Laboratory Medicine Practice Guidelines

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

Edited by Michael J. Bennett

NATIONAL ACADEMY *of* CLINICAL BIOCHEMISTRY
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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

EDITED BY
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Overview

Michael J. Bennett

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.

REFERENCES

1. http://www.cdc.gov/nchs/data/nvsr/nvsr56/nvsr56_12.pdf (accessed 09/19/08)
2. Guthrie R. The origin of newborn screening. Screening, 1; 5–15, 1992
3. Wilson JMG, Jungner G. Principles of screening for disease. Geneva: World Health Organization, 1968
4. National Institutes of Health. Phenylketonuria (PKU): screening and management. NIH consensus statement 17(3); 1–33, 2000
5. Chace DH, Kalas TA, Naylor EW. Use of tandem mass spectrometry for multianalyte screening of dried blood specimens from newborns. Clin Chem 49; 1797–1817, 2003
6. Grosse SD, Khoury MJ, Greene CL, Crider KS, Pollitt RJ. The epidemiology of medium-chain acyl-CoA dehydrogenase deficiency: an update. Genet Med 8; 205–212, 2006
7. http://genes-r-us.uthscsa.edu/ (accessed 09/19/08)

Chapter **1**

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.

REFERENCES

1. American Academy of Pediatrics, Newborn Screening Task Force. (2000) Serving the family from birth to the medical home: newborn screening a blueprint for the future. Pediatrics 106:389–427.
2. Rinaldo P, Zafari S, Tortorelli S, Matern D. (2006) Making the case for objective performance metrics in newborn screening by tandem mass spectrometry. MRDD Research Reviews 12: 255–261.
3. National Newborn Screening and Genetics Resource Center (NNSGRC). (2006) US national newborn screening information system. Available at http://genes-r-us.uthscsa.edu. (accessed 09/19/08)
4. Zytkovicz TH, Fitzgerald EF, Marsden D, et al. (2001) Tandem mass spectrometric analysis for amino, organic, and fatty acid disorders in newborn dried blood spots: a two-year summary from the New England Newborn Screening Program. Clin Chem 47:1945–1955.
5. Chace DH, Kalas TA, Naylor EW. 2003. Use of tandem mass spectrometry for multianalyte screening of dried blood specimens from newborns. Clin Chem 49:1797–1817.
6. Schulze A, Lindner M, Kohlmuller D, et al. 2003. Expanded newborn screening for inborn errors of metabolism by electrospray ionization-tandem mass spectrometry: results, outcome, and implications. Pediatrics. 111:1399–406.
7. Wilcken B, Wiley V, Hammond J, et al. 2003. Screening newborns for inborn errors of metabolism by tandem mass spectrometry. N Engl J Med 348:2304–2312.
8. Hoffmann GF, von Kries R, Klose D, et al. 2004. Frequencies of inherited organic acidurias and disorders of mitochondrial fatty acid transport and oxidation in Germany. Eur J Pediatr 163:76–80.
9. Frazier DM, Millington DS, McCandless SE, et al. 2006. The tandem mass spectrometry newborn screening experience in North Carolina: 1997–2005. J Inherit Metab Dis 29:76–85.
10. Watson MS, Mann MY, Lloyd-Puryear MA, Rinaldo P, Howell RR [editors]. (2006) Newborn screening: Toward a uniform screening panel and system [Executive summary]. Genet Med 8(Supplement):1S–11S.
11. Watson MS, Lloyd-Puryear MA, Mann MY, Rinaldo P, Howell RR [editors]. (2006) Newborn screening: Toward a uniform screening panel and system [Main report]. Genet Med 8 (Supplement):12S–252S.
12. Rinaldo P, Hahn SH, Matern D. 2005. Inborn errors of amino acid, organic acid, and fatty acid metabolism. In: Burtis CA, Ashwood ER, Bruns DE, eds. Tietz Textbook of Clinical Chemistry and Molecular Diagnostics, 4th ed., W.B. Saunders, pp. 2207–2247.
13. Botkin JR, Clayton EW, Fost NC, et al. 2006. Newborn screening technology: proceed with caution. Pediatrics 117:1793–1799.
14. Rinaldo P, Tortorelli S, Matern M. 2004. Recent developments and new applications of tandem mass spectrometry in newborn screening. Curr Opini Pediatr 16:427–432.
15. Sweetman L, Millington DS, Therrell BL, et al. 2006. Naming and counting disorders (conditions) included in newborn screening panels. Pediatrics 117(Pt 2):S308–314.
16. Howell RR. 2006. We need expanded newborn screening. Pediatrics 117:1800–1805.
17. Merinero B, Perez-Cerda C, Ruiz Sala P, Ferrer I, et al. (2006) Persistent increase of plasma butyryl/isobutyrylcarnitine concentrations as marker of SCAD defect and ethylmalonic encephalopathy. J Inherit Metab Dis 29:685.
18. Garcia-Cazorla A, Rabier D, Touati G, Chadefaux-Vekemans B, et al. (2006) Pyruvate carboxylase deficiency: metabolic characteristics and new neurological aspects. Ann Neurol 59:121–127.
19. Tan ES, Wiley V, Carpenter K, Wilcken B. (2007) Non-ketotic hyperglycinemia is usually not detectable by tandem mass spectrometry newborn screening. Mol Genet Metab 90:446–448.
20. Carrozzo R, Dionisi-Vici C, Steuerwald U, Lucioli S, et al. (2007) SUCLA2 mutations are associated with mild methylmalonic aciduria, Leigh-like encephalomyopathy, dystonia and deafness. Brain 130:862–874.
21. Lorey F, Enns G, Cederbaum S, Crombez E, et al. (2007) Should Cobalamin C (Cbl C) be a core target? Evidence for increased prevalence, detection, and effectiveness of treatment intervention

in California newborn screening. Proceedings of the 2007 Newborn Screening and Genetic Testing Symposium, Association of Public Health Laboratories, Minneapolis (MN, p. 36. Also available at http://www.aphl.org.

22. Watson MS. (2006) Current status of newborn screening: decision-making about the conditions to include in screening programs. Ment Retard Dev Disabil Res Rev 12:230–235.
23. Gibson KM, Bennett MJ, Naylor EW, Morton DH. (1998) 3-Methylcrotonyl-coenzyme A carboxylase deficiency in Amish/Mennonite adults identified by detection of increased acylcarnitines in blood spots of their children. J Pediatr. 132:519–523.
24. Schimmenti LA, Crombez EA, Schwahn BC, Heese BA, et al. (2007) Expanded newborn screening identifies maternal primary carnitine deficiency. Mol Genet Metab 90:441–445.
25. Abdenur JE, Chamoles NA, Guinle AE, Schenone AB, et al. (1998) Diagnosis of isovaleric acidaemia by tandem mass spectrometry: false positive result due to pivaloylcarnitine in a newborn screening programme. J Inherit Metab Dis 21:624–630.
26. Lacey JM, Minutti CZ, Magera MJ, Tauscher AL, et al. (2004) Improved specificity of newborn screening for congenital adrenal hyperplasia by second tier steroid profiling using tandem mass spectrometry. Clin Chem 50:621–625.
27. Magera MJ, Gunawardena ND, Hahn SH, Tortorelli S, et al. (2006) Rapid quantitative determination of succinylacetone in dried blood spots by liquid chromatography tandem mass spectrometry. Mol Genet Metab 88:16–21.
28. Pandor A, Eastham J, Beverley C, Chilcott J, et al. (2004) Clinical effectiveness and cost-effectiveness of neonatal screening for inborn errors of metabolism using tandem mass spectrometry: a systematic review. Health Technol Assess 8:1–121.
29. Pandor A, Eastham J, Chilcott J, Paisley S, et al. (2006) Economics of tandem mass spectrometry screening of neonatal inherited disorders. Int J Technol Assess Health Care 22:321–326.
30. Aaron E, Carroll AE, Downs SM. (2006) Comprehensive cost-utility analysis of newborn screening strategies. Pediatrics 117:S287–S295.
31. Waisbren SE, Albers S, Amato S, et al. (2003) Effect of expanded newborn screening for biochemical genetic disorders on child outcomes and parental stress. JAMA 290:2564–2572.
32. Rinaldo P., Zafari S. (2006) Progress report on expanded newborn screening outcomes: The Region 4 MS/MS collaborative project [abstract]. Annual Clinical Genetics Meeting, Program and Abstracts, p. 161 (#335). See also www.region4genetics.org
33. Marcuse EK, Shiffman RN. (2004) Classifying recommendations for clinical practice guidelines. Pediatrics 114:874–877.

Group	Condition (inborn errors of amino acid, fatty acid, and organic acid metabolism) (1)	ACMG code (1)	US incidence (2)	ACMG panel (3)	Primary marker(s) (4)	Informative ratios (5)	Interpretation requires differential diagnosis (6)	Primary or other conditions with same marker(s) (6)	Secondary or other conditions with same markers (6)	Maternal cases detected by NBS (7)	Potential interference by diet, drugs, prematurity (8)	No. of cases (9)	Available 2nd tier test (10)	Evidence level in 2006 ACMG report (1–4) (11)	Evaluation of evidence (LMPG criteria) (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	Argininemia	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 biopterin 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 biopterin 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	Condition (inborn errors of amino acid, fatty acid, and organic acid metabolism) (1)	ACMG code (1)	US incidence (2)	ACMG panel (3)	Primary marker(s) (4)	Informative ratios (5)	Interpretation requires differential diagnosis (6)	Primary or other conditions with same marker(s) (6)	Secondary or other conditions with same markers (6)	Maternal cases detected by NBS (7)	Potential interference by diet, drugs, prematurity (8)	No. of cases (9)	Available 2nd tier test (10)	Evidence level in 2006 ACMG report (1–4) (11)	Evaluation of evidence (LMPG criteria) (12)
FAO	Carnitine palmi-toyl-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 palmi-toyl-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 unsat-urated 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/SCHAD	>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 carboxy-lase 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 dehydroge-nase 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 gluta conic 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 appli-cable to the pri-mary markers		IVA	SCAD, IBG, GA2	-	Yes	7		n/a	**I**

Table 1. Legend

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.

*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 than 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 in 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—Derivitization & Ion Exchange Chromatography

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

Comments/Specific Examples: Derivitization 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 derivitization is preferable since it involves minimal sample handling and produces more consistent results (13).

