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National Academy of Clinical Biochemistry Laboratory Medicine Practice Guidelines: Follow-Up Testing for Metabolic Disease Identified by Expanded Newborn Screening Using Tandem Mass Spectrometry; Executive Summary

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BACKGROUND: Almost all newborns in the US are screened at birth for multiple inborn errors of metabolism using tandem mass spectrometry. Screening tests are designed to be sufficiently sensitive so that cases are not missed. The NACB recognized a need for standard guidelines for laboratory confirmation of a positive newborn screen such that all babies would benefit from equal and optimal follow-up by confirmatory testing.

METHODS: A committee was formed to review available data pertaining to confirmatory testing. The committee evaluated previously published guidelines, published methodological and clinical studies, clinical case reports, and expert opinion to support optimal confirmatory testing. Grading was based on guidelines adopted from criteria derived from the US Preventive Services Task Force and on the strength of recommendations and the quality of the evidence. Three primary methods of analyte measurement were evaluated for confirmatory testing including measurement of amino acids, organic acids, and carnitine esters. The committee graded the evidence for diagnostic utility of each test for the screened conditions.

RESULTS: Ample data and experience were available to make strong recommendations for the practice of analyzing amino acids, organic acids, and acylcarnitines. Likewise, strong recommendations were made for the follow-up test menu for many disorders, particularly those with highest prevalence. Fewer data exist to determine the impact of newborn screening on patient outcomes in all but a few disorders. The guidelines also provide an assessment of developing technology that will fuel a refinement of current practice and ultimate expansion of the diseases detectable by tandem mass spectrometry.

CONCLUSIONS: Guidelines are provided for optimal follow-up testing for positive newborn screens using tandem mass spectrometry. The committee regards these tests as reliable and currently optimal for follow-up testing.
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The criteria routinely cited to justify screening newborns for inherited metabolic conditions include the availability of an ethical, safe, simple, and sensitive test to detect all cases *(1)*. Newborn screening panels have been limited by this constraint for nearly 40 years, since the advent of testing for phenylketonuria (PKU)[8] in dried blood spots *(2, 3)*. In the past decade, methods based on tandem mass spectrometry (MS/MS) have revolutionized the practice of newborn screening. In 2000, 100% of US newborns that were screened were tested for fewer than 10 conditions. Today, nearly all of the 4.3 million newborns in the US are screened for more than 30 conditions *(4)*. Individually, these conditions are quite rare, but the collective inci-

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[8] Nonstandard abbreviations: PKU, phenylketonuria; MS/MS, tandem mass spectrometry; MSUD, maple syrup urine disease; HRSA, Health Resources and Services Administration; ACMG, American College of Medical Genetics; CSF, cerebrospinal fluid; MRM, multiple reaction monitoring; SIM, selected ion monitoring; MCAD, medium-chain acyl-CoA dehydrogenase; LSD, lysosomal storage disease.

dence of disorders of amino, organic, and fatty acid metabolism detectable by MS/MS is approximately 1 in 4000 births *(5)*.

The body of literature dedicated to inborn genetic errors is extensive. The Online Database of Mendelian Inheritance in Man listed nearly 20 000 entries when accessed March 2, 2009. The majority of knowledge regarding diagnosis and natural history of these disorders has been derived after the onset of symptoms. The presenting features of most inborn metabolic diseases are nonspecific and include lethargy, vomiting, characteristic odors, acidosis, and global developmental delay. Some of these disorders, such as PKU and maple syrup urine disease (MSUD), have a variable or slow progressive course and can result in profound neurologic damage before clinical diagnosis and treatment. Other disorders, such as methylmalonic acidemia, glutaric acidemia type I, and fatty acid oxidation defects, may present acutely with life-threatening acidosis, hypoglycemia, seizures, and/or encephalopathy, with the presenting episode being the first chance to identify the disorder.

Although newborn screening presents the opportunity to diagnose and treat these disorders with unprecedented effectiveness, it presents the biochemical genetics laboratory with a challenge: to detect a disease state based primarily on a pattern of metabolic alterations often in the absence of characteristic clinical symptoms and physical signs of the disorder. After a positive newborn screen, the 3 most common tools available for follow-up testing are organic acid analysis in urine and analysis of amino acids and acylcarnitines in plasma or serum. A subset of these tests is normally required to confirm diagnosis. Because these are technically demanding procedures with considerable interlaboratory variability, the recommendations laid out in these guidelines are designed to standardize both the selection and practice of these procedures.

