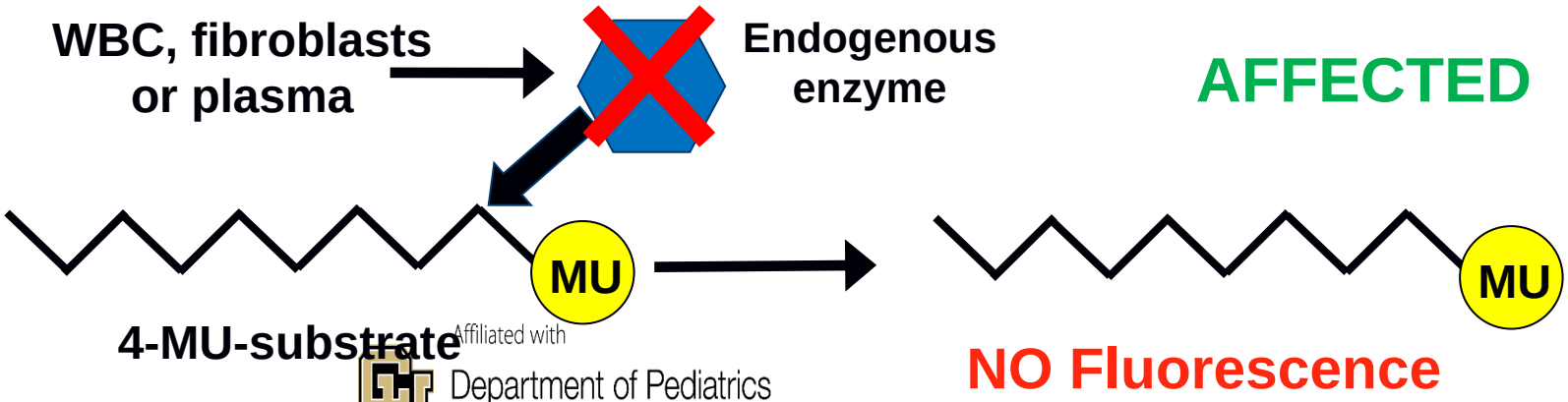
- Commonly 3-5mls of blood is required.
  - Larger volume (10mls) for large panels
  - Patient's white blood cell count can impact the volume of blood required

- Whole blood samples must arrive within 48 hours of testing to preserve sample quality

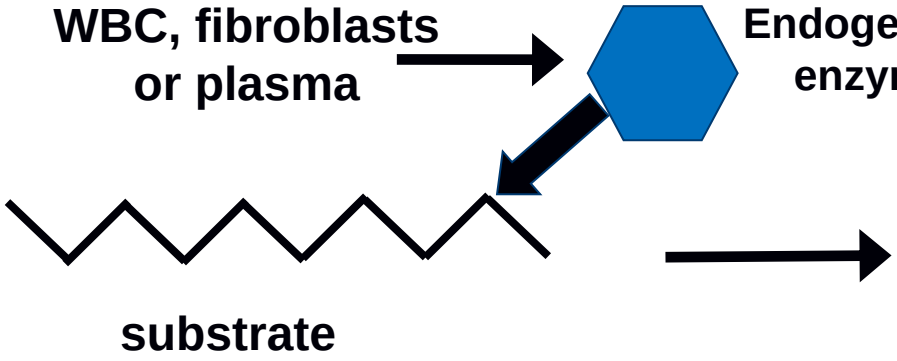
- More options are available for DBS analysis

