

Laboratory Medicine Practice Guidelines

Follow-up Testing for Metabolic Diseases Identified by Expanded Newborn Screening Using Tandem Mass Spectrometry

Edited by Michael J. Bennett



The National Academy of Clinical Biochemistry

Presents

LABORATORY MEDICINE PRACTICE GUIDELINES

**FOLLOW-UP TESTING FOR METABOLIC
DISEASES IDENTIFIED BY EXPANDED
NEWBORN SCREENING USING
TANDEM MASS SPECTROMETRY**

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Overview

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Currently, approximately 4.3 million babies are born annually in the United States (1). Each baby born will have blood drawn and spotted onto a filter paper card for screening purposes for a number of severe biochemical genetic conditions. This blood test is arguably one of the few diagnostic blood tests that will be provided to the entire population.

Whole population newborn screening for metabolic diseases was started in the 1960s and is now accepted in most of the developed world as a standard medical procedure (2). Historically, only a limited number of conditions could be identified on these filter blood spot cards due to technical limitations and lack of ability of screening programs to expand beyond these boundaries. A set of paradigms evolved which determined whether a metabolic disorder should be included in a screening program. The disorders to be included in newborn screening programs were initially required to meet the following criteria:

1. The population frequency of the disease is high enough to warrant screening
2. The untreated natural history of the disease is well-defined
3. The untreated disease clearly confers significant morbidity and/or mortality
4. The disease is treatable and treatment significantly improves outcome
5. Disease testing is safe, simple, and sufficiently sensitive to detect all cases
6. Specific confirmatory testing is available for the disease
7. Testing, treatment, and treatment outcomes are cost-effective with respect to non-treatment of the disease (3).

A number of diseases fulfilled all of these criteria and these conditions formed the basis of most early screening programs. Phenylketonuria (PKU), with an incidence of around 1 case in every 14,000 newborns, set the scene for defining the inclusion criteria. Untreated PKU has a well-described natural history leading to profound mental retardation and a requirement for expensive long-term institutionalization. If detected in the newborn period and early dietary intervention is instigated, clinical outcome is greatly improved and today most individuals with PKU lead normal productive lives. Testing for PKU based upon measurement of blood spot phenylalanine levels is inexpensive and sufficiently sensitive as a screening tool and follow-up confirmatory diagnosis readily available in many certified laboratories (4).

Other genetic diseases that are screened for in most states and in many developed countries include congenital hypothyroidism (incidence, 1 in 4,000), congenital adrenal hyperplasia due to 21-hydroxylase deficiency (incidence, 1 in 10,000 to 1 in 18,000), galactosemia (incidence, 1 in 44,000 to 1 in 80,000), and biotinidase deficiency (incidence, 1 in 60,000). Sickle cell disease (incidence, 1 in 40,000), a hemoglobinopathy, is also included in most programs in the United States.

Recently, technological advances made possible due to the development of tandem mass spectrometry have allowed the initial paradigm for disease inclusion to be expanded to include diagnosis of multiple conditions on the same single blood spot using a single analytical process (5). This process allows us to be able to diagnose several amino acid and urea cycle disorders, including PKU, most of the disorders of mitochondrial fatty acid oxidation and a number of organic acidemias at the same time with a rapid enough turnaround time to allow for whole population screening.

Medium-chain acyl-CoA dehydrogenase (MCAD) deficiency is the most common disorder of fatty acid oxidation with a frequency which occurs in approximately 1 in 14,000 (6). MCAD deficiency is a disorder that, if unrecognized, presents with fasting induced hypoglycemia, hepatic encephalopathy, and in many cases, leads to death. Survivors often have profound residual neurological damage requiring long-term care. If recognized before the onset of symptoms, MCAD deficiency appears to be an eminently treatable condition with good clinical outcomes. Consequently, this disorder fulfills all of the initial criteria for inclusion into whole population screening programs. Tandem mass spectrometry of acylcarnitine species is the only suitable technique for whole population screening for MCAD deficiency using newborn blood spots. The technology, without additional sample preparation and with minimal loss of sensitivity, can also identify many other diseases including PKU, thus reducing the need for a separate screen for PKU.

Although some of the other conditions that can be diagnosed using this technology (to be described in the succeeding sections of this document) appear to be less frequent in the population, they are identifiable simultaneously with no increase in analytical time. As a result of the introduction of tandem mass spectrometry into the newborn screening arena, the numbers of diseases that are potentially identifiable have expanded considerably. Some of these conditions are rare. Some are regarded as having unproven treatability. The sensitivities for detection of some conditions are not always ideal and some of these conditions do not necessarily fulfill the initial criteria for inclusion in a whole population screening program. Thus, necessitating a change in the way in which we approach candidacy for inclusion in these programs.

In addition, the increased numbers of different metabolic diseases that are identifiable by tandem mass spectrometry also increases the complexity of testing required for confirmation of diagnosis. Confirmatory testing is a critical component of the whole process, which may involve additional metabolite measurement, enzyme assay, or molecular testing. We recognize the importance of establishing guidelines for systematic, consistent, and appropriate disease confirmation in the clinical laboratory and also guidelines for monitoring efficacy of therapeutic intervention and patient well-being.

At the time of publication of this document, approximately 98% of all babies born in the United States are provided with expanded newborn screening for metabolic diseases by tandem mass spectrometry. MCAD deficiency is now mandated in 46 states and the District of Columbia, required but not yet implemented in one state, and offered but not mandated in two additional states (7; accessed 09/19/2008). It is highly likely that the process will be taken up by the few remaining states or other providers will provide the service for MCAD deficiency for all babies born in those states before the end of the present decade. In addition, tandem mass spectrometry is being utilized for multiple additional metabolic conditions, which vary by state.

The National Academy of Clinical Biochemistry (NACB) is the American Association for Clinical Chemistry's scientific academy. An important activity of the NACB is to develop laboratory medicine practice guidelines to assist clinical and laboratory practice decisions concerning the diagnosis of specific diseases. NACB recognizes that there is a strong need for evaluation of how these newborn screening laboratory services are provided, and of equal importance, how procedures for adequate follow-up testing should proceed. Screening programs, by definition, should be developed with the highest degree of sensitivity, such that there are fewest possible false-negative cases (missed diagnoses). Follow-up testing should provide the highest degree of specificity so that false positives from the screening process are removed and only true-positive cases are eventually given a diagnosis.

The NACB has convened a panel of experts to evaluate the data supporting the role of expanded newborn screening, to determine optimal methods and performance characteristics for performing the testing, and for optimizing confirmatory follow-up testing procedures for positive screens.

Specific recommendations in this NACB guideline are based whenever possible on relevant published information in the peer-reviewed medical and scientific literature, and from surveys and guidelines produced by other medical academic groups and organizations including the American College of Medical Genetics and the Centers for Disease Control. The strength of the supporting data for each recommendation is determined using the scoring criteria adopted from the US Preventative Services Task Force Recommendations for Preventative Services.

Strength of Recommendations (Modified from US Preventive Services Task Force Recommendations for Preventive Services)

- A. The NACB strongly recommends adoption; there is good evidence that it improves important health outcomes and concludes that benefits substantially outweigh harms.
- B. The NACB recommends adoption; there is at least fair evidence that it improves important health outcomes and concludes that benefits outweigh harms.
- C. The NACB recommends against adoption; there is evidence that it is ineffective or that harms outweigh benefits.
- I. The NACB concludes that the evidence is insufficient to make recommendations; evidence that it is effective is lacking, of poor quality, or conflicting and the balance of benefits and harms cannot be determined.

NACB grades the quality of the overall evidence on a 3-point scale:

- I. Evidence includes consistent results from well-designed, well-conducted studies in representative populations.
- II. Evidence is sufficient to determine effects, but the strength of the evidence is limited by the number, quality, or consistency of the individual studies; generalizability to routine practice; or indirect nature of the evidence.
- III. Evidence is insufficient to assess the effects on health outcomes because of limited number or power of studies, important flaws in their design or conduct, gaps in the chain of evidence, or lack of information.

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Evidence-Based Rationale for Expanded Newborn Screening

Piero Rinaldo, Ronald J. Whitley, William J. Rhead, and W. Harry Hannon

In 2000, the American Academy of Pediatrics (AAP) Newborn Screening Task Force released a report entitled “Newborn Screening: A Blueprint for the Future – A Call for a National Agenda on State Newborn Screening Programs” (1). Tandem mass spectrometry (MS/MS) was mentioned once (p. 395) in the body of the voluminous report, and was recognized correctly as an example of technological advances likely to have a significant impact on the sensitivity, specificity, and scope of newborn screening. However, the positive message was mitigated by a concern described as “the ability to detect individuals with metabolic conditions for which there are no effective treatments at this time.” In retrospect, this conservative assessment is not surprising in view of the fact that when the report was written 100% of US births were screened for fewer than 10 conditions (2), and only a small proportion (7%; MA, ME, NC, SC, WI) was tested for medium-chain acyl-CoA dehydrogenase (MCAD) deficiency using MS/MS. Seven years is a relatively short period of time in public health policy making, so it is remarkable that currently the situation has changed to 98% and 83% of US births being tested for a panel of >20 and >30 conditions, respectively (2). As of April 2008, 98% of US newborns are screened for MCAD deficiency. There is no doubt this unprecedented evolution has been driven by a combination of factors, including public pressure, political action, and increasing attention of mass media to the issue of newborn screening expansion, yet the publication of prospectively collected evidence that has taken place in recent years must be recognized as a primary driver of the ongoing forward progress (2, 4–9). In particular, a critical contribution came as a report from an expert panel assembled by the American College of Medical Genetics (ACMG) (10–11). This effort was commissioned by the Maternal and Child Health Bureau through a contract from the Health Resources and Services Administration (HRSA) to outline a process for the definition, among others outcomes, of a panel of conditions to be recommended for universal and uniform inclusion in state newborn screening programs. The expert panel identified a panel of 29 conditions, a list routinely referred to as the uniform panel. Twenty of the primary conditions are screened for by MS/MS analysis of amino acids and acylcarnitines, and selected ratios (5, 12). An additional 25 conditions, 22 of them also detected by MS/MS, were identified in a cohort of so-called secondary targets. Most of them are part of the differential

diagnosis of one or more conditions in the core panel. The consideration given to the secondary targets has been controversial because, with few exceptions, their incidence, natural history, prospective screening experience, and effectiveness of treatment have not yet been defined (13). However, a defining characteristic of a multiplex platform like MS/MS is the need to perform an elaborate differential diagnosis for most of the metabolites detectable in the amino acid and acylcarnitine profiles (2, 5, 14–15). It seems therefore reasonable to underscore the importance of developing tools for better confirmatory testing and differential diagnosis of all detectable conditions, rather than debating the artificial exclusion of one or more rare conditions that are detected anyway in a profile mode, at no additional cost, and could be misinterpreted as false positives of a better-known condition (16).

Since the publication of the ACMG report, it has become a reality to regard this panel of conditions as the established target of newborn screening by MS/MS, and we concur with such approach even though it is increasingly apparent that there are additional conditions potentially detectable by analysis of the same amino acid and acylcarnitine markers (17–20). This observation suggests a need in the near future to update the list of secondary targets, and possibly upgrade a few of the existing ones to a status of primary target on the basis of new evidence obtained after the implementation of expanded screening by MS/MS (21–22).

To date, a multiplex platform for the simultaneous screening at birth of at least 42 metabolic disorders should be considered an accepted standard of care, of which a full nationwide implementation is likely to be completed very soon (16). Therefore, it is important to shift our collective attention and evaluation of evidence from quantity (how many conditions) to quality (how well we screen for) issues, particularly the monitoring of objective metrics and the definition of targets of adequate performance (2).

Table 1 summarizes the 42 conditions included in the panel recommended by ACMG plus three additional conditions, which are examples of the additional findings to be encountered by MS/MS testing. In addition to an updated list of primary markers and informative ratios, this Table also covers a few emerging aspects of newborn screening by MS/MS, namely the possibility of detecting maternal cases (i.e., an abnormal result of the screening is secondary to a maternal

biochemical phenotype; 23–24) and of observing interference caused by diet, drugs, modality of treatment, and prematurity. Artifacts may be either true elevations of a marker or interference by isobaric compounds (25). Another element of Table 1 is the current status of second tier test availability, an element of increasing importance and recognition in the definition of acceptable targets of performance by MS/MS or any other multiplex platform (2, 26–27). The ability to verify an abnormal result of the primary screening without a recall of the newborn is appealing and likely to increase cost effectiveness (28–30), not to mention the benefit of preventing unnecessary distress of the newborn's family (31). As another, indirect frame of reference, Table 1 includes the number of true-positive cases included as of May 2007 in a cumulative database assembled by a HRSA regional collaborative project entitled "Laboratory Quality Improvement of Newborn Screening by MS/MS" (32). To date, 38 US states and 33 laboratories in 20 countries are actively contributing data to this database. The rationale here is to gauge how likely it will be to collect enough cases within a reasonable period of time to provide an objective, truly evidence-based evaluation of each condition.

Finally, Table 1 shows a summary of validation scores derived from the ACMG report (11). At least two experts rated on a 1 to 4 scale the evidence in support of, or against, the inclusion of a given condition in the recommended panel for four elements (condition, tests, diagnosis, and treatment), according to the levels of evidence defined by the American Academy of Pediatrics (AAP) Steering Committee on Quality Improvement and Management (33). The numeric values presented in Table 1 are the average of these scores (11). This informal assessment of a prior evidence review process was included simply as a point of reference for the strength of the recommendations of this report, shown in the far right column of Table 1, which are based on current LMPG criteria, modified from the US Preventive Services Task Force Recommendations for Preventive Service (33). Seven conditions received an A-I rating, the highest possible, 31 of the remaining 35 conditions included in the ACMG panel are recommended for adoption although the available evidence at this time is limited, or indirect. Four conditions, all but one not yet detected prospectively in a patient by newborn screening, should be re-evaluated regularly to verify that lack of detection could be at least in part a function of improperly set cutoff values.

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Group	Condition (inborn errors of amino acid, fatty acid, and organic acid metabolism)	ACMG code	US incidence	ACMG panel	Primary marker(s)	Informative ratios	Interpretation requires differential diagnosis	Primary or other conditions with same marker(s)	Secondary or other conditions with same markers	Maternal cases detected by NBS	Potential interference by diet, drugs, prematurity	No. of cases	Available 2nd tier test	Evidence level in 2006 ACMG report (1-4)	Evaluation of evidence (LMPG criteria)
(1)	(1)	(1)	(2)	(3)	(4)	(5)	(6)	(6)	(6)	(7)	(8)	(9)	(10)	(11)	(12)
AA	Argininosuccinic acidemia	ASA	<1:100,000	UP	Cit	Cit/Arg	Yes	CIT	CIT II, PC	-	-	13	-	2.50	B-II
AA	Citrullinemia	CIT	<1:100,000	UP	Cit	Cit/Arg	Yes	ASA	CIT II, PC	-	-	73	-	3.00	B-II
AA	Homocystinuria (CBS deficiency)	HCY	<1:100,000	UP	Met	Met/Phe	Yes	-	MET	-	Yes	24	Yes	2.00	B-II
AA	Maple syrup (urine) disease	MSUD	<1:100,000	UP	Val Ile+Leu	Val/Phe (Ile+Leu)/Phe (Ile+Leu)/Ala	-	-	-	-	Yes	99	Yes	2.13	A-II
AA	Phenylketonuria	PKU	>1: 25,000	UP	Phe	Phe/Tyr	Yes	-	H-PHE, BIOPT (BS) & (REG)	Yes	Yes	608	-	2.00	A-I
AA	Tyrosinemia type I	TYR I	<1:100,000	UP	Tyr SUAC	Tyr/Cit	Yes	-	TYR II, TYR III	-	Yes	48	Yes	1.94	A-II
AA	Arginemia	ARG	<1:100,000	ST	Arg	-	-	-	-	-	-	7	-	3.50	B-II
AA	Benign Hyper-phenylalaninemia	H-PHE	<1:100,000	ST	Phe	Phe/Tyr	Yes	PKU	H-PHE, BIOPT (BS) & (REG)	-	-	385	-	n/a	A-I
AA	Citrullinemia type II	CIT II	<1:100,000	ST	Cit	Cit/Arg	Yes	ASA CIT	CIT II, PC	-	-	29	-	2.71	B-II
AA	Defects of bipterin cofactor biosynthesis	BIOPT (BS)	<1:100,000	ST	Phe	Phe/Tyr	Yes	PKU	H-PHE, BIOPT (REG)	-	Yes	3	-	2.00	A-I