Toward this end, the National Academy of Clinical Biochemistry convened a panel of experts in metabolic genetic disorders and their associated complex laboratory methods to examine available peer-reviewed evidence and formulate principles for follow-up testing. The guidelines are based on the examination and interpretation of this evidence as well as the experience and opinion of the experts assembled to examine it. The panel graded the supporting data for each recommendation using criteria adopted from the US Preventive Services Task Force (see below) *(6)*. The document, revised after a public comment period, is divided into 5 sections. The complete guidelines are available at http://www.aacc.org/members/nacb/lmpg/onlineguide/publishedguidelines/newborn/pages/default.aspx. First, the evidence for expanded screening is reviewed (Chapter 1) followed by recommendations for analytic performance (Chapter 2) and selection of tests (Chapter 3) to be applied in the case of positive screening results. The final 2 sections outline the available evidence for improved outcomes (Chapter 4) and future directions in newborn screening (Chapter 5). Although adoption of these recommendations is voluntary, we hope that wide dissemination and adoption will improve the accuracy and efficiency of the process used to confirm metabolic disorders detected by expanded newborn screening programs using tandem mass spectrometry.

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

A: The NACB strongly recommends adoption; there is good evidence that it improves important health outcomes, and the NACB concludes that benefits substantially outweigh harms.
B: The NACB recommends adoption; there is at least fair evidence that it improves important health outcomes, and the NACB 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.

Chapter 1: Evidence-Based Rationale for Expanded Newborn Screening

Among the traditional criteria cited and used to justify screening for a specific disorder are (1) the disease is detectable in the neonatal period before appearance of clinical symptoms; (2) outcomes are changed by early intervention, and (3) a simple, cost-effective, and accu-

rate screening test is available *(1)*. Advances in technology, primarily the development of tandem mass spectrometry, have enabled accurate, cost-effective detection of a broader range of conditions than ever before, spurring debate regarding the expansion of the conditions for which newborns would be screened. Beginning in 2001, an expert panel commissioned by the Health Resources and Services Administration (HRSA) and convened by the American College of Medical Genetics (ACMG) evaluated 84 candidate conditions and, in 2006, published a universal and uniform list of conditions to be included in newborn screening programs. The report details a core group of 29 conditions referred to as the "uniform panel" and a set of 25 secondary targets that may be found as a result of screening for the disorders of the uniform panel *(7)*. Forty-two of these 54 conditions may be detected by amino acid and acylcarnitine profiling using MS/MS.

Evidence supporting the accuracy of screening has been derived from the ACMG expert group, early screening programs implemented in the late 1990s in Australia *(8)* and some of the US *(9, 10)*, and an HRSA-funded collaborative that now consists of 44 US states and 60 laboratories in 35 countries (more information available at www.region4genetics.org) *(4)*. The HRSA regional collaboratives aim to analyze at least 50 cases for each condition and determine appropriate cutoff concentrations, maximizing sensitivity and minimizing false positives. Based on all these accumulated data and experience, the evidence supporting 7 conditions received an A-I rating, 12 received A-II, 19 received B-II, and there was incomplete evidence to rate 4 conditions contained in the uniform panel (Table 1).

Chapter 2: Preanalytical, Analytical, and Postanalytical Issues Related to MS/MS as a Tool for Expanded Newborn Screening

As previously mentioned, the 3 basic tools for confirmatory testing are analyses of amino acids, organic acids, and carnitine esters. Each of these analyses is technically demanding and performed primarily in tertiary referral centers. Many of the recommendations are applicable to all 3 techniques and are an integral component of the best analytic laboratory practices associated with multianalyte profiles (Table 2). Examples of these recommendations include analysis of normal and abnormal quality control specimens (A-I), subscription to proficiency testing programs when available (A-I), establishment of age-specific reference intervals (A-I), and identification of interfering substances (A-I). Each profile should be interpreted in the context of clinical history, physical symptoms, and other laboratory studies by a board-certified doctoral scientist or physician with specialized training in metabolic disease and pertinent analytic testing (A-I). Ideally, follow-up analyses should be performed within 24 h of sample receipt (A-I). Further recommendations specific to amino acid, organic acid, or acylcarnitine profiles are detailed below. Only the strongest recommendations are cited here.