Enzyme testing using 4-MU substrates



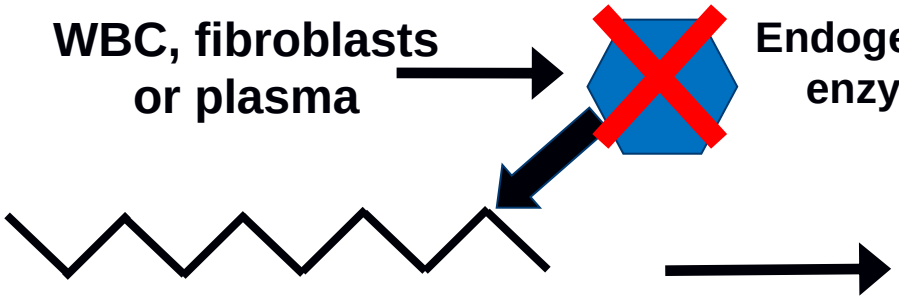


Enzyme testing using MSMS



UNAFFECTED

Detection
by MSMS



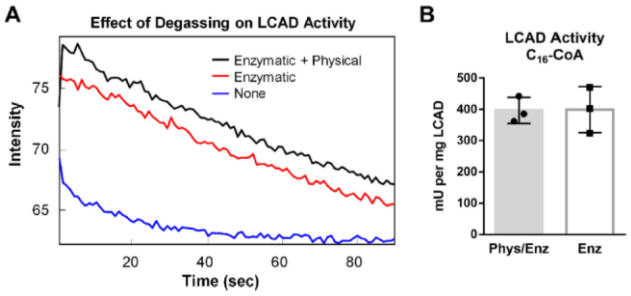
AFFECTED

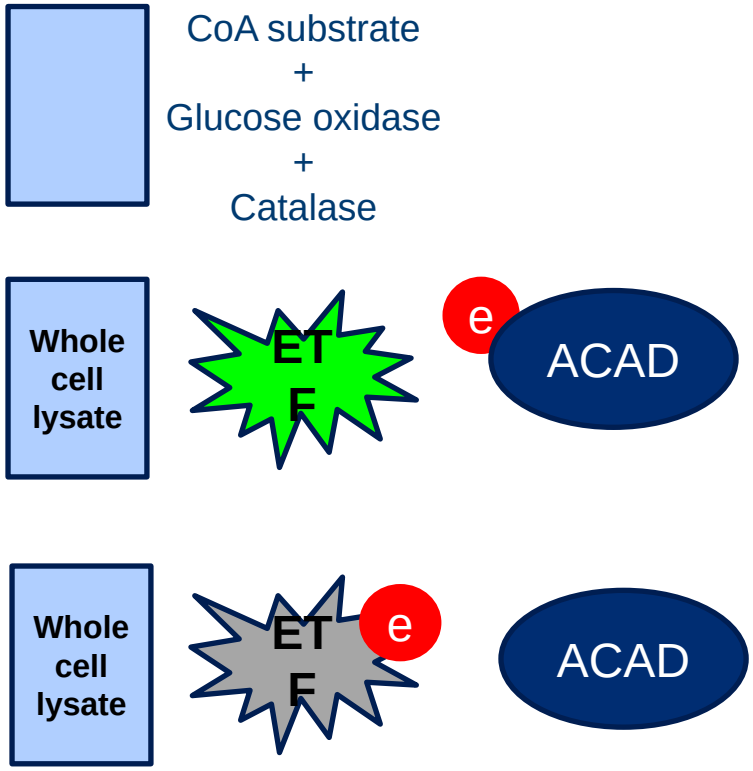
No MSMS signal

Enzyme activity

ETF Fluorescence Reduction Assay

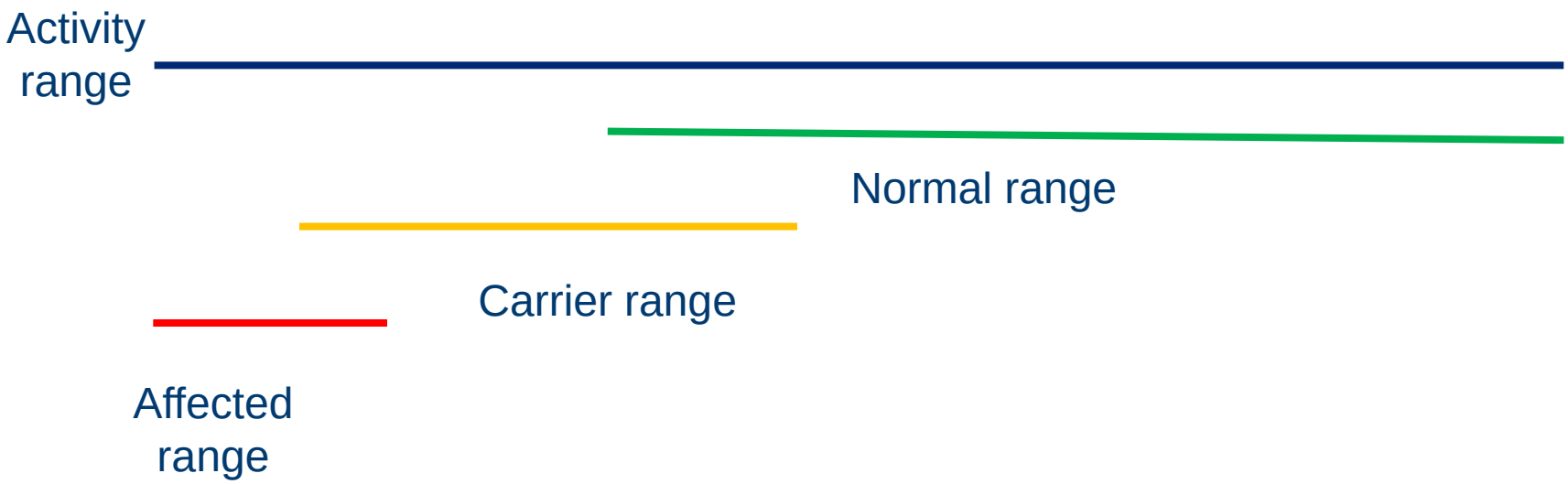
- Electron transferring flavoprotein (ETF)
- Natural electron acceptor for ACADs
- ETF is fluorescent & fluorescence is quenched as ETF accepts electrons from the ACAD
- ACAD activity measured by the reduction of ETF fluorescence
  - The faster the reduction of fluorescence, the more ACAD activity
- Measure specific ACAD by using a different CoA substrate
  - VLCAD = C16-CoA
- Gold standard for measuring ACAD activity





Thanks to Dr. Olivia D'Annibale

Enzyme activity



“False positive” enzyme results

- **For most enzyme assays, the absence/reduction of signal is being associated with the disease state!**
  - A second enzyme should always be measured

- For LSDs common causes are

  - Multiple sulfatase deficiency
  - Mucolipidosis II & III (low in fibroblasts, elevated in plasma)

- Pseudo-deficiency
  - Enzyme activity is deficient in phenotypically normal individuals

Various definitions of pseudodeficiency

Thomas (1994): Enzyme values are sufficiently below the carrier range that carriers are confused with or indistinguishable from affected patients

Genereviews: For MLD the term "pseudodeficiency" refers to very low levels of ARSA enzyme activity in an otherwise healthy individual. The term has been applied to other enzyme deficiency disorders, such as hexosaminidase A deficiency, where specific variants are associated with reduced enzymatic activity when measured using synthetic substrate but have normal enzymatic activity when measured using a natural substrate.