Strength of recommendation: A

Quality of Evidence: I

Specimen Preparation—Derivitization & Tandem Mass Spectrometry

Recommendation: Chemical derivitization 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 underivitized 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 underivitized 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

REFERENCES

1. Shih VE. Amino Acid Analysis. In: Blau N, Duran M, Blaskovics ME, Gibson KM (Eds): *Physician's Guide to the Laboratory Diagnosis of Metabolic Diseases*, Second Edition, Springer, Berlin, 2003, pp. 11–26.
2. Tada K. Non-ketotic hyperglycinemia. In: Fernandes J, Saudubray J-M, Tada K, eds. *Inborn Metabolic Diseases*. Berlin: Springer-Verlag, 1990 323–9.
3. American College of Medical Genetics. F: Clinical biochemical genetics. In: Standards and Guidelines for Clinical Genetics Laboratories. 2006 http://www.acmg.net/Pages/ ACMG_Activities/ stds-2002/f.htm (accessed 09/19/08).
4. Tsai MY, Marshall JG, Josephson MW. Free amino acid analysis of untimed and 24-h urine samples compared. Clin Chem 1980; 26:1804–8.
5. Perry T, Hansen S. Technical pitfalls leading to errors in the quantitation of plasma amino acids. Clin Chim Acta 1969; 25: 53–8.
6. Sahai S, Uhlhaas S. Stability of amino acids in human plasma. Clin Chem Acta 1985; 148:225–9.
7. Levy HL, Madaigan PM, Kum A. Fecal contamination in urine amino acid screening. Artifactual cause of hyperaminoaciduria. Am J Clin Pathol 1969; 51:765–8.
8. Walker V, Mills GA. Quantitative methods for amino acid analysis in biological fluids. Ann Clin Biochem 1995; 32: 28-57.
9. Ambler RP. Standards and accuracy in amino acid analysis. In: Rattenbury JM, ed. *Amino Acid Analysis*. Chichester: Ellis Horwood Ltd, 1981:119–37.
10. Armstrong MD, Stave U. A study of plasma free amino acid levels. I. A study of factors affecting validity of amino acid analyses. Metabolism 1973:22:549–60.
11. Hubbard RW, chambers JG, Sanchez A, Slocum R, Lee P. Amino acid analysis of plasma: studies in sample preparation. J Chromatogr 1988; 431:163–9.
12. Ersser RS, Davey JF. Liquid chromatographic analysis of amino acids in physiological fluids: recent advances. Med Lab sci 1991; 48:59–71.
13. Deyl Z. Profiling of amino acids in body fluids and tissues by means of liquid chromatography. J Chromatogr 1986; 379: 177–250.
14. Chase DH. Mass spectrometry in the clinical laboratory. Chem Rev 2001; 101:445–477.
15. Moodie IM, Shephard GS, Labadarios D. A review of quantitative ion eschange, high performance liquid and gas chromatographic analyses of amino acids in physiological fluids. J High Resol Chromatogr 1989; 12:509–16.
16. Long CL, Geiger JW. Automatic analysis of amino acids: effect of resin cross-linking and operational variables on resolution. Anal Bioch, 1969; 29:265–83.
17. Lee PL, Slocum RH. A high-resolution method for amino acid analysis of physiological fluids containing mixed disulfides. Clin Chem 1988; 34:719–23.
18. Chase DH, Kalas TA, Naylor EW. Use of tandem mass spectometry for multianalyte screening of dried blood specimens form newborns. Clin Chem 2003; 49:1797–1817.
19. Williams AP. General problems associated with the analysis of amino acids by automated in-exchange chromatography. J Chromatogr 1986; 373:175–90.
20. CLSI. Statistical quality control for quantitative measurements: principles and definitions - second edition; approved guideline C24-A3. Wayne, PA: CLSI, 1996.
21. Bremer HJ, Duran M, Kamerling JP, Przyrembel H, Wadman SK. *Disturbances of Amino Acid Metabolism: Clinical Chemistry and Diagnosis*. Baltimore: Urban and Schwarzenberg, 1981.
22. Rinaldo P, Hahn SH, Matern D. (2005) Inborn errors of amino acid, organic acid, and fatty acid metabolism. In: Burtis CA, Ashwood ER, Bruns DE, eds. Tietz Textbook of Clinical Chemistry and Molecular Diagnostics, 4th ed., Elsevier Saunders, St. Louis (Missouri), pp. 2207–2247.
23. Clayton BE, Jenkins P, Round RM. Paediatric Chemical Pathology: Clinical Tests and Reference Ranges. Oxford: Blackwell Scientific Publications, 1980.
24. Parvy P, Bardet J, Rabier D, Kamoun P. Age-related reference values for free amino acids in first morning urine specimens. Clin Chem 1988; 34:2092–5.
25. 42 Code of Federal Regulations (CFR) 493.901, April 23, 2007.
26. Rattenbury JM, Townsend JC. Establishment of an external quality-assessment scheme for amino acid analyses: results from assays of samples distributed during two years. Clin Chem 1990; 36:217–24.
27. Parvy P, Bardet J, Rabier D, Gasquet M, Kamoun P. Intra- and inter-laboratory quality control for assay of amino acids in biological fluids: 14 years of the French experience. Clin Chem 1993; 39:1831–6.
28. Briddon A, Oberholzer VG. Plasma amino acid patterns in critically ill children. J Inher Metab Dis. 1986; 900:254–6.
29. Scriver CR, Sly WS, Childs B, Beaudet AL, Valle D, Kinzer KW, Vogelstein B, eds). The Metabolic and Molecular Bases of Inherited Disease, 8th edition. McGraw-Hill, New York, 2001.
30. Ananth N. Laboratory generated artifacts in plasma amino acid quantitation. Online J Health Allied Scs. 2002; 3:4–7.

APPENDIX

Table 1. Stable Isotope Internal Standards

d_4-Alanine	d_2-Ornithine
d_4,13C-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 H2NCNHNH2
L-Asparagine	133	59 H3CCONH2)
L-Aspartic acid	134	60 (H3CCOOH = acetic acid)
beta-Alanine	90	18 (H2O)
L-Citrulline	176	106 (HCOOH and H2NCONH2)
L-Glutamine/ L-Lysine	147	63 (HCOOH and NH3)
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, H2O)
L-Leucine + L-Isoleucine + L-Hydroxyproline	132	46 (HCOOH = formic acid)
L-Methionine	150	94 (HCOOH and HSCH3)
L-Ornithine	133	63 (HCOOH and NH3)
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 (NH3)
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 – Derivitization

Recommendation: Chemical derivitization 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 underivitized 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) acycarnitine quality assurance program is available mfrom 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

REFERENCES

1. Van Hove JL, Zhang W, Kahler SG, Roe CR, Chen YT, Terada N, et al. Medium-chain acyl-CoA dehydrogenase (MCAD) deficiency: diagnosis by acylcarnitine analysis in blood. Am J Hum Genet 1993; 52:958–66.
2. Chace DH, Hillman SL, Vanhove JLK, Naylor EW. Rapid diagnosis of MCAD deficiency – quantitative analysis of octanoylcarnitine and other acylcarnitines in newborn blood sports by tandem mass spectrometry. Clin Chem 1997; 43:2106–13.
3. Millington DS, Chace DH. Carnitine and acylcarnitines in metabolic disease diagnosis and management. In: Desiderio DM (ed). Mass Spectrometry: Clinical and Biomedical Applications. Volume 1. New York: Plenum Press; 1992, pp 199–219.
4. Chace DH, DiPerna JC, Mitchell BL, Sgroi B, et al. Electrospray tandem mass spectrometry for analysis of acylcarnitines in dried postmortem blood specimens collected at autopsy from infants with unexplained cause of death. Clin Chem 2001; 47:1166–1182.
5. Tortorelli S, Hahn SH, Cowan TM, Brewster TG et al. The urinary excretion of glutarylcarnitine is an informative tool in the biochemical diagnosis of glutaric acidemia type I. Mol Genet Metab 2005; 84:137–143.
6. Rinaldo P, Cowan T, Matern D. Acylcarnitine profile analysis: American College of Medical Genetics Standards and Guidelines for Clinical Genetic Laboratories. Genet Med 2008; 10:151–156
7. Millington DS. Tandem mass spectrometry in clinical diagnosis. In: Blau N, Duran M, Blaskovics ME, Gibson KM (eds) Physician's guide to the laboratory diagnosis of metabolic diseases. Springer, Berlin, 2nd edition, 2003, pp 57–75.
8. Rinaldo P, Hahn S, Matern D. Appendix 55. Inborn errors of amino acid, organic acid, and fatty acid metabolism. In: CA Burtis, ER Ashwood and DE Bruns (Eds.), *Tietz Textbook of Clinical Chemistry*, Fourth Edition, Elsevier Saunders, St. Louis, MO, 2006.
9. Green A, Morgan I, Gray J. *Neonatology and Laboratory Medicine*. ACB Venture Publications. London, 2003.
10. American College of Medical Genetics. B. Personnel Policies. In: *Standards and Guidelines for Clinical Genetics Laboratories*. 2006 http://www.acmg.net/Pages/ACMG_Activities/stds-2002/f.htm
11. Chace DH. Mass spectrometry in the clinical laboratory. Chem Rev. 2001; 101:445–477.
12. Chace DH, Kalas TA, Naylor EW. Use of tandem mass spectrometry for multianalyte screening of dried specimens from newborns. Clin Chem 2003; 49:1797–1817.
13. Nagy N, Takáts Z, Pollreisz F, Szabó T, Vékey K. Direct tandem mass spectrometric analysis of amino acids in dried blood spots without chemical derivatization for neonatal screening; Rapid Communications in Mass Spectrometry, 2003; 17:983–990.
14. Matern D. Acylcarnitines including in vitro loading tests. In: Blau N, Duran M, Gibson KM (eds) Laboratory guide to the methods in biochemical genetics. Berlin: Springer-Verlag, 2008; pp 171–206.
15. Center for Disease Control and Prevention. Using tandem mass spectrometry for metabolic disease screening among newborns: a report of a work group. MMWR 2001; 50 (No. RR-3):1–40.
16. Chace DH, Kalas TA, Naylor EW. The application of tandem mass spectrometry to neonatal screening for inherited disorders of intermediary metabolism. Annu Rev Genomics Hum Genet 2002; 3:17–45.
17. Ghoshal AK, Guo T, Sukhova N, Soldin SJ. Rapid measurement of plasma acylcarnitines by liquid chromatography-tandem mass spectrometry. Clin Chim Acta 2005; 358:104–112.
18. CLSI. Statistical quality control for quantitative measurements: principles and definitions – third edition; approved guideline C24-A3. Wayne, PA:CLSI, 2006.
19. 42 Code of Federal Regulations (CFR) 493.901, April 23, 2007.
20. European Research Network for evaluation and improvement of screening, Diagnosis and treatment of Inherited disorders of Metabolism" (ERNDIM) see http://www.erndimqa.nl/
21. Chace DH, Kalas TA. A biochemical perspective on the use of tandem mass spectrometry for newborn screening and clinical testing. Clin Biochem 2005; 38:296–309.
22. Sweetman L, Millington DS, Therrell BL, Hannon WH et al. Naming and counting disorders (conditions) included in newborn screening panels. Pediatrics. 2006; 117(5 Pt 2):S308–S314.
23. American College of Medical Genetics. F: Clinical biochemical genetics. In: Standards and Guidelines for Clinical Genetics Laboratories. 2006 http://www.acmg.net/Pages/ACMG_Activities/ stds-2002/f.htm
24. Abdenur JE, Chamoles NA, Guinle AE, Schenone AB et al. Diagnosis of isovaleric acidaemia by tandem mass spectrometry: false positive result due to pivaloylcarnitine in a newborn screening programme. J Inherit Metab Dis. 1998; 21:624–630.
25. Vianey-Saban C, Boyer S, Levrat V, Cheillan D, et al. Interference of Cefotaxime in plasma acylcarnitine profile mimicking an increase of 3-hydroxypalmitoleylcarnitine (C16:1-OH) using butyl esters. J Inherit Metab Dis 2004; 27(Suppl. 1):94.