AMINO ACID ANALYSIS

- Heparinized plasma from fasting patients is the preferred specimen type because it can be processed immediately. Serum is an acceptable alternative but not ideal. The extended time required for clot formation at room temperature may lead to artifacts such as glutamine deamination and oxidation of thiol-containing amino acids. Cerebrospinal fluid (CSF) free of blood contamination is useful for investigating some seizure disorders (nonketotic hyperglycinemia), but such disorders are not effectively identified by screening programs. Likewise, urine amino acid analysis is useful as a first-line investigation in a few conditions (e.g., cystinuria) that are generally not detected in expanded screening programs.
- The specimen should be deproteinized before analysis. Sulfosalicylic acid is commonly used for ion-exchange techniques, and methanol is normally used to extract free amino acids for analysis by mass spectrometry. Further sample cleanup may be performed but is not normally necessary.
- Ion-exchange techniques should use a retention time internal standard (e.g., aminoethylcystine) and derivatization for identification and quantification. Derivatization using *o*-phthalaldehyde, phenylisothiocyanate, and ninhydrin may be used. Postcolumn detection with ninhydrin provides consistent results and is used most commonly. Ninhydrin derivatives may be further analyzed at 2 wavelengths (e.g., 570 and 440 nm) to assess peak purity. Calibration is performed with a series of external standards.
- Derivatization, specifically butylation, is recommended for MS/MS analyses to enhance ionization and analytic specificity.
- Chromatography is required for MS/MS techniques to separate key isobaric constituents (e.g., allo-isoleucine, isoleucine, leucine, and hydroxyproline).

ACYLCARNITINE ANALYSIS

- Following the rationale given above for amino acid analysis, plasma is the preferred specimen type for acylcarnitine analysis.
- The sample matrix should be simplified by solid-phase or liquid–liquid extraction. Methanol extraction is most commonly used.

Table 1. Conditions defined by ACMG recommendation for expanded newborn screening by tandem mass spectrometry; strength of evidence graded by Laboratory Medicine Practice Guidelines (LMPG) Committee criteria.

Group	Condition (inborn errors of amino acid, fatty acid, and organic acid metabolism)	US incidence	Strength of evidence
AA[a]	Argininosuccinic acidemia	<1:100 000	B-II
AA	Citrullinemia	<1:100 000	B-II
AA	Homocystinuria (CBS deficiency)	<1:100 000	B-II
AA	MSUD	<1:100 000	A-II
AA	PKU	>1:25 000	A-I
AA	Tyrosinemia type I	<1:100 000	A-II
AA	Argininemia	<1:100 000	B-II
AA	Benign hyperphenylalaninemia	<1:100 000	A-I
AA	Citrullinemia type II	<1:100 000	B-II
AA	Defects of biopterin cofactor biosynthesis	<1:100 000	A-I
AA	Disorders of biopterin cofactor regeneration	<1:100 000	A-I
AA	Hypermethioninemia	<1:100 000	B-II
AA	Tyrosinemia type II	<1:100 000	B-II
AA	Tyrosinemia type III	<1:100 000	B-II
AA	Nonketotic hyperglycinemia	<1:100 000	I
AA	Pyruvate carboxylase deficiency	<1:100 000	I
FAO	Carnitine uptake defect	<1:100 000	A-II
FAO	Long-chain 3-OH acyl-CoA dehydrogenase deficiency	>1:75 000	A-II
FAO	Medium-chain acyl-CoA dehydrogenase deficiency	>1:25 000	A-I
FAO	Trifunctional protein deficiency	<1:100 000	A-II
FAO	Very-long-chain acyl-CoA dehydrogenase deficiency	>1:75 000	A-II
FAO	Dienoyl reductase deficiency	<1:100 000	I
FAO	Carnitine palmitoyl-transferase Ia deficiency (liver)	<1:100 000	B-II
FAO	Carnitine palmitoyl-transferase II deficiency	<1:100 000	B-II
FAO	Glutaric acidemia type II	<1:100 000	B-II
FAO	Medium-/short-chain 3-OH acyl-CoA dehydrogenase deficiency	>1:100 000	I
FAO	Medium-chain ketoacyl-CoA dehydrogenase deficiency	<1:100 000	I
FAO	Short-chain acyl-CoA dehydrogenase deficiency	>1:75 000	I/C-II[b]
FAO	Carnitine/acyl-carnitine translocase deficiency	<1:100 000	B-II
OA	3-Methyl crotonyl-CoA carboxylase deficiency	>1:75 000	B-II
OA	3-Hydroxy 3-methyl glutaric aciduria	<1:100 000	A-II
OA	Beta-ketothiolase deficiency	<1:100 000	A-II
OA	Glutaric acidemia type I	>1:75 000	A-I
OA	Isovaleric acidemia	<1:100 000	A-I
OA	Methylmalonic acidemia (A,B)	<1:100 000	A-II
OA	Methylmalonic acidemia (mutase)	>1:75 000	A-II
OA	Multiple carboxylase deficiency	<1:100 000	B-II
OA	Propionic acidemia	>1:75 000	A-II
OA	2-Methyl 3-hydroxy butyric aciduria	<1:100 000	I
OA	2-Methyl butyryl-CoA dehydrogenase deficiency	<1:100 000	B-II