AA	Disorders of bipterin cofactor regeneration	BIOPT (REG)	<1:100,000	ST	Phe	Phe/Tyr	Yes	PKU	H-PHE, BIOPT (BS)	-	Yes	4	-	2.50	A-I
AA	Hyper-methioninemia	MET	<1:100,000	ST	Met	Met/Phe	Yes	HCY	-	-	Yes	24	Yes	1.75	B-II
AA	Tyrosinemia type II	TYR II	<1:100,000	ST	Tyr	Tyr/Cit	Yes	TYR I	TYR III	-	Yes	18	Yes	2.38	B-II
AA	Tyrosinemia type III	TYR III	<1:100,000	ST	Tyr	Tyr/Cit	Yes	TYR I	TYR II	-	Yes	1	Yes	3.63	B-II
AA	Non ketotic hyperglycinemia	NKHG	<1:100,000	N/A	Gly	Gly/Ala	-	-	-	-	-	24	-	n/a	I
AA	Pyruvate carboxylase deficiency	PC	<1:100,000	N/A	Cit	Cit/Arg	Yes	ASA CIT	CIT II	-	-	2	-	n/a	I
FAO	Carnitine uptake defect	CUD	<1:100,000	UP	C0	(C0+C2+C3 +C16+C18:1)/CIT	Yes	GA I, 3MCC (mat)	-	Yes	Yes	60	-	2.25	A-II
FAO	Long-chain 3-OH acyl-CoA dehydrogenase deficiency	LCHAD	>1:75,000	UP	C16:1-OH C16-OH C18:1-OH C18-OH	C16-OH/C16	Yes	TFP	-	-	Yes	82	-	2.75	A-II
FAO	Medium-chain acyl-CoA dehydrogenase deficiency	MCAD	>1:25,000	UP	C6 C8 C10:1 C10	C8/C2 C8/C10	Yes	-	GA2, MCKAT	-	Yes	807	-	1.63	A-I
FAO	Trifunctional protein deficiency	TFP	<1:100,000	UP	C16:1-OH C16-OH C18:1-OH C18-OH	C16-OH/C16	Yes	LCHAD	-	-	Yes	See LCHAD	-	3.50	A-II
FAO	Very long-chain acyl-CoA dehydrogenase deficiency	VLCAD	>1:75,000	UP	C14:2 C14:1 C14	C14:1/C16	Yes	-	GA2	-	-	164	-	2.58	A-II
FAO	Dienoyl reductase deficiency	DE-RED	<1:100,000	ST	C10:2	C10:2/C10	-	-	-	-	-	1	-	4.00	I

(Continued)

Group	(1) Condition (inborn errors of amino acid, fatty acid, and organic acid metabolism)	(1) ACMG code	(2) US incidence	(3) ACMG panel	(4) Primary marker(s)	(5) Informative ratios	(6) Interpretation requires differential diagnosis	(6) Primary or other conditions with same marker(s)	(6) Secondary or other conditions with same markers	(7) Maternal cases detected by NBS	(8) Potential interference by diet, drugs, prematurity	(9) No. of cases	(10) Available 2nd tier test	(11) Evidence level in 2006 ACMG report (1-4)	(12) Evaluation of evidence (LMPG criteria)
FAO	Carnitine palmitoyl-transferase Ia deficiency (L)	CPT Ia	<1:100,000	ST	C0 (high) C16 (low) C18 (low)	C0/(C16+C18)	-	-	-	-	-	33	-	3.75	B-II
FAO	Carnitine palmitoyl-transferase II deficiency	CPT II	<1:100,000	ST	C16 C18:2 C18:1 C18	C0/(C16+C18)	Yes	-	CAC	-	-	20	-	3.38	B-II
FAO	Glutaric acidemia type II	GA2	<1:100,000	ST	C4-C18 saturated and unsaturated species	All ratios applicable to the primary markers	Yes	MCAD, GA I, IVA	SCAD, IBG, EE	-	-	38	-	3.38	B-II
FAO	Medium/short-chain 3-OH acyl-CoA dehydrogenase deficiency	M/SCH AD	>1:100,000	ST	C4-OH	-	-	-	-	-	-	0	-	4.00	I
FAO	Medium-chain ketoacyl-CoA dehydrogenase deficiency	MCKAT	<1:100,000	ST	C8	C8/C2 C8/C10	Yes	MCAD, GA2	-	-	Yes	0	-	4.00	I
FAO	Short-chain acyl-CoA dehydrogenase deficiency	SCAD	>1: 75,000	ST	C4	C4/C2 C4/C3 C4/C8	Yes	-	GA II, IBG, EE	-	-	251	-	2.63	I/ C-II*
FAO	Carnitine/acyl-carnitine translocase deficiency	CACT	<1:100,000	ST	C16 C18:2 C18:1 C18	C0/(C16+C18)	Yes	-	CPT II	-	-	5	-	2.58	B-II

OA	3-Methyl crotonyl-CoA carboxylase deficiency	3MCC	>1: 75,000	UP	C5OH	C5OH/C8 C5OH/C0	Yes	MCD HMG BKT	2M3HBA 3MGA	Yes	-	255	Yes	2.63	B-II
OA	3-Hydroxy 3-methyl glutaric aciduria	HMG	<1:100,000	UP	C5OH C6DC	C5OH/C8	Yes	3MCC MCD BKT	2M3HBA 3MGA	-	-	16	-	2.13	A-II
OA	Beta-ketothiolase deficiency	BKT	<1:100,000	UP	C5:1 C5OH	C5OH/C8	Yes	3MCC MCD HMG	2M3HBA 3MGA	-	-	9	-	3.50	A-II
OA	Glutaric acidemia type I	GA I	>1:75,000	UP	C5DC	C5DC/C5OH C5DC/C8 C5DC/C16	Yes	-	GA 2	Yes	-	106	-	2.25	A-I
OA	Isovaleric acidemia	IVA	<1:100,000	UP	C5	C5/C0 C5/C2 C5/C3	Yes	-	2MBG, GA2, EE	-	Yes	90	-	1.33	A-I
OA	Methylmalonic acidemia (A,B)	Cbl A,B	<1:100,000	UP	C3	C3/C2 C3/C16	Yes	MUT PA	Cbl C,D	-	Yes	see MUT	Yes	2.75	A-II
OA	Methylmalonic acidemia (Mut)	MUT	>1: 75,000	UP	C3	C3/C2 C3/C16	Yes	Cbl A,B PA	Cbl C,D	-	Yes	161	Yes	2.57	A-II
OA	Multiple carboxylase deficiency	MCD	<1:100,000	UP	C5OH	C5OH/C8	Yes	3MCC HMG BKT	2M3HBA 3MGA	-	-	5	-	2.33	B-II
OA	Propionic acidemia	PA	>1: 75,000	UP	C3	C3/C2 C3/C16	Yes	MUT Cbl A,B	Cbl C,D	-	Yes	73	-	1.50	A-II
OA	2-Methyl 3-hydroxy butyric aciduria	2M3H BA	<1:100,000	ST	C5-OH	C5OH/C8	Yes	3MCC MCD HMG BKT	3MGA	-	-	0	-	3.75	I
OA	2-Methyl butyryl-CoA dehydrogenase deficiency	2MBG	<1:100,000	ST	C5	C5/C0 C5/C2 C5/C3	Yes	IVA	GA2, EE	-	Yes	113	-	2.00	B-II
OA	3-Methyl glutamic aciduria	3MGA	<1:100,000	ST	C5OH	C5OH/C8	Yes	3MCC MCD HMG BKT	2M3HBA	Yes	-	7	-	2.50	B-II
OA	Isobutyryl-CoA dehydrogenase deficiency	IBG	<1:100,000	ST	C4	C4/C2 C4/C3 C4/C8	Yes	-	GA2, SCAD, EE	-	-	37	-	2.13	B-II
OA	Malonic aciduria	MAL	<1:100,000	ST	C3DC	C3DC/C10	-	-	-	-	-	10	-	4.00	B-II
OA	Methylmalonic acidemia (Cbl C,D)	Cbl C,D	<1:100,000	ST	C3	C3/C2 C3/C16 C3/Met	Yes	MUT Cbl A,B PA	-	-	Yes	41	Yes	2.75	A-II
OA	Ethylmalonic encephalopathy	EE	<1:100,000	N/A	C4 C5	All ratios applicable to the primary markers	-	IVA	SCAD, IBG, GA2	-	Yes	7	-	n/a	I

Table 1. Legend

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1. Nomenclature and abbreviations from reference 11 (ACMG main report, Table 1, pp. 5S–6S). PC is a disorder of the gluconeogenesis pathway listed here because of the potential diagnosis by finding of elevated citrulline
 2. From reference 11 (ACMG main report, Fact sheets, pp. 127S–215S)
 3. From reference 11 (ACMG main report, Table 7–8, pp.37S–38S). UP, uniform panel (primary targets); ST, secondary targets; N/A, condition not included in the panel
 4. Abbreviations according to reference 13, with modifications. Informative results are higher than normal unless indicated otherwise (low)
 5. The selection of these ratios are derived from the cumulative experience of the Region 4 collaborative project (Laboratory quality improvement of newborn screening by MS/MS; see www.region4genetics.org), and should be regarded merely as a suggestion.
 6. From reference 11 (ACMG main report, Table 6, p. 37S). (mat) indicates maternal cases
 7. Reported evidence of abnormal NBS results by newborn screening caused by a primary, previously undiagnosed maternal condition
 8. Artifacts include secondary elevations of informative markers due to diet and/or drug therapy. Artifacts may be either true elevations or interference by isobaric compounds
 9. Number of true positive cases included as May 2007 in the database of the Region 4 collaborative project (Laboratory quality improvement of newborn screening by MS/MS; see www.region4genetics.org). This information is provided as an approximated assessment of the evidence being gathered as result of the collaborative project, with no assumptions of estimated prevalence.
 10. A 2nd tier test is considered available when it is performed on a punch of the dried blood spot specimen analyzed by MS/MS, without notification/recall of the newborn
 11. From reference 11 (ACMG main report, Appendix 1, pp. 67S–76S). Values represent the average of 7 to 12 scores for a given condition. n/a, not available
 12. Strength of recommendation, modified from reference 33: US Preventive Services Task Force Recommendations for Preventive Service.
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*Committee was unable to reach a consensus on SCAD deficiency

Chapter 2

Pre-Analytical, Analytical, and Post-Analytical Issues Related to Follow-Up Testing of Positive Newborn Screens

Ronald J. Whitley, W. Harry Hannon, Dennis J. Dietzen, and Piero Rinaldo

Follow-up (confirmatory) testing of positive newborn screens requires a combination of additional methodologies, which may be more specific or more sensitive than the blood spot tandem mass spectrometric process that is used for whole population screening. This may include plasma or serum acycarnitine analysis by tandem mass spectrometry, plasma amino acid analysis by ion-exchange chromatography, and urine organic acid or acylglycine analysis by gas chromatography mass spectrometry. Although measurement of these metabolites has been available for many years, few guidelines for appropriate use of the analytical tools have been developed. Consequently, we have broken down each of the analyses for individual consideration. This has resulted in apparent redundancy in some instances but the Committee decided that this was an essential component for developing clear guidelines. A summary of the recommendations for each of the analytes precedes more detailed discussion of the recommendations. A number of appendices for each section are also listed.

MEASUREMENT OF AMINO ACIDS

Summary of Recommendations

Pre-Analytical Issues/Quality Requirements

1. Plasma (sodium or lithium heparin) is the preferred specimen type.
2. Urine amino acid analysis should be discouraged as a first-tier investigation.
3. Specimen collection requirements should be established by the laboratory and made available to referring physicians upon request.
4. Specimens spotted and dried on filter paper should be transported or mailed to the testing laboratory at ambient temperature. All other specimens should be placed on ice and promptly transported to the laboratory for processing and frozen storage.

Analytical Issues/Quality Requirements

5. The director of the testing laboratory should be a board-certified doctoral scientist or physician with specialized training and/or experience in biochemical genetics.
6. Known concentrations of non-isotopic amino acid reference calibrators should be prepared in an appropriate aqueous matrix.
7. For ion-exchange chromatography, two different compounds eluting in important parts of the chromatogram should be used as internal standards. For tandem mass spectrometry, stable-isotope amino acid internal standards should be used when possible.
8. Specimens should be deproteinized prior to analysis.
9. Chemical derivitization of amino acids is required for detection (e.g., ion-exchange chromatography).
10. Chemical derivitization of amino acids is recommended to enhance assay sensitivity and specificity (e.g., MS/MS).
11. Amino acids should be analyzed quantitatively by a reliable technique, such as automated cation-exchange liquid chromatography.
12. Amino acids should be analyzed quantitatively by a reliable technique, such as electrospray ionization tandem mass spectrometry.
13. Identification of amino acids by ion-exchange chromatography should primarily be based on chromatographic retention time, and retention time relative to an internal standard. Quantitation should be based on the recovery of the internal standard in each specimen compared to the recovery of the internal standard in the calibrators.
14. Qualitative screening methods, such as thin-layer chromatography (TLC), should not be used for amino acid analysis.
15. At least two control mixtures should be analyzed daily to monitor the ongoing performance of the analytic process.

16. Age-matched reference intervals (normal ranges) for reported amino acids should be established or verified by the testing laboratory for the population being investigated.
17. For analytes regulated by Centers for Medicare and Medicaid Services (CMS), the laboratory must participate in a CMS-approved provider proficiency testing (PT) program. Currently, amino acids are not regulated analytes. If the analyte is not regulated, the laboratory must have a mechanism for verifying the accuracy and reliability of its test at least two times per year. Participation in a formal PT provider's service may satisfy this requirement in the absence of a CMS-approved PT provider. The requirement may also be satisfied by an inter-laboratory sample exchange program and/or a custom-designed process within the laboratory. The laboratory must document performance, corrective and preventive actions, and maintain accurate records. PT samples must be handled in a manner identical to the unknown samples. Successful PT or performance assessment schemes are a condition of laboratory accreditation.

Post-Analytical Issues/Quality Requirements

18. Interpretation of test results should be based on relative amino acid levels, pattern recognition, and correlation of positive and negative findings.
19. Test reports should include appropriate patient and specimen information, test results, and clinical interpretation.
20. Substances that have the potential to interfere with the analysis should be identified and taken into account during interpretation.

Pre-Analytical Issues/Quality Requirements

Type of Specimen

Recommendation: Plasma (sodium or lithium heparin) is the preferred specimen type. Serum is generally considered to be less suitable for amino acid analysis.

Comments/Specific Examples: Gel tubes are acceptable. Thrombin-activated tubes are not recommended. Serum may be used but is less ideal; serum specimens generally clot at room temperature, a process that can lead to artifacts from deamination, conversion of arginine to ornithine by red blood cell arginase, release of oligopeptides, and loss of sulfur-containing amino acids due to protein binding (1). Dried blood spots collected on filter paper cards can be used. Cerebral spinal fluid (CSF) is useful in the diagnosis of nonketotic hyperglycemia and other disorders. Analysis of a simultaneous plasma specimen allows calculation of CSF/plasma amino acid ratios (2).

Strength of recommendation: A

Quality of Evidence: I

Type of Specimen

Recommendation: Urine amino acid analysis should be discouraged as a first-tier investigation.

Comments/Specific Examples: Amino acid concentrations are more variable in urine than in plasma due to factors such as renal function and greater interferences from medications. Analysis of urine is primarily indicated for the diagnosis of disorders affecting renal transport (eg, cystinuria, renal Fanconi syndrome) (3, 4).

Strength of recommendation: A

Quality of Evidence: I

Specimen Collection and Processing

Recommendation: Specimen collection requirements should be established by the laboratory and made available to referring physicians upon request.

Comments/Specific Examples: Hemolysis should be avoided when collecting blood specimens, since red blood cells and leukocytes contain high levels of certain amino acids (e.g., glycine, taurine, aspartic acid and glutamic acid). Plasma or serum must be promptly separated from cells (avoid collecting buffy coat material) and refrigerated (<4 hours) or frozen as soon as possible. In infants and newborns, blood should be collected immediately before the next scheduled feeding (2 to 3 hours after last meal) (5, 6). A random urine collection is satisfactory, since urine creatinine is used for normalization. Collection of a 24-hour urine specimen (kept refrigerated during collection) is rarely needed unless a specific disorder is suspected for which plasma is not informative, or unless urine analysis is valuable for the differential diagnosis. Collection of a 24-hour urine should be avoided when a patient is acutely ill, especially pediatric patients. Urine collections should avoid fecal contamination and the addition of preservatives. Urine specimens that are contaminated with bacteria (pH > 7 and/or nitrite positive) should be rejected. Urines should be mixed as soon as possible after collection, and aliquots should be immediately frozen to prevent loss of some amino acids (7). CSF should be collected in tubes without preservatives or anticoagulants free of blood contamination. CSF should be centrifuged to remove and separate cellular material, and the supernatant should be frozen immediately.

Strength of recommendation: A

Quality of Evidence: I

Specimen Transport and Storage

Recommendation: Specimens spotted and dried on filter paper should be transported or mailed to the testing laboratory

at ambient temperature. All other specimens should be placed on ice and promptly transported to the laboratory for processing and frozen storage.

Comments/Specific Examples: Amino acid degradation is largely arrested when specimens are frozen at -20°C for two months or at -80°C for longer periods. Glutamine and asparagine may gradually disappear even in frozen samples, with concomitant increases in glutamic acid and aspartic acid. Specimens that are analyzed at a distant testing laboratory should be transported on dry ice and kept frozen until analysis (8).

Strength of recommendation: A

Quality of Evidence: I

Analytical Issues/Quality Requirements

Calibrators

Recommendation: Known concentrations of non-isotopic amino acid reference calibrators should be prepared in an appropriate aqueous matrix. Performance characteristics (eg, linear range, analytical measurement range, lower limit of detection, imprecision, and accuracy) should be determined for all clinically informative amino acids, when possible.

Comments/Specific Examples: Reference calibrators are commercially available, either individual or pre-mixed, for all amino acids. It may be useful to include amino acids that are not usually present in physiologic specimens, such as alloisoleucine (9).

Strength of recommendation: A

Quality of Evidence: I

Internal Standards

Recommendation: For ion-exchange chromatography, two different compounds eluting in important parts of the chromatogram should be used as internal standards. For tandem mass spectrometry, stable-isotope amino acid internal standards should be used when possible.