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-Palimitoylcarnitine (Hexadecanoyl)	(d_3-C16)
d_3-Octadecanoylcarnitine (Stearoyl)	(d_3-C18)
d_6-Glutarylcarnitine	(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)
Glutarylcarnitine	(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- 2-methyl-3-hydroxybutyryl-	C5-OH	318
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-hydroxydecenoyl-	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 Underivitized 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 ↑	3-Hydroxybutyryl	SCHAD deficiency
C4 ↑	Butyryl	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	Isovaleric acidemia
	Methylbutyryl	Methylbutyryl-CoA dehydrogenase deficiency
C5-OH ↑	3-Hydroxyisovaleryl	β-Methylcrotonyl-CoA carboxylase deficiency Hydroxymethylglutaryl-CoA lyase deficiency Biotinidase deficiency Methylglutaconic aciduria
	2-Methyl-3-Hydroxybutyryl	3-Oxothiolase deficiency 2-Methyl-3-hydroxybutyryl-CoA dehydrogenase deficiency
C5DC ↑	Glutaryl	Glutaric acidemia type I
C8 ↑	Octanoyl	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 spectometry 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 µmol) of creatinine is used. The recommended minimum volume is 0.5 µL 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

Derivitization

Recommendation: Chemical derivitization 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 derivitization is tert-butyldimethylsilylation (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 academia 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 spectometry 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 μmol/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

REFERENCES

1. Chalmers RA, Lawson AM. Analytical chemistry, biochemistry and diagnosis of the organic acidurias. In *Organic Acids in Man*. Chapman and Hall, London, 1982.
2. Hoffmann G, Feyh P. Organic acid analysis. In: Blau N, Duran M, Blaskovics ME, Gibson KM (eds). Physician's Guide to the Laboratory Diagnosis of Metabolic Diseases, Second Edition, Springer, pp. 27–44.
3. Sweetman L. Organic acid analysis. In: *Techniques in Diagnostic Human Biochemical Genetics*. Hommes FA, ed. Wiley-Liss, New York, 1991; 143–176.
4. Bennett M. Recommendations for the measurement of urine organic acids. In: Sherwin JE, Lockitch G, Rosenthal P, et al (au). Laboratory Medicine Practice Guidelines: Maternal-Fetal Risk Assessment and Reference Values in Pregnancy. National Academy Clinical Biochemistry, Washington, DC, 2006; 59–62.
5. American College of Medical Genetics. F: Clinical biochemical genetics. In: Standards and Guidelines for Clinical Genetics Laboratories. 2006 http://www.acmg.net/Pages/ACMG_Activities/ stds-2002/f.htm
6. Lehotay DC, Clarke JTR. Organic acidurias and related abnormalities. *Crit Rev Clin Lab Sci* 32:377–429, 1995.
7. Suh JW, Lee SH, Chung BC. GC-MS determination of organic acids with solvent extraction after cation-exchange chromatography. Clin Chem 1997; 43:2256–2261.
8. Rinaldo P, Hahn S, Matern D. Appendix 55. Inborn errors of amino acid, organic acid, and fatty acid metabolism. In: CA Burtis, ER Ashwood and DE Bruns (Eds.), *Tietz Textbook of Clinical Chemistry*, Fourth Edition, Elsevier Saunders, St. Louis, MO, 2006.
9. Duez P, Kumps A, Mardens Z. GC-MS profiling of urinary organic acids evaluated as a quantitative method. Clin Chem 1996; 42:1609–1615.
10. Mardens Y, Kumps A, Planchon C, Wurth C. Comparison of two extraction procedures for urinary organic acids prior to gas chromatography–mass spectrometry. J Chromatogr 1992; 577:341–346.

11. Shoemaker JD, Elliott WH. Automated screening for urine samples for carbohydrates, organic and amino acids after treatment with urease. J Chromatogr 1991; 562:125–38.
12. Hoffmann G, Aramaki S, Blum-Hoffmann E, Nyhan WL, Sweetman L. Quantitative analysis for organic acids in biological samples: batch isolation followed by gas chromatographic–mass spectrometric analysis. Clin Chem 1989; 35:587–595.
13. Chace DH. Mass spectrometry in the clinical laboratory. Chem Rev 2001; 101:445–477.
14. College of American Pathologists. Cumulative reports of quantitative organic acid analysis in the BGL–A series.
15. CLSI. Statistical quality control for quantitative measurements: principles and definitions – third edition; approved guideline C24-A3. Wayne, PA: CLSI, 20.
16. 42 Code of Federal Regulations (CFR) 493.901, April 23, 2007.
17. Ozand PT, Generoso GG. Organic acidurias: A review. Part 2. J Child Neurol 1991; 288–303.
18. Kumps A, Duez P, Mardens Y. Metabolic, Nutritional, Iatrogenic, and Artifactual Sources of Urinary Organic Acids: A Comprehensive Table. Clin Chem 2002; 48:708–17.

APPENDIX

Table 1. Critical Organic Acid Overlaps

Critical Organic Acid Overlaps
3-hydroxybutyric acid/3-hydroxyisobutyric acid
urea/4-hydoxybutyric 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-hydoxypropionic	3-Hydroxyhexadecanoic	Benzoic	N-Acetylasparatic
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 - Derivitization			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		
Derivitization	A-I		
Gas Chromatogaphy - 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		
Specmen Prep - Derivitization & Ion Exchange Chromatography	A-I		
Specimen Prep - Derivitization & 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

Chapter **3**

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.

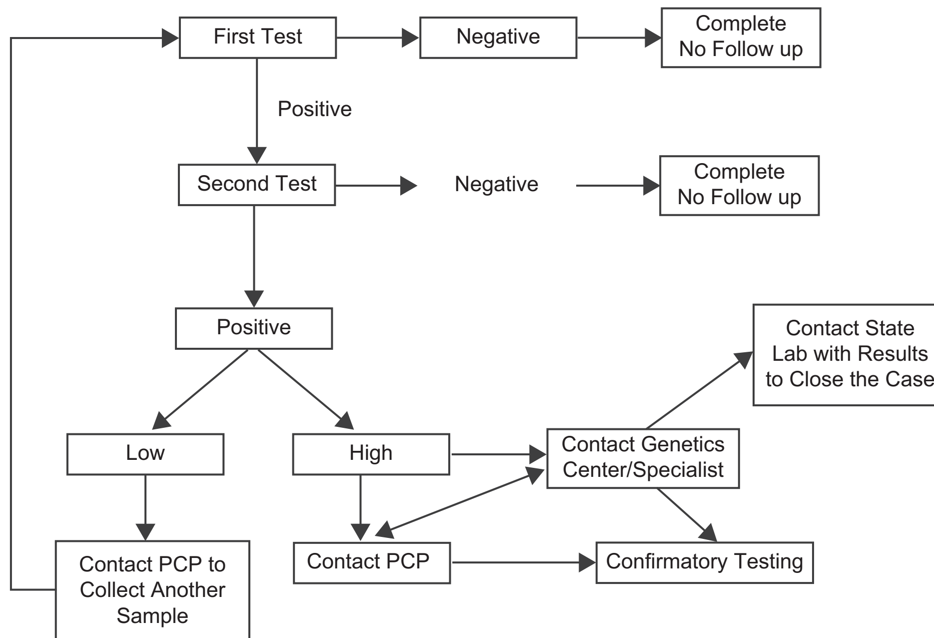

Figure 3-1 Follow-Up Protocol for Newborn Screening

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 biopterin metabolic defects)	Phenylalanine	Plasma amino acids	Phenylalanine	Urine pterin metabolites	A-I	23–26
	Tyrosine		Tyrosine	Dihydropteridine reductase activity		
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.	A-I	44–53
Type 2 (Citrin)	Citrulline			Genetic testing may distinguish I and II.	B-II	
Argininosuccinic acidemia	Citrulline	Plasma/urine amino acids	Argininosuccinate, Citrulline	No additional testing indicated	A-I	54–59
Homocytsinuria	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 sebacic, 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, sebacic 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-methycrotonylglycine 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	3methylcrotonylglycine 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 µM). 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 hyperalimenation 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 mitochdondrial 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 isovalerylglycine. 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 isovalerylglycine and 3-OH isovaleric acid. 2-methylbutyryl CoA dehydrogenase deficiency is characterized by elevated urine concentrations of 2-methylbutyrylglycine in the absence of isovalerylglycine. 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, glutaconic 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-methylacetoacetate 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 (mut^0 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).

REFERENCES

1. Newborn screening: toward a uniform screening panel and system. Genet. Med. 8 Suppl 1:1S–252S.: 1S–252S, 2006
2. Levy HL, Shih VE, Madigan PM, MacCready RA: Transient tyrosinemia in full-term infants. JAMA. 209:249–250, 1969
3. Avery ME, Clow CL, Menkes JH, Ramos A, Scriver CR, Stern L, Wasserman BP: Transient tyrosinemia of the newborn: dietary and clinical aspects. Pediatrics. 39:378–384, 1967
4. Rice DN, Houston IB, Lyon IC, Macarthur BA, Mullins PR, Veale AM, Guthrie R: Transient neonatal tyrosinaemia. J Inherit. Metab Dis. 12:13–22, 1989
5. Niederwieser A, Matasovic A, Tippett P, Danks DM: A new sulfur amino acid, named hawkinsin, identified in a baby with transient tyrosinemia and her mother. Clin. Chim. Acta. 76:345–356, 1977
6. Pelkonen R, Kivirikko KI: Hydroxyprolinemia: an apparently harmless familial metabolic disorder. N. Engl. J Med. 283:451–456, 1970
7. Canepari S, Carunchio V, Girelli AM, Messina A: Determination of argininosuccinate lyase in human serum by ion-pair reversed phase liquid chromatography. Biomed. Chromatogr. 9:171–174, 1995
8. Gallagher JV, Rifai N, Conry J, Soldin SJ: Role of the clinical laboratory in evaluation of argininosuccinate lyase deficiency. Clin. Chem. 37:1384–1389, 1991
9. Coude FX, Ogier H, Marsac C, Munnich A, Charpentier C, Saudubray JM: Secondary citrullinemia with hyperammonemia in four neonatal cases of pyruvate carboxylase deficiency. Pediatrics. 68:914, 1981
10. Al Dirbashi OY, Al Hassnan ZN, Rashed MS: Determination of homocitrulline in urine of patients with HHH syndrome by liquid chromatography tandem mass spectrometry. Anal. Bioanal. Chem. 386:2013–2017, 2006

