Review

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Expanded newborn screening and confirmatory follow-up testing for inborn errors of metabolism detected by tandem mass spectrometry

Abstract

Newborn screening (NBS) of inborn errors of metabolism (IEM) is a coordinated comprehensive system consisting of education, screening, follow-up of abnormal test results, confirmatory testing, diagnosis, treatment, and evaluation of periodic outcome and efficiency. The ultimate goal of NBS and follow-up programs is to reduce morbidity and mortality from the disorders. Over the past decade, tandem mass spectrometry (MS/MS) has become a key technology in the field of NBS. It has replaced classic screening techniques of one-analysis, one-metabolite, one-disease with one analysis, many-metabolites, and many-diseases. The development of electrospray ionization (ESI), automation of sample handling and data manipulation have allowed the introduction of expanded NBS for the identification of numerous conditions on a single sample and new conditions to be added to the list of disorders being screened for using MS/MS. In the case of a screened positive result, a follow-up analytical test should be performed for confirmation of the primary result. The most common confirmatory follow-up tests are amino acids and acylcarnitine analysis in plasma and organic acid analysis in urine. NBS should be integrated with follow-up and clinical management. Recent improvements in therapy have caused some disorders to be considered as potential candidates for NBS. This review covers some of the basic theory of expanded MS/MS and follow-up confirmatory tests applied for NBS of IEM.

Keywords: confirmatory follow-up testing; expanded newborn screening; inborn errors of metabolism; tandem mass spectrometry.

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Definition and aim of newborn screening

Newborn screening (NBS) is the process by which newborns are screened just after birth for a list of disorders that can cause severe illness or death unless detected and treated early [1]. Screening programs are run with the aim to diagnose infants born with certain genetic, metabolic, hormonal and functional diseases and to identify if an infant is more likely than others to have a disease [2]. Inborn errors of metabolism (IEM) having a slow progressive course are not detectable clinically at birth and may result in severe neurologic damage and, in some cases, death before clinical diagnosis and treatment. Some of the IEM may present acute life-threatening symptoms which may lead early identification of the disorder. Most of the symptoms of IEM are non-specific and include lethargy, vomiting, characteristic odors, acidosis, and global developmental delay [3]. NBS programs are designed to provide early diagnosis and treatment before significant and irreversible damage occurs [1–5]. NBS is a coordinated comprehensive system consisting of education, screening (specimen collection, transportation, and testing), follow-up of abnormal and unsatisfactory test results, confirmatory testing and diagnosis, treatment and periodic outcome evaluation, quality assurance and program evaluation, validity of testing systems, efficiency of follow-up and intervention, and assessments of long-term benefits to individuals, families, and society. Public awareness coupled with professional training and family education must be part of the complete NBS system [1, 4, 5]. NBS has been demonstrated to save lives and prevent serious disability. It appears to be cost-effective and represents a public health success [6–10].

NBS laboratory tests are essential laboratory services for screening, detection, diagnosis, and monitoring of IEM, typically, they are biochemical markers related to the disorders being screened [11]. A screened positive result does not mean that the newborn definitely suffers from a

disorder but it does mean that further diagnostic testing is needed and in the case of a screened positive result, a second analytical test, if available, is performed for confirmation of the primary result [1–3, 12].

History

NBS originated in 1960s when Robert Guthrie developed a method to detect phenylketonuria (PKU), using a semi-quantitative bacterial inhibition assay in whole blood samples collected by pricking the heels of infants on a specially designed filter paper. PKU is a disorder which could be managed by dietary adjustment if diagnosed early. Guthrie test detects elevated blood levels of phenylalanine, using the ability of phenylalanine to facilitate bacterial growth in a culture medium with β-2-thienylalanine which inhibits bacterial growth. In the presence of phenylalanine leached from the impregnated filter paper, inhibition is overcome and bacteria grow [13, 14]. The Guthrie test has been applied worldwide to screen for PKU.

In the 1970s, congenital hypothyroidism (CH) was added to the NBS panel as the second disease screened for [14–16]. Over the past four decades, other disorders have been added to the NBS panels and universal NBS programs have become commonplace [14, 15].

Guidelines for deciding whether a particular disorder is a suitable candidate for screening were formulated by Wilson and Jungner in 1968 commissioned by the WHO [17]. They proposed 10 criteria in their publication titled 'Principles and practice of screening for disease' which has become a classic since then [1–5, 14, 15, 17–19]. Although the Wilson and Jungner criteria were not designed specifically for NBS, they are still used, with some modifications, to justify which disorders are included in a universal NBS panel (Table 1).

There is not always agreement on the application of Wilson-Jungner criteria which requires that the disease screened for should be treatable in order to be included into the NBS panel [14, 20]. Fewer than 10 diseases that fully met the criteria proposed by Wilson and Jungner were screened in 2000 in the US [15]. There is a nearly universal consensus on NBS for PKU, CH and cystic fibrosis (CF) [14, 21–23] Up to the beginning of this century, newborns were screened for PKU, CH, CF, galactosemia, sickle cell disease, congenital adrenal hyperplasia (CAH), biotinidase deficiency and hearing impairment [1–5, 16, 24, 25]. Nowadays, nearly all of the newborns in the US are screened for more than 30 conditions. Individually, these conditions are quite rare, but the collective incidence of metabolic disorders is approximately one in 4000 births [3, 26].

The list of disorders for NBS is not uniform and varies from country to country and even from region to region in the same country, depending on funding, regulations, techniques used and prevalence of a specific disorder in the population [27–29]. In spite of economic challenges, many of the low and middle income countries try to implement screening for at least a few disorders and develop a national model program which comprises follow-up, diagnosis, treatment, education and evaluation [30, 31].

In my country, Turkey, a nationwide 'Newborn Screening Program' was started in 1986 for PKU. Birth prevalence of hyperphenylalaninemia was found as 1:4192 and PKU as 1:5049 [32, 33]. Turkey has the highest PKU prevalence at birth, next to Ireland. A screening for CH was added later to the program. The incidence of CH was found to be 1:2659 [34]. Later a screening for biotinidase deficiency was added and the prevalence of biotinidase deficiency was found to be 1:11763. Similarly to most countries in the Mediterranean basin, hemoglobinopathies are an important health problem in Turkey. In 1993, a law entitled 'Fight against hereditary blood diseases especially for thalassemia and hemoglobinopathies' was issued. In 2000, the Turkish National Hemoglobinopathy Council (TNHC) was founded to combine all centers, foundations

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1. The condition sought should be an important health problem.
2. There should be an accepted treatment for patients with recognized disease.
3. Facilities for diagnosis and treatment should be available.
4. There should be a recognizable latent or early symptomatic stage.
5. There should be a suitable test or examination.
6. The test should be acceptable to the population.
7. The natural history of the condition, including development from latent to declared disease, should be adequately understood.
8. There should be an agreed policy on whom to treat as patients.
9. The cost of case-finding (including diagnosis and treatment of patients diagnosed) should be economically balanced in relation to possible expenditure on medical care as a whole.
10. Case-finding should be a continuing process and not a 'once and for all' project.