Comments/Specific Examples: Typical internal standards for ion-exchange chromatography include aminoethylcysteine and glucosaminic acid, which are used to correct for any variation in the operating conditions of the analyzer over time. Stable-isotope internal standards (individual or pre-mixed) are available for tandem mass spectrometry from commercial source (see Appendix, Table 1 for a typical list). Internal standards should be added to all specimens, including calibrators and controls (10).

Strength of recommendation: A

Quality of Evidence: I

Specimen Preparation – Deproteinization/Extraction

Recommendation: Specimens should be deproteinized prior to analysis.

Comments/Specific Examples: For methods employing ion-exchange chromatography, a common method of specimen deproteinization is mixing the specimen and internal standards(s) with a concentrated acid, such as sulfosalicylic acid or trichloroacetic acid, in order to precipitate proteins and other large molecules. The pH of the supernatants or filtrates should be monitored and adjusted if necessary. The supernatant containing the water soluble amino acids can be stored at 4°C for up to 3 days (11). For methods employing tandem mass spectrometry, liquid-liquid and solid-phase extraction procedures are frequently used. Methanol is a common extraction solvent that also serves to deproteinize plasma samples.

Strength of recommendation: A

Quality of Evidence: I

Specimen Preparation—Derivatization & Ion Exchange Chromatography

Recommendation: Chemical derivatization of amino acids is required for detection (eg, ion-exchange chromatography).

Comments/Specific Examples: Derivatization of amino acids for ion-exchange chromatography can be accomplished either pre-column with o-phthalaldehyde or phenylisothiocyanate or post-column using ninhydrin (12). Post-column ninhydrin derivatization is preferable since it involves minimal sample handling and produces more consistent results (13).

Strength of recommendation: A

Quality of Evidence: I

Specimen Preparation—Derivatization & Tandem Mass Spectrometry

Recommendation: Chemical derivatization of amino acids is recommended to enhance assay sensitivity and specificity.

Comments/Specific Examples: Typically, amino acids are derivitized to their butyl esters using hydrogen chloride in butanol and heating at 65°C for 15 minutes. Butylation of amino acids is a useful step to improve detection limits and minimize ion suppression effects. Direct analysis of amino acids without chemical derivitization is also possible (14).

Strength of recommendation: B

Quality of Evidence: II

Detection/Data Acquisition

Recommendation: Amino acids should be analyzed quantitatively by a reliable technique such as automated cation-exchange column liquid chromatography.

Comments/Specific Examples: Ion-exchange chromatography is the most common method of amino acid separation and analysis. Several autosampler/ion exchange/detector configurations are commercially available. Most systems can resolve

and quantitate about 40 amino acid peaks in a 2- to 4-hour analytical run. High purity reagents are essential, and pH is critical to resolution (15–17).

Strength of recommendation: A

Quality of Evidence: I

Detection/Data Acquisition (Tandem Mass Spectrometry)

Recommendation: Amino acids should be analyzed quantitatively by a reliable technique, such as electrospray ionization tandem mass spectrometry.

Comments/Specific Examples: Tandem mass spectrometry is typically used to measure specific amino acids for newborn screening. However, tandem mass spectrometry is increasingly used to measure amino acids quantitatively for diagnosis and therapy assessment. For newborn screening, most acids are measured in the full-scan mode using a neutral loss of mass to charge (m/z) 102. For selected amino acids not adequately detected using a neutral loss scan, selected reaction monitoring scans can be used. For example, arginine can be detected using a neutral loss of m/z 161, citrulline and ornithine using a neutral loss of m/z 119, and glycine using a neutral loss of m/z 56. In the neutral-loss mode for analysis, all precursors sharing a common neutral fragment are detected (18). The precursor molecular weight $[M+H]^+$ ions, corresponding to amino acid butyl esters, are listed in the Appendix, Table 2. Neutral losses of underivatized amino acids are listed in the Appendix, Table 3. For MRM analysis, appropriate parent-product ion pairs are identified for each reported amino acid. Representative MRM transitions for derivitized and underivatized amino acids are listed in the Appendix (Tables 4 and 5, respectively). MRM allows optimization of experimental parameters for each amino acid individually, providing optimum sensitivity and selectivity.

Strength of recommendation: A

Quality of Evidence: I

Chromatogram Analysis and Quantitation (Ion-Exchange Chromatography)

Recommendation: Identification of amino acids by ion-exchange chromatography should primarily be based on chromatographic retention time, and retention time relative to an internal standard. Quantitation should be based on the recovery of the internal standard in each specimen compared with the recovery of the internal standard in the calibrators.

Comments/Specific Examples: Peaks which fall within predetermined limits of known amino acid retention times should be identified. Chromatograms should also be inspected for atypical peaks (eg, alloisoleucine, homocitrulline). If ninhydrin is used for detection, signal ratios at two wavelengths (570 nm and 440 nm) should be determined for proper identification of individual amino acids and for detection of

co-eluting interferences (19). For quantitation, calibration curves can be constructed for each amino acid by plotting the ratio of the peak height (or area) of the amino acid to the peak height (or area) of the corresponding internal standard versus calibrator concentrations.

Strength of recommendation: A

Quality of Evidence: I

Qualitative Analysis

Recommendation: Qualitative screening methods, such as thin-layer chromatography (TLC), should not be used for amino acid analysis.

Comments/Specific Examples: TLC is suitable only for detection of gross abnormalities. Quantitative analysis of plasma, urine, or CSF using more sensitive methods is recommended for the diagnosis and monitoring of disorders characterized by abnormal amino acids (3).

Strength of recommendation: A

Quality of Evidence: I

Quality Control

Recommendation: *At least two control materials should be analyzed daily to monitor the ongoing performance of the analytic process.*

Comments/Specific Examples: Controls should verify assay performance at relevant decision points. One control should contain analyte concentrations above the upper reference limit (ie, positive control), and a second control should be below the upper reference limit (ie, negative control). A third control might contain analyte concentrations at or near the upper reference limit. Valid acceptable ranges for each analyte should be established for each control material. External controls should be run with each new lot number/shipment of analytically critical reagents, after major preventive maintenance, or after change of a critical instrument component. If patient specimens are run in daily batches, these materials should be analyzed with each batch. Internal standard responses for each specimen may also serve as a quality control check (20).

Strength of recommendation: A

Quality of Evidence: I

Reference Intervals

Recommendation: Age-matched reference intervals (normal ranges) for reported amino acids should be established or verified by the testing laboratory for the population being investigated.

Comments/Specific Examples: Reference intervals of several amino acids are characteristically age dependent, thereby requiring test results be compared with a properly defined age group.

Comparison of pediatric results to normative ranges derived from an adult population is not appropriate. If a formal reference interval study is not possible, then the testing laboratory should carefully evaluate published data for its own reference ranges (21–24).

Strength of recommendation: A

Quality of Evidence: I

External Quality Assessment

Recommendation: For analytes regulated by Centers for Medicare and Medicaid Services (CMS), the laboratory must participate in a CMS approved–provider PT program. Currently, amino acids are not regulated analytes. If the analyte is not regulated, the laboratory must have a mechanism for verifying the accuracy and reliability of its test at least two times a year. Participation in a formal proficiency testing (PT) provider’s service may satisfy this requirement in the absence of a CMS-approved PT provider. The requirement may also be satisfied by an interlaboratory sample exchange program and/or a custom-designed process within the laboratory. The laboratory must document performance, corrective and preventive actions, and maintain accurate records. PT samples must be handled in a manner identical to the unknown samples. Successful PT or performance assessment schemes are a condition of laboratory accreditation.

Comments/Specific Examples: PT challenges should include quantitation of amino acids and an assessment of the laboratory’s capability to recognize disease biomarkers and profiles (25–27). The selected PT program or process must use test challenges in the same matrix as the patient sample. The College of American Pathologists (CAP) offers a PT program for amino acid profile analysis in a plasma matrix. The Newborn Screening Quality Assurance Program at the Centers for Disease Control and Prevention (CDC) offers dried blood spot PT and quality control programs for amino acids measured for identification of newborn disorders. Also an amino acid quality assurance program using lyophilized, spiked human serum is available from ERNDIM (the European Research Network for evaluation and improvement of screening, Diagnosis and treatment of Inherited disorders of Metabolism).

Strength of recommendation: A

Quality of Evidence: I

Post-Analytical Issues/Quality Requirements

Test Interpretation

Recommendation: Interpretation of test results should be based on relative amino acid levels, pattern recognition, and correlation of positive and negative findings.

Comments/Specific Examples: Disease states are often characterized by a pattern of elevated amino acids in comparison to

age-matched reference ranges. Amino acid elevation(s) or overall profiles should be evaluated in the context of clinical findings and/or additional test results. Interpretation of abnormal findings can be difficult and requires considerable knowledge not only of inherited metabolic disorders, but also of numerous physiological factors that affect amino acid concentrations (28–29).

Strength of recommendation: A

Quality of Evidence: I

Patient Reports

Recommendation: Test reports should include appropriate patient and specimen information, test results, and clinical interpretation.

Comments/Specific Examples: Laboratory reports should be designed to convey patient results effectively to a non-expert physician. This includes documentation of the analytical method used and clinical interpretation of the test results. The purpose for performing the test should also be described (eg, screening follow-up). Identification of all relevant amino acids should be listed, and quantitative results should be reported with appropriate age-matched reference intervals. A detailed interpretive report of abnormal results should include an overview of the significance of the test results, correlation to available clinical information and/or additional test results, differential diagnosis, and recommendations for further confirmatory biochemical testing (eg, enzyme assay, molecular analysis). When no abnormalities are detected, test results can be reported qualitatively (3).

Strength of recommendation: A

Quality of Evidence: I

Interferences/Artifacts

Recommendation: Substances that have the potential to interfere with the amino acid analysis should be identified and taken into account during interpretation.

Comments/Specific Examples: Many medications and dietary artifacts affect test results (eg, increase in glycine due to valproate therapy) (1). Bacterial contamination enhances conversion of glutamine and asparagine to glutamic and aspartic acids, and conversion of cystathionine to homocystine, mimicking homocystinuria; decreases of glycine, alanine, proline, and other amino acids may be seen in urine. Tryptophan may be lost due to deproteinization, while delayed deproteinization may cause loss of disulfide-containing amino acids. The same effect occurs during clotting, making serum unsuitable for these amino acids. Hemolysis and/or contamination of plasma and serum with blood cells may lead to increased levels of several amino acids (30).

Strength of recommendation: A

Quality of Evidence: I

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APPENDIX

Table 1. Stable Isotope Internal Standards

d ₄ -Alanine	d ₂ -Ornithine
d ₄ , ¹³ C-Arginine	d ₅ -Phenylalanine
d ₂ -Citrulline	¹³ C ₆ -Tyrosine
¹⁵ N, ²⁻¹³ C-Glycine	d ₈ -Valine
d ₃ -Leucine	d ₃ -Aspartate
d ₃ -Methionine	d ₃ -Glutamate

Table 2. Neutral Losses of Amino Acid Butyl Esters ([M+H]⁺ ions are shown)

Amino Acid	[M+H] ⁺ Ions (m/z)	Neutral Losses
L-Alanine	146	102
L-Arginine	231	161
L-Asparagine		
L-Aspartic acid	246	
beta-Alanine		
L-Citrulline	232	119 (102, 17)
L-Lysine	203	
L-Glutamic acid	260	
Glycine	132	
L-Histidine	212	
L-Hydroxyproline		
L-Leucine + L-Isoleucine + L-Hydroxyproline	188	
L-Methionine	206	102
L-Ornithine	189	
L-Phenylalanine	222	102
L-Proline	172	
L-Serine	162	
L-Threonine	176	
L-Tryptophan	261	
L-Tyrosine	238	
L-Valine	174	

Table 3. Neutral Losses of Underivitized Amino Acids ([M+H]⁺ ion masses are shown)

Amino Acid	[M+H] ⁺ Precursor Ions (m/z)	Neutral Losses
L-Alanine	90	46 (HCOOH = formic acid)
L-Arginine	175	105 (HCOOH and H ₂ NCNHNH ₂)
L-Asparagine	133	59 H ₃ CCONH ₂)
L-Aspartic acid	134	60 (H ₃ CCOOH = acetic acid)
beta-Alanine	90	18 (H ₂ O)
L-Citrulline	176	106 (HCOOH and H ₂ NCONH ₂)
L-Glutamine/ L-Lysine	147	63 (HCOOH and NH ₃)
L-Glutamic acid	148	46 (HCOOH = formic acid)
Glycine	76	46 (HCOOH = formic acid)
L-Histidine	156	46 (HCOOH = formic acid)
L-Hydroxyproline	132	64 (HCOOH, H ₂ O)
L-Leucine + L-Isoleucine + L-Hydroxyproline	132	46 (HCOOH = formic acid)
L-Methionine	150	94 (HCOOH and HSCH ₃)
L-Ornithine	133	63 (HCOOH and NH ₃)
L-Phenylalanine	166	46 (HCOOH = formic acid)
L-Proline	116	46 (HCOOH = formic acid)
L-Serine	106	46 (HCOOH = formic acid)
L-Threonine	120	46 (HCOOH = formic acid)
L-Tryptophan	205	17 (NH ₃)
L-Tyrosine	182	46 (HCOOH = formic acid)
L-Valine	118	46 (HCOOH = formic acid)

Table 4. Representative MRM Transitions for Butyl Esters of Amino Acids

Amino Acid	MRM Transition (Q1/Q3)
L-Alanine	146/44
L-Arginine	231/70
L-Asparagine	
L-Aspartic acid	246/144
beta-Alanine	
L-Citrulline	
L-Lysine	203/84
L-Glutamic acid	260/158
Glycine	132/76
L-Histidine	212/110
L-Hydroxyproline	
L-Leucine	188/43
L-Isoleucine	188/69
L-Methionine	206/104
L-Ornithine	189/70
L-Phenylalanine	222/120
L-Proline	172/70
L-Serine	162/60
L-Threonine	176/74
L-Tryptophan	261/244
L-Tyrosine	238/136
L-Valine	174/72

Table 5. Representative MRM Transitions for Underivitized Amino Acids

Amino Acid	MRM Transition (Q1/Q3)
L-Alanine	90/44
L-Arginine	175/70
L-Asparagine	133/74
L-Aspartic acid	134/74
beta-Alanine	90/72
L-Citrulline	176/70
L-Glutamine/L-Lysine	147/84
L-Glutamic acid	148/84
Glycine	76/30
L-Histidine	156/110
L-Hydroxyproline	132/68
L-Leucine + L-Isoleucine + L-Hydroxyproline	132/86
L-Methionine	150/56
L-Ornithine	133/70
L-Phenylalanine	166/120
L-Proline	116/70
L-Serine	106/60
L-Threonine	120/74
L-Tryptophan	205/188
L-Tyrosine	182/136
L-Valine	118/72

MEASUREMENT OF ACYLCARNITINES

Summary of Recommendations

Pre-Analytical Issues/Quality Requirements

1. Preferred specimen type for testing of symptomatic and asymptomatic individuals is plasma (sodium or lithium heparin) or serum.
2. Specimen collection requirements should be established by the laboratory and made available to referring physicians upon request.
3. Specimens spotted and dried on filter paper should be transported or mailed to the testing laboratory at ambient temperature. All other specimen types should be transported to the testing laboratory on dry ice and kept frozen until analysis.
4. Specimens should be collected and transported to the testing laboratory within 24/48 hours. Specimen analysis should be performed with a 24-hour turnaround time.

Analytical Issues/Quality Requirements

5. The director of the testing laboratory should be a board-certified doctoral scientist or physician with specialized training and/or experience in biochemical genetics.
6. Instrument optimization, method validation, and clinical testing should be performed by personnel with specialized training in the operation of MS/MS.

7. Stable-isotope acylcarnitine internal standards should be used. These labeled internal standards should be identical to the acylcarnitine of interest.
8. Known concentrations of non-isotopic acylcarnitine reference calibrators should be prepared in an appropriate matrix. Performance characteristics (eg, linear range, analytical measurement range, lower limit of detection) should be determined for all clinically informative acylcarnitines, when possible.
9. At least two control materials should be analyzed with every batch of specimens tested to monitor the ongoing performance of the analytic process.
10. Reference intervals (normal ranges) for reported acylcarnitines should be established or verified by the testing laboratory for the population being investigated.
11. Chemical derivitization of acylcarnitines may be necessary to enhance assay sensitivity and specificity.
12. Acylcarnitines should be isolated from the specimen matrix prior to detection.
13. Acylcarnitines should be analyzed by electrospray ionization tandem mass spectrometry (MS/MS) in the positive-ion mode, using either precursor-ion scan or multiple reaction monitoring (MRM), or both.
14. Chromatographic separation should be considered when separation of acylcarnitine isomers is desired or clinically relevant.
15. If the analyte is regulated by Centers for Medicare and Medicaid Services (CMS), the laboratory must participate in a CMS approved-provider PT program. If the analyte is not regulated, the laboratory must have a mechanism for verifying the accuracy and reliability of its test at least two times a year. Participation in a formal proficiency testing (PT) provider's service may satisfy this requirement in the absence of CMS-approved PT provider. An interlaboratory specimen exchange program may also satisfy the requirement and/or a custom-designed process within the laboratory. The laboratory must document performance, corrective and preventive actions, audits, and maintain accurate records.

Post-Analytical Issues/Quality Requirements

16. Interpretation of test results should be based on both pattern recognition and correlation of quantitative findings.
17. Test reports should contain appropriate patient and specimen information.
18. Substances that have the potential to interfere with the analysis should be identified.
19. Signal intensities should be used to produce quantitative information.

