ACMG TECHNICAL STANDARD | Genetics inMedicine

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

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Clinical laboratory geneticists are encouraged to document in the patient's record the rationale for the use of a particular procedure or test, whether or not it is in conformance with this technical standard. They also are advised to take notice of the date any particular technical standard was adopted, and to consider other relevant medical and scientific information that becomes available after that date. It also would be prudent to consider whether intellectual property interests may restrict the performance of certain tests and other procedures.

Acylcarnitine analysis is a useful test for identifying patients with inborn errors of mitochondrial fatty acid β-oxidation and certain organic acidemias. Plasma is routinely used in the diagnostic workup of symptomatic patients. Urine analysis of targeted acylcarnitine species may be helpful in the diagnosis of glutaric acidemia type I and other disorders in which polar acylcarnitine species accumulate. For newborn screening applications, dried blood spot acylcarnitine analysis can be performed as a multiplex assay with other analytes, including amino acids, succinylacetone, guanidinoacetate, creatine, and lysophosphatidylcholines. Tandem mass spectrometric methodology, established more than 30 years ago, remains a valid approach for acylcarnitine analysis. The method involves flow-injection analysis of esterified or underivatized acylcarnitines species and detection using a precursor-ion scan. Alternative methods utilize liquid chromatographic separation of isomeric and isobaric species and/or detection by selected reaction monitoring. These technical standards were developed as a resource for diagnostic laboratory practices in acylcarnitine analysis, interpretation, and reporting.

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BACKGROUND

Acylcarnitine profile (ACP) analysis is performed for the biochemical detection of disorders of mitochondrial fatty acid β-oxidation (FAO) and organic acid metabolism.[1,2] ACP results can be diagnostic for some disorders, such as medium-chain acyl-CoA dehydrogenase (MCAD) deficiency; however, additional testing may be needed in other cases to obtain a precise diagnosis.[1–3] The conditions revealed by acylcarnitine analysis have in common the accumulation of short, medium, and/or long-chain acyl-CoA species, which are substrates for one of several carnitine acyl-CoA transferases expressed in different intracellular compartments.[4,5] The resulting acylcarnitine species are measured in the following clinical situations: (1) evaluation of symptomatic patients, (2) evaluation of asymptomatic (at-risk) siblings of known patients, (3) newborn screening and follow-up testing, (4) prenatal diagnosis, (5) postmortem evaluation.

Laboratories providing this analysis as a clinical service most often do so using flow-injection analysis–tandem mass spectrometry (FIA-MS/MS) as the analytical platform.[6,7] Newer MS/MS methods that include a liquid chromatographic (LC) step to separate isobars and isomers are also used.[8–12] Other reported methodologies include gas chromatography–mass spectrometry (GC-MS),[13] radio high-performance LC,[14,15] and capillary electrophoresis.[16] As is the case for all complex metabolic profiles, appropriate

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acylcarnitine analysis requires both analytical proficiency and in-depth interpretation of results with informative reporting.

CLINICAL DESCRIPTION OF DISORDERS IDENTIFIED THROUGH ACYLCARNITINE ANALYSIS

ACP analysis detects disorders of mitochondrial FAO and organic acid metabolism. Examples of disorders that may be detected by acylcarnitine analysis are listed in Tables 1 and 2. Inborn errors of mitochondrial FAO disorders may present at any age, from birth to adulthood. They are associated with life-threatening episodes of metabolic decompensation after a period of inadequate caloric intake and/or intercurrent illness. Typical manifestations may include hypoketotic hypoglycemia, liver disease, skeletal myopathy and cardiomyopathy, and sudden unexpected death.[17] ACP abnormalities have also been described in peroxisomal disorders associated with impaired very long–chain fatty acid (VLCFA) β-oxidation.[18,19] However, this latter group of disorders are better diagnosed by plasma VLCFA and branched-chain fatty acid analysis.[20]

Organic acidemias are a heterogeneous group of inborn errors of metabolism (IEM).[21] Classic organic acidemias typically present with recurrent episodes of acute life-threatening illness, hypo- or hypertonia, failure to thrive, and developmental delay. Common acute manifestations include vomiting, lethargy, coma, and seizures.

With rare exceptions, the diagnosis of these conditions is almost exclusively a laboratory process, of which acylcarnitine analysis is a key component. A comprehensive metabolic evaluation may also include the analysis of plasma amino acids, urine organic acids, and plasma carnitine (free and total). Depending on the results of these studies, additional testing may be warranted.

Table 1 Inborn errors of metabolism detected by acylcarnitine profile analysis.