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Table 1. Conditions defined by ACMG recommendation for expanded newborn screening by tandem mass spectrometry; strength of evidence graded by Laboratory Medicine Practice Guidelines (LMPG) Committee criteria. *(Continued from page 1618)*

Group	Condition (inborn errors of amino acid, fatty acid, and organic acid metabolism)	US incidence	Strength of evidence
OA	3-Methyl glutaconic aciduria	<1:100 000	B-II
OA	Isobutyryl-CoA dehydrogenase deficiency	<1:100 000	B-II
OA	Malonic aciduria	<1:100 000	B-II
OA	Methylmalonic acidemia (Cbl C,D)	<1:100 000	A-II
OA	Ethylmalonic encephalopathy	<1:100 000	I

[a] AA, disorder of amino acid catabolism and transport; FAO, disorder of fatty acid oxidation; OA, organic aciduria; CBS, cystathionine beta-synthase.
[b] No consensus reached.

- Chemical derivatization is recommended to enhance analytic sensitivity and molecular specificity. Vast analytic experience and expertise exist for the measurement of acylcarnitine butyl esters.
- Carnitine esters should be analyzed by electrospray ionization tandem mass spectrometry in either multiple reaction monitoring (MRM) or precursor ion scanning mode, or both. Chromatographic separation of isobaric carnitine esters is not normally required during initial follow-up studies. In the presence of interfering substances (e.g., pivalic acid, cefotaxime), chromatographic separation of acylcarnitines or use of other complementary techniques such as urine organic acid analysis may be necessary to augment diagnostic information.
- Pure forms of the >30 carnitine esters measured are not available, and full external calibration is not possible. Quantification should be accomplished by calculating the signal intensity of a specific ester to that of an appropriate deuterated internal standard. Quantification of carnitine esters that lack identical internal standards should be performed by using the ester with the internal standard with the nearest appropriate chain length (e.g., quantifying C16:1 with deuterated C16). Linearity, imprecision, and limits of detection must be frequently validated under the conditions used.

ORGANIC ACID ANALYSIS

- Urine is the specimen of choice. Random urine collections are acceptable. Diagnostic organic acids are concentrated in urine, making detection easier in this matrix as opposed to blood or CSF.
- Analysis should be normalized to urine creatinine concentration.
- Organic acids should be isolated from the matrix by extraction and derivatized before analysis. Ethyl acetate extraction and formation of trimethylsilyl derivatives are the most common strategies.
- Organic acids should be analyzed by capillary GC-MS.
- Analysis may be performed quantitatively or semiquantitatively. Abundant molecular species may be detected by a full ion scan from m/z 50 to 550. Selected ion monitoring (SIM) should be used to increase the sensitivity of detecting less abundant but clinically important molecular species (e.g., 3-hydroxyglutaric and 4-hydroxybutryic acids). A migration toward quantitative methods that increasingly rely on stable isotopes will improve the detection of subtle metabolic abnormalities and reduce laboratory-to-laboratory variability in organic acid measurement.
- Organic acids should be identified by retention time and mass spectra. Many diagnostically important compounds must be distinguished from coeluting organic acids (e.g., ethylmalonic/phosphoric acid, 3-hydroxybutyric/3-hydoxyisobutyric acid, and 2-ketoglutaric/3-hydroxyglutaric acid).