Pre-Analytical Issues/Quality Requirements

Type of Specimen

Recommendation: Preferred specimen type for testing of symptomatic and asymptomatic individuals is plasma (sodium or lithium heparin) or serum.

Comments/Specific Examples: Gel tubes are acceptable. Dried blood spots collected on filter paper cards can be used (1–4). Urine is less frequently used but may be valuable in investigation of inconclusive/borderline urine organic acid and plasma acylcarnitine profiles (eg, detecting glutaric academia type I, biotinidase deficiency, and isobutyryl-CoA dehydrogenase deficiency) (5). Post-mortem screening can be performed on dried blood spots or bile (4). Cell-free supernatant of amniotic fluid can be used for prenatal diagnosis of selected inborn errors of metabolism.

Strength of recommendation: A

Quality of Evidence: I

Specimen Collection and Processing

Recommendation: Specimen collection requirements should be established by the laboratory and made available to referring physicians upon request.

Comments/Specific Examples: Acylcarnitines are unstable, and plasma/serum should be separated from cells and frozen as soon as possible. Typical specimen testing volumes are listed in the Appendix Table 1. Clinical information is needed for appropriate interpretation. Additional required information includes age, sex, diet (eg, total parenteral nutrition therapy), drug therapy, and family history (6). No strong evidence of diurnal variation, so specimens can be collected at any time of day. Acylcarnitine levels vary with patient age (7).

Strength of recommendation: B

Quality of Evidence: II

Specimen Transport and Storage

Recommendation: Specimens spotted and dried on filter paper should be transported or mailed to the testing laboratory at ambient temperature. All other specimen types should be transported to the testing laboratory on dry ice and kept frozen until analysis.

Comments/Specific Examples: Compounds are stable indefinitely when stored at -80°C . Long-term storage at room temperature causes progressive losses, with degradation of short-chain acylcarnitines occurring faster than long-chain species (6, 8).

Strength of recommendation: B

Quality of Evidence: II

Emergency Specimen Collection

Recommendation: Specimens should be collected and transported to the testing laboratory as quickly as possible. Specimen analysis should be performed within 24 hours or fewer.

Comments/Specific Examples: In life-threatening situations, where an abnormality is thought to be likely (either from family history, results of preliminary investigations or clinical presentation), there should be no delay in specimen transport and/or delay in specimen analysis. At the earliest opportunity, the testing laboratory should be contacted to discuss appropriate investigations. If possible, specimens should be collected before death (9).

Strength of recommendation: B

Quality of Evidence: II

Analytical Issues/Quality Requirements

Laboratory Director

Recommendation: The director of the testing laboratory should be a board-certified doctoral scientist or physician with specialized training and/or experience in biochemical genetics.

Comments/Specific Examples: The laboratory director is responsible for the technical and scientific oversight of the testing laboratory and for the interpretation and reporting of results. At a minimum, the director should meet the personnel standards for high complexity as defined by CLIA '88. Board certification in clinical chemistry, clinical pathology, and/or biochemical genetics is recommended (10).

Strength of recommendation: A

Quality of Evidence: I

Testing Personnel

Recommendation: Instrument optimization, method validation, and clinical testing should be performed by personnel with specialized training in the operation of MS/MS.

Comments/Specific Examples: Operators of MS/MS instruments should hold a minimum of a Bachelor of Science degree in a laboratory science or medical technology. Appropriate training and ongoing competency requirements for testing personnel performing acylcarnitine analysis should be established and documented (15).

Strength of recommendation: A

Quality of Evidence: I

Stable-Isotope Internal Standards

Recommendation: Stable isotope acylcarnitines internal standards should be used. These labeled internal standards should be identical to the acylcarnitine of interest.

Comments/Specific Examples: Stable isotope internal standards (individual and pre-mixed) are available from a variety of commercial sources. Typical internal standards for a complete carnitine/acylcarnitine profile are listed in Appendix (Table 2). Commercial suppliers should provide concentration

and stability data. If an acylcarnitine standard is not available in labeled form, then a close mass homologue should be substituted. For example, d3-C16 can be used to estimate the concentrations of C16, C16:1, C16OH, and C16:1OH (assumes acylcarnitines of same chain length have similar performance characteristics). Labeled internal standards should be validated by determining recoveries from weighed, non-isotopic acylcarnitine calibrators. Labeled internal standards should be analyzed with each specimen (6).

Strength of recommendation: A

Quality of Evidence: I

Calibrators

Recommendation: Known concentrations of non-isotopic acylcarnitine reference calibrators should be prepared in an appropriate matrix. Performance characteristics (eg, linear range, analytical measurement range, lower limit of detection) should be determined for all clinically informative acylcarnitines, when possible.

Comments/Specific Examples: Reference calibrators (Appendix, Table 3) are not available for all acylcarnitines, particularly hydroxyl and unsaturated species. Quantitation of these compounds should be extrapolated by application of the calibration for the nearest species of similar structure. Performance characteristics should be verified on a regular basis as specified by regulatory agencies. The matrix used for calibrators should be free of endogenous carnitine and acylcarnitines (6).

Strength of recommendation: A

Quality of Evidence: I

Specimen Preparation – Derivatization

Recommendation: Chemical derivatization of acylcarnitines is recommended to enhance assay sensitivity and specificity.

Comments/Specific Examples: Typically, acylcarnitines are derivitized to their butyl esters using hydrogen chloride in butanol and heating at 65°C for 15 minutes (11, 12, 14). Direct analysis of acylcarnitines without derivitization is also possible, and alternative derivitization methods including methylation are occasionally used (13).

Strength of recommendation: B

Quality of Evidence: II

Specimen Preparation – Deproteinization/Extraction

Recommendation: Acylcarnitines should be isolated from the specimen matrix prior to detection.

Comments/Specific Examples: Liquid-liquid and solid-phase extraction procedures are frequently used. Methanol is a common extraction solvent which also serves to deproteinize

plasma samples. Blood spots are dry samples, and a certain degree of accuracy and precision is lost because of variations in blood volumes in the disks and in extraction efficiency (12, 16).

Strength of recommendation: A

Quality of Evidence: I

Detection/Data Acquisition

Recommendation: Acylcarnitines should be analyzed by electrospray ionization tandem mass spectrometry (MS/MS) in the positive-ion mode, using either precursor-ion scan or multiple reaction monitoring (MRM), or both.

Comments/Specific Examples: In the precursor-ion mode for analysis of butylated derivatives, all precursors of products m/z 85 within a mass range between 200 to 500 m/z are recorded. The parent ions (molecular weight $[M+H]^+$ ions), corresponding to the butyl ester derivatives, are listed in the Appendix, Table 4. Advantages of the precursor-ion full scan mode include evaluation of all potential acylcarnitines, detection of common drug artifacts, presence of interfering compounds, and evidence of poor derivitization. For MRM analysis, appropriate parent-product ion pairs are identified for each reported acylcarnitine. Representative MRM transitions for underivatized acylcarnitines are listed in the Appendix, Table 5. MRM allows optimization of experimental parameters for each acylcarnitine individually, providing optimum sensitivity and selectivity. In addition to being more specific and thus more precise, MRM may also allow for selective measurement of particular acylcarnitines (6, 7, 12).

Strength of recommendation: A

Quality of Evidence: I

Chromatographic Separation

Recommendation: Liquid chromatographic separation should be considered when separation of acylcarnitine isomers is desired or clinically relevant.

Comments/Specific Examples: Accurate determination of individual acylcarnitine isomers by MS/MS requires chromatographic separation (6, 17).

Strength of recommendation: B

Quality of Evidence: II

Quality Control

Recommendation: At least two control materials should be analyzed with every batch of specimens tested to monitor the ongoing performance of the analytic process.

Comments/Specific Examples: One control should contain analyte concentrations above the upper reference limit (ie, positive control), and a second control should be below the upper reference limit (ie, negative control). A third control might

contain analyte concentrations at or near the upper reference limit. Valid acceptable ranges should be established for each control material (18).

Strength of recommendation: A

Quality of Evidence: I

Reference Intervals

Recommendation: Reference intervals (normal ranges) for reported acylcarnitines should be established or verified by the testing laboratory for the population being investigated.

Comments/Specific Examples: Reference intervals must be age matched. Comparison of pediatric results to normative ranges derived from an adult population is not appropriate. If a formal reference interval study is not possible, then the testing laboratory should carefully evaluate published data for its own reference ranges (8, 14).

Strength of recommendation: A

Quality of Evidence: I

External Quality Assessment

Recommendation: If the analyte is regulated by Centers for Medicare and Medicaid Services (CMS), the laboratory must participate in a CMS approved–provider PT program. Currently, acylcarnitines are not regulated analytes. If the analyte is not regulated, the laboratory must have a mechanism for verifying the accuracy and reliability of its test at least two times a year. Participation in a formal proficiency testing (PT) provider’s service may satisfy this requirement in the absence of a CMS-approved PT provider. An interlaboratory sample exchange program may also satisfy the requirement and/or a custom-designed process within the laboratory. The laboratory must document performance, corrective and preventive actions, and maintain accurate records. PT samples must be handled in a manner identical to the unknown samples. Successful PT or performance assessment schemes are a condition of laboratory accreditation.

Comments/Specific Examples: Proficiency testing (PT) challenges should include quantitation of acylcarnitines and an assessment of the laboratory’s capability to recognize disease biomarkers and profiles. The selected PT program or process must use test challenges in the same matrix as the patient sample. The College of American Pathologists (CAP) offers a PT program for acylcarnitine profile analysis in a plasma matrix. The Newborn Screening Quality Assurance Program at the Centers for Disease Control and Prevention (CDC) offers dried blood spot PT and quality control programs for acylcarnitines measured for identification of newborn disorders. Also a qualitative blood spot (35 to 50mL) acylcarnitine quality assurance program is available from ERNDIM (the European Research Network for evaluation and improvement of screening,

Diagnosis and treatment of Inherited disorders of Metabolism; 19, 20).

Strength of recommendation: A

Quality of Evidence: I

Post-Analytical Issues/Quality Requirements

Test Interpretation

Recommendation: Interpretation of test results should be based on both pattern recognition and correlation of quantitative findings.

Comments/Specific Examples: Disease states are often characterized by a pattern of elevated acylcarnitines in comparison to age-matched reference ranges (see Appendix, Table 6). Calculation of ratios (eg, C8/C10 ratio in MCAD deficiency) may also be useful in the interpretation of abnormal results (6, 21, 22).

Strength of recommendation: A

Quality of Evidence: I

Patient Reports

Recommendation: Test reports should contain appropriate patient and specimen information, test results, and clinical interpretation.

Comments/Specific Examples: Interpretation of abnormal results should include an overview of the significance of the test results, quantitative results with reference ranges, a correlation of available clinical information, elements of differential diagnosis, and recommendations for additional biochemical testing (23).

Strength of recommendation: A

Quality of Evidence: I

Interferences/Artifacts

Recommendation: Substances that have the potential to interfere with the analysis should be identified.

Comments/Specific Examples: Several drug interferences are known to induce false positive test results (eg, pivalic acid, valproic acid, cefotaxime). Other exogenous compounds (eg, drug metabolites, food additives, intravenous fluids containing dextrose) may result in the appearance of peaks with m/z values in close proximity to, or overlapping with, acylcarnitines of diagnostic significance. Dietary artifacts are also possible, usually related to food intake enriched with fatty acids (eg, ketogenic diet, MCT oil; 7, 24, 25).

Strength of recommendation: A

Quality of Evidence: I

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APPENDIX

Table 1. Typical Sample Testing Volumes

Sample Type	Testing Volume
Amniotic fluid (cell-free supernatant)	20 µL
Amniocyte culture medium	50 µL*
Bile (liquid specimen)	20 µL**
Bile dried spot (postmortem)	one 1/8" punch**
Blood dried spot (neonatal, postmortem)	one 1/8" or 3/16" punch
Cell culture medium	50 µL*
Plasma/serum	20 µL
Urine	Variable; standardized to creatinine

*Spotted on filter paper. **May require dilution if profile is abnormal.

Table 2. Stable Isotope Internal Standards

d ₉ -free carnitine	(d ₉ -C0)
d ₃ -Acetylcarnitine	(d ₃ -C2)
d ₃ -Propionylcarnitine	(d ₃ -C3)
d ₃ -Butyrlcarnitine	(d ₃ -C4)
d ₉ -Isovalerylcarnitine	(d ₉ -C5)
d ₃ -Hexanoylcarnitine	(d ₃ -C6)
d ₃ -Octanoylcarnitine	(d ₃ -C8)
d ₃ -Decanoylcarnitine	(d ₃ -C10)
d ₃ -Dodecanoylcarnitine (Lauroyl)	(d ₃ -C12)
d ₃ -Myristoylcarnitine (Tetradecanoyl)	(d ₃ -C14)
d ₃ -Palmitoylcarnitine (Hexadecanoyl)	(d ₃ -C16)
d ₃ -Octadecanoylcarnitine (Stearoyl)	(d ₃ -C18)
d ₆ -Glutaryl carnitine	(d ₆ -C5DC)

Table 3. Reference Calibrators

Free carnitine	(C0)
Acetylcarnitine	(C2)
Propionylcarnitine	(C3)
Butyrlcarnitine	(C4)
Isovalerylcarnitine	(C5)
Hexanoylcarnitine	(C6)
Octanoylcarnitine	(C8)
Decanoylcarnitine	(C10)
Lauroylcarnitine	(C12)
Myristoylcarnitine	(C14)
Palimitoylcarnitine	(C16)
Octadecanoylcarnitine	(C18)
Glutaryl carnitine	(C5DC)

Table 4. [M+H]⁺ Ions of Acylcarnitine-Butyl-Esters

Acyl-carnitine	Chain Length	[M+H] ⁺
Acetyl-	C2	260
Acrylyl-	C3:1	272
Propionyl-	C3	274
Formiminoglutamate (FIGLU)		287
Isobutyryl-/Butyryl-	C4	288
Tiglyl-	C5:1	300
Isovaleryl-/2-methylbutyryl-	C5	302
3-hydroxybutyryl	C4-OH	304
Hexanoyl-	C6	316
3-hydroxyisovaleryl-	C5-OH	318
2-methyl-3-hydroxybutyryl-		
Heptanoyl-	C7	330
3-hydroxyhexanoyl-	C6-OH	332
Octenoyl-	C8:1	342
Octanoyl-	C8	344
Malonyl-	C3-DC	360
Decadienoyl-	C10:2	368
Decenoyl-	C10:1	370
Decanoyl-	C10	372
Methylmalonyl-	C4-DC	374
3-hydroxydecanoyl-	C10:1-OH	386
Glutaryl-/3-hydroxydecanoyl-	C5-DC/ C10-OH	388
Dodecenoyl-	C12:1	398
Dodecanoyl- (lauroyl-)	C12	400
3-hydroxydodecenoyl-	C12:1-OH	414
3-hydroxydodecanoyl-	C12-OH	416
Tetradecadienoyl-	C14:2	424
Tetradecenoyl-	C14:1	426
Tetradecanoyl- (myristoyl-)	C14	428
3-hydroxytetradecenoyl-	C14:1-OH	442
3-hydroxytetradecenoyl-	C14-OH	444
Hexadecenoyl-	C16:1	454
Hexadecanoyl- (palmitoyl-)	C16	456
3-hydroxyhexadecenoyl-	C16:1-OH	470
3-hydroxyhexadecanoyl-	C16-OH	472
Octadecadienoyl-	C18:2	480
Octadecenoyl-	C18:1	482
Octadecanoyl- (stearyl-)	C18	484
3-hydroxyoctadecadienoyl-	C18:2-OH	496
3-hydroxyoctadecenoyl-	C18:1-OH	498
3-hydroxyoctadecanoyl-	C18-OH	502

Table 5. Representative Multiple Reaction Monitoring (MRM) Transitions for Underivatized Acylcarnitines

Acyl-carnitine	Chain Length	MRM Transition
Acetyl-	C2	204/85
Propionyl-	C3	218/85
Isobutyryl-/Butyryl-	C4	232/85
Isovaleryl-/2-methylbutyryl-	C5	246/85
Hexanoyl-	C6	260/85
Octanoyl-	C8	288/85
Decanoyl-	C10	316/85
Dodecanoyl- (lauroyl-)	C12	344/85
Tetradecanoyl- (myristoyl-)	C14	372/85
Hexadecanoyl- (palmitoyl-)	C16	400/85

Table 6. Basic Acylcarnitine Patterns Associated With Various Disease States

Designation	Acylcarnitine Name	Disorder
C0 ↓	Free Carnitine	Carnitine transporter deficiency Secondary carnitine deficiencies, insufficient dietary intake, renal tubulopathy, medications including valproate, other metabolic diseases (e.g., in organic acidemias and FAOs)
C0 ↑	Free Carnitine	CPTI deficiency (with C16, C18 ↓) Secondary to rhabdomyolysis
C3 ↑	Propionyl	Propionic acidemia Methylmalonic acidemias Multiple carboxylase deficiency (with C5-OH)
C4-OH ↑ C4 ↑	3-Hydroxybutyryl Butyryl	SCHAD deficiency SCAD deficiency Formiminoglutamic aciduria (with more prominent peak at m/z 287) Multiple acyl-CoA dehydrogenase deficiency (with C5, and other longer chain species)
	Isobutyryl	Isobutyryl-CoA dehydrogenase deficiency
C5 ↑	Isovaleryl Methylbutyryl	Isovaleric acidemia Methylbutyryl-CoA dehydrogenase deficiency
C5-OH ↑	3-Hydroxyisovaleryl	β-Methylcrotonyl-CoA carboxylase deficiency Hydroxymethylglutaryl-CoA lyase deficiency Biotinidase deficiency Methylglutaconic aciduria 3-Oxothiolase deficiency
	2-Methyl-3-Hydroxybutyryl	2-Methyl-3-hydroxybutyryl-CoA dehydrogenase deficiency
C5DC ↑ C8 ↑	Glutaryl Octanoyl	Glutaric acidemia type I MCAD deficiency (with C6, C10, C10:1)
C14:1 ↑	Tetradecenoyl	VLCAD deficiency (with C14, C14:2)
C16 ↑	Palmitoyl	CPTII deficiency (with C18:2, C18:1, C18) Carnitine/acyl carnitine translocase deficiency (with C18:2, C18:1, C18)
C16-OH ↑	3-Hydroxypalmitoyl	LCHAD deficiency (with C18:1-OH, C18-OH) Trifunctional protein deficiency (with C16:1-OH, C18:1-OH, C18-OH)

MEASUREMENT OF ORGANIC ACIDS**Summary of Recommendations****Pre-Analytical Issues/Quality Requirements**

1. Urine is the preferred specimen type for organic acid analysis.
2. Specimen collection requirements should be established by the laboratory and made available to referring physicians upon request.
3. Urine specimens should be promptly transported to the laboratory for processing and frozen storage.