Fatty acid oxidation disorders	OMIM[a]	Gene
Carnitine uptake defect	212140	*SLC22A5*
Carnitine palmitoyltransferase I (CPT I) deficiency	255120	*CPT1A*
Carnitine-acylcarnitine translocase (CACT) deficiency	212138	*SLC25A20*
Carnitine palmitoyltransferase II (CPT II) deficiency	608836, 600649, 255110	*CPT2*
Short-chain acyl-CoA dehydrogenase (SCAD) deficiency	201470	*ACADS*
Medium-chain acyl-CoA dehydrogenase (MCAD) deficiency	201450	*ACADM*
Very long–chain acyl-CoA dehydrogenase (VLCAD) deficiency	201475	*ACADVL*
Long-chain L-3-hydroxyacyl-CoA dehydrogenase (LCHAD) deficiency	609016	*HADHA*
Trifunctional protein (TFP) deficiency	609015	*HADHA*, *HADHB*
Multiple acyl-CoA dehydrogenase (MAD) deficiency (glutaric acidemia type II):	231680	
i α-ETF		*ETFA*
ii β-ETF		*ETFB*
iii ETF-ubiquinone oxidoreductase		*ETFDH*
3-Hydroxyacyl-CoA dehydrogenase (HADH) deficiency	231530	*HADH*
Dienoyl-CoA reductase deficiency caused by mitochondrial NAD kinase 2 deficiency	616034	*NADK2*

Medium-chain 3-ketoacyl-CoA thiolase (MCKAT; MIM 602199) deficiency has been reported in one patient[61] and it is not known whether this disorder can be detected by acylcarnitine analysis.
[a]Phenotype MIM number.

Table 2 Inborn errors of metabolism detected by acylcarnitine profile analysis.

Organic acid disorders	OMIM[a]	Gene
Ethylmalonic encephalopathy	602473	*ETHE1*
Glutaryl-CoA dehydrogenase deficiency (glutaric acidemia type I)	231670	*GCDH*
Glutamate formiminotransferase deficiency (formiminoglutamic aciduria)	229100	*FTCD*
3-Hydroxyisobutyryl-CoA hydrolase deficiency	250620	*HIBCH*
3-Hydroxy-3-methylglutaryl-CoA lyase (HMG-CoA lyase) deficiency	246450	*HMGCL*
Isobutyryl-CoA dehydrogenase deficiency	611283	*ACAD8*
Isovaleryl-CoA dehydrogenase deficiency (isovaleric acidemia)	243500	*IVD*
β-Ketothiolase (2-methylacetoacetyl-CoA thiolase, or 3-oxothiolase)	203750	*ACAT1*
Malonyl-CoA decarboxylase deficiency	248360	*MLYCD*
2-Methylbutyryl-CoA dehydrogenase (short/branched-chain acyl-CoA dehydrogenase (SBCAD) deficiency)	610006	*ACADSB*
3-Methylcrotonyl-CoA carboxylase (3-MCC) deficiency	210200, 210210	*MCCC1*, *MCCC2*
3-Methylglutaconyl-CoA hydratase deficiency	250950	*AUH*
2-Methyl-3-hydroxybutyryl-CoA dehydrogenase (MHBD) deficiency (HSD10 mitochondrial disease)	300438	*HSD17B10*
Methylmalonic acidemia (MMA):		
i Methylmalonyl-CoA mutase deficiency	251000	*MUT*
ii Methylmalonyl-CoA racemase deficiency	251120	*MCEE*
iii Cobalamin metabolism disorders		
cblA	251100	*MMAA*
cblB	251110	*MMAB*
cblC	277400	*MMACHC*, *PRDX1*
cblD	277410	*MMADHC*
cblF	277380	*LMBRD1*
cblJ	614857	*ABCD4*
cblX	309541	*HCFC1*
Multiple carboxylase deficiency caused by deficiency of:		
i Holocarboxylase synthetase	253270	*HLCS*
ii Biotinidase	253260	*BTD*
Propionyl-CoA carboxylase deficiency (propionic acidemia)	606054	*PCCA*, *PCCB*
Succinyl-CoA ligase deficiency		
i Mitochondrial DNA depletion syndrome 5	612073	*SUCLA2*
ii Mitochondrial DNA depletion syndrome 9	245400	*SUCLG1*

[a]Phenotype MIM number.

PREVALENCE

The combined incidence of these disorders is estimated to be between 1:5,000 and 1:10,000 live births.[22]

MODE OF INHERITANCE

A majority of IEM detectable by acylcarnitine analysis are inherited as autosomal recessive traits. Two X-linked disorders are potentially detected by acylcarnitine analysis. The first is HSD10 mitochondrial disease (MIM 300438), which is caused by a deficiency of a mitochondrial ribonuclease P subunit, encoded by *HSD17B10* (MIM 300256). This multifunctional protein has dehydrogenase activity toward a range of substrates, including 2-methyl-3-hydroxybutyryl-CoA.[23] The second is cobalamin disorder type X (MIM 309541), caused by a deficiency of a global transcriptional coregulator encoded by *HCFC1*.[24,25] It should be noted that acylcarnitine analysis may have low sensitivity for detecting HSD10 mitochondrial disease,[23] and the sensitivity of acylcarnitine analysis to detect cobalamin disorder type IX has not been fully evaluated.