Chapter 3: Follow-Up of Positive Screens

Before expanded newborn screening, biochemical investigations were normally prompted by a multitude of nonspecific symptoms, including vomiting, lethargy, tachypnea, seizures, and encephalopathy. In symptomatic patients, biochemical abnormalities are not often subtle. Diagnostic metabolites may be present at concentrations an order of magnitude greater than those found in normal, age-matched children. In contrast, biochemical studies prompted by abnormal newborn screening results are not accompanied by clear-cut symptoms. In fact, most infants are asymptomatic at the time of sampling. Consequently, laboratory tech-

Table 2. Strength of recommendations for analytical procedures required for optimal follow-up testing for positive newborn screens.			
	Amino acids	**Organic acids**	**Acylcarnitines**
Pre-analytical			
Specimen type	Heparinized plasma (A-I)	Random urine (A-1)	Heparinized plasma (A-1)
Collection/processing	Separate plasma promptly; 4 °C up to 4 h; −20 °C if analysis to be delayed >4 h (A-1)	Store at 4 °C up to 24 h; −20 °C if analysis delayed >24 h (A-1)	Separate plasma promptly and freeze at −20 °C unless analysis is immediate (B-II)
Analytical			
Technique	Ion exchange chromatography/ ninhydrin detection (A-1); LC-MS/MS[a] (A-1)	GC/MS (A-1)	LC-MS/MS (A-1)
Sample preparation	Deproteinization required; acid for ion exchange (A-1), methanol for MS/MS (B-II)	Liquid–liquid or solid-phase extraction; ethyl acetate most common (A-1)	Sample should be deproteinized (methanol use is common) (A-1)
Derivatization	Postcolumn for colorimetric detection using ion exchange protocols (A-1); butylation for MS/MS (B-II)	Trimethylsilyl derivatives for GC compatibility and libraries of known mass spectra (A-1); routine oximation of ketones (B-II)	Derivatization (butylation) to enhance sensitivity and analytic specificity (B-II)
Calibration	Full external calibration for each quantified amino acid (A-1)	Full external calibration for each quantified organic acid (A-1)	Full external calibration not possible; quantify using internal standard of nearest appropriate chain length (A-1)
Internal standard	Small number used for retention time drift in ion exchange techniques (A-1); comprehensive array of isotopologs for isotope-dilution quantification in LC/MS/MS protocols (A-1)	Small number used for retention time drift in semiquantitative profiles (A-1); comprehensive inclusion of isotopologs for isotope-dilution quantitative profiles (A-1)	Broad chain length coverage and use of deuterated isotopologs of key diagnostic species when possible (A-1)
Chromatography	Necessary to resolve isobars (leucine, isoleucine, allo-isoleucine) (A-1)	Fused silica capillary (e.g., DB-1) (A-1)	Not necessary unless interfering substance suspected (B-II)
Data acquisition	Integrated peak areas MRM mode for MS/MS (A-1)	Full scan *m/z* 50–550; SIM for clinically important analytes present at low concentration (A-1)	Precursor ion scan or MRM mode (A-1)

[a] LC-MS/MS, liquid chromatography/tandem mass spectrometry.

niques used for follow-up studies must be able to detect more subtle metabolic abnormalities. Because the rarity of these conditions precludes large studies of diagnostic accuracy, we examined primary literature summarizing studies in asymptomatic individuals as well as accumulated follow-up data. This exercise yielded general recommendations for the follow-up process (Fig. 1) as well as specific follow-up test menus tailored to specific abnormal newborn screening results (Table 3).