Analytical Issues/Quality Requirements

4. The director of the testing laboratory should be a board-certified doctoral scientist or physician with specialized training and/or experience in biochemical genetics.
5. The urine creatinine concentration should be measured and used to standardize specimen preparation and to determine relative concentration.
6. One or more different compounds should be used as internal standards.
7. Known concentrations of non-isotopic reference calibrators that elute at various points during the chromatographic run should be prepared in an appropriate matrix. Performance characteristics (eg, linear range, analytical measurement range, lower limit of detection) should be determined for all quantified organic acids, when possible, and verified on a regular basis.
8. Oximation of ketoacids may be performed routinely or as a reflex testing under specific clinical circumstances (eg, suspicion of maple syrup urine disease or tyrosinemia type I).
9. Organic acids should be isolated from the specimen matrix prior to analysis.
10. Chemical derivitization of organic acids is required for analysis by gas chromatography-mass spectrometry.
11. Capillary gas chromatography-mass spectrometry (GC-MS) should be used for analysis of organic acids in urine.
12. A temperature ramp should be used to elute organic acids with low volatility.
13. Mass spectral data should be collected in scan mode and presented as a “total ion current” (TIC) chromatogram.
14. Organic acids should be identified by evaluation of mass spectra.
15. For quantitative analysis without isotope dilution, the data should be acquired either in scan mode with integration of extracted ion chromatograms, or in SIM mode.

16. Isotope ratio mass spectrometry using stable-isotope labeled internal standards should be used for accurate quantitation of selected organic acids.
17. A quality control program based on the analysis of normal and abnormal controls should be implemented and performed with patient specimens.
18. For quantitative reporting, reference intervals (normal ranges) for organic acids should be established or verified by the testing laboratory for the population being investigated.
19. For analytes regulated by Centers for Medicare and Medicaid Services (CMS), the laboratory must participate in a CMS approved-provider PT program. Currently, organic acids are not regulated analytes. If the analyte is not regulated, the laboratory must have a mechanism for verifying the accuracy and reliability of its test at least two times a year. Participation in a formal proficiency testing (PT) provider's service may satisfy this requirement in the absence of a CMS-approved PT provider. An interlaboratory sample exchange program may also satisfy the requirement and/or a custom-designed process within the laboratory. The laboratory must document performance, corrective and preventive actions, and maintain accurate records. PT samples must be handled in a manner identical to the unknown samples. Successful PT or performance assessment schemes are a condition of laboratory accreditation.

Post-Analytical Issues/Quality Requirements

20. Interpretation of organic acid profiles should be based on pattern recognition and correlation of positive and negative findings.
21. Test reports should include appropriate patient and specimen information, test results, and clinical interpretation.
22. Substances that have the potential to interfere with the organic acid analysis should be identified and taken into account during interpretation.

Pre-Analytical Issues/Quality Requirements

Type of Specimen

Recommendation: Urine is the preferred specimen type for organic acid analysis.

Comments/Specific Examples: In rare instances, organic acids can be analyzed in heparinized plasma, cerebral spinal fluid (CSF), or vitreous humor. Collection of body fluids other than urine is indicated when a urine specimen cannot be obtained (eg, post-mortem). Quantitation of specific CSF metabolites has been reported to be helpful in identifying organic acid disorders in some patients (eg, "cerebral" lactic acidemias, disorders of biotin metabolism, glutaryl-CoA deficiency; 1–5).

Strength of recommendation: A

Quality of Evidence: I

Specimen Collection and Processing

Recommendation: Specimen collection requirements should be established by the laboratory and made available to referring physicians upon request.

Comments/Specific Examples: Any random or timed urine specimen may be collected.

Ideally, urine specimens should be obtained during the time of acute illness and metabolic decompensation (eg, first urine void after emergency admission), since abnormal metabolite levels often decrease to near-normal concentrations if specimens are collected only when the patient is apparently healthy. Urine specimens should be collected in clean containers without preservatives, and samples should be frozen as soon as possible after collection (4). Urine specimens collected and dried on filter paper strips can also be used (6).

Strength of recommendation: A

Quality of Evidence: I

Specimen Transport and Storage

Recommendation: Liquid urine specimens should be promptly transported to the laboratory for processing and frozen storage.

Comments/Specific Examples: Urine organic acids are stable for very long periods of time (several years) if stored at -20°C or colder. Freezing is not necessary if analysis is immediate. Specimens may be stored refrigerated for 24 hours after collection, but urines stored at ambient (room) temperature cause are unacceptable. Specimens that are analyzed at a distant testing laboratory should be shipped on dry ice via overnight courier. If freezing is not possible, or if dry ice is not available, then preservatives (eg, sodium azide, thymol, chloroform) may be added to prevent bacterial growth. Plasma, CSF, or vitreous humor should be frozen soon after collection (4). Specimens dried on filter paper strips may be transported or mailed to the testing laboratory at ambient temperature (7).

Strength of recommendation: A

Quality of Evidence: I

Analytical Issues/Quality Requirements

Specimen Preparation

Recommendation: The urine creatinine concentration should be measured and used to standardize specimen preparation and to determine relative concentration.

Comments/Specific Examples: A volume of thawed, thoroughly mixed urine equivalent to a constant amount of creatinine is used. Typically, a volume of urine corresponding to 0.25 mg (2.2 μ mol) of creatinine is used. The recommended minimum volume is 0.5 μ L and the maximum is 5.0 mL (8).

Strength of recommendation: A

Quality of Evidence: I

Internal Standard

Recommendation: One or more different compounds should be used as internal standards.

Comments/Specific Examples: The internal standard should not be a metabolite that might be detected in normal or pathologic urine, nor should it co-chromatograph with significant metabolites. Examples of internal standards include heptadecanoic acid, 2-phenylbutyric acid, and tropic acid. The final concentration of internal standard should be chosen to generate a peak on the total ion chromatogram that is clearly recognizable (9).

Strength of recommendation: A

Quality of Evidence: I

Calibrators/Calibration

Recommendation: Known concentrations of non-isotopic reference calibrators that elute at various points during the chromatographic run should be prepared in an appropriate matrix. Performance characteristics (eg, linear range, analytical measurement range, lower limit of detection) should be determined for all quantified organic acids, when possible, and verified on a regular basis.

Comments/Specific Examples: To generate calibration curves, calibration mixtures consisting of known standard compounds (eg, 10 to 15 analytes) should be prepared and carried through the entire sample preparation procedure. The use of standards not carried through the extraction procedure is discouraged since they do not control for different extraction efficiencies of various acids. Concentrations of reference calibrators should be corrected for endogenous organic acids present in the urine matrix (9).

Strength of recommendation: A

Quality of Evidence: I

Oximation

Recommendation: Oximation of ketoacids may be performed routinely, or as a reflex testing under specific clinical circumstances (eg, suspicion of maple syrup urine disease or tyrosinemia type I).

Comments/Specific Examples: The addition of an oximating reagent, such as hydroxylamine hydrochloride, serves to stabilize

alpha-keto acids that are present in urine. Important ketoacids include pyruvate, succinylacetone, and certain intermediates of branched chain amino acid metabolism (eg, 2-ketoisocaproic acid, 2-keto-3-methylvaleric, and 2-ketoisovaleric acids present in maple syrup urine disease; 1).

Strength of recommendation: B

Quality of Evidence: II

Specimen Extraction

Recommendation: Organic acids should be isolated from the specimen matrix prior to analysis.

Comments/Specific Examples: Liquid-liquid and solid-phase extraction procedures are frequently used. In a typical analysis, urine plus internal standard is acidified to pH 1-2 and extracted into an equal volume of an organic solvent. Ethyl acetate is most commonly used. For greatest efficiency, the specimen may be extracted up to five times. The addition of saturating amounts of sodium chloride prior to the extraction process may reduce the extraction of urea, which can interfere with the identification of other organic acids (10). Alternatively, the addition of urease prior to extraction reduces urea interference (11). Solid phase ion-exchange chromatography can also be used for specimen extraction (12). The addition of ammonia prior to evaporation may avoid loss of volatile acids.

Strength of recommendation: A

Quality of Evidence: I

Derivatization

Recommendation: Chemical derivatization of organic acids is required for analysis by gas chromatography-mass spectrometry.

Comments/Specific Examples: Most databases for organic acid spectra are based upon spectra generated procedure, organic acids in dried urine extracts are mixed with N5O5-bis-(trimethylsilyl) trifluoroacetamide with 1% trimethylchlorosilane (BSTFA/TCMS) and heated at 80°C for 30 minutes. A possible alternative to TMS derivatization is tert-butyl-dimethylsilylation (3).

Strength of recommendation: A

Quality of Evidence: I

Gas Chromatography-Mass Spectrometry

Recommendation: Capillary gas chromatography-mass spectrometry (GC-MS) should be used for analysis of organic acids in urine.

Comments/Specific Examples: The only acceptable method of analysis for urine organic acids is GC-MS. Gas chromatography alone without positive identification of compounds of complex

profiles should be discouraged. Tandem mass spectrometry may be used for analysis of targeted compounds but is not recommended for screening. Several GC-MS instrument configurations are commercially available. Mass spectra are obtained using electron impact (EI) ionization using quadrupole filter mass spectrometers (2).

Strength of recommendation: A

Quality of Evidence: I

Column

Recommendation: Capillary GC columns should be used to separate organic acids.

Comments/Specific Examples: A variety of capillary GC columns are used to separate organic acids with equivalent efficiency. Columns are typically 15 to 30 meters in length, 0.2 to 0.5 mm in internal diameter, and coated with a 0.1 to 1.0 μm layer of a liquid stationary phase (eg, OV-1, OV-17, DB-1, and SE-30). Sample injection onto the column should be in the split mode with a 1 to 3 μL injection and a split ratio of at least 1:15 to prevent column overload (4).

Strength of recommendation: A

Quality of Evidence: I

Column Temperature

Recommendation: A temperature ramp should be used to elute organic acids with low volatility.

Comments/Specific Examples: The initial oven temperature, hold time, rate of temperature ramp, highest temperature, and final fold time will determine the total run time, which is typically 30 to 60 minutes (4).

Strength of recommendation: A

Quality of Evidence: I

Data Acquisition—Qualitative Analysis

Recommendation: Mass spectral data should be collected in scan mode and presented as a total ion current (TIC) chromatogram.

Comments/Specific Examples: Data should be acquired in scan mode with a full-scale scan every 0.5 seconds. The range of ions scanned should be from m/z 50 to m/z 500 to 550. The abscissa (x -axis) of the TIC chromatogram represents the elution times of separated or co-eluting peaks from the gas chromatograph. The ordinate (y -axis) is the relative abundance of each compound in the analyzed specimen. Four variables determine the final appearance of the chromatogram: the volume of urine used for extraction, the final volume after derivitization, the injection volume, and the injection mode (split ratio; 4).

Strength of recommendation: A

Quality of Evidence: I

Compound Identification

Recommendation: Organic acids should be identified by evaluation of mass spectra.

Comments/Specific Examples: Compound identification is essential to diagnosing disorders of organic acid metabolism. The identification of each individual peak relies primarily on comparisons of mass spectral patterns with those of pure reference compounds. Spectral match should be greater than 80% in the presence of a known co-chromatographing peak in order to provide positive identification. A limited number of commercial libraries containing mass spectra of TMS derivatives of organic acids are available, but laboratories should also build their own in-house libraries based on experience and availability of organic acid standards and samples from patients with known organic acidurias. The methods and criteria for peak identification must be documented by the laboratory. Evaluation of chromatographic retention times, or relative retention times to an internal standard, is also useful, particularly for correct recognition of isomers or structurally similar compounds. Each chromatogram should be examined carefully for very small peaks which could indicate a clinically significant abnormality (eg, orotic acid, hexanoylglycine, 4-hydroxybutyrate, 3-hydroxyglutarate, succinylacetone). Attention should also be paid to areas of the chromatogram where complete or partial overlap of peaks might affect interpretation (see the Appendix Table 1 for a list of critical overlaps; 4). One hundred of the nearly 500 organic acids that have been identified in urine are listed in the Appendix (Table 2).

Strength of recommendation: A

Quality of Evidence: I

Data Acquisition—Quantitative Analysis Without Isotope Dilution

Recommendation: For quantitative analysis without isotope dilution, the data should be acquired either in scan mode with integration of extracted ion chromatograms, or in selected ion monitoring (SIM) mode.

Comments/Specific Examples: SIM is the standard method for compound specific analysis with improved sensitivity. Quantitation of organic acid concentrations should be performed in relation to known reference calibrators using at least one internal standard. Calibration curves should be constructed by plotting the ratio of the peak area of a (unique) organic acid ion to the peak area of an ion specific to the internal standard versus calibrator concentrations (13). Methods of organic acid analysis vary among testing laboratories, and quantitative values are often not comparable. Quantitative analysis is of value when monitoring therapy in some conditions, for instance, to evaluate the effects of vitamin B12 in patients with methylmalonic acidemia resulting from cobalamin defects. For this purpose, the data needs to be consistent from laboratory to laboratory to provide portability of resulting. Quantitative organic acid analysis is not yet sufficiently portable in the absence of isotope-labeled internal standards as evaluated from data accumulated by the College of American

Pathologists where the coefficient of variation of quantitative data is unacceptably high. At this time, the strongest recommendation for quantitative analysis without isotope dilution is to perform the analysis in only one laboratory for patient monitoring purposes (14).

Strength of recommendation: B

Quality of Evidence: II

Data Acquisition – Quantitative Analysis with Isotope Dilution

Recommendation: Isotope ratio mass spectrometry using stable isotope–labeled internal standards should be used for accurate quantitation of selected organic acids.

Comments/Specific Examples: In some cases, methods of higher sensitivity and specificity may be needed to overcome the limitations of standard organic acid analysis, particularly for the investigation of non-acutely ill patients whose organic acid concentrations are lower than 10 $\mu\text{mol/mol}$ creatinine. Data collection for this purpose should be in the selected ion monitoring mode using at least two ions for both internal standards and native compound. GC MS methods that use SIM and stable-isotope labeled internal standard are often used for quantitative analysis of acylglycines, methyl malonic acid (MMA), and orotic acid. In some high-volume testing laboratories, liquid chromatography tandem mass spectrometry (LC-MS/MS) methods are replacing GC-MS procedures (eg, determination of plasma and urine MMA; 5).

Strength of recommendation: A

Quality of Evidence: I

Quality Control

Recommendation: A quality control program based on the analysis of normal and abnormal controls should be implemented and performed with patient specimens.

Comments/Specific Examples: Quality control samples must use or simulate the urine matrix of the patient test samples as close as possible. Quality control samples must be handled in a manner identical to that used for patient samples in order to accurately reflect the performance of the assay. One control should contain analyte concentrations above the upper reference limit (ie, positive control), and a second control should be below the upper reference limit (ie, negative control). An optional third control might contain analyte concentrations at or near the upper reference limit. Valid acceptable ranges for each analyte should be established for each control material. The internal standard(s) in each specimen serves as a quality control check for each specimen (15).

Strength of recommendation: A

Quality of Evidence: I

Reference Intervals

Recommendation: For quantitative reporting, reference intervals (normal ranges) for organic acids should be established or verified by the testing laboratory for the population being investigated.

Comments/Specific Examples: Reference intervals of several organic acids in urine are characteristically age dependent, thereby requiring that quantitative results be compared with a properly defined age group. If a formal reference interval study is not possible, then the testing laboratory should carefully evaluate published data for its own reference ranges. Quantitation of urine organic acids is nearly always expressed relative to the concentration of creatinine (2, 4, 8).

Strength of recommendation: A

Quality of Evidence: I

External Quality Assessment

Recommendation: For analytes regulated by Centers for Medicare and Medicaid Services (CMS), the laboratory must participate in a CMS approved–provider PT program. Currently, organic acids are not regulated analytes. If the analyte is not regulated, the laboratory must have a mechanism for verifying the accuracy and reliability of its test at least two times a year. Participation in a formal proficiency testing (PT) provider's service may satisfy this requirement in the absence of a CMS-approved PT provider. An interlaboratory sample exchange program may also satisfy the requirement and/or a custom-designed process within the laboratory. The laboratory must document performance, corrective and preventive actions, and maintain accurate records. PT samples must be handled in a manner identical to the unknown samples. Successful PT or performance assessment schemes are a condition of laboratory accreditation.