NTSAD - A “pseudodeficiency allele” reduces enzyme activity but does not cause a disease.

Enzymes (LSD disorders) with known pseudodeficiency

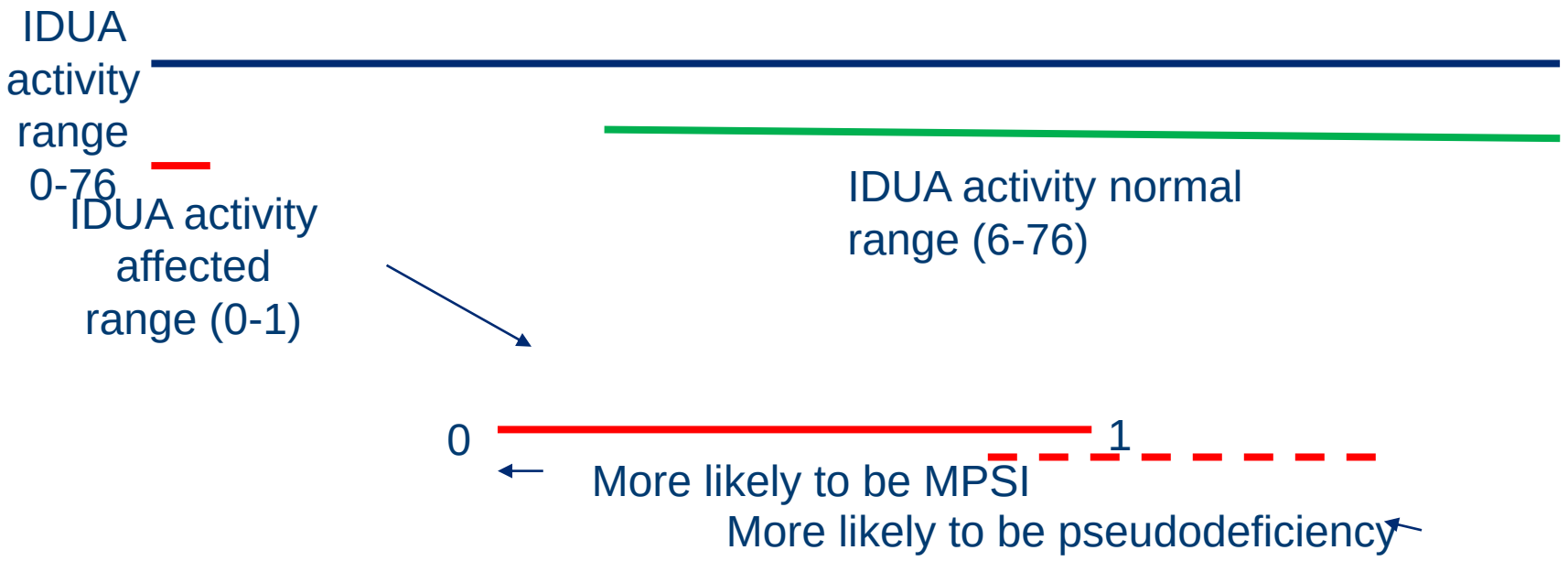
- Arylsulfatase A (Metachromatic Leukodystrophy)
- Beta hexosaminidase A (Tay Sachs)
- Beta hexosminidase A and B (Sandhoff)
- Glucocerebrosidase (Krabbe)
- Alpha galactosidase (Fabry)
- Alpha glucosidase (Pompe)
- Alpha fucosidase (Fucosidosis)
- Beta glucuronidase (Sly)
- Beta galactosidase (GM1 gangliosidosis)
- Arylsulfatase B (Maroteaux-Lamy syndrome)
- Alpha iduronidase (Hurler syndrome, MPSI)

Mechanisms of pseudodeficiency

- Substrate specificity differences between natural and artificial substrates
- Reduced mRNA expression
- Changes in glycosylation
- Reduced stability
- Reduction in enzyme activity

- Or a combination of several!

How to study pseudodeficiency?



Should the assay be recalibrated / redesigned to assess low activity?

IDUA expression analysis

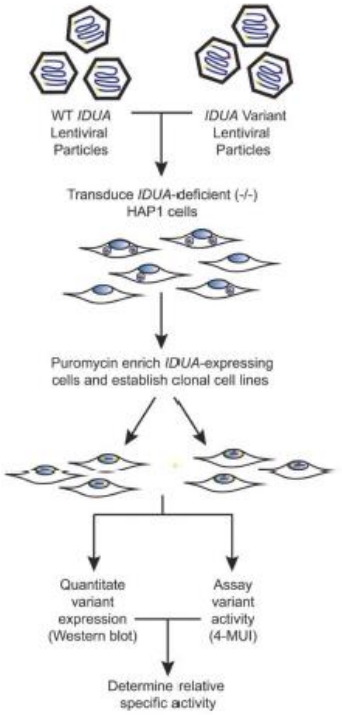
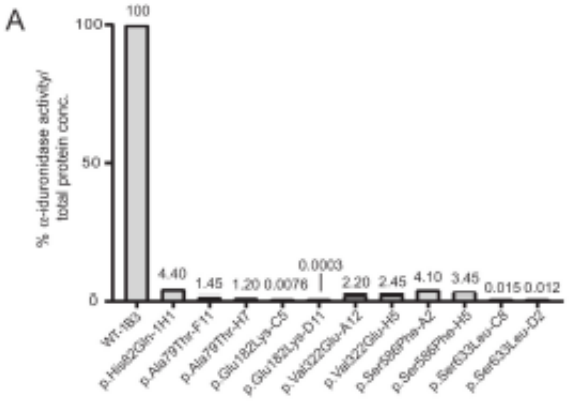
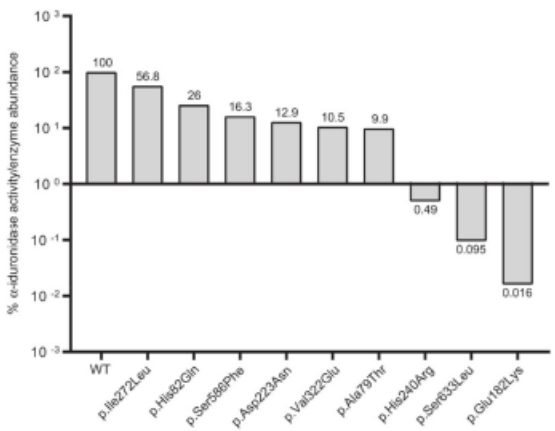