The laboratory is central to the follow-up process. Laboratory personnel should be among the first notified of the impending referral of a screen-positive patient to arrange for test selection, prompt testing, or sample referral (A-I). The initial techniques used should serve 2 purposes: (1) to confirm the presence of a biochemical abnormality and (2) to identify the metabolic defect with enough precision to allow imple-

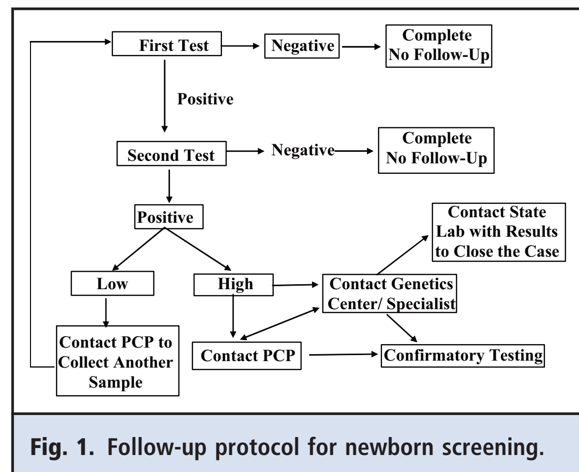

Fig. 1. Follow-up protocol for newborn screening.

Table 3. Recommended follow-up testing procedures for positive newborn screens.

	Screening marker	Follow-up analyses	Follow-up markers	Additional testing	Evidence
Disorders of amino acid catabolism and transport					
Phenylketonuria (includes benign hyperphenylalaninemia and biopterin metabolic defects)	Phenylalanine tyrosine	Plasma amino acids	Phenylalanine tyrosine	Urine pterin metabolites; dihydropteridine reductase activity	A-I
Tyrosinemia					
Type 1	Tyrosine	Urine organic acids	Succinylacetone	No additional testing indicated	A-I
Type 2		Plasma amino acids	Tyrosine >1000 μmol/L on presentation		A-I
MSUD	Isoleucine + leucine + alloisoleucine	Plasma amino acids	Isoleucine, leucine, valine, alloisoleucine	No additional testing indicated	A-I
Citrullinemia					
Type 1 (ASA synthase)	Citrulline	Plasma/urine amino acids	Citrulline, argininosuccinate	Ammonia, bilirubin, ALP,[a] GGT	A-I
Type 2 (citrin)	Citrulline			Genetic testing may distinguish 1 and 2	B-II
Argininosuccinic acidemia	Citrulline	Plasma/urine amino acids	Argininosuccinate, citrulline	No additional testing indicated	A-I
Homocystinuria	Methionine	Immunoassay; plasma/urine amino acids; urine organic acids	Homocysteine, methionine, homocystine, methylmalonic acid	Folate/vitamin B12 status should be investigated; disorders of cobalamin metabolism should also be considered	A-I
Disorders of fatty acid oxidation					
Medium-chain acyl CoA dehydrogenase deficiency (MCAD)	C6, C8, C10 acylcarnitine	Acylcarnitine analysis	C6, C8, C10 acylcarnitine species	Molecular genetic analysis for predominant A985G MCAD mutation	A-I
		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	A-II
		Urine organic acids	Increased long-/medium-chain dicarboxylic acids with limited ketosis	Genetic analysis may discriminate acute from later-onset form	
Long-chain hydroxyacyl CoA dehydrogenase deficiency (LCHAD)/ TFP deficiency	C16-OH, C18-OH, C18:1-OH acylcarnitine	Acylcarnitine analysis	C16-OH, C18-OH, C18:1OH acylcarnitine species	80%–90% of alleles display G1528C; distinction from TFP deficiency requires isolated activity assay	A-II
		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 urine organic acids equivocal	B-II
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
Primary carnitine deficiency	Free (C0) carnitine	Free + total carnitine	Low total and free carnitine; urine carnitine elevated	Secondary?; low fibroblast carnitine uptake	B-II

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Table 3. Recommended follow-up testing procedures for positive newborn screens. *(Continued from page 1621)*

	Screening marker	Follow-up analyses	Follow-up markers	Additional testing	Evidence
Organic acidurias					
β-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
3-OH-3-methylglutaryl (HMG) CoA 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
Glutaric aciduria					
Type 1	C5DC (glutaryl)		Glutaric, 3-OH glutaric		A-I
Type 2	Acylcarnitine 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 deficiency	A-I
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
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
Propionic acidemia	C3-acylcarnitine	Urine organic acids	3-OH propionic, tiglyl-glycine, methylcitrate	B12 studies	A-I
Methylmalonic acidemia	C3-acylcarnitine	Urine organic acids	Methylmalonic, 3-OH propionic, tiglylglycine, methylcitrate	Complementation analysis; B12 studies	A-I
Isovaleric acidemia	C5-acylcarnitine	Urine organic acids	3-OH isovaleric acid, isovaleryl glycine	No additional testing indicated	A-II