Comments/Specific Examples: Proficiency testing (PT) challenges should evaluate analytical proficiency and provide an assessment of the laboratory's capability to recognize disease biomarkers and profiles. The selected PT program or process must use test challenges in the same matrix as the patient sample. The College of American Pathologists (CAP) offers a PT program for organic acids in a human urine matrix. The European Research Network for evaluation and improvement of screening, Diagnosis and treatment of Inherited disorders of Metabolism (ERNDIM) offers a quality assurance program for quantitative organic acids in urine (lyophilized spiked human urine). The Newborn Screening Quality Assurance Program at the Centers for Disease Control and Prevention (CDC) covers the dried blood spot matrix, which is the routine matrix for detection of newborn screening disorders (16).

Strength of recommendation: A

Quality of Evidence: I

Post-Analytical Issues/Quality Requirements

Interpretation of Organic Acid Profiles

Recommendation: Interpretation of organic acid profiles should be based on pattern recognition of abnormal organic acids and correlation of positive and negative findings.

Comments/Specific Examples: Meaningful interpretation of organic acid profiles is based on the overall pattern of organic acids present in abnormal quantities, not on individual abnormal values. Diagnostic specificity of organic acids may vary considerably, and the disease pattern may not always be obvious. For example, the organic acid profile in fatty acid oxidation disorders can appear normal when the patient is asymptomatic, yet can be vastly abnormal during an acute episode. Experience in interpreting both qualitative and quantitative reports is essential. The rarity of some organic acidurias means that very few laboratories have a great depth of experience. Accurate interpretation also depends on the availability of clinical information, especially family history and relevant diet and drug history. Informative profiles may not always be detected in disorders where excretion of diagnostic organic acids depend on the residual activity of the defective enzyme, the dietary load of precursors, and the anabolic status of a patient. An abnormal organic acid profile is not sufficient to establish conclusively a diagnosis, and confirmation by an independent method is recommended whenever practical (eg, acylcarnitine and amino acid analyses, in vitro enzyme assay, molecular analysis; 8, 16).

Strength of recommendation: A

Quality of Evidence: I

Patient Reports

Recommendation: Test reports should include appropriate patient and specimen information, test results, and clinical interpretation.

Comments/Specific Examples: Identification of all relevant compounds should be provided on the report. Some laboratories provide extensive quantitative reports, but there is no evidence that this format is clinically useful. Quantitative reports should be reported with appropriate age-matched reference intervals. When no clinically significant abnormalities are detected, the report may be interpreted in qualitative terms (eg, “normal”, “unremarkable” or “no specific abnormality detected”). When abnormal results are detected, a detailed interpretive report should include an overview of the significance of the test results, a correlation of available clinical information, elements of a differential diagnosis, recommendations for additional confirmatory biochemical testing, and contact information for the reporting laboratory (5).

Strength of recommendation: A

Quality of Evidence: I

Interferences/Artifacts

Recommendation: Substances that have the potential to interfere with the organic acid analysis should be identified and taken into account during interpretation.

Comments/Specific Examples: A number of different conditions that influence organic acid results. Dietary factors such as total parenteral nutrition, medium-chain triglyceride supplementation, and ketogenic diets produce significant artifactual changes. Medications, such as valproic acid, aspirin, and many others, can produce a wide variety of artifactual peaks. Many of these conditions should not hinder analysis provided the testing laboratory is provided a patient history that includes diet (eg, total parenteral nutrition) and drug therapy. The most common artifactual changes are due to bacterial contamination, which can be prevented by proper handling of specimens (18).

Strength of recommendation: A

Quality of Evidence: I

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APPENDIX

Table 1. Critical Organic Acid Overlaps

3-hydroxybutyric acid/3-hydroxyisobutyric acid
 urea/4-hydroxybutyric acid
 ethylmalonic acid/phosphoric acid
 3-methylglutaconic acid/3-hydroxyadipic lactone
 tiglylglycine/3-methylcrotonylglycine
 hexanoylglycine/4-hydroxyphenylacetic acid
 2-ketoglutarate/3-hydroxyglutarate

Table 2. Some Organic Acids Detected by GC-MS

2-Ethyl-3-hydroxypropionic	3-Hydroxyhexadecanoic	Benzoic	N-Acetylaspartic
2-Hydroxy-3-methylvaleric	3-Hydroxyisobutyric	Butyrylglycine	N-Acetyltyrosine
2-Hydroxyadipic	3-Hydroxyisovaleric	cis-4-Decenoic	Octanoic
2-Hydroxybutyric	3-Hydroxyoctanoic	Citric	Oleic
2-Hydroxydecanedioic	3-Hydroxypropionic	Decanoic	Orotic
2-Hydroxyglutaric	3-Hydroxytetradecanoic	Ethylmalonic (EMA)	Palmitic
2-Hydroxyisocaproic	3-Hydroxyvaleric	Fumaric	Palmitoleic
2-Hydroxyisovaleric	3-Methyladipic	Glutaconic	Phenylacetic
2-Hydroxyphenylacetic	3-Methylcrotonylglycine	Glutaric	Phenyllactic
2-Methyl-3-hydroxybutyric	3-Methylglutaconic	Glyceric	Phenylpropionylglycine
2-Methyl-3-hydroxyvaleric	3-MethylGlutaric	Glycolic	Phenylpyruvic
2-Methylacetoacetic	4-Hydroxybutyric	Glyoxylic	Pimelic
2-Methylbutyrylglycine	4-Hydroxycyclohexylacetic	Hexanoylglycine	Propionylglycine
2-Oxo-3-methylvaleric	4-Hydroxyisovaleric	Hippuric	Pyruvic
2-Oxoadipic	4-Hydroxyphenylacetic	Isobutyrylglycine	Sebacic
2-Oxoglutaric or alpha-ketoglutaric	4-Hydroxyphenyllactic	Isocitric	Stearic
2-Oxoisocaproic	4-Hydroxyphenylpyruvic	Isovalerylglycine	Suberic
2-Oxoisovaleric	5-Hydroxyhexanoic	Lactic	Suberylglycine
3-Hydroxy-3methylglutaric (HMG)	5-Hydroxymethyluracil	Lauric	Succinic
3-Hydroxyadipic	5-Oxoproline	Linoleic	Succinylcetone
3-Hydroxybutyric	Acetoacetic	Malic	Thymine
3-Hydroxydecanedioic	Aconitic	Malonic	Tiglylglycine
3-Hydroxydecanoic	Adipic	Methylcitric	Uracil
3-Hydroxydodecanoic	Azelaic	Methylmalonic (MMA)	Xanthin
3-Hydroxyglutaric		Methylsuccinic	
		Mevalonic	
		Myristic	

Summary of Strengths of Recommendations for This Section

	Amino Acids	Organic Acids	Acylcarnitines
PRE-ANALYTICAL			
Type of Specimen	A-I	A-I	A-I
Type of Specimen (urine amino acids)	A-II		
Specimen Collection and Processing	A-I	A-I	B-II
Specimen Transport and Storage	A-I	A-I	B-II
ANALYTICAL			
Laboratory Director			A-I
Testing Personnel			A-I
Acylcarnitines			
Stable Isotope Internal Standards			A-I
Internal Standards			A-I
Calibrators			A-I
Specimen Prep - Derivatization			B-II
Specimen Prep - Deproteinization/Extraction			A-I
Detection/Data Acquisition			A-I
Chromatographic Separation			B-II
Organic Acids			
Specimen Prep	A-I		
Internal Standards	A-I		
Calibrators/Calibration	A-I		
Oximation	B-II		
Specimen Extraction	A-I		
Derivatization	A-I		
Gas Chromatography - Mass Spectrometry	A-I		
Column	A-I		
Column Temperature	A-I		
Data Acquisition - Qualitative Analysis	A-I		
Compound Identification	A-I		
Data acquisition - Quantitative Analysis Without Isotope Dilution	B-II		
Data acquisition - Quantitative Analysis With Isotope Dilution	A-I		
Amino Acids			
Calibrators	A-I		
Internal standards	A-I		
Specimen Prep - Deproteinization/Extraction	A-I		
Specimen Prep - Derivatization & Ion Exchange Chromatography	A-I		
Specimen Prep - Derivatization & Tandem Mass Spectrometry	B-II		
Detection/Data Acquisition (Ion Exchange Chromatography)	A-I		
Detection/Data Acquisition (Tandem Mass Spectrometry)	A-I		
Chromatogram Analysis and Quantitation (Ion Exchange Chromatography)	A-I		
Qualitative Analysis	A-I		
Quality Control	A-I	A-I	A-I
Reference Intervals	A-I	A-I	A-I
External Quality Assessment	A-I	A-I	A-I
POST-ANALYTICAL			
Test Interpretation	A-I	A-I	A-I
Patient Reports	A-I	A-I	A-I
Interferences/Artifacts	A-I	A-I	A-I

Follow-Up of Positive Screening Results

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With the rapid expansion of newborn screening to include more newborns and more pathologic conditions comes an additional pool of individuals presenting to primary care providers, community hospitals, or tertiary care genetic centers for follow-up evaluation. With the exception of conditions that have historically been included in screening programs, this population presents with potential disorders that have not previously been encountered with great frequency in asymptomatic patients. For example, in the symptomatic phase, urea cycle disorders would present in the context of severe hyperammonemic encephalopathy, methylmalonic aciduria with acute acidosis and encephalopathy, and medium-chain acyl CoA dehydrogenase (MCAD) deficiency with hypoketotic hypoglycemia and liver failure. Metabolic findings in acute circumstances are usually pronounced and enable unequivocal diagnosis. In today's newborn screening environment, there is a high likelihood that neonates will present in an asymptomatic clinical phase of the illness requiring that laboratory tests and those that interpret them detect ever more subtle metabolic abnormalities.

In this context, the biochemical genetics laboratory plays a pivotal role in recommending and interpreting initial studies as well as dictating the necessity and type of further testing. The focus of this Chapter, therefore, is not on the diagnostic accuracy of newborn screening per se, but on the sensitivity and specificity of the sophisticated testing applied to infants referred for follow-up of a positive newborn screening result. Primary literature and expert opinion has guided the testing recommendations to follow. The rarity of these disorders generally precludes large, randomized studies of diagnostic accuracy. An exhaustive review of the literature, primarily case reports and series, were reviewed for evidence of biochemical abnormalities in affected, generally asymptomatic children.

GENERAL REQUIREMENTS OF THE FOLLOW-UP PROCESS

Abnormal screening results should be followed up promptly to avoid morbidity and mortality. The protocol for initial testing of blood spots varies among screening laboratories. A commonly employed protocol for newborn screening by MS/MS is illustrated in Figure 3-1. A sample is tested using MS/MS with a specific cut-off. If the sample value is below the cut-off, no further testing is done and the result is reported as negative.

When the value of a particular marker or set of markers exceeds the requisite cut-off, a second sample from the original blood spot is tested. If the second value is below the cut-off, the result is reported as negative and no further testing is done. If the value is above the cut-off, the case is categorized as low risk or high risk depending on the concentration of the marker. In the low risk category, another sample from the infant is collected and tested without the involvement of a genetic specialist due to the reasonable likelihood that the result is false positive.

If the result falls into a high-risk category, the primary care physician and a specialist are contacted and immediate follow-up commences. The follow-up includes clinical evaluation and confirmatory testing, the subject of this Chapter. Primary care physicians may be the first persons to be contacted and should be familiar with the initial management and follow-up. They should also be prepared to explain the positive results to the family and make arrangement for the referral.

Several desirable attributes characterize the role of the laboratory in the referral of the newborn. First, laboratory personnel should be among the first responders notified about the referral. Biochemical genetics laboratories typically operate in batch mode during normal working hours. Procedures often require many hours or even days to complete. Early notification will facilitate prompt laboratory analysis. Second, testing should be properly pointed to the suspected disorder(s). Children will be referred on the basis of an elevated marker. Only those tests that provide relevant, timely contribution to the narrowing the differential diagnosis should be undertaken. Recommendations for these pointed investigations are presented in Table 3-1, which is organized by disorder. The discussion that follows considers the scope of other disorders that should be dictated by each of the markers in the screen. Results of laboratory analyses should be interpreted promptly by an experienced laboratory director in consultation with the clinical team responsible for clinical assessment of the newborn. Finally, the follow-up circle should be closed by providing results to newborn screening centers to enhance understanding of the diagnostic performance of screening tests.

GENERAL RECOMMENDATIONS

For the reasons outlined above, we recommend adoption of the following practices with the highest level of evidence: A-I.

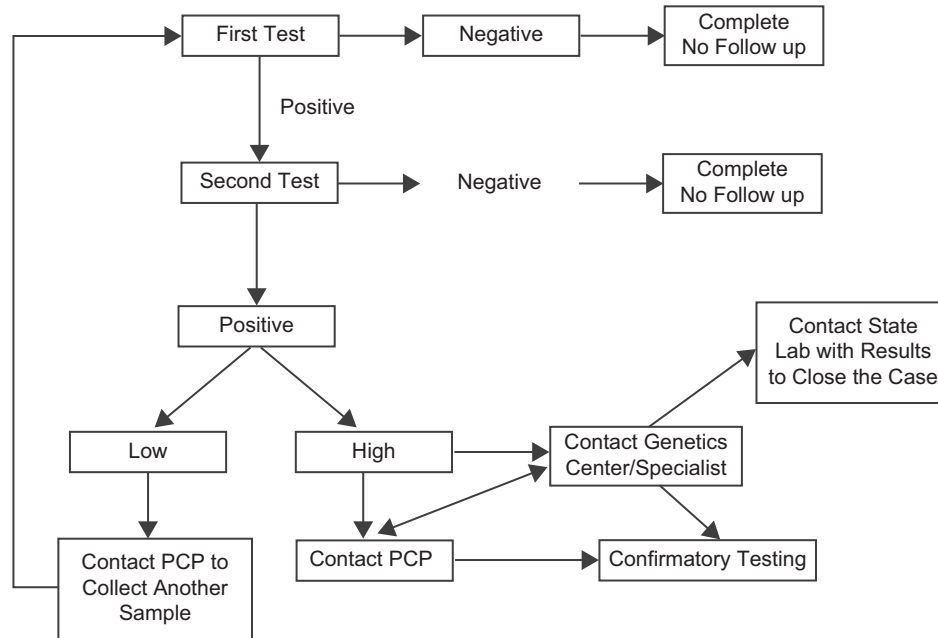


Figure 3-1 Follow-Up Protocol for Newborn Screening

1. Analytical methods used in the diagnosis of inborn metabolic disease must become more sensitive to subtle but pathologic alterations in metabolite concentrations. This includes but is not limited to appreciation of age-specific concentrations in well populations and changes in metabolite concentrations associated with maturation of organ systems, prematurity, diet, and other pathologic states not caused by inborn metabolic disease.
2. Metabolic profiles should be promptly interpreted by personnel expert in the requisite sophisticated analytic procedures and with knowledge of the biochemistry and pathophysiology of a broad spectrum of inborn metabolic disorders.
3. Metabolic laboratory personnel should be among the first notified of the impending referral of a screen-positive infant to arrange for test selection, prompt testing, or sample referral.
4. Initial follow-up testing should be targeted to providing a diagnosis with adequate definition to dictate the necessity for first-line therapy as rapidly as possible as outlined within this Chapter.
5. Follow-up laboratories should provide feedback to newborn screening centers to allow for continuous improvement in the diagnostic accuracy of screening tests.

DISEASE-SPECIFIC FOLLOW-UP TESTING FOR PRIMARY TARGETS

Table 3-1 includes recommendations organized by the 21 primary targets defined by the American college of Medical

Genetics (ACMG) working group (1) and detected by tandem MS protocols. Those primary ACMG targets not detected by tandem MS (eg, galactosemia, hemoglobinopathy, congenital hypothyroidism) are not considered here. When there is ample published evidence and experience to indicate that specific disorders will be detected in asymptomatic but affected infants, the recommendation is graded A-I. In cases where evidence is scant or indicates substantial episodic detection of abnormal metabolites, the recommendation is graded A-II. Finally, when evidence indicates that testing will detect specific disorders in symptomatic patients but evidence is lacking in regard to the asymptomatic infant, these recommendations are graded B-II.

SECONDARY TARGETS

In addition to the primary targets of screening tests, other so-called secondary disease targets are also contained in the differential diagnosis. In this section, the role of follow-up testing in narrowing the differential diagnosis is discussed and organized according to specific screening markers rather than disease state as in Table 3-1.

Amino Acid Markers

In general, initial follow-up testing requires comprehensive amino acid analysis done most frequently by cation exchange chromatography and photometric detection of amino acid-ninhydrin chromophores. In some instances, additional analyses noted are required to establish a specific diagnosis. When utility is clear, enzyme activity measurement and/or genetic analyses that provide important confirmatory information are also noted.