Table 1 Wilson and Jungner criteria used to justify screening for a specific disorder.

and associations into a single organization. The TNHC comprises seven subcommittees dealing with registration, education, screening, prenatal diagnosis, conventional treatment, bone marrow transplantation and social issues. In 2003, the hemoglobinopathy scientific committee was set up, a guidebook was published and a national hemoglobinopathies control program (HCP) was started in the high-risk provinces [35]. Prevalence of metabolic diseases in Turkey is high compared to figures from different countries. PKU, maple syrup urine disease, methylmalonic acidemia, hereditary urea cycle defects and galactosemia are the most commonly seen metabolic diseases. In pilot studies, galactosemia deficiency was found as 1:23775. Turkey has a high rate of consanguineous marriages and the Quinquennial Turkish Demographic and Health Survey (TDHS) showed that the rate of consanguinity has been stable at 20%–25% since 1983. This shows that high consanguinity can be a contributing factor to high incidence of IEM [36].

Expanded newborn screening

New platform technologies like tandem mass spectrometry (MS/MS) enable accurate and cost-effective identification of numerous disorders on a single sample through a single analytical process, some of which meet, while others fail to meet the Wilson-Jungner criterium of being treatable [3, 18, 37, 38]. The ability of MS/MS to measure simultaneously many metabolites has deeply changed the older NBS approach 'one test for one disorder' to 'one test for many disorders', i.e., a multiplex test. This has led to the revision of the traditional Wilson and Jungner guidelines [1, 17]. Disorders without effective treatments have been included into NBS panels detected by MS/MS with minimal extra effort, time and cost [14]. MS/MS allows other disorders to be added for screening without a need for additional samples or analysis time. The initial cost of MS/MS might be quite high, but the analytical cost per sample for screening approximately 30 disorders is US $10–20, and the addition of another disorder to the panel may be <$1 [19]. In recent decades, screening methods and tests are being developed more quickly than the treatment of the pathologies. Detection of disorders having no treatment may not help the newborn, but can help the family providing early diagnosis and in planning future pregnancies [14, 39].

The new developments in MS/MS and electrospray ionization (ESI) allowed rapid and high throughput analyses of samples extracted from dried blood spots (DBS) collected for NBS for a large number of IEM [15, 40–44].

Today, almost all developed countries have expanded NBS programs that screen from approximately 20 to over 40 inherited metabolic diseases by MS/MS [15]. In contrast to the US, European countries with high standard medical care, such as UK and France, still screen for <5–10 IEM. The current UK NBS program is relatively conservative in scope, screening for PKU, CH, sickle-cell disorders (hemoglobinopathies), CF and medium chain acylCoA dehydrogenase deficiency (MCADD). In France, five diseases are subject to NBS: PKU, CAH, CH, sickle cell disease, and CF. The European Union (EU) conducted a survey in 2010/2011 in the 28 EU member states, four EU candidate states (Croatia, FYROM, Iceland, Turkey), three potential EU candidate states (Bosnia Herzegovina, Montenegro, Serbia), and two EFTA states (Norway and Switzerland) to collect data to gain some insight into the current situation. The results indicated that there are large variations in the design of the NBS programs in the European countries [45, 46]. The ultimate goal of EU is to have a uniform screening panel, as has been achieved in the US, resulting in equal screening opportunities for all European newborn infants [4].

The lack of uniformity in the NBS program even in the same country and the emerging technologies necessitated assessment of the current NBS and future directions of the NBS programs [12]. In 2001, an expert panel commissioned and convened by the American College of Medical Genetics (ACMG) evaluated 84 candidate disorders and published in 2006, a universal and uniform list of disorders for NBS. The report defined a uniform panel of 29 core disorders and 25 secondary disorders. Forty-two of these 54 disorders are detected using MS/MS (Table 2). The rest of the disorders (Hb SS-sickle cell anemia, Hb S/β-thalassemia, Hb S/C disease, various other hemoglobinopathies, CH, CAH, biotinidase deficiency, galactosemia due to GALT deficiency, galactosemia due to GALE deficiency Galactosemia due to GALK deficiency, congenital hearing loss, CF) are detected by other methods. The screening panel recommended by ACMG allows new disorders to be added to the NBS program as new treatment options become available. With the recent addition of severe combined immunodeficiency (SCID), the core panel has reached 30 disorders [1–5, 12, 15, 25, 47–51].

The National Academy of Clinical Biochemistry (NACB) recommended adoption of the following 'Strength of Recommendations' for IEM detected using MS/MS [3]:

A: The NACB strongly recommends adoption.
B: The NACB recommends adoption.
C: The NACB recommends against adoption.
D: The NACB no consensus reached.

Amino acid metabolism disorders

Core conditions

	Strength of evidence	Incidence	Clinical symptoms	Treatment	Prognosis
Argininosuccinic aciduria (ASA) (argininosuccinate lyase deficiency) (ASL)	B-II	1:70 000	Most commonly symptoms begin in the first days of life with brain swelling due to ammonia, coma, failure to thrive, lethargy, coma, ataxia, permanent neurological injury, and, sometimes, death.	Early diagnosis and treatment can be lifesaving; treatment consists of protein restriction, avoiding fasting, prevention of ammonia build-up, nutritional supplements, and in some cases, liver transplant.	Variable, despite treatment episodic hyperammonemia and developmental delay
Citrullinemia, type I (CIT) (argininosuccinate synthase deficiency) (ASS)	B-II	1:60 000	Symptoms begin during the newborn period or later in infancy; seizures, failure to thrive, lethargy, ataxia, coma, brain damage and death	With early diagnosis and treatment, normal development is possible. Protein restriction, prevention of ammonia build-up, nutritional supplements	Despite treatment Severe developmental delay is common
Maple syrup urine disease (MSUD) (branch-chain α-ketoacid dehydrogenase complex deficiency)	A-II	1:200,000	Failure to thrive, progressive neurological injury, coma, seizures	Diet restricted from branched chain amino acids and protein, supplementation of thiamin	Without treatment, babies do not survive the first month; good outcome if identified and treated early
Homocystinuria (HCY) (cystathionine β synthase deficiency) (CBS)	B-II	1:150 000	Intellectual disabilities, eye problems, skeletal abnormalities, thrombosis, and stroke	Pyridoxine (vitamin B6) and B12, low methionine, cystine supplemented diet and betaine for pyridoxine non-responders	Good in vitamin B6 responsive cases, variable in others
Phenylketonuria (PKU) (phenylalanine hydroxylase deficiency >98%, BH4 synthesis/regeneration defects <2%)	A-I	1:25 000	Mental retardation, autism, hyperactivity, seizures, behavioral problems	Phenylalanine restricted diet, BH4 supplementation	Almost normal development
Tyrosinemia, type I (TYR-I) (Fumarylacetoacetate hydrolase deficiency) (FAH)	A-II	1:100 000	Hepatic and renal failure, nerve damage, hypophosphatemic rickets and death	Drug treatment (NTBC inhibits 4-hydroxyphenyl pyruvate dioxygenase), tyrosine and protein restricted diet	Variable, increased risk for hepatocellular carcinoma, drug treatment prevents liver and kidney damage