11. Gaull GE, Tallan HH: Methionine adenosyltransferase deficiency: new enzymatic defect associated with hypermethioninemia. Science. 186:59–60, 1974
12. Stockler S, Isbrandt D, Hanefeld F, Schmidt B, von Figura K: Guanidinoacetate methyltransferase deficiency: the first inborn error of creatine metabolism in man. Am. J Hum. Genet. 58: 914–922, 1996
13. Kaminska JE, Fox IH: Decreased S-adenosylhomocysteine hydrolase in inborn errors of purine metabolism. J Lab Clin. Med. 96:141–147, 1980
14. Ferrier B, Martin M, Janbon B, Baverel G: Transport of beta-hydroxybutyrate and acetoacetate along rat nephrons: a micropuncture study. Am. J Physiol. 262:F762–F769, 1992
15. Andresen BS, Dobrowolski SF, O'Reilly L, Muenzer J, McCandless SE, Frazier DM, Udvari S, Bross P, Knudsen I, Banas R, Chace DH, Engel P, Naylor EW, Gregersen N: Medium-chain acyl-CoA dehydrogenase (MCAD) mutations identified by MS/MS-based prospective screening of newborns differ from those observed in patients with clinical symptoms: identification and characterization of a new, prevalent mutation that results in mild MCAD deficiency. Am. J Hum. Genet. 68:1408–1418, 2001
16. Andresen BS, Olpin S, Poorthuis BJ, Scholte HR, Vianey-Saban C, Wanders R, Ijlst L, Morris A, Pourfarzam M, Bartlett K, Baumgartner ER, deKlerk JB, Schroeder LD, Corydon TJ, Lund H, Winter V, Bross P, Bolund L, Gregersen N: Clear correlation of genotype with disease phenotype in very-long-chain acyl-CoA dehydrogenase deficiency. Am. J Hum. Genet. 64:479–494, 1999
17. Gregersen N, Andresen BS, Bross P: Prevalent mutations in fatty acid oxidation disorders: diagnostic considerations. Eur. J Pediatr. 159 Suppl 3:S213–8.:S213–S218, 2000
18. Sims HF, Brackett JC, Powell CK, Treem WR, Hale DE, Bennett MJ, Gibson B, Shapiro S, Strauss AW: The molecular basis of pediatric long chain 3-hydroxyacyl-CoA dehydrogenase deficiency associated with maternal acute fatty liver of pregnancy. Proc. Natl. Acad. Sci. U.S.A. 92:841–845, 1995
19. Tiranti V, D'Adamo P, Briem E, Ferrari G, Mineri R, Lamantea E, Mandel H, Balestri P, Garcia-Silva MT, Vollmer B, Rinaldo P, Hahn SH, Leonard J, Rahman S, Dionisi-Vici C, Garavaglia B, Gasparini P, Zeviani M: Ethylmalonic encephalopathy is caused by mutations in ETHE1, a gene encoding a mitochondrial matrix protein. Am. J Hum. Genet. 74:239–252, 2004
20. Nezu J, Tamai I, Oku A, Ohashi R, Yabuuchi H, Hashimoto N, Nikaido H, Sai Y, Koizumi A, Shoji Y, Takada G, Matsuishi T, Yoshino M, Kato H, Ohura T, Tsujimoto G, Hayakawa J, Shimane M, Tsuji A: Primary systemic carnitine deficiency is caused by mutations in a gene encoding sodium ion-dependent carnitine transporter. Nat. Genet. 21:91–94, 1999
21. Abdenur JE, Chamoles NA, Guinle AE, Schenone AB, Fuertes AN: Diagnosis of isovaleric acidaemia by tandem mass spectrometry: false positive result due to pivaloylcarnitine in a newborn screening programme. J Inherit. Metab Dis. 21:624–630, 1998
22. Qureshi AA, Rosenblatt DS, Cooper BA: Inherited disorders of cobalamin metabolism. Crit Rev. Oncol. Hematol. 17:133–151, 1994
23. Chace DH, Millington DS, Terada N, Kahler SG, Roe CR, Hofman LF: Rapid diagnosis of phenylketonuria by quantitative analysis for phenylalanine and tyrosine in neonatal blood spots by tandem mass spectrometry. Clin. Chem. 39:66–71, 1993
24. Chace DH, Sherwin JE, Hillman SL, Lorey F, Cunningham GC: Use of phenylalanine-to-tyrosine ratio determined by tandem mass spectrometry to improve newborn screening for phenylketonuria of early discharge specimens collected in the first 24 hours. Clin. Chem. 44:2405–2409, 1998
25. Ceglarek U, Muller P, Stach B, Buhrdel P, Thiery J, Kiess W: Validation of the phenylalanine/tyrosine ratio determined by tandem mass spectrometry: sensitive newborn screening for phenylketonuria. Clin. Chem. Lab Med. 40:693–697, 2002
26. Spiekerkoetter U, Sun B, Zytkovicz T, Wanders R, Strauss AW, Wendel U: MS/MS-based newborn and family screening detects asymptomatic patients with very-long-chain acyl-CoA dehydrogenase deficiency. J Pediatr. 143:335–342, 2003
27. Tuchman M, Freese DK, Sharp HL, Whitley CB, Ramnaraine ML, Ulstrom RA, Najarian JS, Ascher N, Buist NR, Terry AB: Persistent succinylacetone excretion after liver transplantation in a patient with hereditary tyrosinaemia type I. J Inherit. Metab Dis. 8:21–24, 1985
28. Laine J, Salo MK, Krogerus L, Karkkainen J, Wahlroos O, Holmberg C: The nephropathy of type I tyrosinemia after liver transplantation. Pediatr. Res. 37:640–645, 1995
29. Holme E, Lindstedt S: Neonatal screen for hereditary tyrosinaemia type I. Lancet. 340:850, 1992
30. Holme E, Lindstedt S: Tyrosinaemia type I and NTBC (2-(2-nitro-4-trifluoromethylbenzoyl)-1,3-cyclohexanedione). J Inherit. Metab Dis. 21:507–517, 1998
31. Kvittingen EA, Halvorsen S, Jellum E: Deficient fumarylacetoacetate fumarylhydrolase activity in lymphocytes and fibroblasts from patients with hereditary tyrosinemia. Pediatr. Res. 17:541–544, 1983
32. Gagne R, Lescault A, Grenier A, Laberge C, Melancon SB, Dallaire L: Prenatal diagnosis of hereditary tyrosinaemia: measurement of succinylacetone in amniotic fluid. Prenat. Diagn. 2:185–188, 1982
33. Allard P, Grenier A, Korson MS, Zytkovicz TH: Newborn screening for hepatorenal tyrosinemia by tandem mass spectrometry: analysis of succinylacetone extracted from dried blood spots. Clin. Biochem. 37:1010–1015, 2004
34. Tallab TM: Richner-Hanhart syndrome: importance of early diagnosis and early intervention. J Am. Acad. Dermatol. 35:857–859, 1996
35. Rehak A, Selim MM, Yadav G: Richner-Hanhart syndrome (tyrosinaemia-II) (report of four cases without ocular involvement). Br. J Dermatol. 104:469–475, 1981
36. Pelet B, Antener I, Faggioni R, Spahr A, Gautier E: Tyrosinemia without liver or renal damage with plantar and palmar keratosis and keratitis (hypertyrosinemia type II). Helv. Paediatr. Acta. 34:177–183, 1979
37. Goldsmith LA, Kang E, Bienfang DC, Jimbow K, Gerald P, Baden HP: Tyrosinemia with plantar and palmar keratosis and keratitis. J Pediatr. 83:798–805, 1973
38. Garibaldi LR, Siliato F, de M, I, Scarsi MR, Romano C: Oculocutaneous tyrosinosis. Report of two cases in the same family. Helv. Paediatr. Acta. 32:173–180, 1977
39. Chitayat D, Balbul A, Hani V, Mamer OA, Clow C, Scriver CR: Hereditary tyrosinaemia type II in a consanguineous Ashkenazi Jewish family: intrafamilial variation in phenotype; absence of parental phenotype effects on the fetus. J Inherit. Metab Dis. 15:198–203, 1992
40. Strauss KA, Morton DH: Branched-chain Ketoacyl Dehydrogenase Deficiency: Maple Syrup Disease. Curr. Treat. Options. Neurol. 5:329–341, 2003
41. Morton DH, Strauss KA, Robinson DL, Puffenberger EG, Kelley RI: Diagnosis and treatment of maple syrup disease: a study of 36 patients. Pediatrics. 109:999–1008, 2002