METHODS

This laboratory technical standard was informed by a review of the literature, including any current guidelines, and expert opinion. Resources consulted included PubMed (search terms: acylcarnitine quantitation, acylcarnitine analysis, separation, UPLC, peroxisomal, prenatal, urine), Clinical and Laboratory Standards Institute (CLSI) guidelines[26–28] and CLIA regulations, and the Centers for Disease Control and Prevention (CDC) *Morbidity and Mortality Weekly Report* on Good Laboratory Practices for Biochemical Genetics Testing and Newborn Screening for Inherited Metabolic Disorders.[29] When the literature provided conflicting evidence about a topic or when there was insufficient evidence, the authors used expert opinion to inform the recommendations. Expert opinion included the coauthors of the document, members of the Biochemical Genetics Subcommittee of the Lab QA Committee, as well as the experts consulted outside the workgroup and acknowledged in this document. Any conflicts of interest for workgroup members or consultants are listed. The American College of Medical Genetics and Genomics (ACMG) Laboratory Quality Assurance Committee reviewed the document providing further input on the content, and a final draft was delivered to the ACMG Board of Directors for review and approval to send out for member comment. The final draft of the document was posted on the ACMG website and an email link was sent to ACMG members inviting all to provide comment. All members' comments were assessed by the authors; additional evidence was also included, and our recommendations were amended as deemed appropriate. Member comments and author responses were reviewed by a representative of the Laboratory Quality Assurance Committee and the ACMG Board. The final document was approved by the ACMG Board of Directors. This updated standard replaces the previous document "Acylcarnitine profile analysis" by Rinaldo et al.[30]

PREANALYTICAL REQUIREMENTS

Specimen requirements

Carnitine and its esters are found in virtually all biological fluids. The clinical utility of an acceptable biological fluid will depend upon the purpose of the analysis. Concentrations of acylcarnitine species vary between sample types and plasma concentrations may not be reflective of tissue concentrations.[10] Typical volumes of different sample types are shown in Table 3.

Plasma/serum

Heparinized plasma is the preferred sample type for the diagnostic evaluation of symptomatic patients and asymptomatic at-risk individuals. EDTA plasma or serum may also be accepted by testing laboratories as alternative sample types.

Dried blood spot

Dried blood spots (DBS) are used for newborn screening, and have utility in the clinical diagnostic setting, providing an alternative to plasma. In comparison with plasma or serum, blood spots have higher concentrations of long-chain acylcarnitines and certain other species such as C2, C3, C4-DC, and C5-OH, and lower free carnitine (C0).[31] DBS may therefore have a greater sensitivity for detecting carnitine palmitoyltransferase (CPT) IA deficiency (MIM 255120), a disorder characterized by reduced concentrations of long-chain acylcarnitines and elevated free carnitine.[31] In contrast, plasma may have greater sensitivity for diagnosing disorders associated with an accumulation of long-chain acylcarnitines, such as CPT II deficiency (MIM 600649, 608836, 255110) and mitochondrial trifunctional protein deficiency (MIM 609015).[31,32]

Bile or bile spot

Postmortem screening can be performed in bile as a first-level evaluation for sudden or unexpected death.[33,34] Bile can be

Table 3 Typical sample testing volumes for acylcarnitine profile analysis.

Sample type	Typical testing volume
Amniotic fluid (cell-free supernatant)	20 µL
Amniocyte culture medium	50 µL[a]
Bile (liquid specimen)	2 µL[b]
Bile dried spot (postmortem)	one 1/8-inch punch[b]
Blood dried spot (neonatal, postmortem)	one 1/8-inch or 3/16-inch punch
Cell culture medium	50 µL[a]
Plasma/serum	20 µL
Urine	Variable; standardized to creatinine

[a]Spotted on filter paper.
[b]May require dilution if profile is abnormal.

spotted onto filter paper and analyzed in the same manner as DBS.