Secondary conditions

	Strength of evidence	Incidence	Clinical symptoms	Treatment	Prognosis
Argininemia (ARG) (arginase deficiency)	B-II	1:350 000	Irritability, growth failure, anorexia, vomiting, progressive spastic quadriplegia, seizures, developmental delay, mental retardation	Protein restriction, low arginine and phenylbutyrate	Variable
Citrullinemia, type II	B-II	1:100 000	Neonatal intrahepatic cholestasis, jaundice, fatty liver. Symptoms include confusion, restlessness, memory loss, abnormal behaviors (such as aggression, irritability, and hyperactivity), seizures, and coma	Liver transplant in the adult form; protein restriction, arginine may help to ameliorate the symptoms	The neonatal form may resolve. The adult form progresses to death

(Table 2 continued)

	Strength of evidence	Incidence	Clinical symptoms	Treatment	Prognosis
Hypermethioninemia	B-II	1:100 000	Often no symptoms. Some exhibit intellectual disability and other neurological problems	Protein restriction, B6 supplements.	Delays in growth and learning
Benign hyperphenylalaninemia	A-I	1:100 000	Developmental abnormalities, eczema, and vomiting	Phenylalanine restricted diet, BH4 supplementation	Almost normal development
Defect of biopterin cofactor biosynthesis	A-I	1:100 000	Developmental delays, seizures (epilepsy), behavioral troubles trouble regulating body temperature, poor sucking during feeding, hypotonia	Phenylalanine restricted diet, BH4 supplementation, dopamine	Good outcome if identified and treated early
Disorders of biopterin cofactor regeneration	A-I	1:100 000	Developmental delays, seizures (epilepsy), behavioral troubles trouble regulating body temperature, poor sucking during feeding, hypotonia,	Phenylalanine restricted diet, BH4 supplementation, dopamine	Good outcome if identified and treated early
Tyrosinemia, type II (TYR II) (tyrosine transaminase deficiency)	B-II	1:250 000	Corneal ulcers, hyperkeratosis, cognitive reduction, mental retardation, microcephaly and failure to thrive	Diet restricted in tyrosine and phenylalanine	Generally good
Tyrosinemia, type III	B-II	1:100 000	Mild mental retardation, seizures, and periodic loss of balance and coordination (intermittent ataxia)	Diet low in phenylalanine, methionine and tyrosine	Generally good
Fatty acid oxidation disorders Core conditions					
Carnitine uptake defect (CUD) (carnitine transporter defect)	A-II	1:75 000 1:40 000 in Japan	Hypoglycemia, coma, lethargy, hypotonia, liver dysfunction, cardiomyopathy	Avoid fasting, low fat high carbohydrate diet, carnitine supplement	Generally good
Medium-chain acyl-CoA dehydrogenase deficiency (MCAD)	A-I	1:25 000	Hypoglycemia, coma, lethargy, hypotonia, liver dysfunction, respiratory arrest, vomiting	Avoid fasting, low fat high carbohydrate diet	Good
Very long-chain acyl-CoA dehydrogenase deficiency (VLCAD)	A-II	1:75 000	Non-ketotic hypoglycemia, cardiomyopathy, hepatic dysfunction, skeletal myopathy, sudden death in infancy with hepatic steatosis, exercise induced myalgia, 'adult' onset recurrent myoglobinuria	Avoid fasting and prolonged exercise, low fat high carbohydrate diet, carnitine supplement, symptomatic support	Outcome depends on age of onset and presence or absence of multi-system dysfunction
Long-chain-L-3-hydroxyacyl-CoA dehydrogenase deficiency (LCHAD/MTP)	A-II	1:75 000	Hypoglycemia, coma, lethargy, cardiomyopathy, hypotonia, liver dysfunction, seizures, progressive myopathy and polyneuropathy, pigmentary retinopathy, intestinal pseudo-obstruction, coma, and sudden infant death	Avoid fasting, low fat high carbohydrate diet and supplements. Pregnant women have increased risk of developing acute fatty liver of pregnancy and pregnancy complications	Variable
Trifunctional protein deficiency	A-II	1:100 000	Fever, nausea, diarrhea, vomiting hypoglycemia, poor appetite, muscle weakness, delays in walking and talking, absent reflexes	Avoid fasting, low fat high carbohydrate diet and L-carnitine supplement	Variable

(Table 2 continued)