Figure 1. Schematic of the biochemical platform.

Table 1. List of IDUA variants and their classification within this study.

Variant	Classification	References
p.Ala79Thr	pseudodeficient	[20]
p.His82Gln	pseudodeficient	[20]
p.Glu182Lys	Pathogenic (Hurler)	[21]
p.Asp223Asn	pseudodeficient	[20]
p. His240Arg	Pathogenic (Scheie)	[16,17]
p.Ile272Leu	VUS	N.R.
p.Val322Glu	pseudodeficient	[20]
p.Ser586Phe	VUS	[15]
p.Ser633Leu	Pathogenic (Scheie)	[16–18]





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MPSI variant expression analysis



- Drs. Steet, Flanagan-Steet, Pollard have funding from the MPS society to study novel variants identified via NBS
- They are developing a rapid expression system to study novel variants and to quickly calibrate them as likely pseudodeficiency.
- MPSI but also MPSII
- In addition to enzyme activity, they will be assessing lysosomal function and GAG storage
- Contact them (rsteet@ggc.org) for more information

Genetics in Medicine (2022) ■, 1–15

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ACMG TECHNICAL STANDARD

Measurement of lysosomal enzyme activities: A technical standard of the American College of Medical Genetics and Genomics (ACMG)

Erin T. Strovel[1], Kristina Cusmano-Ozog[2], Tim Wood[3], Chunli Yu[4,5]; on behalf of the ACMG Laboratory Quality Assurance Committee[6,*]

Table 2 Common pseudodeficiency alleles

Gene	Reference Sequence	Variant	GMAF	Effect of Variants in Enzyme Studies	References
ARSA	NM_000487.5	c.*96A>G	0.04992	Loss of polyadenylation signal decreases the amount of 2.1 kb mRNA by 90%; 3.7 kb and 4.8 kb mRNA species are not affected	Harvey et al,[22] Gieselmann et al[23]
		c.1055A>G p.Asn352Ser	0.22484	Loss of one of the N-glycosylation sites may result in aberrant targeting to the lysosome and reduces activity by approximately 50% c.[*96A>G;1055A>G];[*96A>G;1055A>G] reduces activity by ~90%	
ARSB	NM_000046.4	c.1072G>A p.Val358Met	0.28554	c.1072G>A reduces activity by approximately 30% c.1151G>A reduces activity by approximately 40%	Garrido et al[24]
		c.1151G>A p.Ser384Asn	0.01957	c.[1072G>A;1151G>A] reduces activity by approximately 80%	
GAA	NM_000152.4	c.1726G>A p.Gly576Ser	0.03714	c.[1726G>A];[1726G>A] reduces activity by approximately 85%	Tajima et al[25]
	NM_000152.3	c.2065G>A p.Glu689Lys	0.07808	c.[2065G>A];[2065G>A] reduces activity by approximately 50% and is a common allele in China and Japan c.[1726G>A;2065G>A];[1726G>A;2065G>A] is a common allele with activity levels in the affected range	Suzuki et al,[26] Kroos et al[27]
GALC	NM_000153.4	c.550C>T p.Arg184Cys	0.02716	Common and benign variants that attenuate GALC activity but do not cause disease	Orsini et al[12]
		c.742G>A p.Asp248Asn	0.07947		
		c.1685T>C p.Ile562Thr	0.44748	In cis with other variants, c.1685T>C further reduces activity by approximately 50% and is a common variant in Africa	Hosain et al[28]
GLA	NM_000169.2	c.937G>T p.Asp313Tyr	0.00212	c.937G>T reduces activity by approximately 40% at a neutral pH	Yasuda et al[29]
GLB1	NM_000404.3	c.1561C>T p.Arg521Cys	0.07288	c.1561C>T reduces activity by approximately 70% and is a common variant in Brazil	Caciotti et al[30]
		c.1783C>T p.Arg595Trp	0.00020	c.1783C>T reduces activity by approximately 50% and is a common variant in the Basque population	Gort et al[31]
HEXA	NM_000520.5	c.739C>T p.Arg247Trp	0.00040	c.739C>T and c.745C>T reduce activity by approximately 70%	Cao et al[32]
	NM_000520.4	c.745C>T p.Arg249Trp	0.00016[a]		
IDUA	NM_000203.4	c.235G>A p.Ala79Thr	0.01018	c.235G>A is a common variant in Africa Decreased DBS and WBC activity with normal urine GAGs	Wasserstein et al[33]
		c.246C>G p.His82Gln	0.00120	c.246G>A is a common variant in Africa Decreased DBS activity with normal urine GAGs	Donati et al[34]
		c.667G>A p.Asp223Asn	0.00260	Decreased DBS and WBC with normal urine GAGs	Wasserstein et al[33]
		c.898G>A p.Ala300Thr	0.00004[b]	c.898G>A has reduced activity with 4-MU substrate but normal fibroblast studies	Aronovich et al[35]
	NM_000203.5	c.965T>A p.Val322Glu	0.00180	Decreased DBS and WBC activity with normal urine GAGs	Wasserstein et al[33]

Make sure the assay is designed for and can answer your clinical question!