Urine

Urinary acylcarnitine analysis can be useful in the workup of organic acidemias and FAO disorders[35] associated with accumulations of short- or medium-chain acylcarnitine species,[36–39] which are efficiently excreted in the urine. This is most often applied in the case of glutaric acidemia type I, in which an elevation of glutarylcarnitine may be more readily discernible in urine compared with DBS or plasma.[39] Long-chain acylcarnitines have low solubility and hence low concentrations in urine; urinary acylcarnitine analysis is not helpful for the diagnosis of long-chain mitochondrial FAO disorders. However, utility for diagnosing patients with certain peroxisomal disorders has been suggested.[19] In patients with peroxisomal biogenesis disorders and D-bifunctional protein deficiency, an increase was reported in urinary long-chain dicarboxylic acid acylcarnitines, and to a lesser extent in very long–chain monocarboxylic acid species.[19]

Cultured fibroblasts

ACP performed in cultured fibroblasts incubated with fatty acid substrates and carnitine can be useful for the diagnosis of FAO disorders.[2,40,41]

Amniotic fluid

Acylcarnitine analysis in amniotic fluid has been used for the prenatal diagnosis of certain IEM.[42,43] This testing may be useful when there is a family history of IEM and molecular studies were not obtained or were uninformative in the proband, and when performed in combination with one or more additional independent tests.[1]

Conditions of sample collection, shipping, handling, and storage

Plasma, serum, whole blood, and urine samples collected at the time of acute illness have the greatest sensitivity for detecting fatty acid oxidation disorders and organic acidemias. At ambient temperature, plasma acylcarnitines are stable for less than two days and in DBS for less than two weeks. At colder temperatures, specimens may be preserved for longer. Short-chain species are more unstable compared with long-chain species. Performing laboratories should define appropriate handling, shipping, and storage guidelines for their specific application.

Exogenous variables

Dietary factors such as medium-chain triglyceride (MCT)–containing formulas, fasting, ketogenic diet, carnitine supplementation, and total parenteral nutrition may influence acylcarnitine profiles. Carnitine insufficiency lowers the concentrations of acylcarnitines in body fluids and may decrease the sensitivity of ACP testing. Conversely, carnitine supplementation enhances blood concentrations and urinary excretion of acylcarnitines,[38] and may complicate interpretation of profiles. Renal impairment may cause an increase in short and medium-chain dicarboxylic acid acylcarnitine species (S. Young, unpublished observations, DUHS Biochemical Genetics Laboratory), whereas liver dysfunction may cause an increase in long-chain dicarboxylic acid acylcarnitine species.[6]

Clinical indications for testing

There are a variety of clinical indications for ACP analysis. Most commonly, ACP analysis is obtained following an abnormal newborn screen. ACP analysis can be helpful when an organic acidemia is suspected; findings often include metabolic acidosis, poor feeding, and lethargy or encephalopathy. Note that for an organic acidemia, urine organic acid analysis is the preferred method for diagnosis.[44] For disorders of fatty acid oxidation, typical clinical indications include hypoglycemia (in the absence of ketonuria), rhabdomyolysis, and cardiomyopathy. ACP analysis should also be considered in the setting of low free carnitine, to evaluate for the possibility of secondary carnitine deficiency, as well as in cases where the acyl to free carnitine ratio is elevated.

Due to an increased risk of long-chain 3-hydroxyacyl-CoA dehydrogenase or trifunctional deficiency, and possibly other fatty acid oxidation disorders, ACP analysis may be obtained in neonates with maternal complications of acute fatty liver of pregnancy and/or hemolysis, elevated liver enzymes, and low platelet count (HELLP syndrome).[45,46] Both CPT II deficiency and multiple acyl-CoA dehydrogenase deficiency (MADD) can present in the neonatal period with cystic kidneys and other malformations;[47] as these early presentations are often fatal, it is imperative to obtain the correct diagnosis for genetic counseling.

ACP analysis should be considered in cases of sudden infant death or other unexpected death at any age, particularly if there was fasting or vomiting in the preceding hours, a history of Reye syndrome, or if fatty infiltration of the liver is noted on autopsy. ACP analysis may be obtained in first-degree relatives following the diagnosis of an FAO disorder or organic acidemia in the proband. Finally, ACP analysis may be followed over time in patients with known disorders of fatty acid oxidation or organic acidemias for monitoring the disease and compliance with treatment.

ANALYSIS OF ACYLCARNITINES

Sample preparation

Acylcarnitine analysis may be performed as a dedicated standalone diagnostic test, or as a component of a multiplexed method for newborn screening that includes analysis of other analytes such as amino acids, succinylacetone, guanidinoacetate, creatine, and very long–chain lysophospholipids.[7,48,49] Acylcarnitines are most often analyzed as butyl-esters,[2] methyl-esters,[32] or without derivatization.[49] Esterification of the carboxylic acid groups results in a fixed quaternary positive charge on the carnitine moiety of acylcarnitine species. This increases the sensitivity of the method in

positive-ion mode, especially for dicarboxylic acids. However, this approach requires specimen heating under acidic conditions, resulting in hydrolysis of acylcarnitine ester bonds, reducing the levels of acylcarnitines and increasing the pool of free carnitine.[50] Therefore, the derivatization conditions must be carefully controlled to minimize loss of acylcarnitines. The use of stable-isotope internal standards is also important to compensate for any losses that do occur.