	Strength of evidence	Incidence	Clinical symptoms	Treatment	Prognosis
Secondary conditions					
Short-chain acyl-CoA dehydrogenase deficiency (SCAD)	I/C-II	1:75 000	Poor feeding, vomiting, failure to thrive, seizures progressive muscle weakness (lipid storage myopathy), developmental delay	Avoid fasting, low fat high carbohydrate diet	Variable
Medium/short-chain L-3-hydroxyacyl-CoA dehydrogenase deficiency	I	1:100 000	Poor appetite, vomiting, diarrhea, lethargy, hypotonia, liver problems, hypoglycemia, hyperinsulinism	Avoid fasting, low fat high carbohydrate diet and L-carnitine supplement.	Variable
Multiple acyl-CoA dehydrogenase deficiency (MADD) or glutaric acidemia-type 2	B-II	1:1000 000	Variable, from lethal neonatal acidosis with renal cysts and brain dysplasia to mild adult lipid storage myopathy	Avoid fasting, low fat high carbohydrate diet, carnitine, riboflavin supplement	Variable
Medium-chain ketoacyl-CoA thiolase deficiency	A-II	1:100 000	Vomiting, weight loss, poor appetite, diarrhea, trouble breathing, hypoketotic hypoglycemia, lethargy, seizures, coma, hepatomegaly and acute liver disease	Avoid fasting, low fat high carbohydrate diet and L-carnitine supplement	Variable. Good outcome if identified and treated early
2,4-Dienoyl-CoA reductase deficiency	I	1:100 000	Vomiting, weight loss, poor appetite, diarrhea, trouble breathing, hypoketotic hypoglycemia, lethargy, seizures, sepsis, hypotonia, coma	Avoid fasting, low fat high carbohydrate diet and L-carnitine supplement	Variable
Carnitine palmitoyltransferase I deficiency (CPT I)	B-II	<1:1000 000	Hypoglycemia, coma, lethargy, vomiting, hepatomegaly	Avoid fasting, low fat high carbohydrate diet	Good
Carnitine palmitoyltransferase II deficiency (CPT II)	B-II	<1:1000 000	Hypoglycemia, coma, lethargy, cardiomyopathy, myoglobinuria, hypotonia, liver dysfunction	Avoid fasting, low fat high carbohydrate diet	Variable
Carnitine acylcarnitine Translocase (CACT) deficiency	B-II	<1:1000 000	Hypoglycemia, coma, lethargy, cardiomyopathy, hypotonia, liver dysfunction	Avoid fasting, low fat high carbohydrate diet, carnitine supplement	Symptoms continue usually despite treatment
Organic acid disorders					
Core Conditions					
Propionic acidemia (PA; PROP) (propionyl-CoA carboxylase deficiency	A-II	1:75 000	Episodes of ketosis and acidosis, failure to thrive, vomiting, anorexia, hypotonia, dermatitis, brain damage, coma and death	Protein restriction (limited intake of valine, isoleucine, methionine and threonine), and nutritional supplements (carnitine)	Despite treatment developmental delays, seizures, dystonia, cerebral atrophy, frequent infections and heart problems are common
Methylmalonic acidemia due to methylmalonyl-CoA mutase deficiency	A-II	1:75 000	Episodic ketoacidosis, failure to thrive, vomiting, anorexia, hypotonia, dermatitis, renal failure, cardiomyopathy, recurrent pancreatitis	Protein restricted diet (limited valine, isoleucine, methionine and threonine intake), supplementation of cobalamin (B12), and carnitine	Variable, despite treatment some die within first year or develop brain damage

(Table 2 continued)

	Strength of evidence	Incidence	Clinical symptoms	Treatment	Prognosis
Methylmalonic acidemia due to cobalamin C, D, F deficiency	A-II	1:100 000	Episodic ketoacidosis, failure to thrive, spastic quadriparesis, seizures, brain damage, paralysis, coma and death	Cobalamin (B12), carnitine and protein restriction	Most patients respond to cobalamin therapy
Isovaleric acidemia (IVA) (isovaleryl-CoA dehydrogenase)	A-I	1:100 000	Episodic vomiting, lethargy, sweaty feet odor, coma, permanent neurological damage, and death	Protein and leucine restriction, glycine and L-carnitine intake	Variable
3-Methylcrotonyl-CoA carboxylase deficiency (3-MCC)	B-II	1:75 000	Highly variable, normal phenotype, Reye-like illness, hypotonia, brain damage, seizures, liver failure	Protein and isoleucine restriction	Variable
3-Hydroxy-3-methylglutaric aciduria (HMG) (3-hydroxy-3-methylglutaryl-CoA lyase deficiency	A-II	1:100 000	Vomiting, lethargy, seizures, hypotonia, hypoketotic hypoglycemia, hepatomegaly (fatty liver), metabolic decompensation	Leucine restriction, carnitine supplementation, avoid fasting	Generally good
Holocarboxylase synthetase deficiency (multiple CoA carboxylase deficiency) (HCS; MCD)	B-II	1:100 000	Ketosis, vomiting, alopecia, skin rash, hair loss, hypotonia, seizure brain damage, coma and death	Biotin	Good. Early diagnosis and treatment with biotin allows normal growth and development
ß-Ketothiolase deficiency (ßKT) (mitochondrial acetoacetyl-CoA thiolase deficiency)	A-II	1:100 000	Episodic ketosis, acidosis, vomiting, lethargy, coma, brain damage and death	Protein and isoleucine restriction	Variable, generally good
Glutaric aciduria type I (GA-I) (glutaryl-CoA dehydrogenase deficiency)	A-II	1:50 000	Dystonia, macrocephaly, episodic encephalopathy, subdural hemorrhage, retinal hemorrhages	Protein restriction to reduce lysine and tryptophan intake, L-carnitine supplement, avoid fasting	Variable, some patients develop normally while some others have permanent brain damage
Secondary conditions					
Methylmalonic acidemia with homocystinuria	A-II	1:00 000	Microcephaly, hydrocephalus, poor appetite and growth, delay in learning, mental retardation, vision, heart and kidney problems, skin rashes	Low-protein diet. Vitamin B12, L-Carnitine, Betaine supplement, avoid fasting	Variable, some patients may have life-long learning problems or mental retardation. Develop psychiatric disorders
Malonic aciduria	B-II	1:100 000	Delayed development, hypotonia, seizures, diarrhea, vomiting, hypoglycemia, cardiomyopathy and metabolic acidosis	Avoid fasting, low fat high carbohydrate diet	Variable but it can be lethal in the neonatal period
Isobutyrylglycinuria (Isobutyryl-CoA dehydrogenase deficiency)	B-II	1:100 000	Symptoms appear later in infancy and include failure to thrive, dilated cardiomyopathy, seizures, anemia, and very low blood levels of carnitine	Low-protein diet. Vitamin B12, L-carnitine, betaine supplement, avoid fasting	Variable

(Table 2 continued)

	Strength of evidence	Incidence	Clinical symptoms	Treatment	Prognosis
2-methylbutyryl glycinuria (2MBG) (2-methylbutyryl-CoA dehydrogenase deficiency)	B-II	<1:1000 000	Muscular atrophy, mental retardation, lethargy, apnea, tachycardia, fever, nausea, vomiting, hypoglycemia, metabolic acidosis	Protein and isoleucine restriction	Variable, some patients develop normally while some others have neurological deficit
3-methylglutaconic aciduria (3MGA) (3-methylglutaconyl-CoA hydratase deficiency) Type I	B-II	<1:1000 000	Choreoathetosis, spastic paraparesis, dementia, delayed speech and motor development	Protein and leucine restriction, carnitine supplement	Neurological disease with variable severity
3-Methyl glutaconic aciduria type II (Barth syndrome)	B-II	<1:1000 000	Normal cognitive development, mitochondrial cardiomyopathy, short stature, skeletal myopathy, and recurrent infections	Symptomatic therapy	Variable, high morbidity and mortality
3-Methylglutaconic aciduria type III	B-II	1:10 000 among Iraqi Jewish kindred	Optic atrophy, choreoathetosis, spastic paraplegia, ataxia	Supportive therapy	Poor
3-Methylglutaconic aciduria type IV	B-II	<1:1000 000	Neonatal respiratory distress, inguinal hernia, cryptorchidism, subaortic stenosis, biventricular hypertrophy, severe mental retardation, cerebellar dysgenesis, neonatal hypotonia, absent reflexes	Symptomatic therapy	Poor
2-Methyl-3-hydroxybutyric aciduria	I	1:100 000	Metabolic acidosis, hypoglycemia, hypotonia, seizures, movement problems, retinal degeneration, and hearing loss, severe neurodegeneration in males	Avoid fasting, low protein and fat and high carbohydrate diet	Development of intellectual disabilities are common even if they receive treatment