Macromolecules

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Accuracy of FGF-21 and GDF-15 for the diagnosis of mitochondrial disorders: A meta-analysis

Yan Lin[1], Kunqian Ji[1], Xiaotian Ma[2], Shuangwu Liu[1], Wei Li[1], Yuying Zhao[1] & Chuanzhu Yan[1,2,3]

[1]Research Institute of Neuromuscular and Neurodegenerative Diseases and Department of Neurology, Qilu Hospital, Cheeloo College of Medicine, Shandong University, Jinan, Shandong, 250000, China
[2]Mitochondrial Medicine Laboratory, Qilu Hospital (Qingdao), Shandong University, Qingdao, Shandong, 266035, China
[3]Brain Science Research Institute, Shandong University, Jinan, Shandong, 250000, China

Detecting lysosomal storage disorders by glycomic profiling using liquid chromatography mass spectrometry

Justin Mak[a,*], Tina M. Cowan[a,b]

[a] *Clinical Biochemical Genetics Laboratory, Stanford Health Care, United States of America*
[b] *Department of Pathology, Stanford University Medical Center, United States of America*

Metabolite studies in *HIBCH* and *ECHS1* defects: Implications for screening

Heidi Peters[c], Sacha Ferdinandusse[d], Jos P. Ruiter[d], Ronald J.A. Wanders[d], Avihu Boneh[b,c], James Pitt[a,b,*]

[a] *Victorian Clinical Genetics Services, Murdoch Children's Research Institute, Royal Children's Hospital, Melbourne Australia*
[b] *Department of Paediatrics, University of Melbourne, Australia*
[c] *Metabolic Research, Murdoch Children's Research Institute, Royal Children's Hospital, Melbourne Australia*
[d] *Departments of Clinical Chemistry and Pediatrics, Laboratory Genetic Metabolic Diseases, Academic Medical Centre, University of Amsterdam, Amsterdam, The Netherlands*

Anal Biochem. 2013 Nov 15;442(2):178-85. doi: 10.1016/j.ab.2013.07.037. Epub 2013 Aug 6.

Serum N-glycan and O-glycan analysis by mass spectrometry for diagnosis of congenital disorders of glycosylation

Baoyun Xia[1], Wenyue Zhang, Xueli Li, Rong Jiang, Tisa Harper, Renpeng Liu, Richard D Cummings, Miao He

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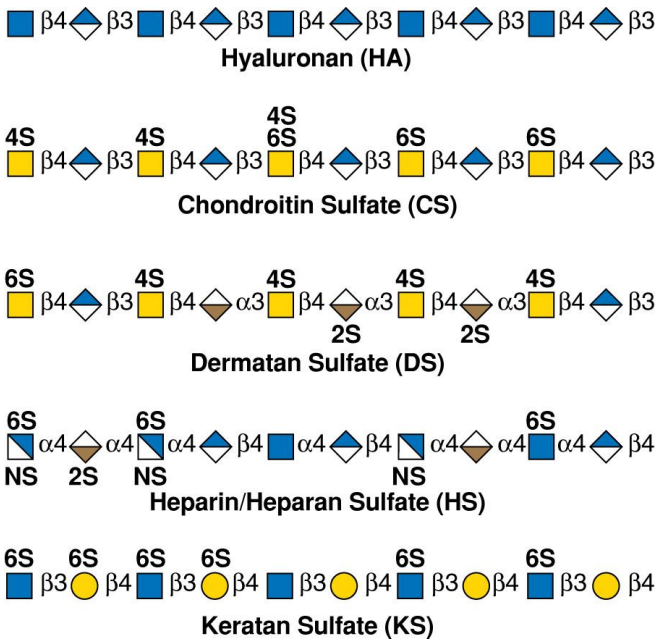
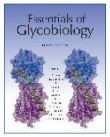
Glycosaminoglycans Consist of Repeating Disaccharide Units



Chapter 17, Figure 2. Essentials of Glycobiology, Third Edition

Symbol Nomenclature for Glycans (SNFG)