42. Chace DH, Hillman SL, Millington DS, Kahler SG, Roe CR, Naylor EW: Rapid diagnosis of maple syrup urine disease in blood spots from newborns by tandem mass spectrometry. Clin. Chem. 41:62–68, 1995
43. Schadewaldt P, Bodner-Leidecker A, Hammen HW, Wendel U: Significance of L-alloisoleucine in plasma for diagnosis of maple syrup urine disease. Clin. Chem. 45:1734–1740, 1999
44. Whelan DT, Brusso T, Spate M: Citrullinemia: phenotypic variations. Pediatrics. 57:935–941, 1976
45. McMurray WC, Mohyuddin F, Rathbun JC: Citrullinuria. Rev. Neuropsiquiatr. 27:287–93.:287–293, 1964
46. Johnson DW: Analysis of amino acids as formamidene butyl esters by electrospray ionization tandem mass spectrometry. Rapid Commun. Mass Spectrom. 15:2198–2205, 2001
47. Stadler S, Gempel K, Bieger I, Pontz BF, Gerbitz KD, Bauer MF, Hofmann S: Detection of neonatal argininosuccinate lyase deficiency by serum tandem mass spectrometry. J Inherit. Metab Dis. 24:370–378, 2001
48. Marsden D: Expanded newborn screening by tandem mass spectrometry: the Massachusetts and New England experience. Southeast Asian J Trop. Med. Public Health. 34 Suppl 3:111–4.: 111–114, 2003
49. Ohura T, Kobayashi K, Tazawa Y, Nishi I, Abukawa D, Sakamoto O, Iinuma K, Saheki T: Neonatal presentation of adult-onset type II citrullinemia. Hum. Genet. 108:87–90, 2001
50. Tazawa Y, Kobayashi K, Ohura T, Abukawa D, Nishinomiya F, Hosoda Y, Yamashita M, Nagata I, Kono Y, Yasuda T, Yamaguchi N, Saheki T: Infantile cholestatic jaundice associated with adult-onset type II citrullinemia. J Pediatr. 138:735–740, 2001
51. Tamamori A, Okano Y, Ozaki H, Fujimoto A, Kajiwara M, Fukuda K, Kobayashi K, Saheki T, Tagami Y, Yamano T: Neonatal intrahepatic cholestasis caused by citrin deficiency: severe hepatic dysfunction in an infant requiring liver transplantation. Eur. J Pediatr. 161:609–613, 2002
52. Kobayashi K, Sinasac DS, Iijima M, Boright AP, Begum L, Lee JR, Yasuda T, Ikeda S, Hirano R, Terazono H, Crackower MA, Kondo I, Tsui LC, Scherer SW, Saheki T: The gene mutated in adult-onset type II citrullinaemia encodes a putative mitochondrial carrier protein. Nat. Genet. 22:159–163, 1999
53. Saheki T, Kobayashi K: Mitochondrial aspartate glutamate carrier (citrin) deficiency as the cause of adult-onset type II citrullinemia (CTLN2) and idiopathic neonatal hepatitis (NICCD). J Hum. Genet. 47:333–341, 2002
54. Batshaw ML, Berry GT: Use of citrulline as a diagnostic marker in the prospective treatment of urea cycle disorders. J Pediatr. 118:914–917, 1991
55. Sander J, Janzen N, Sander S, Steuerwald U, Das AM, Scholl S, Trefz FK, Koch HG, Haberle J, Korall H, Marquardt I, Korenke C: Neonatal screening for citrullinaemia. Eur. J Pediatr. 162:417–420, 2003
56. Kleijer WJ, Garritsen VH, Linnebank M, Mooyer P, Huijmans JG, Mustonen A, Simola KO, Arslan-Kirchner M, Battini R, Briones P, Cardo E, Mandel H, Tschiedel E, Wanders RJ, Koch HG: Clinical, enzymatic, and molecular genetic characterization of a biochemical variant type of argininosuccinic aciduria: prenatal and postnatal diagnosis in five unrelated families. J Inherit. Metab Dis. 25:399–410, 2002
57. Widhalm K, Koch S, Scheibenreiter S, Knoll E, Colombo JP, Bachmann C, Thalhammer O: Long-term follow-up of 12 patients with the late-onset variant of argininosuccinic acid lyase deficiency: no impairment of intellectual and psychomotor development during therapy. Pediatrics. 89:1182–1184, 1992
58. Zytkovicz TH, Fitzgerald EF, Marsden D, Larson CA, Shih VE, Johnson DM, Strauss AW, Comeau AM, Eaton RB, Grady GF: Tandem mass spectrometric analysis for amino, organic, and fatty acid disorders in newborn dried blood spots: a two-year summary from the New England Newborn Screening Program. Clin. Chem. 47:1945–1955, 2001
59. Chace DH, Kalas TA, Naylor EW: Use of tandem mass spectrometry for multianalyte screening of dried blood specimens from newborns. Clin. Chem. 49:1797–1817, 2003
60. Gaustadnes M, Ingerslev J, Rutiger N: Prevalence of congenital homocystinuria in Denmark. N. Engl. J Med. 340:1513, 1999
61. Sokolova J, Janosikova B, Terwilliger JD, Freiberger T, Kraus JP, Kozich V: Cystathionine beta-synthase deficiency in Central Europe: discrepancy between biochemical and molecular genetic screening for homocystinuric alleles. Hum. Mutat. 18:548–549, 2001
62. Mudd SH, Skovby F, Levy HL, Pettigrew KD, Wilcken B, Pyeritz RE, Andria G, Boers GH, Bromberg IL, Cerone R, .: The natural history of homocystinuria due to cystathionine beta-synthase deficiency. Am. J Hum. Genet. 37:1–31, 1985
63. Refsum H, Fredriksen A, Meyer K, Ueland PM, Kase BF: Birth prevalence of homocystinuria. J Pediatr. 144:830–832, 2004
64. Fowler B, Jakobs C: Post- and prenatal diagnostic methods for the homocystinurias. Eur. J Pediatr. 157 Suppl 2:S88–93.:S88–S93, 1998
65. Chace DH, Hillman SL, Millington DS, Kahler SG, Adam BW, Levy HL: Rapid diagnosis of homocystinuria and other hypermethioninemias from newborns' blood spots by tandem mass spectrometry. Clin. Chem. 42:349–355, 1996
66. Rhead WJ: Newborn screening for medium-chain acyl-CoA dehydrogenase deficiency: a global perspective. J Inherit. Metab Dis. 29:370–377, 2006
67. Rinaldo P, O'Shea JJ, Coates PM, Hale DE, Stanley CA, Tanaka K: Medium-chain acyl-CoA dehydrogenase deficiency. Diagnosis by stable-isotope dilution measurement of urinary n-hexanoylglycine and 3-phenylpropionylglycine. N. Engl. J Med. 319:1308–1313, 1988
68. Chace DH, DiPerna JC, Mitchell BL, Sgroi B, Hofman LF, Naylor EW: Electrospray tandem mass spectrometry for analysis of acylcarnitines in dried postmortem blood specimens collected at autopsy from infants with unexplained cause of death. Clin. Chem. 47:1166–1182, 2001
69. Chace DH, Hillman SL, van Hove JL, Naylor EW: Rapid diagnosis of MCAD deficiency: quantitative analysis of octanoylcarnitine and other acylcarnitines in newborn blood spots by tandem mass spectrometry. Clin. Chem. 43:2106–2113, 1997
70. van Hove JL, Zhang W, Kahler SG, Roe CR, Chen YT, Terada N, Chace DH, Iafolla AK, Ding JH, Millington DS: Medium-chain acyl-CoA dehydrogenase (MCAD) deficiency: diagnosis by acylcarnitine analysis in blood. Am. J Hum. Genet. 52:958–966, 1993
71. Clayton PT, Doig M, Ghafari S, Meaney C, Taylor C, Leonard JV, Morris M, Johnson AW: Screening for medium chain acyl-CoA dehydrogenase deficiency using electrospray ionisation tandem mass spectrometry. Arch. Dis. Child. 79:109–115, 1998
72. Pourfarzam M, Morris A, Appleton M, Craft A, Bartlett K: Neonatal screening for medium-chain acyl-CoA dehydrogenase deficiency. Lancet. 358:1063–1064, 2001
73. Wood JC, Magera MJ, Rinaldo P, Seashore MR, Strauss AW, Friedman A: Diagnosis of very long chain acyl-dehydrogenase deficiency from an infant's newborn screening card. Pediatrics. 108:E19, 2001

74. Brown-Harrison MC, Nada MA, Sprecher H, Vianey-Saban C, Farquhar J, Jr., Gilladoga AC, Roe CR: Very long chain acyl-CoA dehydrogenase deficiency: successful treatment of acute cardiomyopathy. Biochem. Mol. Med. 58:59–65, 1996
75. Matern D, Strauss AW, Hillman SL, Mayatepek E, Millington DS, Trefz FK: Diagnosis of mitochondrial trifunctional protein deficiency in a blood spot from the newborn screening card by tandem mass spectrometry and DNA analysis. Pediatr. Res. 46:45–49, 1999
76. van Hove JL, Kahler SG, Feezor MD, Ramakrishna JP, Hart P, Treem WR, Shen JJ, Matern D, Millington DS: Acylcarnitines in plasma and blood spots of patients with long-chain 3-hydroxyacyl-coenzyme A dehydrogenase defiency. J Inherit. Metab Dis. 23:571–582, 2000
77. Rinaldo P, Matern D, Bennett MJ: Fatty acid oxidation disorders. Annu. Rev. Physiol. 64:477–502.:477–502, 2002
78. Tein I, Haslam RH, Rhead WJ, Bennett MJ, Becker LE, Vockley J: Short-chain acyl-CoA dehydrogenase deficiency: a cause of ophthalmoplegia and multicore myopathy. Neurology. 52:366–372, 1999
79. Corydon MJ, Vockley J, Rinaldo P, Rhead WJ, Kjeldsen M, Winter V, Riggs C, Babovic-Vuksanovic D, Smeitink J, De Jong J, Levy H, Sewell AC, Roe C, Matern D, Dasouki M, Gregersen N: Role of common gene variations in the molecular pathogenesis of short-chain acyl-CoA dehydrogenase deficiency. Pediatr. Res. 49:18–23, 2001
80. Young SP, Matern D, Gregersen N, Stevens RD, Bali D, Liu HM, Koeberl DD, Millington DS: A comparison of in vitro acylcarnitine profiling methods for the diagnosis of classical and variant short chain acyl-CoA dehydrogenase deficiency. Clin. Chim. Acta. 337:103–113, 2003
81. Coates PM, Hale DE, Finocchiaro G, Tanaka K, Winter SC: Genetic deficiency of short-chain acyl-coenzyme A dehydrogenase in cultured fibroblasts from a patient with muscle carnitine deficiency and severe skeletal muscle weakness. J Clin. Invest. 81:171–175, 1988
82. Bhala A, Willi SM, Rinaldo P, Bennett MJ, Schmidt-Sommerfeld E, Hale DE: Clinical and biochemical characterization of short-chain acyl-coenzyme A dehydrogenase deficiency. J Pediatr. 126:910–915, 1995
83. Koeberl DD, Young SP, Gregersen NS, Vockley J, Smith WE, Benjamin DK, Jr., An Y, Weavil SD, Chaing SH, Bali D, McDonald MT, Kishnani PS, Chen YT, Millington DS: Rare disorders of metabolism with elevated butyryl- and isobutyrylcarnitine detected by tandem mass spectrometry newborn screening. Pediatr. Res. 54:219–223, 2003
84. Bennett MJ, Spotswood SD, Ross KF, Comfort S, Koonce R, Boriack RL, Ijlst L, Wanders RJ: Fatal hepatic short-chain L-3-hydroxyacyl-coenzyme A dehydrogenase deficiency: clinical, biochemical, and pathological studies on three subjects with this recently identified disorder of mitochondrial beta-oxidation. Pediatr. Dev. Pathol. 2:337–345, 1999
85. Bennett MJ, Russell LK, Tokunaga C, Narayan SB, Tan L, Seegmiller A, Boriack RL, Strauss AW: Reye-like syndrome resulting from novel missense mutations in mitochondrial medium- and short-chain l-3-hydroxy-acyl-CoA dehydrogenase. Mol. Genet. Metab. 89:74–79, 2006
86. Molven A, Matre GE, Duran M, Wanders RJ, Rishaug U, Njolstad PR, Jellum E, Sovik O: Familial hyperinsulinemic hypoglycemia caused by a defect in the SCHAD enzyme of mitochondrial fatty acid oxidation. Diabetes. 53:221–227, 2004
87. Yang SY, He XY, Schulz H: 3-Hydroxyacyl-CoA dehydrogenase and short chain 3-hydroxyacyl-CoA dehydrogenase in human health and disease. FEBS J. 272:4874–4883, 2005
88. Clayton PT, Eaton S, Aynsley-Green A, Edginton M, Hussain K, Krywawych S, Datta V, Malingre HE, Berger R, van dB, I: Hyperinsulinism in short-chain L-3-hydroxyacyl-CoA dehydrogenase deficiency reveals the importance of beta-oxidation in insulin secretion. J Clin. Invest. 108:457–465, 2001
89. Luo MJ, Mao LF, Schulz H: Short-chain 3-hydroxy-2-methylacyl-CoA dehydrogenase from rat liver: purification and characterization of a novel enzyme of isoleucine metabolism. Arch. Biochem. Biophys. 321:214–220, 1995
90. Treacy EP, Lambert DM, Barnes R, Boriack RL, Vockley J, O'brien LK, Jones PM, Bennett MJ: Short-chain hydroxyacyl-coenzyme A dehydrogenase deficiency presenting as unexpected infant death: A family study. J Pediatr. 137:257–259, 2000
91. Treem WR, Stanley CA, Finegold DN, Hale DE, Coates PM: Primary carnitine deficiency due to a failure of carnitine transport in kidney, muscle, and fibroblasts. N. Engl. J Med. 319:1331–1336, 1988
92. Wilcken B, Wiley V, Sim KG, Carpenter K: Carnitine transporter defect diagnosed by newborn screening with electrospray tandem mass spectrometry. J Pediatr. 138:581–584, 2001
93. Tein I, De Vivo DC, Bierman F, Pulver P, De Meirleir LJ, Cvitanovic-Sojat L, Pagon RA, Bertini E, Dionisi-Vici C, Servidei S, .: Impaired skin fibroblast carnitine uptake in primary systemic carnitine deficiency manifested by childhood carnitine-responsive cardiomyopathy. Pediatr. Res. 28:247–255, 1990
94. Fukao T, Zhang GX, Sakura N, Kubo T, Yamaga H, Hazama A, Kohno Y, Matsuo N, Kondo M, Yamaguchi S, Shigematsu Y, Kondo N: The mitochondrial acetoacetyl-CoA thiolase (T2) deficiency in Japanese patients: urinary organic acid and blood acylcarnitine profiles under stable conditions have subtle abnormalities in T2-deficient patients with some residual T2 activity. J Inherit. Metab Dis. 26:423–431, 2003
95. Fukao T, Scriver CR, Kondo N: The clinical phenotype and outcome of mitochondrial acetoacetyl-CoA thiolase deficiency (beta-ketothiolase or T2 deficiency) in 26 enzymatically proved and mutation-defined patients. Mol. Genet. Metab. 72:109–114, 2001
96. Renom G, Fontaine M, Rolland MO, Duprey J, Degand PM, Dobbelaere D: A new case of 2-methylacetoacetyl-CoA thiolase deficiency? J Inherit. Metab Dis. 23:751–753, 2000
97. Gibson KM, Feigenbaum AS: Phenotypically mild presentation in a patient with 2-methylacetoacetyl-coenzyme A (beta-keto)thiolase deficiency. J Inherit. Metab Dis. 20:712–713, 1997
98. Fontaine M, Briand G, Ser N, Armelin I, Rolland MO, Degand P, Vamecq J: Metabolic studies in twin brothers with 2-methylacetoacetyl-CoA thiolase deficiency. Clin. Chim. Acta. 255:67–83, 1996
99. Aramaki S, Lehotay D, Sweetman L, Nyhan WL, Winter SC, Middleton B: Urinary excretion of 2-methylacetoacetate, 2-methyl-3-hydroxybutyrate and tiglylglycine after isoleucine loading in the diagnosis of 2-methylacetoacetyl-CoA thiolase deficiency. J Inherit. Metab Dis. 14:63–74, 1991
100. Faull KF, Bolton PD, Halpern B, Hammond J, Danks DM: The urinary organic acid profile associated with 3-hydroxy-3-methylglutaric aciduria. Clin. Chim. Acta. 73:553–559, 1976
101. Gibson KM, Breuer J, Nyhan WL: 3-Hydroxy-3-methylglutaryl-coenzyme A lyase deficiency: review of 18 reported patients. Eur. J Pediatr. 148:180–186, 1988