Table 2   Core and secondary conditions recommended by ACMG; strength of evidence graded by Laboratory Medicine Practice Guidelines (LPMG) Committee criteria, incidence, clinical symptoms, treatment and prognosis of MS/MS detectable disorders for expanded NBS.

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

I: Evidence includes consistent results from well-designed and 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.

Based on the accumulated data, seven IEM in the uniform panel detected by MS/MS received an A-I rating, 12 received A-II, 19 received B-II, and four received I [3]. Brief clinical information (incidence, clinical symptoms, treatment and prognosis) of MS/MS detectable IEM is summarized in Table 2.

Dried blood sample preparation and techniques for expanded newborn screening using tandem mass spectrometry

The process of NBS starts with the collection of blood samples from newborns on a high quality cotton fiber-based absorbent paper (WhatmanTM) [15]. Better results are obtained from samples collected by heel prick in comparison to cord blood. The heel is first warmed up to approximately 42°C for a few minutes to increase the blood flow, followed by cleaning and puncturing the skin by a sterile lancet. After wiping the first drop, several filter paper circles are filled completely with blood by gently touching the filter paper against the oozing blood. It is important not to squeeze the heel to avoid hemolysis, over saturate the filter paper and touch it to avoid contamination [19]. Filter paper is allowed to dry in air and dried blood sample (DBS) is sent to the laboratory for analysis [14, 15, 52, 53]. Timing of the blood sample collection is important and sampling at 24–72 h of age is recommended [14, 15, 54]. If the newborn is discharged before 24 h, a filter paper sample should be collected before discharge and then repeated when the infant is seen by the pediatrician later [19]. Information regarding prematurity, birth weight, neonatal jaundice, parenteral nutrition, transfusions and type of feed should be recorded on the NBS card to help interpretation of the results. Some analytes are relatively unstable and heat, humidity, light and delays in transport can cause degradation and potential FNs [14, 15].

The use of urine as a NBS sample has been dropped in favor of DBS due to additional need for a blood sample for CH testing and better sensitivity of blood phenylalanine levels. However, urine samples have started to be used recently in some multiplex MS/MS testing to detect some IEM preferentially to DBS [14, 55].

Storage periods of DBS cards vary between laboratories. Stored cards are a valuable resource and might be used for diagnostic purposes, retrospective genetic diagnosis and forensic identification when there is no other DNA-containing material available [14].

Automated machines are used in many laboratories for punching DBS. Instrument availability and run time are the limiting factors for turn-around time [15, 56]. A 5 mm diameter DBS is mixed with labeled internal standards, extracted, derivatized and analyzed in a MS/MS instrument [15, 40, 44]. The quantity of analytes is determined using software supplied by the MS manufacturer which automatically flags all the abnormal results. The Clinical Laboratory Standards Institute (CLSI) recently published a document entitled 'Newborn screening by tandem mass spectrometry; an approved guideline (I/LA32-A)' describing the details of MS/MS methods such as method validation, measurement of linearity, limits of detection, precision, maximizing sensitivity and minimizing FPs [15, 57].

Every NBS laboratory must determine normal ranges, cut-off values, and ratios for analytes by measuring 1000–2000 normal DBS samples. The cut-off value is set above 99% of the normal range, and below the lowest 10% of the abnormal range [15]. Since the number of affected newborns detected for a specific disease will be very small, it is useful to collaborate with other laboratories to establish normal ranges of analytes by analyzing at least 50 cases for each NBS disease [15, 26]. The number of diseases screened for should not be related to the number of markers which can be measured, but, instead, should be closely linked with the follow-up processes for NBS screen positives [15].

In the last 10 years reagents including the software to process the data generated by MS/MS have become commercially available leading to the development of a simpler, but more expensive methodology. The laboratory establishes reference ranges, cut-off values and starts running samples for NBS. Instead of commercial reagents, some NBS laboratories purchase only isotope-labeled and unlabeled compounds and prepare their reagents using

the method published originally by Chace et al. for MS/MS [15, 44].

The most commonly used instrument for NBS is a triple quadrupole MS/MS. It consists of two mass spectrometers (MS/MS) separated by a collision cell. Two mass spectrometers in tandem (MS/MS) enable control of the formation of molecular and fragment ions. Before injection, metabolites are extracted from DBS samples using methanol and stable isotope internal standards are used as controls. Formation of butyl derivatives is commonly used to improve sensitivity and specifity. The recent MS/MS instruments simplify the assay procedure and samples are directly introduced into the ESI source without chromatography. The early method of Millington using fast atom bombardment to produce ionization was difficult to automate. This limitation was overcome with the development of ESI and high throughput testing suitable for NBS was developed afterwards [14, 58–60]. Ionization of both underivatized and derivatized analytes is achieved by ESI. The sample is sprayed through a small tube into a strong electric field in the presence of a flow of warm nitrogen gas to assist desolvation and formation of ions. The ionization process involves transfer of a charge to the solvent droplets, evaporation of the solvent and, finally, production of positively and negatively charged ions. This vaporized and ionized mixture enters the first MS which functions as a separating device and allows only the ion(s) of interest to pass through. Ions passing through the first MS are called precursor or parent ions which enter the collision cell where the fragmentation takes place. Fragmentation is achieved by putting an inert collision gas such as nitrogen or argon into the collision cell. The fragments generated in the collision cell are called product or daughter ions. The mass of these fragments are measured in the second MS. Fragments in the second MS are correlated with the intact molecules produced in the first MS. This process enables unique MS such as precursor ion scans and neutral loss scans. Since certain compound classes share common fragment ions of neutral fragment molecules, a special analysis is set up to detect and measure only precursor ions with a particular fragment without detecting molecules that are not of interest. Several metabolites are measured selectively in a 2-min assay. This process avoids time consuming chromatographic separations that can take up to 30 min using a GC– or LC–MS system. MS/MS is usually operated in multiple reaction monitoring (MRM) mode, but it can also be operated in scanning mode to measure full amino acid and acyl carnitine profiles [14, 19, 61, 62].