Table 3-1 Disease-Specific Follow-Up Testing Recommendations

Disorders of Amino Acid Catabolism and Transport	Screening Marker	Follow-Up Analyses	Follow-Up Markers	Additional Testing	Evidence	References
Phenylketonuria (includes benign hyperphenylalaninemia, and bipterin metabolic defects)	Phenylalanine Tyrosine	Plasma amino acids	Phenylalanine Tyrosine	Urine pterin metabolites Dihydropteridine reductase activity	A-I	23–26
Tyrosinemia Type 1	Tyrosine	Urine organic acids	Succinylacetone	No additional testing indicated	A-I	27–39
Type 2		Plasma amino acids	Tyrosine >1000 µM on presentation		A-I	
Maple Syrup Urine Disease	Isoleucine + leucine + alloisoleucine	Plasma amino acids	Isoleucine, Leucine, valine, alloisoleucine	No additional testing indicated	A-I	40–43
Citrullinemia Type 1 (ASA Synthase)	Citrulline	Plasma/Urine amino acids	Citrulline Argininosuccinate,	Ammonia, bilirubin, Alk Phos, GGT. Genetic testing may distinguish I and II.	A-I B-II	44–53
Type 2 (Citrin)	Citrulline					
Argininosuccinic acidemia	Citrulline	Plasma/urine amino acids	Argininosuccinate, Citrulline	No additional testing indicated	A-I	54–59
Homocystinuria	Methionine	Immunoassay	Homocysteine	Folate/Vitamin B12 status should be investigated.	A-I	60–65
		Plasma/urine amino acids	Methionine, Homocystine	Disorders of cobalamin cobalamin metabolism should also be considered.		
		Urine organic acids	Methylmalonic acid			
Disorders of Fatty Acid Oxidation	Screening Marker	Follow-Up Analyses	Follow-Up Markers	Additional Testing	Evidence	References
Medium Chain Acyl CoA Dehydrogenase	C6, C8, C10 acylcarnitine	Acylcarnitine analysis	C6, C8, C10 acylcarnitine species predominant A985G MCAD mutation	Molecular genetic analysis for	A-I	15, 66–72
Deficiency (MCAD)		Urine organic acids	Medium chain dicarboxylic acids, ketone bodies (low) hexanoylglycine, suberylglycine, 3-phenylpropionylglycine			
Very Long Chain Acyl CoA Dehydrogenase Deficiency (VLCAD)	C14:0, C14:1 acylcarnitine	Acylcarnitine analysis	C14:0, C14:1, C16:0, C16:1, C18:0, C18:1 acylcarnitine species	Activity assay generally not available. Genetic analysis may discriminate acute from later onset form.	A-II	16, 26, 73–74
		Urine organic acids	Increased long/medium chain dicarboxylic acids with limited ketosis.			
Long Chain Hydroxyacyl CoA Dehydrogenase Deficiency (LCHAD)/TFP deficiency	C16-OH, C18:1 OH acylcarnitine	Acylcarnitine analysis	C16-OH, C18-OH, C18-OH, acylcarnitine species	80–90% of alleles display G1528C. Distinction from TFP deficiency requires isolated activity assay	A-II	18, 75–77

(Continued)

Table 3-1 Disease-Specific Follow-Up Testing Recommendations (Cont'd)

Disorders of Fatty Acid Oxidation	Screening Marker	Follow-Up Analyses	Follow-Up Markers	Additional Testing	Evidence	References
		Urine organic acids	Increased 3-hydroxy-dicarboxylic aciduria with limited ketosis			
Short Chain Acyl CoA Dehydrogenase Deficiency (SCAD)	C4 carnitine	Urine organic acids	Ethylmalonic and methylsuccinic acids with normal ketosis. Butyrylglycine	Fibroblast fatty acid oxidation profile indicated when UOA equivocal.	B-II	78–83
Medium/Short Chain Hydroxyacyl CoA Dehydrogenase Deficiency (M/SCHAD)	C4-OH carnitine	Urine organic acids	3-OH adipic, 3-OH sebatic, 3-OH suberic acids. 3-OH glutarate	White cell enzyme assay, molecular testing	B-II	84–90
Primary Carnitine Deficiency	Free (C0) Carnitine	Free + Total Carnitine	Low total and free carnitine. Urine carnitine elevated	Secondary? Low fibroblast carnitine uptake	B-II	91–93
Organic Acidurias	Screening Marker	Follow-Up Analyses	Follow-Up Markers	Additional Testing	Evidence	References
B-ketothiolase Deficiency (T2)	C5-OH, C5:1 acylcarnitine	Urine organic acids	2-methyl-3-OH butyric, tiglylglycine, AcAc, 2 MeAcAC, butanone, 3-OH butyric	Episodic plasma glucose, anion gap. Enzyme assay (fibroblast) to confirm.	A-II	94–99
3-OH-3-Methylglutaryl CoA (HMGCoA) Lyase Deficiency	C5-OH, C6-DC, C6OH-DC acylcarnitine	Urine organic acids	3-OH-3-methylglutaric, 3-methylglutaric, 3-methylglutaconic, 3-OH isovaleric acids	<100% sensitivity. Mildly abnormal metabolite excretion dictates activity study	A-II	100–104
Glutaric Aciduria Type 1	C5DC (glutaryl) acylcarnitine		Glutaric, 3-OH glutaric		A-I	105–121
Type 2	C5DC, C5, C5OH, C6, C8, C10-C16 acylcarnitine	Urine organic acids	Glutaric, 2-OH glutaric, adipic, suberic, sebatic ethylmalonic, 3-OH isovaleric, isobutyric	No additional testing indicated. ETF activity is not readily available.	A-I	
Biotinidase Deficiency	C5-OH, C3 acylcarnitine	Biotinidase activity + urine organic acids	3-OH propionic, 3-OH isovaleric, tiglylglycine, 3-methylcrotonylglycine methylcitrate	Selected carboxylase activities + biotin to exclude multiple carboxylase def.	A-I	122–130
Multiple Carboxylase Deficiency	C5-OH, C3 acylcarnitine	Urine organic acids + plasma acylcarnitine	3-OH propionic, 3-OH isovaleric, tiglylglycine methylcitrate, 3-MCC (glycine), lactate	Biotinidase activity. Isolated carboxylase activities + biotin.	A-II	129–136
3-Methylcrotonyl CoA Carboxylase Deficiency	C5-OH acylcarnitine	Urine organic acids + plasma acylcarnitine	3-methylcrotonylglycine 3-OH isovaleric acid. 3-OH isovaleryl-carnitine.	Concurrent testing for maternal source and repeat testing for clearance indicated.	A-I	137–144
Propionic acidemia	C3-acylcarnitine	Urine organic acids	3-OH propionic, tiglyl-glycine, methylcitrate	B12 studies.	A-I	145–150
Methylmalonic acidemia	C3-acylcarnitine	Urine organic acids	Methylmalonic, 3-OH propionic, tiglylglycine, methylcitrate	Complementation analysis. B12 studies	A-I	150–155
Isovaleric Acidemia	C5-acylcarnitine	Urine organic acids	3-OH isovaleric acid, isovaleryl glycine	No additional testing indicated	A-II	156–162

Phenylalanine. Elevated phenylalanine is primarily caused by defects in phenylalanine hydroxylase (classic phenylketonuria) but may be benign or due to defects in the synthesis or recycling of tetrahydrobiopterin, a cofactor necessary for conversion of phenylalanine to tyrosine and the synthesis of various neurotransmitters. Assessment of plasma amino acids showing elevated phenylalanine and normal or reduced tyrosine confirms the diagnosis of hyperphenylalaninemia and dictates dietary phenylalanine restriction. Further investigations (eg, urine pterin metabolites) not typically available at referral centers are required to assess the integrity of tetrahydrobiopterin metabolism and the utility of tetrahydrobiopterin (BH_4) supplementation.

Tyrosine. Elevated tyrosine indicates disrupted tyrosine catabolism at the level of fumarylacetoacetate hydrolase (type I), tyrosine aminotransferase (type II), or the very rare deficiency of hydroxyphenylpyruvate dioxygenase (type III). Tyrosine elevations in Type II disease are pronounced while elevations in Type I and Type III disease may be marginal. Screening results are confounded by transient elevations in tyrosine due to slow maturation of the liver catabolic pathway, particularly in premature infants (2–4). Both plasma amino acids and urine organic acids are indicated for follow-up. Urine specimens from affected neonates will uniformly contain the tyrosine metabolites 4-hydroxyphenyllactate and 4-hydroxyphenylpyruvate. The additional finding of succinylacetone in urine is pathognomonic for Type I disease. Type II disease is characterized by massive elevations of plasma tyrosine ($>1,000 \mu\text{M}$). Biochemical evidence for the very rare Type III disease includes demonstration of 4-hydroxycyclo-hexylacetic acid in urine and the unusual amino acid, hawkinsin, in serum and/or urine (5).

Leucine. Screening techniques utilizing tandem MS do not distinguish leucine from isoleucine, alloisoleucine, or hydroxyproline. Leucine may also be modestly elevated in fasting ketosis. All these possibilities must be considered in follow-up analysis. Plasma amino acid analysis that distinguishes each of the branched-chain amino acids (leucine, isoleucine, alloisoleucine, and valine) from each other and from hydroxyproline is essential. Modest elevations of branched chain amino acids and ketosis are consistent with a catabolic state. The detection of alloisoleucine along with significant elevations of the branched-chain amino acids is diagnostic of MSUD. Demonstration of elevated branched-chain keto-acids (α -ketoisovaleric, α -ketoisocaproic, and α -ketomethylvaleric acids) is not necessary to establish the diagnosis. Hydroxyprolinemia is probably a benign disorder (6). Confirmation of MSUD by amino acid analysis dictates prompt dietary therapy.

Citrulline. Citrulline is the product of the ornithine transcarbamoylase reaction and a substrate for argininosuccinate (ASA) synthase. Elevation of citrulline indicates deficiency of ASA synthase (citrullinemia) or a deficiency of ASA lyase. Comprehensive amino acid analysis is necessary to distinguish these disorders. Detection of ASA by standard LC/ninhydrin amino acid analyzers is not straightforward. It appears as three species with distinct retention times: two cyclic anhydrides and the uncyclized tricarboxylic acid (7, 8). Analysis of both serum

and urine is indicated. In early stages of these disorders, elevations can be appreciated in urine before plasma elevations are readily apparent. Citrulline is also elevated due to deficiency of citrin, the mitochondrial glutamate-aspartate antiporter. In addition to hyperammonemia, this disorder is characterized by hyperbilirubinemia associated with cholestasis. Citrulline, along with lysine, alanine, and proline, may also be elevated in pyruvate carboxylase deficiency (9). Profound lactic acidemia and hypoglycemia should prompt consideration of this disorder.

Methionine. Methionine is primarily used as a marker to detect deficiency of cystathionine- β -synthetase (CBS). Methionine is one step removed from the primary substrate for CBS, homocysteine. Follow-up testing should therefore include plasma amino acid analysis and plasma homocysteine. The latter can be rapidly done with widely available immunoassays developed to assess risk for cardiovascular disease. Elevated homocysteine is detected in plasma amino acid analysis as its oxidized homodimer, homocystine. Immunoassays for detection of homocysteine are available on most automated chemistry platforms. Methionine measurement by LC/ninhydrin based techniques can be confounded by co-elution of homocitrulline (found, for example, in HHH syndrome) (10). This cause of elevated methionine should be excluded in all cases of elevated methionine. In less frequent situations, plasma methionine may be elevated without elevation of homocystine or homocysteine. Causes of this profile include deficiencies of methionine adenosyltransferase (11), guanidinoacetate methyltransferase (12), and adenosylhomocysteine hydrolase (13). Mild elevation of methionine may also occur due to hyperalimination or liver disease.

Acylcarnitine Markers

Urine organic acid analysis plays a major role in the initial follow-up testing of most suspected inborn errors marked by specific acylcarnitines. In many instances, however, diagnostic urinary organic acid patterns are only evident during episodes of disease exacerbation. Recognition of subtle abnormalities is a particular challenge for testing the asymptomatic infant with abnormal newborn screening results. Plasma acylcarnitine analyses are less subject to episodic fluctuation and are, therefore, also recommended during the initial follow-up of all positive screens, which indicate elevations of specific carnitine esters. Again, when utility is clear, complementation studies, enzyme activity analyses, and genetic studies are noted.

Medium chain, saturated acylcarnitine species (C8, C6, C10). Of these three species, C8 is elevated to the greatest extent. The differential diagnosis for this screening result is limited to MCAD deficiency. Follow-up analyses should include a confirmatory plasma acylcarnitine profile and urine organic acid analysis. Abnormalities in the acylcarnitine profile are typically constant whereas abnormalities of urine organic acids may be episodic and evident only during acute illness. Affected neonates excrete abnormal quantities of medium chain dicarboxylic acids (adipic, suberic, sebacic) with inappropriately low ketone bodies. Several abnormal glycine conjugates are also reliably detected in most organic acid protocols.

These include hexanoylglycine, suberylglycine, and 3-phenylpropionylglycine. The latter compound, derived from gut bacterial metabolism, is typically not found in urine from neonates with limited bacterial flora. Interpretation of urine organic acid profiles can be complicated by fasting and by dietary consumption of medium chain triglyceride. The concentration of ketones in urine is an important consideration in follow-up urine organic acid analysis. MCAD deficiency inhibits most but not all ketosis so the urine from affected individuals is relatively hypoketotic in the context of dicarboxylic aciduria. The absence of urine ketones does not exclude the presence of ketones in blood, as significant ketonemia (3 to 5 mM) may exist before evidence of ketosis appears in the urine (14). MCAD deficiency is reliably confirmed by molecular genetic testing for the A985G mutation. This mutation accounts for 63% of mutated alleles in cases detected by newborn screening (15).

C14:1-carnitine. Elevations of this marker suggest deficiency of very long chain acyl CoA dehydrogenase deficiency (VLCAD). Like potential MCAD deficiency, appropriate follow-up includes plasma acylcarnitine analysis and urine organic acid analysis. The acylcarnitine profile contains elevations of saturated and monounsaturated C14, C16, and C18 species. Urine organic acids in affected individuals contain both long and medium chain dicarboxylic acids with inappropriately low ketone bodies present. Unlike MCAD deficiency, biochemical findings may not adequately confirm diagnosis. In these circumstances, fibroblast fatty acid oxidation studies or molecular genetic analysis may be required. The former are not readily available and are time consuming. Genetic analysis for VLCAD deficiency is often more accessible and helpful in discriminating severe, neonatal onset disease caused by truncating mutations that result in little functional protein from later onset, milder disease caused by single amino acid changes, insertions, or deletions (16). Western blots for VLCAD protein may also provide some indication of severity.

C16-OH. Long chain hydroxyacyl CoA dehydrogenase (LCHAD) is one of three activities associated with the mitochondrial trifunctional protein (TFP). TFP consists of long chain enoyl CoA hydratase and long chain ketoacyl thiolase activities in addition to LCHAD. First-line follow-up testing should include plasma acylcarnitine analysis and urine organic acid analysis. For confirmation, the former contains elevated levels of long chain monocarboxylic (chain length >10) hydroxyacylcarnitine species and the latter is notable for medium and long chain (C6 to C14) hydroxydicarboxylic aciduria and C6 to C10 dicarboxylic aciduria. The G1528C mutation of the α subunit of TFP is found in 80% to 90% of all mutant alleles (17). Isolated LCHAD deficiency may be distinguished from generalized TFP deficiency by activity measurement but does not alter treatment. The presence of fetal LCHAD carries a high risk of obstetrical complications such as acute fatty liver of pregnancy and the Hemolysis, Elevated Liver enzymes and Low Platelets (HELLP) syndrome (18).

C4 carnitine. Confirmatory testing for elevated C4 carnitine requires distinction among three disorders: isobutyryl CoA dehydrogenase (IBDH) deficiency, short chain acyl CoA dehydrogenase (SCAD) deficiency, and ethylmalonic acid

(EMA) encephalopathy. IBDH deficiency is caused by a defect in the terminal steps of valine catabolism, SCAD deficiency by a defect in terminal oxidation of fatty acids and EMA encephalopathy by a defect in the *ETHE1* gene, the function of which is poorly defined (19). Urine organic acid analysis is the most effective means to differentiate these disorders. IBDH deficiency is distinguished from the others by elevated excretion of isobutyric acid and the absence of ethylmalonic acid. Increased ethylmalonic acid is observed in both SCAD deficiency and EMA encephalopathy. In the latter condition, however, increased EMA excretion is associated with increased excretion of isovaleryl glycine. In classic cases of the former, increased EMA excretion is often observed along with elevated excretion of methylsuccinic acid. Genetic analyses of each of the causative genes are available but the occurrence of prevalent mutations and genotype/phenotype correlations are not well known. Fibroblast fatty acid oxidation studies may clarify the role of SCAD in a potential defect. Direct enzymatic activity measurements of IBDH are complicated by overlapping substrate specificities of other short-branched chain CoA dehydrogenases. The function of the *ETHE1* product is not known so enzymatic activity measurement is not yet possible.

C4-OH carnitine. The differential diagnosis of elevated C4-OH carnitine is limited to the poorly characterized medium/short-chain acyl CoA dehydrogenase (M/SCHAD) deficiency. Urine organic acid analysis revealing elevated excretion of both hydroxylated and non-hydroxylated C6-C10 dicarboxylic acids strongly suggests this disorder. Activity measurement in tissue or fibroblast cultures is of limited availability but may augment the diagnosis.

Low C0 (free) carnitine. Primary carnitine deficiency results from the inability to reabsorb carnitine from the glomerular filtrate. Diagnosis is confirmed by the presence of low free and total carnitine in blood and inappropriately high concentrations in urine. Interpretation of results in the newborn period may be complicated by maternal status. High maternal carnitine intake may mask deficiency in the newborn and maternal deficiency may, in turn, cause secondary deficiency in the newborn. Interpretation requires adequate dietary history. Transporter activity measurement and genetic (*OCTN2*) (20) analysis are not readily available but are essential for diagnostic confirmation and differentiation from secondary causes of tubular carnitine loss.