102. Wilkinson SP, Wysocki SJ, Hahnel R: Diagnosing 3-hydroxy-3-methylglutaric aciduria. Clin. Chem. 32:1615–1616, 1986
103. Wysocki SJ, Hahnel R: 3-Hydroxy-3-methylglutaryl-coenzyme a lyase deficiency: a review. J Inherit. Metab Dis. 9:225–233, 1986
104. Barash V, Mandel H, Sella S, Geiger R: 3-Hydroxy-3-methylglutaryl-coenzyme A lyase deficiency: biochemical studies and family investigation of four generations. J Inherit. Metab Dis. 13:156–164, 1990
105. Kyllerman M, Skjeldal OH, Lundberg M, Holme I, Jellum E, von Dobeln U, Fossen A, Carlsson G: Dystonia and dyskinesia in glutaric aciduria type I: clinical heterogeneity and therapeutic considerations. Mov Disord. 9:22–30, 1994
106. Kolker S, Christensen E, Leonard JV, Greenberg CR, Burlina AB, Burlina AP, Dixon M, Duran M, Goodman SI, Koeller DM, Muller E, Naughten ER, Neumaier-Probst E, Okun JG, Kyllerman M, Surtees RA, Wilcken B, Hoffmann GF, Burgard P: Guideline for the diagnosis and management of glutaryl-CoA dehydrogenase deficiency (glutaric aciduria type I). J Inherit. Metab Dis. 30:5–22, 2007
107. Morton DH, Bennett MJ, Seargeant LE, Nichter CA, Kelley RI: Glutaric aciduria type I: a common cause of episodic encephalopathy and spastic paralysis in the Amish of Lancaster County, Pennsylvania. Am. J Med. Genet. 41:89–95, 1991
108. Merinero B, Perez-Cerda C, Font LM, Garcia MJ, Aparicio M, Lorenzo G, Martinez PM, Garzo C, Martinez-Bermejo A, Pascual C, I, .: Variable clinical and biochemical presentation of seven Spanish cases with glutaryl-CoA-dehydrogenase deficiency. Neuropediatrics. 26:238–242, 1995
109. Kolker S, Garbade SF, Greenberg CR, Leonard JV, Saudubray JM, Ribes A, Kalkanoglu HS, Lund AM, Merinero B, Wajner M, Troncoso M, Williams M, Walter JH, Campistol J, Marti-Herrero M, Caswill M, Burlina AB, Lagler F, Maier EM, Schwahn B, Tokatli A, Dursun A, Coskun T, Chalmers RA, Koeller DM, Zschocke J, Christensen E, Burgard P, Hoffmann GF: Natural history, outcome, and treatment efficacy in children and adults with glutaryl-CoA dehydrogenase deficiency. Pediatr. Res. 59:840–847, 2006
110. Hoffmann GF, Bohles HJ, Burlina A, Duran M, Herwig J, Lehnert W, Leonard JV, Muntau A, Plecko-Starting FK, Superti-Furga A,.: Early signs and course of disease of glutaryl-CoA dehydrogenase deficiency. J Inherit. Metab Dis. 18:173–176, 1995
111. Hedlund GL, Longo N, Pasquali M: Glutaric acidemia type 1. Am. J Med. Genet. C. Semin. Med. Genet. 142:86–94, 2006
112. Bjugstad KB, Goodman SI, Freed CR: Age at symptom onset predicts severity of motor impairment and clinical outcome of glutaric acidemia type 1. J Pediatr. 137:681–686, 2000
113. Strauss KA, Puffenberger EG, Robinson DL, Morton DH: Type I glutaric aciduria, part 1: natural history of 77 patients. Am. J Med. Genet. C. Semin. Med. Genet. 121:38–52, 2003
114. Christensen E, Ribes A, Merinero B, Zschocke J: Correlation of genotype and phenotype in glutaryl-CoA dehydrogenase deficiency. J Inherit. Metab Dis. 27:861–868, 2004
115. Lindner M, Kolker S, Schulze A, Christensen E, Greenberg CR, Hoffmann GF: Neonatal screening for glutaryl-CoA dehydrogenase deficiency. J Inherit. Metab Dis. 27:851–859, 2004
116. Dusheiko G, Kew MC, Joffe BI, Lewin JR, Mantagos S, Tanaka K: Recurrent hypoglycemia associated with glutaric aciduria type II in an adult. N. Engl. J Med. 301:1405–1409, 1979
117. Jakobs C, Sweetman L, Wadman SK, Duran M, Saudubray JM, Nyhan WL: Prenatal diagnosis of glutaric aciduria type II by direct chemical analysis of dicarboxylic acids in amniotic fluid. Eur. J Pediatr. 141:153–157, 1984
118. Poplawski NK, Ranieri E, Harrison JR, Fletcher JM: Multiple acyl-coenzyme A dehydrogenase deficiency: diagnosis by acylcarnitine analysis of a 12-year-old newborn screening card. J Pediatr. 134:764–766, 1999
119. Russell AP, Schrauwen P, Somm E, Gastaldi G, Hesselink MK, Schaart G, Kornips E, Lo SK, Bufano D, Giacobino JP, Muzzin P, Ceccon M, Angelini C, Vergani L: Decreased fatty acid beta-oxidation in riboflavin-responsive, multiple acylcoenzyme A dehydrogenase-deficient patients is associated with an increase in uncoupling protein-3. J Clin. Endocrinol. Metab. 88:5921–5926, 2003
120. Yamaguchi S, Shimizu N, Orii T, Fukao T, Suzuki Y, Maeda K, Hashimoto T, Previs SF, Rinaldo P: Prenatal diagnosis and neonatal monitoring of a fetus with glutaric aciduria type II due to electron transfer flavoprotein (beta-subunit) deficiency. Pediatr. Res. 30:439–443, 1991
121. Yamaguchi S, Orii T, Suzuki Y, Maeda K, Oshima M, Hashimoto T: Newly identified forms of electron transfer flavoprotein deficiency in two patients with glutaric aciduria type II. Pediatr. Res. 29:60–63, 1991
122. Wallace SJ: Biotinidase deficiency: presymptomatic treatment. Arch. Dis. Child. 60:574–575, 1985
123. Haagerup A, Andersen JB, Blichfeldt S, Christensen MF: Biotinidase deficiency: two cases of very early presentation. Dev. Med. Child Neurol. 39:832–835, 1997
124. Wolf B, Norrgard K, Pomponio RJ, Mock DM, McVoy JR, Fleischhauer K, Shapiro S, Blitzer MG, Hymes J: Profound biotinidase deficiency in two asymptomatic adults. Am. J Med. Genet. 73:5–9, 1997
125. Wolf B, Pomponio RJ, Norrgard KJ, Lott IT, Baumgartner ER, Suormala T, Ramaekers VT, Coskun T, Tokatli A, Ozalp I, Hymes J: Delayed-onset profound biotinidase deficiency. J Pediatr. 132:362–365, 1998
126. Heard GS, Wolf B, Jefferson LG, Weissbecker KA, Nance WE, McVoy JR, Napolitano A, Mitchell PL, Lambert FW, Linyear AS: Neonatal screening for biotinidase deficiency: results of a 1-year pilot study. J Pediatr. 108:40–46, 1986
127. Wolf B, Heard GS, Weissbecker KA, McVoy JR, Grier RE, Leshner RT: Biotinidase deficiency: initial clinical features and rapid diagnosis. Ann. Neurol. 18:614–617, 1985
128. Wolf B, Heard GS, Jefferson LG, Proud VK, Nance WE, Weissbecker KA: Clinical findings in four children with biotinidase deficiency detected through a statewide neonatal screening program. N. Engl. J Med. 313:16–19, 1985
129. Suormala TM, Baumgartner ER, Wick H, Scheibenreiter S, Schweitzer S: Comparison of patients with complete and partial biotinidase deficiency: biochemical studies. J Inherit. Metab Dis. 13:76–92, 1990
130. Hoffman TL, Simon EM, Ficicioglu C: Biotinidase deficiency: the importance of adequate follow-up for an inconclusive newborn screening result. Eur. J Pediatr. 164:298–301, 2005
131. Yang X, Aoki Y, Li X, Sakamoto O, Hiratsuka M, Kure S, Taheri S, Christensen E, Inui K, Kubota M, Ohira M, Ohki M, Kudoh J, Kawasaki K, Shibuya K, Shintani A, Asakawa S, Minoshima S, Shimizu N, Narisawa K, Matsubara Y, Suzuki Y: Structure of human holocarboxylase synthetase gene and mutation spectrum of holocarboxylase synthetase deficiency. Hum. Genet. 109:526–534, 2001
132. Suormala T, Fowler B, Duran M, Burtscher A, Fuchshuber A, Tratzmuller R, Lenze MJ, Raab K, Baur B, Wick H, Baumgartner R: Five patients with a biotin-responsive defect in holocarboxylase formation: evaluation of responsiveness to