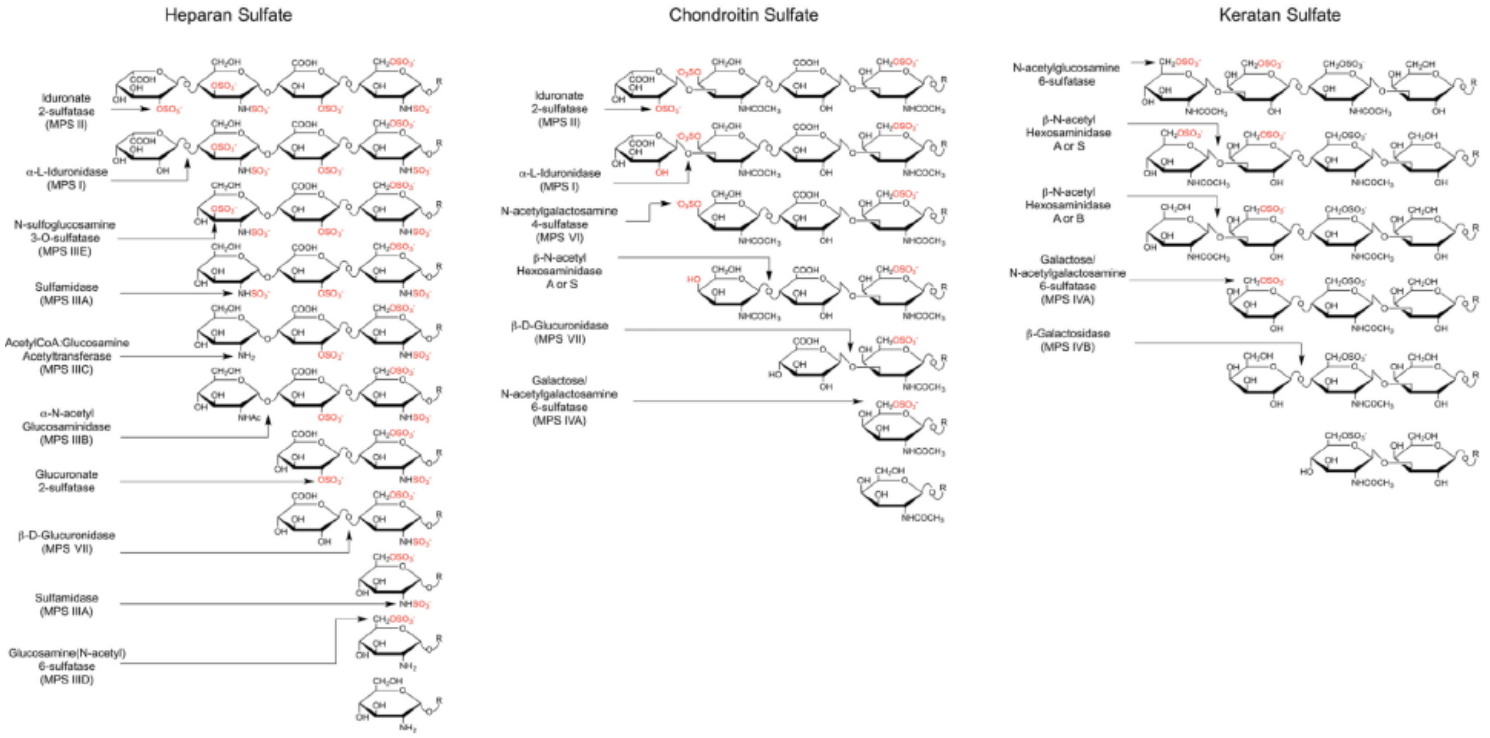
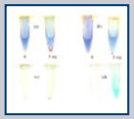
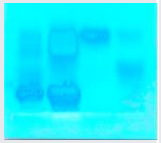


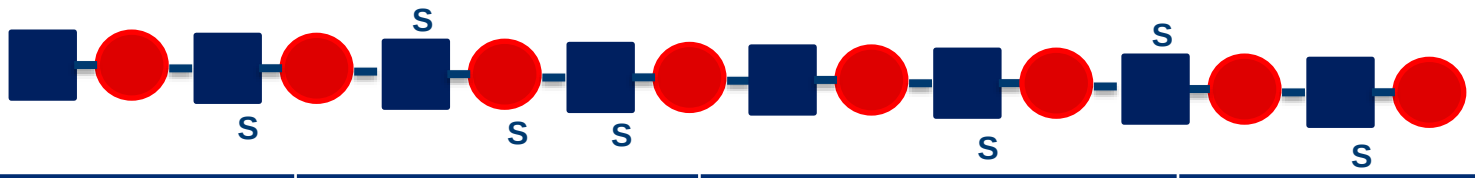
Fig. 1.
Glycosaminoglycan catabolism. The schemes show the different enzymatic activities required for the sequential catabolism of a hypothetical NREs from heparan sulfate, dermatan sulfate and keratan sulfate. It should be noted that the glucuronic acid 2-O-sulfatase in heparan sulfate degradation has been demonstrated in vitro, but has not yet been identified genetically.
Scheme modified from [3] according to findings from Lawrence et al. [18] and Kowalewski et al. [87].

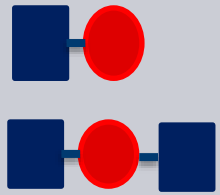
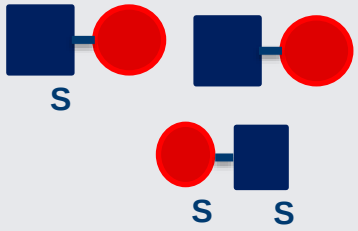
Urinary GAG measurements

	Method	Quantitative	Benefit	Limitation
Intact GAGs	Dye binding assay (total glycosaminoglycans) 	Yes	• Standardized across labs • Widely available • Low cost	• Does not tell you which GAG species is elevated • False positives common • False negatives well-documented
	Qualitative GAG analysis  	No	• Identifies which GAG species are elevated • Low cost	• Not quantitative • Subjective analysis • Keratan sulfate can be difficult to detect • Requires large sample volume

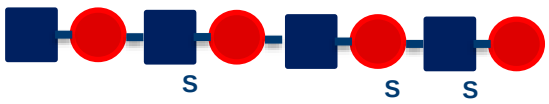
GAG fragments	Mass spectrometry 	Yes	• Can quantitate each GAG species
			• High sensitivity
			• Applicable to several sample types
			• Useful for treatment monitoring
			• Minimal sample volume required
				• Not standardized across labs
				• Requires expensive equipment
				• Time consuming (STAT analysis may be difficult)
				• Several methods
				  • Chemical cleavage
				  • Enzyme digestion
				  • NRE analysis