Plasma specimens are diluted with a mixture of deuterium-labeled internal standards dissolved in aqueous organic solvent mixtures, with or without organic acid modifiers (e.g., formic acid). Commonly used internal standards include [$^2H_3$]-labeled C2, C3, C4, C8, C14, C16; [$^2H_9$]-labeled C5 and C14; and [$^2H_6$]-C5-DC. Other internal standards can be used, but care must be taken not to interfere with the signal of a clinically relevant species (e.g., the butyl-ester of d_3-C6 at m/z 319 is only 1 Da from m/z 318, the molecular ion of hydroxyisovalerylcarnitine). Samples are centrifuged to remove protein precipitate and the supernatant containing extracted acylcarnitines is transferred to another tube and dried under nitrogen. Acylcarnitines are derivatized to their butyl-esters with 3 M HCl in *n*-butanol and heating at 65 °C for 15 minutes. Samples are again dried under nitrogen and reconstituted with a solvent matrix. DBS are similarly prepared; acylcarnitines are extracted from one or more punches from the spot using methanol or acetonitrile containing internal standards.

Analytical methods

Flow-injection analysis–tandem mass spectrometry with precursor-ion scan

Acylcarnitine analysis by FIA positive-ion electrospray–tandem mass spectrometry (MS/MS) utilizes a precursor-ion scan to separate mono- and dicarboxylic acid species, 2 to 18 carbons in length. Butylated and underivatized acylcarnitines are detected by precursors of m/z 85 with a scan from approximately m/z 200 to 500. Methylated acylcarnitines are detected using a precursor-ion scan of m/z 99. This mode of detection confers sufficient sensitivity and specificity to the method without the need for chromatographic separation, allowing for a rapid analysis. FIA combined with a precursor-ion scan has several advantages over methods that utilize a selected reaction monitoring (SRM) mode of detection, with or without chromatography. The whole profile of acylcarnitine species with masses within the scan range can be evaluated. Additionally, common drug artifacts, interfering compounds, and evidence of poor derivatization can be more readily detected. For example, the parent-product ion pair for butylated C4-acylcarnitine species (m/z 288 > 85) can have a contribution from the [M + 1] isotope of butylated formiminoglutamate, which has a $[M+H]^+$ of m/z 287.[51] A contribution from glutamate to the m/z 260 > 85 transition can result in an inaccurate quantification of acetylcarnitine (C2) by the butylation method.[52] The use of a more specific transition (m/z 260 > 141) can be used to more accurately quantify butylated acetylcarnitine.[53] Flow-injection methods do not allow separation of isomeric and isobaric species, which limits the diagnostic specificity of profiles generated by these methods. However, when performed in combination with other tests, such as urine organic acid or acylglycine analysis, the differential diagnosis suggested by flow-injection acylcarnitine profiles can usually be discerned. The isobaric pairs differ depending on the derivatization method (Supplemental Table 1). For example, C3-DC and C4-OH are isobaric by the underivatized method, C3-DC and C5-OH by the methylation method, and C5-DC and C10-OH by the butylation method. With flow-injection methods, when there is an elevated signal for an isobaric pair, a reflex test using an alternative derivative can be used to identify which acylcarnitine species are elevated. Acylcarnitine species and the corresponding mass of the derivatized (butylated or methylated) or nonderivatized molecular ions are listed in Supplemental Table 1. Tolerance limits for the identification by mass must be defined by the laboratory.

LC-MS/MS methods

Liquid chromatography methods are used to enable more sensitive and specific acylcarnitine identification. In this approach, specimens are subjected to LC prior to MS/MS identification with the goal of separating isomeric or isobaric acylcarnitines and interferents. These methods can be performed on underivatized specimens, simplifying sample preparation and allowing concurrent quantification of free carnitine. Reversed phase LC generates the highest chromatographic resolution of acylcarnitines,[8,9] but hydrophilic interaction liquid chromatography (HILIC) methods have also been described that provide relatively rapid analysis times with simple sample preparations.[10,11] LC-MS/MS acylcarnitine chromatograms are highly complex in comparison with flow-injection profiles. For example, C8-carnitine alone has 26 different hypothetical structural isomers, each potentially having unique chromatographic properties.[12] Numerous other acylcarnitines have one or more chiral centers, including all hydroxylated acylcarnitines. These enantiomeric compounds generate multiple peaks by some LC-MS/MS methods.[9] Pure analytic standards are not available for the majority of these compounds, challenging the confident assignment and quantification of the full range of acylcarnitines potentially detectable by LC-MS/MS methods. Even fewer isotopically labeled acylcarnitine standards exist, making it difficult to account for matrix effects. Considering these challenges, chromatographic methods are best used in the setting of focused reflex testing or research studies.