biotin therapy in vivo and comparative biochemical studies in vitro. Pediatr. Res. 41:666–673, 1997
133. Suormala T, Wick H, Bonjour JP, Baumgartner ER: Rapid differential diagnosis of carboxylase deficiencies and evaluation for biotin-responsiveness in a single blood sample. Clin. Chim. Acta. 145:151–162, 1985
134. Sakamoto O, Suzuki Y, Li X, Aoki Y, Hiratsuka M, Holme E, Kudoh J, Shimizu N, Narisawa K: Diagnosis and molecular analysis of an atypical case of holocarboxylase synthetase deficiency. Eur. J Pediatr. 159:18–22, 2000
135. Sakamoto O, Suzuki Y, Li X, Aoki Y, Hiratsuka M, Suormala T, Baumgartner ER, Gibson KM, Narisawa K: Relationship between kinetic properties of mutant enzyme and biochemical and clinical responsiveness to biotin in holocarboxylase synthetase deficiency. Pediatr. Res. 46:671–676, 1999
136. Aoki Y, Li X, Sakamoto O, Hiratsuka M, Akaishi H, Xu L, Briones P, Suormala T, Baumgartner ER, Suzuki Y, Narisawa K: Identification and characterization of mutations in patients with holocarboxylase synthetase deficiency. Hum. Genet. 104:143–148, 1999
137. Ficicioglu C, Payan I: 3-Methylcrotonyl-CoA carboxylase deficiency: metabolic decompensation in a noncompliant child detected through newborn screening. Pediatrics. 118:2555–2556, 2006
138. Visser G, Suormala T, Smit GP, Reijngoud DJ, Bink-Boelkens MT, Niezen-Koning KE, Baumgartner ER: 3-methylcrotonyl-CoA carboxylase deficiency in an infant with cardiomyopathy, in her brother with developmental delay and in their asymptomatic father. Eur. J Pediatr. 159:901–904, 2000
139. Stadler SC, Polanetz R, Maier EM, Heidenreich SC, Niederer B, Mayerhofer PU, Lagler F, Koch HG, Santer R, Fletcher JM, Ranieri E, Das AM, Spiekerkotter U, Schwab KO, Potzsch S, Marquardt I, Hennermann JB, Knerr I, Mercimek-Mahmutoglu S, Kohlschmidt N, Liebl B, Fingerhut R, Olgemoller B, Muntau AC, Roscher AA, Roschinger W: Newborn screening for 3-methylcrotonyl-CoA carboxylase deficiency: population heterogeneity of MCCA and MCCB mutations and impact on risk assessment. Hum. Mutat. 27:748–759, 2006
140. Layward EM, Tanner MS, Pollitt RJ, Bartlett K: Isolated biotin-resistant 3-methylcrotonyl-CoA carboxylase deficiency presenting as a Reye syndrome-like illness. J Inherit. Metab Dis. 12:339–340, 1989
141. Rolland MO, Divry P, Zabot MT, Guibaud P, Gomez S, Lachaux A, Loras I: Isolated 3-methylcrotonyl-CoA carboxylase deficiency in a 16-month-old child. J Inherit. Metab Dis. 14:838–839, 1991
142. Murayama K, Kimura M, Yamaguchi S, Shinka T, Kodama K: Isolated 3-methylcrotonyl-CoA carboxylase deficiency in a 15-year-old girl. Brain Dev. 19:303–305, 1997
143. van Hove JL, Rutledge SL, Nada MA, Kahler SG, Millington DS: 3-Hydroxyisovalerylcarnitine in 3-methylcrotonyl-CoA carboxylase deficiency. J Inherit. Metab Dis. 18:592–601, 1995
144. Koeberl DD, Millington DS, Smith WE, Weavil SD, Muenzer J, McCandless SE, Kishnani PS, McDonald MT, Chaing S, Boney A, Moore E, Frazier DM: Evaluation of 3-methylcrotonyl-CoA carboxylase deficiency detected by tandem mass spectrometry newborn screening. J Inherit. Metab Dis. 26:25–35, 2003
145. Dhondt JL, Farriaux JP, Amedee MO, Cartigny B, Sweetman L, Duran M: Antenatal diagnosis of propionic acidaemia by methylcitrate determination. J Inherit. Metab Dis. 4:49–50, 1981
146. Aramaki S, Lehotay D, Nyhan WL, MacLeod PM, Sweetman L: Methylcitrate in maternal urine during a pregnancy with a fetus affected with propionic acidaemia. J Inherit. Metab Dis. 12:86–88, 1989
147. Merinero B, DelValle JA, Jimenez A, Garcia MJ, Ugarte M, Solaguren R, Lopez O, Condado I: Late onset type of propionic acidaemia: case report and biochemical studies. J Inherit. Metab Dis. 4:71–72, 1981
148. Wolf B, Paulsen EP, Hsia YE: Asymptomatic propionyl CoA carboxylase deficiency in a 13-year-old girl. J Pediatr. 95:563–565, 1979
149. Naylor EW, Chace DH: Automated tandem mass spectrometry for mass newborn screening for disorders in fatty acid, organic acid, and amino acid metabolism. J Child Neurol. 14 Suppl 1:S4–8.:S4–S8, 1999
150. Chace DH, DiPerna JC, Kalas TA, Johnson RW, Naylor EW: Rapid diagnosis of methylmalonic and propionic acidemias: quantitative tandem mass spectrometric analysis of propionylcarnitine in filter-paper blood specimens obtained from newborns. Clin. Chem. 47:2040–2044, 2001
151. Horster F, Hoffmann GF: Pathophysiology, diagnosis, and treatment of methylmalonic aciduria-recent advances and new challenges. Pediatr. Nephrol. 19:1071–1074, 2004
152. Baumgarter ER, Viardot C: Long-term follow-up of 77 patients with isolated methylmalonic acidaemia. J Inherit. Metab Dis. 18:138–142, 1995
153. Matsui SM, Mahoney MJ, Rosenberg LE: The natural history of the inherited methylmalonic acidemias. N. Engl. J Med. 308:857–861, 1983
154. Coulombe JT, Shih VE, Levy HL: Massachusetts Metabolic Disorders Screening Program. II. Methylmalonic aciduria. Pediatrics. 67:26–31, 1981
155. la Marca G, Malvagia S, Pasquini E, Innocenti M, Donati MA, Zammarchi E: Rapid 2nd-tier test for measurement of 3-OH-propionic and methylmalonic acids on dried blood spots: reducing the false-positive rate for propionylcarnitine during expanded newborn screening by liquid chromatography-tandem mass spectrometry. Clin. Chem. 53:1364–1369, 2007
156. Ensenauer R, Vockley J, Willard JM, Huey JC, Sass JO, Edland SD, Burton BK, Berry SA, Santer R, Grunert S, Koch HG, Marquardt I, Rinaldo P, Hahn S, Matern D: A common mutation is associated with a mild, potentially asymptomatic phenotype in patients with isovaleric acidemia diagnosed by newborn screening. Am. J Hum. Genet. 75:1136–1142, 2004
157. Vockley J, Parimoo B, Tanaka K: Molecular characterization of four different classes of mutations in the isovaleryl-CoA dehydrogenase gene responsible for isovaleric acidemia. Am. J Hum. Genet. 49:147–157, 1991
158. Budd MA, Tanaka K, Holmes LB, Efron ML, Crawford JD, Isselbacher KJ: Isovaleric acidemia. Clinical features of a new genetic defect of leucine metabolism. N. Engl. J Med. 277:321–327, 1967
159. Mayatepek E, Kurczynski TW, Hoppel CL: Long-term L-carnitine treatment in isovaleric acidemia. Pediatr. Neurol. 7:137–140, 1991
160. de Sousa C, Chalmers RA, Stacey TE, Tracey BM, Weaver CM, Bradley D: The response to L-carnitine and glycine therapy in isovaleric acidaemia. Eur. J Pediatr. 144:451–456, 1986
161. Duran M, van Sprang FJ, Drewes JG, Bruinvis L, Ketting D, Wadman SK: Two sisters with isovaleric acidaemia, multiple attacks of ketoacidosis and normal development. Eur. J Pediatr. 131:205–211, 1979
162. Tanaka K, Orr JC, Isselbacher KJ: Identification of beta-hydroxyisovaleric acid in the urine of a patient with isovaleric acidemia. Biochim. Biophys. Acta. 152:638–641, 1968

Chapter **4**

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.

REFERENCES

1. Schoen EJ, Baker JC, Colby CJ, Trinh T. Cost-Benefit Analysis of Universal Tandem Mass Spectrometry for Newborn Screening. Pediatrics 2002, 110(4):781–786.
2. Pollitt RJ. International Perspectives on Newborn Screening. J Inherit Metab Dis 2006, 29:390–396.
3. Insinga RP, Laessig RH, Hoffman GL. Newborn Screening with Tandem Mass Spectrometry: Examining its Cost-Effectiveness in the Wisconsin Newborn Screening Panel. J Peds 2002, 141(4):524–531.
4 Pandor A, Eastham J, Beverley C, Chilcott J, Paisley S. Clinical Effectiveness and Cost-Effectiveness of Neonatal Screening for Inborn Errors of Metabolism Using Tandem Mass Spectrometry: A Systematic Review. Health Technology Assessment 2004, 8:12 (Executive Summary).
5. Haas M, Chaplin M, Joy P, Wiley V, Black C, Wilcken B. Healthcare Use and Costs of Medium-chain Acyl-CoA Dehydrogenase Deficiency in Australia: Screening Versus No Screening. J Pediatr 2007, 151:121–6.
6. Cipriano LE, Rupar CA, Zaric GS. The Cost-Effectiveness of Expanding Newborn Screening for up to 21 Inherited Metabolic Disorders Using Tandem Mass Spectrometry: Results from a Decision-Analytic Model. Value in Health 2007, 10(2):83–97.
7. van der Hilst CS, Derks TGJ, Reijngoud DJ, Smit PA, TenVergert EM. Cost-Effectiveness of Neonatal Screening for Medium Chain Acyl-CoA Dehydrogenase Deficiency: The Homogeneous Population of the Netherlands. J Pediatr 2007, 151:115–20.
8. McCandless SE. A Primer on Expanded Newborn Screening by Tandem Mass Spectrometry. Prim Care Clin Office Pract 2004, 31:583–604.
9. Schulz A, Lindner M, Kohlmuller D, Olgemuller K, Mayatepek E, Hoffmann GF. Expanded Newborn Screening for Inborn Errors of Metabolism by Electrospray Ionization-Tandem Mass Spectromery: Results, Outcome, and Implications. Pediatr 2003, 111(6):1399–1406.
10. Green NS, Dolan SM, Murray TH. Newborn Screening: Complexities in Universal Genetic Testing. Amer J Pub Health 2006, 96(11):1955–58.
11. Dionsi-Vici C, Deodato F, Roschinger W, Rhead WJ, Wilcken B. ‘Classical’ Organic Acidurias, Propionic Aciduria, Methylmalonic Aciduria and Isovaleric Aciduria: Long-Term Outcome and Effects of Expanded Newborn Screening Using Tandem Mass Spectrometry. J Inherit Metab Dis 2006, 29:383–389.
12. Waisbren SE. Newborn Screening for Metabolic Disorders. JAMA 2006, 296(8):993–995.
13. Turgeon C, Magera MJ, Allard P, Tortorelli S, Gavrilov D, Oglesbee D, Raymond K, Rinaldo P, Matern D. Combined newborn screening for succinylacetone, amino acids, and acylcarnitines in dried blood spots. Clin Chem 54, 657–664, 2008.
14. Wilcken B. The Consequences of Extended Newborn Screening Programmes: Do We Know Who Needs Treatment? J Inherit Metab Dis, Online, February 2008.
15. van Maldegem BT, Duran M, Wanders RJA, Niezen-Koning KE, Hogeveen M, Ijlst L, Waterham HR, Wijburg FA. Clinical, Biochemical, and Genetic Heterogeneity in Short-Chain Acyl-Coenzyme A Dehydrogenase Deficiency. JAMA 2006, 296(8):943–52.
16. Nennstiel-Ratzel U, Arenz S, Maier EM, Knerr I, Baukotter J, Roschinger W, Liebl B, Hadron HB, Roscher AA, von Kries R. Reduced Incidence of Severe Metabolic Crisis or Death in Children with Medium Chain Acyl-CoA Dehydrogenase Deficiency Homozygous for c.985A>G Identified by Neonatal Screening. Mol Genet Met 2005, 85:157–159.
17. Wilcken B, Haas M, Joy P, Wiley V, Chaplin M, Black C, Fletcher J, McGill J, Boneh A. Outcome of Neonatal Screening for Medium-Chain Acyl-CoA Dehydrogenase Deficiency in Australia: A Cohor Study. Lancet 2007, 369(9555):37–42.
18. Grosse SD, Khoury MJ, Greene CL, Crider KS, Pollitt RJ. The Epidemiology of Medium Chain Acyl-CoA Dehydrogenase Deficiency: An Update. Genet Med 2006, 8(4):205–212.
19. Simon E, Fingerhut R, Baumkotter J, Konstantopoulou V, Ratschmann R, Wendel U. Maple Syrup Urine Disease: Favourable Effect of Early Diagnosis by Newborn Screening on the Neonatal Course of the Disease. J Inherit Metab Dis 2006, 29:532–537.
20. Lindner M, Ho S, Fang-Hoffmann GF, Kolker S. Neonatal Screening for Glutaic Aciduria Type I: Strategies to Proceed. J Inherit Metab Dis 2006, 29:378–382.
21. Strauss KA, Puffenberger EG, Robinson DL, Morton DH. Type I Glutaric Aciduria, Part 1: Natural History of 77 Patients. Am J Med Gen (Semin. Med. Genet.) 2003, 121C:38–52.
22. Hsieh CT, Hwu WL, Huang YT, Huang AC, Wang SF, Hu MH, Chien YH. Early Detection of Glutaric Aciduria Type I by Newborn Screening in Taiwan. J Formos Med Assoc 2008, 107(2):139–144.
23. Kolker S, Garbade SF, Boy N, Maier EM, Meissner T, Muhlhausen C, Hennermann JB, Lucke T, Haberle J, Baumkotter J, Haller W, Muller E, Zschocke J, Burgard P, Hoffmann G. Decline of Acute Encephalopathic Crises in Children with Glutaryl-CoA Dehydrogenase Deficiency Identified by Newborn Screening in Germany. Pediatric Research 2007, 62(3):357–363.