GAG analysis via MSMS

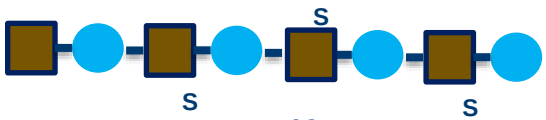


Method	What are you measuring	Benefit	Limitation
Chemical digestion (methanolysis or butanolysis)		• Relatively simple process • Reagents are commercially available	• Requires tight control of chemical reaction • Removes modifications
Enzyme digestion		• By leaving modifications more information may be gathered	• Several choices of which disaccharides to measure • Availability of commercially available enzymes
Biomarkers and/or non reducing ends		• Highly specific to each disorder • Very little present in samples from normal individuals	• Limited literature on clinical utility • Standards for all compounds not avaliable

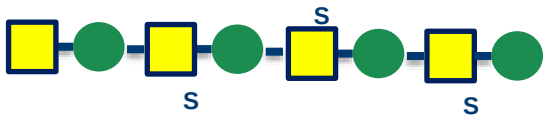
Intact GAG analysis via MSMS (Internal disaccharide analysis)



Dermatan sulfate – D0a4



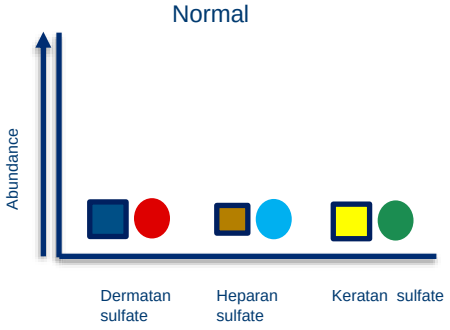
Heparan sulfate – D0A0, D0S0

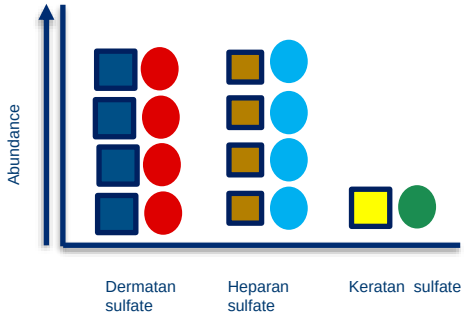


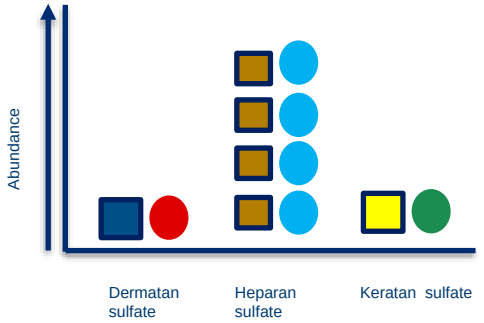
Keratan sulfate – g0A6, g6A6

↓ Enzyme digestion or chemical cleavage

↓ Separation and quantification of disaccharides via LCMSMS







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GAG biomarkers (endogenous NRE)

- Samples are not digested.
- Assay measures small oligosaccharides that are disease specific
- Endogenous NRE method, endogenous biomarkers

Table S1. LC-ESI-MS/MS conditions for the oligosaccharides.

oligosaccharide	RT	MRM	DP	CE	CXP	MPS subtype
ΔUA-GalNAc-4S IS	2.8	788/534	-70	-35	-25	
UA-HNAc (1S)	2.9/3.2	806/295	-84	-45	-25	I, II
UA-HN-UA(1S)	3.3	940/331	-80	-44	-25	I, VII
HNAc (1S)	3.6	630/256	-80	-45	-25	IIID, IVA, VI
HNAc (2S)	3.0	710/256	-80	-45	-25	VI
HNAc-UA (1S)	3.8	806/331	-84	-45	-25	IVA, VI
$(HNAc\text{-}UA)_2$ (1S)	3.0	1185/931	-80	-45	-25	IIIB
$(HNAc\text{-}UA)_2$ (2S) $[M\text{-}H]^{2-}$	3.2	632/298	-80	-45	-25	IIID, IVA, VI
HN-UA (1S)	3.5	764/331	-80	-45	-25	IIIA
$(HN\text{-}UA)_2$-HNAc (2S) $[M\text{-}H]^{2-}$	2.6	691/605	-40	-24	-25	IIIC
$(Hex\text{-}HNAc)_2$ (2S)	2.4	1240/256	-87	-41	-25	IVB

PMP-derivatised oligosaccharides were infused at a flow rate of 5 µL/min for identification. Ion source temperature 250 °C, ion spray voltage -4500V, nitrogen was used as the curtain gas, 25 psi, collision gas, high, gas 1, 30 units and gas 2, 40 units. Product ion scans were used to identify PMP-oligosaccharides and MRM transitions in MPS urine. Retention times (RT) - in minutes - were determined over an LC gradient as detailed above.