[a] ALP, alkaline phosphatase; GGT, γ-glutamyl transferase; TFP, trifunctional protein; ETF, electron transfer flavoprotein.

mentation of appropriate therapy. Interpretation of analytic results must distinguish pathologic alterations from changes in metabolite concentrations associated with maturation of organ systems, prematurity, diet, and other concurrent illnesses. Finally, the laboratory plays a dual role in closing the follow-up loop. First, results of follow-up testing are provided to newborn screening collaboratives to maintain sensitivity of the screening process while minimizing the false-positive rate. Second, laboratory personnel should be aware of newborn screening statistics such as local incidence and recall rate to provide optimal feedback to clinicians involved in the follow-up process.

Many aspects of the follow-up process are illustrated by neonates who have increased blood propionylcarnitine concentrations. Elevations of propionylcarnitine (C3) may indicate deficiency of propionyl CoA carboxylase, holocarboxylase synthetase, methylmalonyl CoA mutase, cobalamin metabolism, or dietary cobalamin. Expert opinion and peer-reviewed data *(11–15)* strongly support (A-I) a follow-up protocol that includes a plasma acylcarnitine analysis along with urine organic acid analysis and serum cobalamin concentration to discriminate accurately among these possibilities and enable institution of proper acute therapy. Amino acid analyses do not contribute to clarifying the initial differential diagnosis but do play a chronic therapeutic role if the eventual diagnosis dictates protein/amino acid restriction. Table 3 includes an itemized list of disorders included in the ACMG panel, screening markers suggestive of disease, recommended follow-up analyses and informative metabolites used to confirm or exclude the diagnosis, and the level of evidence supporting the recommended protocol.

Chapter 4: Patient Outcomes from Expanded Newborn Screening

The ability of tandem mass spectrometry to detect multiple diagnostic markers in a single analytic run has greatly expanded the number of conditions detectable

Table 4. Evaluation of clinical outcomes resulting from early diagnosis of metabolic diseases due to expanded newborn screening by MS/MS.

	Condition	Abbreviation	
1	Medium-chain acyl CoA dehydrogenase deficiency	MCAD	A-I
2	PKU (including benign hyperphenylalaninemia and BH4[a] 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 acyl CoA dehydrogenase deficiency	VLCAD	A-II
7	MSUD	MSUD	A-I
8	Long-chain hydroxy acyl CoA dehydrogenase deficiency	LCHAD	B-II
9	Glutaric acidemia (all forms)	GA	
	Type 1	GA1	A-I
	Type 2	GA2	B-II
10	3-OH-3-methylglutaryl (HMG) CoA 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 Acyl CoA dehydrogenase deficiency	SCAD	C-II/I[b]
23	Medium-/short-chain hydroxyacyl Coa DH deficiency	SCHAD	B-III

[a] BH4, tetrahydrobiopterin.
[b] The committee could not reach a consensus.

in newborns. The ability to detect these disorders is not, however, the ultimate goal of newborn screening programs. Rather, reduction of morbidity and mortality from the disorder is the aim. Effective treatment, a mild reversible phenotype, and a sufficient latent period before full development of the disease phenotype bode well for positive patient outcomes. Some fatty acid oxidation disorders, biotinidase deficiency, and 3-methylcrotonyl CoA carboxylase deficiency are examples of these "milder" disorders. On the other hand, propionic acidemia, methylmalonic acidemia, and type II glutaric aciduria often present catastrophically before results of newborn screening analyses are available. Improving outcomes in these situations is more challenging. Outcome assessment is complicated by the rarity of these disorders, lack of standardized care, and the variability of the expression of the same enzymatic defect in different patients. We assessed outcomes of expanded newborn screening in this context.