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	
	Type 1	*GA1*	A-I
	Type 2	*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
	Mutase	*MUT*	A-II
	Cbl	*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	
	Type 1	*TYR 1*	B-III
	Type 2	*TYR 2*	B-III
	Type 3	*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

Chapter **5**

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 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.

REFERENCES

1. Chamoles NA, Blanco M, Gaggioli D. Fabry disease: enzymatic diagnosis in dried blood spots on filter paper. Clin Chim Acta 308; 195–196, 2001
2. Umapathysivam K, Hopwood JJ, Meikle PJ. Determination of acid alpha-glucosidase activity in blood spots as a diagnostic test for Pompe disease. Clin Chem 47; 1378–1383, 2001
3. Chamoles NA, Blanco MB, Gaggioli D, Casentini C. Hurler-like phenotype: enzymatic diagnosis in dried blood spots on filter paper. Clin Chem 47; 2098–2102, 2001
4. Chamoles NA, Blanco M, Gaggioli D, Casentini C. Gaucher and Niemann-Pick diseases-enzymatic diagnosis in dried blood spots on filter paper: retrospective diagnoses in newborn-screening cards. Clin Chim Acta 317; 191–197, 2002
5. Chamoles NA, Blanco M, Gaggioloi D, Casentini C. Tay-Sachs and Sandhoff diseases: enzymatic diagnosis in dried blood spots on filter paper: retrospective diagnoses in newborn screening cards. Clin Chim Acta 318; 133–137, 2002
6. Dean CJ, Bockmann MR, Hopwood JJ, Brooks DA, Meikel PJ. Detection of mucopolysaccharidosis type II by measurement of iduronidate-2-sulfatase in dried blood spots and plasma samples. Clin Chem 52; 643–649, 2006
7. Civallero G, Michelin K, de Mari J, Viapiana M, Coelho JC, Giugliani R. Twelve different enzyme assays on dried-blood filter paper samples for detection of patients with selected inherited lysosomal storage diseases. Clin Chim Acta 372; 98–102, 2006
8. Li Y, Brockmann K, Turecek F, Scott CR, Gelb MH. Tandem mass spectrometry for the direct assay of enzymes in dried blood spots: application to newborn screening for Krabbe disease. Clin Chem 50; 638–640, 2004
9. Li Y, Scott CR, Chamoles NA, Ghavami A, Pinto BM, Turecek F, Gelb MH. Direct multiplex assay of lysosomal enzymes in dried blood spots for newborn screening. Clin Chem 50; 1785–1796, 2004
10. Wang D, Eadala B, Sadilek M, Chamoles NA, Turecek F, Scott CR, Gelb MH. Tandem mass spectrometric analysis of dried blood spots for screening of mucopolysaccharidosis I in newborns. Clin Chem 51; 898–900, 2005
11. Gelb MH, Turecek F, Scott CR, Chamoles NA. Direct multiplex assay of enzymes in dried blood spots by tandem mass spectrometry for the newborn screening of lysosomal storage disorders. J Inherit Metab Dis 29; 397–404, 2006
12. Tandem mass spectrometry for the direct assay of enzymes in dried blood spots: application to newborn screening for mucopolysaccharidosis II (Hunter disease). Clin Chem 53; 137–140, 2007
13. Turecek F, Scott CR. Gelb MH. Tandem mass spectrometry in the detection of inborn errors of metabolism for newborn screening. Methods Mol Biol 359; 143–157, 2007
14. Wraith JE. Limitations of enzyme replacement therapy: current and future. J Inherit Metab Dis 29; 442–447, 2006

15. Starzyk K, Richards S, Yee J, Smith SE, Kingma W. The long-term international safety experience of imiglucerase therapy for Gaucher disease. Mol Genet Metab 90; 157–163, 2007
16. Sidransky E, LaMarca ME, Ginns EI. Therapy for Gaucher disease: don't stop thinking about tomorrow. Mol Genet Metab 90; 122–125, 2007 (commentary on reference 15)
17. Schiffmann R, Askari H, Timmons M, Robinson C, Benko W, brady RO, Ries M. weekly enzyme replacement therapy may slow decline of renal function in patients with Fabry disease who are on long-term biweekly dosing. J Am Soc Nephrol 18; 1576–1583, 2007
18. Germain DP, Waldeck S, Banikazemi M, Bushinsky DA, Charrow J, Desnick RJ, Lee P, Loew T, Vedder AC, Abichandani R, Wilcox WR, Guffon N. Sustained long-term renal stabilization after 54 months of agalsidase beta therapy in patients with Fabry disease. J Am Soc Nephrol 18; 1547–1557, 2007
19. Thomas JA, Jacobs S, Kierstein J, Van Hove J. Outcome after three years of laronidase enzyme replacement therapy in a patient with Hurler syndrome. J Inherit Metab Dis 29; 762, 2006
20. Sifuentes M, Dorosshow R, Hoft R, Mason G, Walot I, Diament M, Ojkazaki S, Huff K, Cox GF, Swiedler SJ, Kakkis ED. A follow up study of MPSI patients treated withlaronidase enzyme replacement therapy for 6 years. Mol Genet Metab 90; 171–180, 2007
21. Tokic V, Barisic I, Huzjak N, Petkovic G, Fumic K, Pasche E. Enzyme replacement therapy in two patients with an advanced severe (Hurler) phenotype of mucopolysaccharidosis I. Eur J Pediatr 166; 727–732, 2007
22. Allard P, Grenier A, Korson MS, Zytkovicz TH. Newborn screening for hepatorenal tyrosinemia by tandem mass spectrometry: analysis of succinylacetone extracted from dried blood spots. Clin Biochem 37; 1010–1015, 2004
23. Sander J, Janzen N, Peter M, sander S, Steuerwald U, Holtkamp U, Schwahn B, Mayatepek E, Trefz FK, Das AM. Newborn screening for hepatorenal tyrosinemia: tandem mass spectrometric quantification of succinylacetone. Clin Chem 52, 482–487, 2006
24. Magera MJ, Gunawardena ND, Hahn SH, Tortorelli S, Mitchell GA, Goodman SI, Rinaldo P, Matern D. Quantitative determination of succinylacetone in dried blood spots for newborn screening of tyrosinemia type 1. Mol genet Metab 88; 16–21, 2006
25. Johnson DW, Gerace R, Ranieri E, Trinh MU, Fingerhut R. Analysis of succinylacetone, as a Girard T derivative, in urine and dried blood spots by flow injection electrospray ionization tandem mass spectrometry. Rapid Commun Mass Spectrom 21; 59–63, 2007
26. Turgeon C, Magera MJ, Allard P, Tortorelli S, Gavrilov D, Oglesbee D, Raymond K, Rinaldo P, Matern D. Combined newborn screening for succinylacetone, amino acids, and acylcarnitines in dried blood spots. Clin Chem 54, 657–664, 2008
27. Minutti CZ, Lacey JM, Magera MJ, Hahn SH, McCann M, Schulze A, Cheillan D, Dorche C, Chace DH, Lymp JF, Zimmerman D, Rinaldo P, Matern D. Steroid profiling by tandem mass spectrometry improves the positive predictive value of newborn screening for congenital adrenal hyperplasia. J Clin Endocrinol Metab 89; 3687–3693, 2004
28. Lacy JM, Minutti CZ, Magera MJ, Tauscher AL, Casetta B, McCann M, Lymp J. Hahn SH, Rinaldo P, Matern D. Improved specificity of newborn screening for congenital adrenal hyperplasia by second-tier steroid profiling using tndem mass spectrometry. Clin Chem 50; 621–625, 2004
29. Mushtaq I, Logan S, Morris M, Johnson AW, Wade AM, Kelly D, Clayton PT. Screening of newborn infants for cholestatic hepatobiliary disease with tandem mass spectrometry. Brit Med J 319; 471–477, 1999
30. Bodamer OA, Bloesch SM, Gregg AR, Stockler-Ipsiroglu S, O'Brien WE. Analysis of guanidinoacetate and creatine by isotope dilution tandem mass spectrometry. Clin Chim Acta 308; 173–178, 2001
31. Cardicci C, Santagata S, Leuzzi V, Carducci C, Artiola C, Giovanniello T, Battini R, Antonozzi I. Quantitative determination of guanidinoacetate and creatine in dried blood spots by flow-injection analysis-electrospray tandem mass spectrometry. Clin Chim Acta 364; 180–187, 2006

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