Quality control, accuracy, reliability, and precision of the assays using tandem mass spectrometry

Quality control (QC) is performed to ensure that the results reported are reliable, accurate and precise. NBS laboratories must run positive and negative controls which test the instrument as well as sample preparation performance. The precision and accuracy of the tests by MS/MS are monitored by measuring controls containing three different concentrations of selected analytes. Controls containing analytes near the cut-off values should be used because accuracy is very important near the cut-off values. All samples that are above the cut-off value for any analyte should be re-analyzed to ensure that the abnormal result is not due to pre- or analytical error. The recovery of specific analytes and the coefficient of variability are measured on different days at three different levels [15].

NBS laboratories should participate in external control programs and exchange samples with reference laboratories. There are several sources of reference materials and external quality assessment programs for NBS such as Center for Disease Control and Prevention's (CDC's) Newborn Screening Quality Assurance Program (NSQAP); College of American Pathologists (CAP); European Research Network for Evaluation and Improvement of Screening, Diagnosis, Treatment of Inherited Disorders of Metabolism (ERNDIM); and Reference Institute for Bioanalytics. They provide QC material for the markers of all the disorders listed in the recommended uniform screening panel. In 2009, the NSQAP provided QC services for DBS testing to 459 NBS laboratories in 63 countries for 50 newborn disorders [11, 63].

Plotting and monitoring controls using Levy-Jennings graphics are useful for observing and monitoring trends and shifts. It is also used to monitor the total or current ion abundance (TIC) of the internal standard added to each sample. After the analysis is completed, each run is checked by a number of criteria including an inspection of the TIC of each sample, a check that the internal standard and all controls are within the expected range. If the acceptance criteria are met, then abnormal results are reviewed to determine which sample is to be reported as an abnormal screen result, and for which disease. Failed runs do not meet one or more of the acceptance criteria and are analyzed for a possible explanation of the failure, and the run is repeated after identification and solution of the problem. It is helpful to determine the overall performance of the NBS laboratory to monitor true and false

positives (TP and FP), true and false negatives (TN and FN), as well as the positive-predictive value (PPV) and the negative-predictive value (NPV) for each disease, which are defined as: PPV=TP/(TP+FP) and NPV=TN/(FN+TN), respectively [15, 26].

It has been reported that 10%–40% of FP NBS results are due to low birth weight (LBW <2500 g), prematurity (defined as <37 weeks of gestation), sickness in the newborn period, alimentation by total parenteral nutrition (TPN), or medications. A second sample is screened at a later time when the baby has been cut-off from TPN for a week. The reason for high false-screen positives in preterm, LBW and sick newborns is not clear, and has been addressed in one of the CLSI institute guidelines [15, 57]. Collection of blood samples is recommended as the first DBS on admission (<24 h), the second between 24 h and 72 h and a third at a few weeks of age before discharge from the hospital [15, 64].

Small concentration gradients occur during the spreading and drying of blood on the filter paper which causes approximately 10% imprecision of any DBS test. Due to the imprecision of screening tests on DBS, it is important to note that NBS is not diagnostic and any positive result requires confirmation, preferably with a new sample and a different test method [1–5, 14, 15].

Estimated error associated with using DBS as the sample for NBS is approximately ±30% mostly due to the variance of blood volume in DBS which will be extracted from the filter paper. The recovery of analytes from DBS is the most imprecise part of the five steps in the NBS methodology which includes specimen collection, preparation, MS/MS analysis, interpretation and reporting [15]. Methanol is used usually as the extraction solvent for extracting carnitine esters and amino acids from DBS. Amino acids at physiological concentrations are recovered 100% with methanol, but higher concentrations of amino acids are not recovered completely. It was reported that a mixture of methanol and water (90/10 or 80/20; v/v) is a better extraction solvent for polar amino acids at higher concentrations [15].

Optimization of test performance of MS/MS used for NBS is described in detail in the document I/LA-32-A recently published by CLSI [15, 57]. The method used for specimen preparation (free or derivatized acids) and the conditions used for derivatization have an impact on test results and must be carefully controlled. Free acids give lower ion abundance compared to derivatized acids, but new instruments are sensitive enough that this reduction in response is not a limiting factor for NBS. Derivatization of amino acids and acylcarnitines give higher ion abundances in MS, but derivatization is usually performed under acidic conditions which may cause hydrolysis

of acylcarnitines. In a recent study, a simple and reliable method has been used for the detection of amino acids without derivatization [65]. It must be verified that the concentrations of free carnitine and shorter chain acylcarnitines are not altered by the conditions used to derivatize the samples [66]. Most of NBS laboratories use reliable and sensitive tandem quadrupole instruments, and specialized software which controls instrumentation and calculates concentrations of analytes using a set of acceptance rules, distinguishing normal results from abnormal or out-of-range results which are identified and flagged for reporting [15, 57].

Every additional metabolite added to the NBS panel is associated with a small FP rate which results in an overall significant FP rate, collectively. Expanded NBS by MS/MS increased the number of tests screened leading to an increase in the number of FPs. The number of infants having FP results in NBS with MS/MS in the US in 2005 was estimated to be 51 059 assuming that the specificity of NBS tests is approximately 99.9% [67]. It is possible to keep the rates of FP results <0.5% with appropriate selection of cut-offs. Several metabolite ratios can also be used to improve PPV. For example, C3 carnitine has a relatively poor PPV, and a ratio of C3 carnitine to C2 carnitine can be used to improve PPV [14, 68].

Reporting and interpretation of screening results

NBS test results are mostly negative, which means that the infant does not have any IEM for which laboratory tests are performed. Results are reported either as analytes measured or diseases screened for. If all the results are normal, then the results are entered into the Laboratory Information system [15]. Screen positive results are repeated from the original DBS and when the repeated test reveals a positive result, NBS laboratories provide a written interpretive comment for each screen positive result which requires experience and knowledge for all of the aspects of the NBS process, the disorder screened and the factors that can cause FP results. Screen positive results should be confirmed by other tests including plasma amino acid, acylcarnitine and urine organic acid profiles to avoid false abnormalities which may not be due to a metabolic disease [3]. When a primary analyte is significantly elevated, the interpretation is usually called a screen positive for that particular disease. Analyte ratios and a secondary analyte usually support a screen positive result when the primary marker is elevated. However, when a secondary analyte or

only a ratio is abnormal without a significant increase in a primary analyte, the interpretation is more cautious. If the results from the confirmatory tests are positive, expert pediatricians provide management, counseling, and follow-up of the infants [3, 15, 38, 57, 65, 69].