C5 carnitine. The differential diagnosis for elevated C5-carnitine includes deficiencies of isovaleryl CoA dehydrogenase, 2-methylbutyryl CoA dehydrogenase (short/branched chain CoA dehydrogenase) or administration of antibiotics containing pivalic (2,2-dimethylpropionic) acid (21). Discrimination between these possibilities is accomplished by urine organic acid analysis. Isovaleric acidemia is characterized by increased excretion of isovaleryl glycine and 3-OH isovaleric acid. 2-methylbutyryl CoA dehydrogenase deficiency is characterized by elevated urine concentrations of 2-methylbutyrylglycine in the absence of isovaleryl glycine. The absence of both of these acylglycines suggests a false positive screening result.

C5DC carnitine. Isolated elevation of this species suggests the deficiency of the conversion of glutaryl CoA to crotonyl

CoA by glutaryl CoA dehydrogenase (glutaric aciduria type I). In follow-up testing, the presence of elevated concentrations of glutaric acid, 3-OH glutaric acid and in some patients, glutaric acid is diagnostic for this disorder. The absence of these compounds generally excludes this diagnosis. However, in the presence of other clinical abnormalities associated with glutaric aciduria type I (eg, macrocephaly) measurement of fibroblast glutaryl CoA dehydrogenase activity or analysis of the corresponding gene may be indicated to definitively exclude this diagnosis. C5DC is also elevated in glutaric aciduria type II (also known as multiple acyl CoA dehydrogenation deficiency MADD). Type II is distinguished from Type I glutaric aciduria by elevation of multiple additional acylcarnitine species and urine organic acids. Elevated acylcarnitine species include C5, C5OH, C6, C8, and C10 to C16 carnitine esters. Urine organic acids in glutaric aciduria type II include 2-OH rather than 3-OH glutaric acid, medium chain dicarboxylic acids, and ethylmalonic, 3-OH isovaleric, and isobutyric acids.

C5-OH carnitine. Elevated blood concentrations of this species dictate a broad differential diagnosis that includes deficiencies of 3-methylcrotonylcarboxylase (MCC), 3-hydroxy-3-methylglutaryl (HMG)-CoA lyase, β -ketothiolase (T2), 2-methyl-3-hydroxybutyryl CoA dehydrogenase (MHBD), 3-methylglutaconyl CoA hydratase (MGA), biotinidase, or holocarboxylase synthetase. Initial discrimination between these disorders is accomplished by analysis of urine organic acids. 3-MCC deficiency is characterized by excretion of 3-methylcrotonylglycine and variable amounts of 3-methylcrotonic acid, and 3-OH isovaleric acid. A large number of false positive screening results have been attributed to transplacental transfer of 3-methylcrotonyl-carnitine from affected mothers. It is necessary to distinguish 3-MCC deficiency from errors affecting propionyl CoA, acetyl CoA, and pyruvate carboxylase. In so-called multiple carboxylase deficiency, urine specimens contain elevated concentrations of lactate, 3-OH propionic, methylcitrate, tiglylglycine, and 3-OH isovaleric acid in addition to 3-methylcrotonylglycine. Direct assay of biotinidase, holocarboxylase synthetase, or isolated carboxylase activities is required to clarify the etiology of the disorder. HMG CoA lyase deficiency and thiolase deficiency are disorders of ketone body metabolism. In the former condition, urine specimens contain elevated amounts of HMG, its immediate precursor, 3-methylglutaconic acid, and 3-OH isovaleric acid. The latter condition affects both ketone body utilization and isoleucine catabolism. Urine contains large quantities of ketone bodies in the context of normoglycemia, 2-methyl-3-hydroxybutyric acid, and tiglylglycine. 2-methyl-lacetoacetate and butanone may be variably detected because of their volatility. Finally, 3-methylglutaconic aciduria is characterized by excretion of large amounts of 3-methylglutaconic and 3-OH isovaleric acids.

C3 carnitine. Isolated elevation of C3 carnitine suggests a disorder in the anaplerotic pathway of propionic acid to its disposition as succinate in the citric acid cycle. Potential disturbances in this pathway include propionyl CoA carboxylase, methylmalonyl CoA mutase, or multiple errors in

cobalamin metabolism. Again, urine organic acids play a major role in the initial distinction. Propionic acid is not routinely detected in urine organic acid analysis but propionylglycine and a less volatile metabolite, 3-OH propionic acid are detected. At high concentrations, 3-OH propionyl CoA acid may outcompete acetyl CoA in the citrate synthase reaction and form methylcitrate. Further upstream, tiglylglycine may also be detected. Methylmalonyl CoA mutase deficiency is notable for the hyperexcretion of methylmalonic acid in addition to 3-OH propionate, methylcitrate, and tiglylglycine. Methylmalonic aciduria may be caused by mutations in methylmalonyl CoA mutase (*mut⁰* or *mut⁻*) but also by various disturbances in cobalamin metabolism. Plasma homocysteine measurement is useful in defining the role of cobalamin metabolism. Methylmalonic aciduria without elevation of homocysteine may be caused by defective synthesis of adenosylcobalamin, the cofactor for methylmalonyl CoA mutase (*cbl A* and *B* subtype). These subtypes are often responsive to vitamin B12 administration. Methylmalonic aciduria, with elevated plasma homocysteine, suggests a defect in the formation of adenosylcobalamin and methylcobalamin, the latter being a requisite cofactor for the conversion of homocysteine to methionine. These disorders belong to the *cbl C, D, or F* subtypes. Fibroblast complementation analysis is required for definitive classification of the metabolic defect (22).

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Patient Outcomes From Early Diagnosis by Expanded Newborn Screening

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Assessing patient outcomes from expanded newborn screening is difficult and requires solid data on the following points (1–7). The epidemiology and natural history of the disease should be well understood, including clinical progression from latent to overt disease. There should also be a clear disease marker in the latent, preclinical phase and there should be effective treatment for patients identified through either newborn screening or early symptomatology. All other cost effective primary prevention measures should have been implemented and recognized as inadequate to reduce the morbidity and mortality due to the condition. There should be effective treatment for patients identified through early detection and clear evidence that early treatment leads to better outcomes. Treatment should be consensus based, using the best clinical evidence to institute appropriate and effective early treatments (8–10). Obviously, optimal evidence comes from high-quality randomized controlled trials proving that screening for the disorder is effective in reducing mortality or morbidity; such trials are extremely difficult to perform in the newborn screening context (2, 4). Clearly, the benefits of newborn screening should outweigh the physical, medical, and psychological harm caused by the initial test, follow-up diagnostic procedures, and treatment.

However, meeting these conditions for all the disorders screened for via tandem mass spectrometry newborn screening is problematic. A number of these disorders, including maple syrup urine disease, the severe form of glutaric acidemia, type 2, propionic acidemia, and several of the methylmalonic acidemias can present with catastrophic presentations very early in life, with clinically severe metabolic decompensations at age 2 days to five days, well before the 5 to 7 days required in many programs for completion of tandem mass spectrometry newborn screening in the reference laboratory. The rarity of these disorders, and the genetic and biochemical heterogeneity of the methylmalonic acidemias in particular, render assessment of patient outcomes and treatment effectiveness very difficult for these diseases (2, 6, 10, 11, 12).

In addition, other disorders, such as type 1 tyrosinemia may be very difficult to confirm using our current tandem mass spectrometry screening technology, although new technologies are being developed to address these difficulties (13). At the other extreme, a variety of disorders, notably, short chain acyl-CoA dehydrogenase deficiency (SCAD), 2-methylbutyryl-CoA dehydrogenase deficiency, and 3-methylcrotonyl-CoA

carboxylase deficiency, have far milder and more variable clinical phenotypes than thought at their first identifications in the past (1–4, 8, 10, 13). Indeed, the available evidence derived from prospective detection of SCAD deficiency demonstrates a significant majority of patients showing no apparent evidence of disease after short- and medium-term follow-up. Furthermore, there is as yet no clear understanding that signs and symptoms in patients diagnosed with SCAD deficiency are related to the defect. This Committee is in agreement that outcomes measurement will be difficult to evaluate given the lack of clinical consensus and acknowledge the opinions that some groups have recommended the exclusion of SCAD deficiency from the panel of conditions targeted by newborn screening programs (14, 15). But the Committee did not reach a consensus on grading or strength of evidence. Therefore, we have graded SCAD as I/C-II. However, elevated levels of C4 acylcarnitine are also associated with isobutyryl-CoA dehydrogenase deficiency, and ethylmalonic encephalopathy, disorders for which outcomes are severe or not yet established. Reporting of elevated C4 acylcarnitine is still recommended.

With these concerns in mind, only a handful of disorders screened for by tandem mass spectrometry meet the criteria of clinical effectiveness as measured by improved patient outcomes in neonatally detected patients. Arranged in descending order of the strength of evidence, these diseases include medium chain acyl-CoA dehydrogenase deficiency (MCAD: A-I; 16–18), maple syrup urine disease (MSUD: A-I; 19), glutaric acidemia type 1 (GA-1: A-I; 20–23), and the so-called “classical” organic acidemias, propionic acidemia, methylmalonic acidemia, and isovaleric acidemia (A- II; 11). Cost effectiveness and health care use analyses confirm the utility of newborn screening for these disorders (1, 3–7). The remaining disorders have lower quality of evidence and carry weaker recommendations. In fact, as noted above, several disorders, such as short chain acyl-CoA dehydrogenase and 2-methylbutyryl-CoA dehydrogenase deficiencies, are sufficiently mild and clinically benign that they could be considered for removal from tandem mass spectrometry newborn screening panels. Accumulation, pooling, and analysis of data from the tandem mass spectrometry newborn screening programs around the world will lead to firmer and better evidence based conclusions concerning these conditions with weaker recommendations in the years to come.

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APPENDIX

Table 1. List of Conditions, Abbreviations, and Levels of Evidence

Condition		Abbreviation	
1	Medium Chain Acyl CoA Dehydrogenase Deficiency	MCAD	A-I
2	Phenylketonuria (inc. benign hyper PHE, & BH4 defects)	PKU	A-I
3	Biotinidase Deficiency	BIOT	B-II
4	Congenital Adrenal Hyperplasia	CAH	A-I
5	Isovaleric acidemia	IVA	A-II
6	Very Long Chain AcylCoA Dehydrogenase Deficiency	VLCAD	A-II
7	Maple Syrup Urine Disease	MSUD	A-I
8	Long Chain Hydroxy AcylCoA Dehydrogenase Deficiency	LCHAD	B-II
9	Glutaric Acidemia (all forms)	GA	
	<i>Type 1</i>	GA1	A-I
	<i>Type 2</i>	GA2	B-II
10	HMGCoA Lyase Deficiency	HMG	A-II
11	Trifunctional Protein Deficiency	TFP	A-II
12	Multiple Carboxylase Deficiency	MCD	B-III
13	Methylmalonic Acidemia (all forms)	MMA	A-II
	<i>Mutase</i>	MUT	A-II
	<i>Cbl</i>	CBL	A-II
14	Homocystinuria	HCY	B-III
15	3-Methylcrotonyl CoA Carboxylase Deficiency	MCC	C-II
16	Propionic Acidemia	PA	A-II
17	Primary Carnitine Deficiency	PCD	B-II
18	Thiolase Deficiency	KT	B-II
19	Citrullinemia	CIT	B-III
20	Argininosuccinic Acidemia	ASA	B-III
21	Tyrosinemia (all forms)	TYR	
	<i>Type 1</i>	TYR 1	B-III
	<i>Type 2</i>	TYR 2	B-III
	<i>Type 3</i>	TYR 3	B-III
22	Short Chain AcylCoA Dehydrogenase Deficiency	SCAD	C-II/I*
23	Medium/Short Chain Hydroxyacyl CoA DH deficiency	SCHAD	B-III

*Committee unable to reach a consensus on SCAD deficiency

Future Directions in Expanded Newborn Screening for Metabolic Diseases by Tandem Mass Spectrometry

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The present status of expanded newborn screening for metabolic diseases comprises a panel of 29 recommended conditions, referred to as the uniform panel, for which sufficient evidence is available for inclusion in a whole population screen. Twenty of these conditions can presently be diagnosed using tandem mass spectrometry. An additional 25 conditions (22 detectable by tandem mass spectrometry (MS/MS) comprise a secondary cohort for which evidence is less strong. The paradigm shift towards comprehensive screening using multiple reaction monitoring (MRM) analysis (multiple conditions identified on a single analytical run) has provided a basis for the development of MS/MS methods for early detection of many additional metabolic diseases. Often treatment options are not as well defined as for the uniform panel, but for some conditions better treatment options appear to be improving outcomes. Most of these treatments have not yet generated sufficient prospective evidence to make recommendations at this time. Therefore, for most of the conditions outlined in this section, the strength of the evidence is insufficient to make recommendations. However, MS/MS methods based on newborn blood spots are constantly being developed to diagnose additional conditions and groups of metabolic diseases. In many instances this provides “proof of principle”, and presently, we cannot justify inclusion in a whole population screening program. Given the rapid speed of implementation of MS/MS, it is likely that additional conditions will be added in the future and that update of this Laboratory Medicine Practice Guideline will become necessary. This section addresses the emerging evidence for developing tandem MS methodology for multiple additional conditions and evaluates the human clinical evidence for outcome measures based on presently available treatment options.

LYSOSOMAL STORAGE DISORDERS

The natural history of most lysosomal storage disorders is that of progressive and irreversible neurodegeneration with variable visceral and connective tissue abnormalities. Some of the “milder” forms of these diseases, such as the non-neuronopathic form of Gaucher disease and Fabry disease, result in chronic debilitating disease that progresses throughout adulthood, necessitates multiple hospital admissions, and can result in premature death. Diagnosis has historically been based upon

observation of clinical phenotype and complex enzyme measurement using tissues such as white blood cell preparations or cultured skin fibroblasts. Consequently, these conditions were considered to be not amenable to whole population analysis. Recently, many lysosomal enzymes have been shown to have remarkable stability when collected and stored on newborn screening cards (1–7). A method has been developed for the simultaneous measurement of a number of lysosomal enzymes using tandem MS and multiple reaction monitoring (8–13).

At the same time there are increasing numbers of treatment options for some of these conditions that early data from clinical trials of symptomatically diagnosed patients suggests improves the outcome. Enzyme replacement therapy (ERT) has now been available for the milder, later, presenting non-neuronopathic form of Gaucher disease for more than 10 years and shows promise. There are no studies demonstrating therapeutic benefit in individuals diagnosed with this form of Gaucher disease in the newborn period but the availability of a screening tool is likely to provide an opportunity for study of presymptomatic individuals. Despite the lack of evidence to show that there is a clinical benefit to early newborn diagnosis, the state of New York has already introduced mass screening for Krabbe disease based on tandem mass spectrometry (National Newborn Screening and Genetics Resource Center, accessed April 14, 2008).

Table 2 highlights additional groups of conditions for which diagnostic methods based on blood spot analysis and direct flow injection tandem mass spectrometry have been identified but not yet considered for whole population screening. The longer term potential for additional diseases to be included into statewide and national whole population screening programs appears to be limited only the availability of sufficient data on diagnostic sensitivity and specificity and by the availability of sufficient numbers of mass spectrometers and appropriately trained operators.

TYROSINEMIA TYPE 1

The diagnosis of hepatorenal tyrosinemia (tyrosinemia type 1) poses particular problems using present paradigms. Although included in the uniform panel, there are many causes of falsely elevated blood spot tyrosine levels including prematurity and/or

Table 1. Lysosomal Storage Disorders That Can Be Diagnosed by Tandem Mass Spectrometry

Condition	Enzyme	ERT Treatment	Strength of Evidence for Treatability	References
Gaucher disease	beta-glucosidase	Yes	B-I for non-neuronopathic form	14–16
Fabry disease	alpha-galactosidase	Yes	I	17–18
Krabbe disease	galactosyl-ceramidase	Animal model only	I	
Hurler/Scheie disease	alpha-L-iduronidase	Yes	I	19–21
Pompe disease	acid alpha glucosidase	Yes	I	
Hunter disease	iduronidate sulfatase	Development	I	
Niemann-Pick A/B	sphingomyelinase	Development	I	

Note. Treatment for neuronopathic Gaucher disease has not been shown to be effective.

Table 2. Metabolic Diseases for Which Tandem Mass Spectrometric Methods Are Available but Not Yet Applied to Whole Population Screening

Disease	Metabolite	Reference	Notes
Tyrosinemia type 1	Succinylacetone	22–26	
Congenital adrenal hyperplasia due to 21-hydroxylase deficiency	17-hydroxyprogesterone, cortisol, and 17-HP/cortisol ratio	27–28	Used as a second tier test to increase positive predictive value
Cholestatic hepatobiliary disorders	Conjugated bile acids	29	
Disorders of creatine synthesis	Guanidinoacetate and creatine	30–31	

immature hepatic handling of tyrosine (transient tyrosinemia of the newborn) and hepatic disease resulting from congenital infections or other metabolic diseases. Methods have recently been developed for the measurement of the pathognomonic marker for tyrosinemia type 1, succinylacetone, using flow injection tandem mass spectrometry whilst simultaneously measuring amino acids and acylcarnitines. This process requires an additional extraction and derivatization process. It is predicted that this approach will increase the specificity of diagnosis for this form of tyrosinemia. Prospective data is awaited prior to making any recommendations.

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