Table 1 Oligosaccharides in control and MPS urine

	Control	MPS I	MPS II	MPS IIIA	MPS IIIB	MPS IIIC	MPS IID	MPS IVA	MPS IVB	MPS VI	MPS VII
n	630	19	13	16	12	3	1	13	2	12	2
HNAc (1S)	10 (0.2–49)	12 (4–36)	12 (8–28)	14 (9–27)	11 (5—16)	15 (12–29)	**123**	**81 (39–261)**	7 (5–9)	**180 (71–1234)**	3 (2.5–3.5)
HNAc (2S)	0.6 (0.02–2.3)	0.8 (0.3–1.9)	0.7 (0.3–1.7)	0.8 (0.5–1.3)	0.8 (0.4–1.0)	0.8 (0.5–1.9)	1.0	0.8 (0.4–1.8)	0.4 (0.3-0.6)	**13 (7.3–107)**	0.6 (0.4–0.8)
HNAc-UA (1S)	n.d. (n.d.–0.12)	n.d. (n.d.–0.06)	n.d. (n.d.–0.07)	0.03 (n.d.–0.07)	n.d.(n.d.-0.08)	n.d.(n.d.-0.04)	0.04	**0.56 (0.24–5.55)**	n.d. (N/A)	1.45 (0.35–7.35)	0.06 (0.06–0.07)
UA-HNAc (1S) early RT	n.d. (n.d.–0.04)	**1.5 (0.4–2.9)**	n.d.(N/A)	n.d. (n.d.–0.06)	n.d. (N/A)	n.d. (n.d.–0.01)	n.d.	n.d. (N/A)	n.d. (N/A)	n.d. (n.d.–0.08)	n.d. (N/A)
UA-HNAc (1S) late RT	n.d. (n.d.–0.04)	0.16 (0.03–0.48)	**0.19 (0.13–0.31)**	n.d. (n.d.–0.05)	n.d. (N/A)	n.d. (N/A)	0.02	n.d. (N/A)	n.d. (N/A)	n.d. (n.d.–0.02)	n.d. (N/A)
HN-UA (1S)	n.d. (n.d.–0.04)	0.01 (n.d.-0.3)	0.02 (n.d.-0.1)	**1.1 (0.1–2.0)**	n.d. (n.d.–0.02)	n.d. (n.d.–0.05)	0.1	n.d. (n.d.–0.01)	n.d. (N/A)	n.d. (n.d.–0.01)	n.d. (N/A)
UA-HN-UA (1S)	n.d. (n.d.–0.03)	0.04 (n.d.–0.25)	n.d. (N/A)	n.d. (N/A)	**0.27 (0.16–0.58)**	n.d. (N/A)	0.04	n.d. (n.d.–0.04)	n.d. (N/A)	n.d. (N/A)	**0.10 (0.08–0.11)**
$(HNAc\text{-}UA)_2$ (1S)	n.d. (n.d.–0.01)	n.d. (n.d.–0.02)	n.d. (N/A)	n.d. (N/A)	**0.27 (0.16–0.58)**	n.d. (N/A)	0.04	n.d. (n.d.–0.04)	n.d. (N/A)	0.01 (n.d.–0.29)	n.d. (N/A)
$(HNAc\text{-}UA)_2$ (2S)	n.d. (n.d.–0.7)	0.1 (n.d.-0.7)	0.1 (n.d.-0.2)	n.d. (n.d.–0.2)	0.1 (n.d.-0.4)	0.1 (n.d.-0.3)	**3.0**	n.d. (n.d.-0.9)	0.2 (0.1–0.2)	0.9 (0.4–2.4)	n.d. (N/A)
$(Hex\text{-}HNAc)_2$ (2S)	n.d. (n.d.–0.03)	n.d. (n.d.–0.01)	n.d. (n.d.–0.01)	n.d. (N/A)	n.d. (N/A)	n.d. (N/A)	n.d.	n.d. (N/A)	**0.23 (0.03–0.43)**	n.d. (n.d.–0.04)	n.d. (N/A)
(HN-UA)2-HNAc (S)	n.d. (n.d.–0.02)	n.d. (n.d.–0.02)	n.d. (N/A)	0.01 (n.d.–0.02)	n.d. (N/A)	**0.20 (0.19–0.33)**	n.d.	n.d. (n.d.–0.01)	n.d. (N/A)	n.d. (N/A)	n.d. (N/A)

Values are the median concentration (mmol/mol creatinine) for each group with the range shown in parentheses. Bold results depict the signature oligosaccharide
MPS, mucopolysaccharidosis; *n.d.*, below the limit of quantification

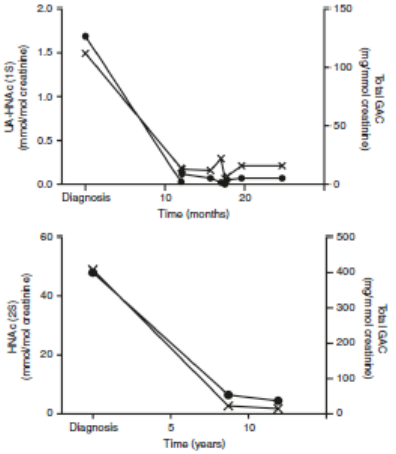

Fig. 1 Signature oligosaccharide and GAG concentrations in urine following enzyme replacement. Top and bottom panels depict an MPS I and MPS VI patient, respectively. Initial urine sample from both patients is at diagnosis, providing a single pretreatment concentration with filled circles representing the signature oligosaccharide and crosses total GAG. *GAG*, glycosaminoglycan; *MPS*, mucopolysaccharidosis.

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The future of biochemical laboratory testing (outside of NBS)

Clinical trials

Biomarker

Functional testing

Treatment

Summary

- Biochemistry is often complex so it is good to rely on tables and charts for quick references.
- More pieces of information you have the better. Combine biochemistry with molecular and clinical to make a diagnosis
- When in doubt, contact the laboratory director or laboratory staff with questions or concerns.

Thanks!

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