Second tier tests

The FP results and relatively poor PPV for some MS/MS tests have led to the development of a number of second-tier tests. Each of the second-tier tests requires a separate testing protocol and rapid turn-around of results is required [14]. The second tier tests available and incorporated into the NBS programs are for tyrosinemia I, propionic acidemia, maple syrup urine disease (MSUD), CAH, hyperammonemia-hyperornithinemia-hyperhomocitrullinemia (HHH) syndrome, galactosemia and lysosomal storage diseases [14, 15, 24, 25, 70–74].

Lysosomal storage diseases by tandem mass spectrometry

The lysosomal storage diseases (LSD) are a group of rare, inherited metabolic diseases that result from defects in lysosomal function as a consequence of deficiency or absence of specific enzymes which are responsible for the breakdown of unwanted substrates. LSDs include more than 50 genetic disorders which vary in severity and prevalence resulting in the accumulation of substrates that would normally be degraded by the lysosomal enzymes. Fifteen LSDs account for approximately 75% of patients with LSDs. The incidence of all LSDs is about 1:5000–1:10 000 [15, 18, 69, 75, 76]. In the absence of a family history, presymptomatic detection of LSD can be achieved only through NBS testing. Some LSDs are treatable as therapeutic options become available including enzyme replacement therapy, bone marrow transplantation and gene therapy [19, 77].

ACMG did not include any of the LSDs into the NBS program in the US in 2006 due to the absence of proven and high throughput screening assays [1, 4, 5, 15]. In a recent paper, three LSDs were evaluated – Pompe disease, Fabry disease, and Krabbe disease which fail to meet some of the critical Wilson and Jungner criteria and thus are not ready for inclusion in universal NBS panels. It was concluded that screening for these conditions should only be performed in the research context with institutional review board approval and parental permission [18].

In recent years, the use of MS/MS in the detection of LSDs has been expanded based on the availability of therapy and development of multiplex assays. Six LSDs have been started screening in the US within a single MS/MS run using different substrates and internal standard combinations: Gaucher disease; Pompe disease; Fabry disease; Niemann-Pick disease; mucopolysaccharidosis I; and Krabbe disease [15, 29, 77, 78]. It has been shown that the enzymes tested for LSDs remain active in DBS. The amount of product generated was calculated using the ratio of product to internal standard, incubation time, volume of blood and amount of internal standard [15, 78–81]. The LSD assays are performed using direct injection into the MS/MS system. This allows for short run times and high sample throughput, but increases the need for sample cleanup [15, 56]. MS/MS methods have been optimized for LSDs recently for high throughput screening minimizing the sample volume needed from the DBS and using selected reaction monitoring (SRM) for all products and internal standards having unique SRM transitions [15, 82]. Calibrated standards for LSD assays are not commercially available [15, 83]. Reference ranges for LSDs must be established using samples from unaffected normal infants. Carriers of LSDs are expected to have 50% of the enzyme activity of the unaffected normal infants which usually partially overlaps with the normal range. Therefore, the detection of carriers for LSDs using MS/MS enzyme assays is not very reliable [15, 78]. The Center for Disease Control (CDC) in the US has developed QC material for five LSDs (Fabry, Gaucher, Krabbe, Niemann–Pick A/B and Pompe) [15, 84].

Confirmatory follow-up testing procedures for positive newborn screens

An advanced diagnostic laboratory is needed for confirmation of the screen positive results. After a positive newborn screen, the three most common analyses available for confirmatory follow-up testing are amino acids and acylcarnitine analysis in plasma or serum and organic acid analysis in urine [3]. Following a screen positive result, a subset of these tests is performed to confirm diagnosis (Table 3). In order to provide reliable, accurate and precise results, recommendations for three confirmatory

	Primary screening tests (MS/MS)	Confirmatory/follow-up tests	Findings of confirmatory tests for diagnosis
Amino acid metabolism disorders			
Argininosuccinic aciduria (ASA) (argininosuccinate lyase deficiency) (ASL)	↑Citrulline	Plasma NH3, UAA, PAA, enzyme assay	↑NH3, ↑argininosuccinic acid on UAA and PAA, ↓fibroblast/liver ASL activity
Argininemia (ARG) (arginase deficiency)	↑Arginine	Plasma NH3, PAA, enzyme assay	↑NH3, ↑arginine on PAA, ↓hepatic arginase activity
Citrullinemia, type I (CIT) (argininosuccinate synthase deficiency) (ASS)	↑Citrulline	Plasma NH3, PAA	↑NH3, ↑citrulline on PAA, ↓fibroblast/liver ASS activity
Homocystinuria (HCY) (cystathionine β-synthase deficiency) (CBS)	↑Methionine	PAA, UAA, UOA	↑Blood and urine homocyst(e)ine, methionine on PAA and UAA; ↑urine methylmalonic acid on UOA in cobalamin synthesis defects
Maple syrup urine disease (MSUD) (branch-chain α-ketoacid dehydrogenase complex deficiency)	↑Total 'Leucine, isoleucine, alloisoleucine' and ↑Valine	PAA, urine DNPH, UOA	↑Leucine, isoleucine, alloisoleucine and valine on PAA; positive DNPH; ↑branched chain α-keto and hydroxyl acids on UOA
Phenylketonuria (PKU) (phenylalanine hydroxylase deficiency) (>98%), BH4 synthesis defects/regeneration defects (<2%)	↑Phenylalanine, ↑phenylalanine/tyrosine ratio	PAA, urine and/or blood or CSF neopterin and biopterin	↑Phenylalanine on PAA, ↑phenylalanine/tyrosine ratio; abnormal urinary and/or blood or CSF pterins in BH4 synthesis defects
Tyrosinemia, type I (TYR-I) (Fumarylacetoacetate hydrolase deficiency) (FAH)	↑Tyrosine	PAA, UOA	↑Tyrosine and methionine on PAA; ↑succinylacetone and tyrosine metabolites on UOA
Tyrosinemia, type II (TYR II) (tyrosine aminotransferase deficiency)	↑Tyrosine	PAA, UOA	↑Tyrosine on PAA; ↑tyrosine metabolites without increased succinylacetone on UOA
Fatty acid oxidation disorders			
Carnitine uptake defect (CUD) (carnitine transporter defect)	↓C16–C18 acylcarnitines, ↓free carnitine	PACP, urine carnitine, CK, glucose, NH3	↓C16–C18 acylcarnitines on PACP; ↑urine carnitine; ↓free carnitine, ↑CK, ↓glucose, ↑NH3 on plasma
Medium-chain acyl-CoA dehydrogenase deficiency (MCAD)	↑C6–C10 acylcarnitines	PACP, UOA, CK, glucose, NH3	↑C6–C10 acylcarnitines on PACP; ↑dicarboxylic acids, hexanoylglycine, phenylpropionylglycine and suberylglycine on UOA; ↑CK, ↓glucose, ↑NH3 on plasma
Very-long-chain acyl-CoA dehydrogenase deficiency (VLCAD)	↑C14, C14:1,C14:2 acylcarnitines, ↓Free carnitine	PACP, CK, glucose, NH3	↑Long chain (C14:0, C14:1, C16:0, C16:1, C18:0, C18:1) acylcarnitines on PACP; ↑CK, ↓glucose,↑NH3 on plasma
Long-chain-L-3-hydroxyacyl-CoA dehydrogenase deficiency (LCHAD/MTP)	↑Long chain 3-hydroxy acylcarnitines (C16-OH, C18-OH, C18:1-OH)	PACP, UOA, CK, glucose, NH3	↑Long chain 3-hydroxy acylcarnitines (C16-OH, C18-OH, C18:1-OH) on PACP; ↑3-OH dicarboxylic acids on UOA; ↓glucose, ↑NH3 on plasma
Short-chain acyl-CoA dehydrogenase deficiency (SCAD)	↑C4 carnitine	PACP, UOA	↑C4 carnitine on PACP; ↑ethylmalonic, methylsuccinic with normal ketosis, butyrylglycine on UOA
Medium/short-chain L-3-hydroxyacyl-CoA dehydrogenase deficiency (M/SCHAD)	↑C4-OH carnitine	UOA	↑3-OH adipic, ↑3-OH sebacic, ↑3-OH suberic acids, ↑3-OH glutarate
Multiple acyl-CoA dehydrogenase deficiency (MADD) or glutaric acidemia-type 2	↑Multiple acylcarnitines	PACP, UOA, CK, glucose, NH3	↑Multiple acylcarnitines on PACP; ↑glutaric, ethylmalonic, dicarboxylic acids, hexanoylglycine, phenylpropionylglycine and suberylglycine on UOA; ↑CK, ↓glucose, ↑NH3 on plasma