METHOD VALIDATION

Each laboratory must validate the performance characteristics of their specific analytical protocol and periodically verify its performance in accordance with local regulations. In the United States, this includes CLIA and the College of American Pathologists (CAP) requirements. Examples of published method validation approaches are provided by

CLSI in several different documents (e.g., C24-A3: Statistical Quality Control for Quantitative Measurement Procedures: Principles and Definitions, 4th edition[27] and C26-A: Liquid Chromatography–Mass Spectrometry Methods; Approved Guidelines[28]) and ACMG technical standards, such as this one. Laboratories should also devise procedures to address analytical values outside of their established criteria for performance and for clinical specimens with ambiguous stability.

Calibration and quantitation

Stable-isotope internal standards are available from a variety of commercial sources. These standards must be validated by determining their recoveries from weighed, nonisotopic acylcarnitine standards for as many analytes as possible. Internal standards must be periodically revalidated at a regular frequency to account for natural decay. Reference standards are not available for all acylcarnitine species detected by a profile analysis, such as the hydroxylated acylcarnitine species. Quantitation of these compounds should be extrapolated by application of the calibration for the nearest species of similar structure and m/z. The laboratory should determine their method's linear range, analytical measurement range, and lower limit of detection for all clinically informative acylcarnitines, when possible. These performance characteristics should be verified on a regular basis as specified by CLIA.

Reference intervals

Laboratory-specific, age-appropriate reference intervals for all reported acylcarnitines should be established and periodically validated per applicable regulations (e.g., CLSI EP28-A3c: Defining, Establishing, and Verifying Reference Intervals in the Clinical Laboratory, Approved Guideline, 3rd edition).[26] When literature-based intervals are used, they must be verified by the laboratory before implementation. For all applications, reference intervals should be established on the appropriate tissue, collection tube, and matrix. For instance, the comparison of a pediatric result to a blood spot, plasma, or serum normative interval derived from an adult population is inappropriate. Free carnitine and short-chain acylcarnitine species are increased in postmortem blood samples[34] and reference intervals for this sample type should be generated using postmortem control samples.

Instrument quality cross-check

When more than one mass spectrometric instrument is used to complete quantitative analyses for acylcarnitines, instrument comparisons should be routinely performed, and compatibility confirmed at regular intervals. This comparison should include instruments implemented as a backup for emergency analyses. The process to confirm acceptable performance can include quality control (QC) comparisons, proficiency testing (PT) evaluations, or interlaboratory comparisons at the laboratory's discretion.

Testing personnel

Appropriate training and ongoing competency requirements for laboratory personnel performing ACP analysis must be established and documented. MS/MS-based applications, instrument optimization, method validation, and clinical testing must be performed by personnel with specialized training in the operation of MS/MS.

Quality control

A positive and negative control should be prepared and analyzed with every batch of patient samples tested. The internal standard mix should also be analyzed separately with each batch.

A QC program based on the quantitative analysis of normal and abnormal control specimens should be implemented on a regular basis. Target ranges for QCs should be established for each metabolite and used to accept or reject a given run. The internal standards in each specimen serve as a QC check for each specimen. The use of Westgard rules for clinical specimen analysis further controls the parameters for quality patient diagnosis and reporting.[54] A laboratory director or designee should perform a monthly review and approval of control values.

Proficiency testing

Laboratories should participate in a PT program at time intervals as required by regulating agencies. PT performance should be monitored and insufficiencies requiring further investigation identified. Remedial action for laboratories uncovered by PT performance should be documented. The CAP provides a commercially available, external proficiency testing service for quantitative plasma acylcarnitine analysis. The European Research Network for Evaluation and Improvement of Screening, Diagnosis and Treatment of Inherited Disorders of Metabolism (ERNDIM) also has a PT scheme for plasma and DBS acylcarnitines. The CDC provides a PT scheme for DBS. Scheduled interlaboratory comparison (alternative PT) can be used when external proficiency testing is unavailable for certain tissues and matrices.

TEST INTERPETATION AND REPORTING

Interpretation

Results should be reviewed and interpreted by an ABMGG-certified biochemical geneticist or other qualified individual. Informative results are usually characterized by a pattern of one or more elevated acylcarnitine species in comparison with age-matched reference ranges. Interpretation of results is based on pattern recognition, rather than on individual abnormal values (see Fig. 1). The differential diagnosis of abnormal results of species potentially representing multiple isomers (C4, C5, C5-OH) is critical and cannot be overstated.[2,3] Basic acylcarnitine patterns associated with various disease states are listed in Fig. 1. In addition to the primary markers listed, calculation of ratios is useful in the interpretation of abnormal results (Fig. 1). Examples of