The results of this outcome analysis are presented in Table 4. Given the relative infancy of expanded newborn screening it is not surprising that evidence supports screening for only a very few conditions; these include medium-chain acyl-CoA dehydrogenase

(MCAD) deficiency (A-I) *(16–18)*, PKU (A-I) *(19–22)*, MSUD (A-I) *(23)*, and glutaric aciduria type I (A-I) *(24–27)*, as well as propionic, methylmalonic, and isovaleric acidemias (A-II) *(28)*. Other conditions suffer from a variety of gaps in the amount or quality of evidence and carry weaker recommendations. These gaps include small numbers of affected patients (e.g., medium-/short-chain hydroxyacyl CoA dehydrogenase deficiency), very mild, ill-defined phenotypes (e.g., short-chain acyl CoA dehydrogenase deficiency), poor screening accuracy (e.g., tyrosinemia type 1), and lack of treatment options (e.g., glutaric aciduria type II).

On the positive side, the state of this evidence is dynamic and continues to accumulate. Pooling of data from worldwide programs and thoughtful analysis in terms of screening cutoffs, treatment approaches, and their clinical impact will allow periodic reassessment of the efficacy of screening. Hasty conclusions regarding inclusion or removal of markers for specific conditions are not appropriate in the ever-changing screening environment, since development of screening markers and treatments need not be synchronous. Should efforts to detect serious disorders (e.g., lysosomal storage diseases) stop because effective treatment does not currently exist? Should some apparently minor conditions (e.g., short-chain acyl-CoA dehydrogenase deficiency) be excluded from screening because our current experience indicates that these disorders have almost no discernible phenotype? Such disorders may be important modifiers of other disease processes as children age and therefore should be preserved pending thorough longitudinal evaluation of adequate numbers of such patients.

Chapter 5: Future Directions in Expanded Newborn Screening for Metabolic Disorders

Newborn screening programs will certainly continue to evolve, with tandem mass spectrometry as the basis for expansion. Development of effective disease markers detectable in blood spots by mass spectrometry lowers or removes the "ease of detection" hurdle for many disorders and will enable prospective evaluation of treatments and eventual outcomes for these disorders. New markers and new analytic methods beyond the measurement of amino acids and acylcarnitines are already driving this evolution. Assessment of enzyme activity, steroids, organic acids, and bile acids promise to enhance the scope of disease detection.

The most immediate group of disorders under consideration for widespread screening is the lysosomal storage diseases (LSDs). These disorders were low-priority targets in the ACMG expert panel, in part because detection was not considered feasible, cost-effective, and simple *(7)*. However, enzyme activities specific for these storage diseases are stable in blood spots and can be assessed using appropriate synthetic substrates and tandem mass spectrometry *(29–31)*. At the same time, enzyme replacement therapy for a number of these conditions is available or is in development. Such therapy has demonstrated some efficacy in patients with well-established disease but lacks impact on the central nervous system manifestations that most of these disorders produce *(32–35)*. Early detection in concert with new therapies now provide an opportunity to investigate the potential of early enzyme replacement on disease progression. Currently, only the state of New York in the US has implemented population screening of a single LSD, Krabbe disease. Recommendations for implementation of LSD screening must await the outcome of these early trials.

Tyrosinemia type 1 represents the converse of the current status of LSDs. Although effective treatment exists, the measurement of blood spot tyrosine does not effectively discriminate affected neonates from normal ones. The pathognomonic metabolite of tyrosinemia type 1 is succinylacetone, rather than tyrosine. Methods to detect succinylacetone in dried blood spots under analytic conditions comparable to those for amino acids and acylcarnitines have recently been developed to circumvent this issue *(36, 37)*. This approach has been implemented in some laboratories to reduce false-positive screening rates, but proof from prospective screening of large populations is still lacking.

Tandem mass spectrometry promises to be a universal platform for identifying inborn errors detectable using not only small molecules, but also proteins. The expanding menu of small molecules also includes steroids for detection of congenital adrenal hyperplasia *(38, 39)*, bile acids for identifying hepatobiliary diseases *(40)*, and creatine and guanadinoacetate for diagnosing creatine synthetic and transport disorders *(41, 42)*. Feasibility of detecting larger molecules has also been demonstrated. Peptides derived from ceruloplasmin may enable diagnosis of Wilson disease in dried blood spots *(43)*. These additional analytic approaches based on MS/MS would require little new equipment and allow cost-effective expansion of conditions detectable in newborn screening programs. Evidence is currently incomplete, but accumulating.

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