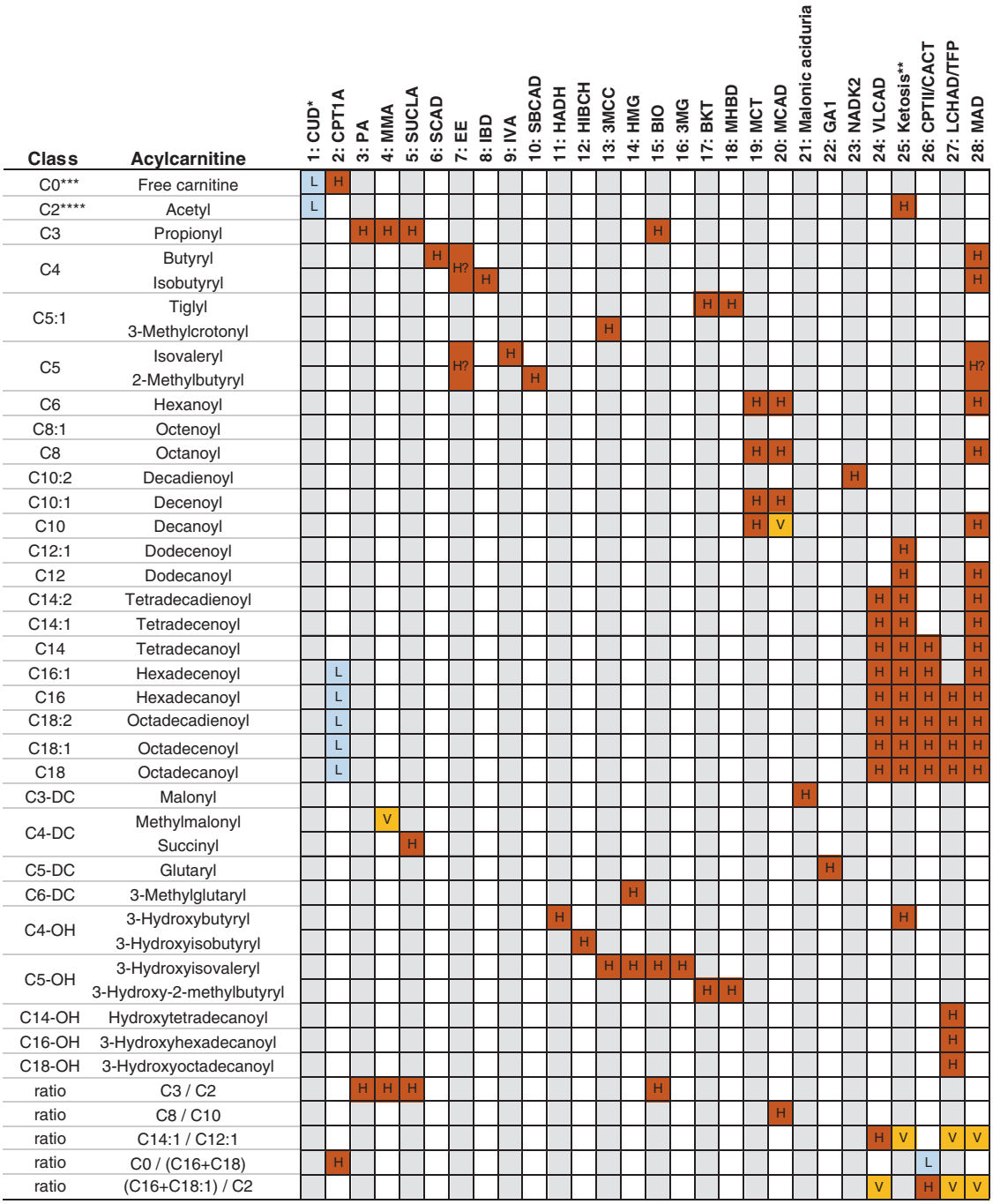

Fig. 1 Common acylcarnitine patterns associated with various disease states. *H* high, *H?* high but specific isomeric species are not clearly defined for this disorder, *L* low, *V* level varies from high-normal to elevated, *In CUD other acylcarnitine species are generally low. **C14:1/C12:1 is typically in the high-normal range or slightly elevated (< 3). ***For many listed disorders, C0 can be low due to secondary carnitine depletion. ****Low C2 may also be observed in other fatty acid oxidation and amino acid metabolism disorders, secondary to factors such as low flux through the β-oxidation pathway, and/or carnitine depletion. Note, in SUCLA2 deficiency, C4-DC elevations were shown to be succinylcarnitine, whereas methylmalonylcarnitine was within normal reference limits.[60] 1: *CUD* carnitine uptake deficiency, 2: *CPT1A* carnitine palmitoyltransferase I deficiency, 3: *PA* propionic acidemia, 4: *MMA* methylmalonic acidemia, 5: *SUCLA* succinyl-CoA ligase deficiency, 6: *SCAD* short-chain acyl-CoA dehydrogenase, 7: *EE* ethylmalonic encephalopathy, 8: *IBD* isobutyryl-CoA dehydrogenase deficiency, 9: *IVA* isovaleryl-CoA dehydrogenase deficiency, 10: *SBCAD* short/branched-chain acyl-CoA dehydrogenase deficiency, 11: *HADH* 3-hydroxyacyl-CoA dehydrogenase, 12: *HIBCH* 3-hydroxyisobutyryl-CoA hydrolase deficiency, 13: *3-MCC* 3-methylcrotonyl-CoA carboxylase deficiency, 14: *HMG* HMG-CoA lyase deficiency, 15: *BIO* biotinidase or holocarboxylase synthetase deficiency, 16: *3MG* 3-methylglutaconyl-CoA hydratase deficiency (AUH), 17: *BKT* β-ketothiolase deficiency, 18: *MHBD* 2-methyl-3-hydroxybutyryl-CoA dehydrogenase deficiency, 19: *MCT* supplementation with medium-chain triglycerides, 20: *MCAD* medium-chain acyl-CoA dehydrogenase deficiency, 21: *Malonic aciduria* malonyl-CoA decarboxylase deficiency, 22: *GA1* glutaric aciduria type 1, 23: *NADK2* dienoyl-CoA reductase deficiency caused by mitochondrial NAD kinase 2 deficiency, 24: *VLCAD* very long–chain acyl-CoA dehydrogenase deficiency, 26: *CPT II/CACT* carnitine palmitoyltransferase II/carnitine-acylcarnitine translocase deficiency, 27: *LCHAD/TFP* long-chain L-3-hydroxyacyl-CoA dehydrogenase/trifunctional protein deficiency, 28: *MAD* multiple acyl-CoA dehydrogenase deficiency.

commonly used ratios include C3/C2, which is elevated in propionic and methylmalonic acidemia and related disorders; C8/C10, which is elevated in MCAD deficiency; and C14:1/C12:1, which is elevated in very long–chain acyl-CoA dehydrogenase (VLCAD) deficiency, although mild elevations of this ratio may also be observed in long-chain 3-hydroxyacyl-CoA dehydrogenase (LCHAD) and trifunctional protein (TFP) deficiencies, and in ketosis. Informative profiles may not always be detected in disorders where the accumulation of diagnostic species is a reflection of the residual activity of the defective enzyme, the dietary load of carnitine and precursors, and the anabolic or catabolic status of the patient. Modest abnormalities should be correlated to the patient's carnitine levels as a status of carnitine insufficiency could affect the outcome of this analysis. It is important to correlate ACP results with other laboratory findings, such as the urine organic acid profile and routine chemistries, and the demographic and clinical status of the patient. For instance, in premature infants, elevations of short and medium-chain dicarboxylic acid species may be observed, presumably secondary to renal immaturity. In adults with late-onset CPT II or VLCAD deficiency that presents as a myopathic phenotype, elevations of long-chain species can be subtle.

Several drugs may induce or mimic abnormal levels of one or more known acylcarnitine species;[55,56] these include pivalic acid (a C5 isomer), valproic acid (a C8 isomer), cefotaxime (a C16:1-OH isobar by the butyl-ester method). Other exogenous compounds could result in the appearance of atypical species, some of them at m/z values in close proximity to or even overlapping acylcarnitines of diagnostic significance. Examples include 2-ethylhexanoic acid from plasticizers used in tubing used for extracorporeal membrane oxygenation and other procedures,[57] and intravenous fluids containing dextrose.[58] Dietary artifacts are also possible, usually related to food intake enriched with fatty acids (MCT oil, ketogenic diet).[59]

Reporting

Important elements to include in patient reports are appropriate patient and specimen information, analyte values reported against reference limits, and interpretive comments. As discussed above, interpretations should integrate other lab findings (e.g., organic acids, free and total carnitine, and/or amino acids), and take into consideration the clinical and dietary history, when applicable and available. When abnormal results are detected, the interpretation should include an overview of the results and their significance, quantitative results with age-appropriate reference ranges, a correlation to available clinical information, elements of differential diagnosis, recommendations for additional biochemical testing and in vitro confirmatory studies (e.g., enzymatic analysis, molecular analysis), and a phone number to reach the reporting laboratory with additional questions.

SUPPLEMENTARY INFORMATION

The online version of this article (https://doi.org/10.1038/s41436-020-00990-1) contains supplementary material, which is available to authorized users.

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DISCLOSURE

All authors (M.J.M., D.O., K.C-O., and S.Y.) work for clinical laboratories that offer acylcarnitine analysis as a clinical service.

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