(Table 3 continued)

	Primary screening tests (MS/MS)	Confirmatory/ follow-up tests	Findings of confirmatory tests for diagnosis
Carnitine palmitoyltransferase deficiency type II (CPT II)	↑ C16–C18 acylcarnitines, ↓ free carnitine	PACP, CK, glucose, NH3	↑ C16–C18 acylcarnitines on PACP; ↓ free carnitine, ↑ CK, ↓ glucose, ↑ NH3 on plasma
Primary carnitine deficiency	Free (C0) carnitine	↓ Free and total carnitine	↓ Total and free carnitine on PACP; ↑ urine carnitine
Organic acid disorders			
Propionic acidemia (PA; PROP) (propionyl-CoA carboxylase deficiency)	↑ C3-acylcarnitine	UOA, PACP	↑ 3-Hydroxypropionate, methylcitrate, propionylglycine; ↑ propionylcarnitine (C3) on PACP
Holocarboxylase synthetase deficiency (multiple CoA carboxylase deficiency) (HCS; MCD)	↑ C5-OH, C3 acylcarnitine	UOA, PACP	↑ 3-OH-isovaleric, 3-methylcrotonylglycine, methylcitrate, 3-OH-propionic, lactate, pyruvate, acetoacetate, 3-OH-butyrate on UOA; ↑ C3-acylcarnitine, ↑ 3-hydroxyisovalerylcarnitine (C5-OH acylcarnitine) on PACP
Methylmalonic acidemia (methylmalonyl-CoA mutase deficiency, Cobalamin C, D, F deficiency)	↑ C3-acylcarnitine	UOA, PACP	↑ Methylmalonic, 3-hydroxypropionate, methylcitrate, propionylglycine on UOA; ↑ propionylcarnitine (C3-acylcarnitine) on PACP
3-Methylglutaconic aciduria (3MGA) (3-methylglutaconyl-CoA hydratase deficiency)	↑ 3-Hydroxyisovaleryl carnitine (C5-OH)	UOA, PACP	↑ 3-Hydroxyisovaleric, 3-methylglutaconic, 3-methylglutaric on UOA; ↑ 3-hydroxyisovalerylcarnitine (C5-OH) on PACP
2-Methylbutyrylglycinuria (2MBG) (2-methylbutyryl-CoA dehydrogenase deficiency)	↑ 2-Methylbutyryl carnitine (C5)	UOA	↑ 2-Methylbutyrylglycine on PACP
3-Methylcrotonyl-CoA carboxylase deficiency (3-MCC)	↑ 3-Hydroxy isovalerylcarnitine (C5-OH) acyl carnitine	UOA, PACP	↑ 3-Hydroxyisovaleric acid, 3-methylcrotonylglycine on UOA; ↑ 3-hydroxyisovalerylcarnitine (C5-OH) on PACP
ß-Ketothiolase deficiency (ßKT) (mitochondrial acetoacetyl-CoA thiolase deficiency)	↑ Tiglylcarnitine (C5:1), ↑ 3-hydroxy-2-methylbutyryl carnitine (C5-OH)	UOA, PACP	↑ 2-Methyl-3- hydroxybutyrate, 2-methylacetoacetic, tiglylglycine on UOA; ↑ tiglylcarnitine (C5:1), ↑ 3-hydroxy-2-methylbutyryl Carnitine (C5-OH) on PACP
Isovaleric academia (IVA) (isovaleryl-CoA dehydrogenase	↑ Isovalerylcarnitine (C5)	UOA, PACP	↑ Isovalerylglycine, 3-hydroxyisovaleric acid on UOA; ↑ isovalerylcarnitine (C5) on PACP
Glutaric aciduria type I (GA-I) (glutaryl-CoA dehydrogenase deficiency)	↑ Glutarylcarnitine (C5-dicarboxylic)	UOA, PACP	↑ Glutaric acid, 3-hydroxygluratic acid, glutaconic acid on UOA; ↑ Glutarylcarnitine (C5-dicarboxylic) on PACP
3-Hydroxy-3-methylglutaric aciduria (HMG) (3-hydroxy-3-methylglutaryl-CoA lyase deficiency	↑ C5-OH, C6-DC, C6OH-DC acylcarnitine	UOA	↑ 3-OH-3-methylglutaric, 3-methylglutaric, 3-methylglutaconic, 3-OH isovaleric acids

Table 3   Primary screening and confirmatory follow-up tests and findings for MS/MS detectable newborn metabolic disorders. PAA, plasma amino acids; UAA, urine amino acids; UOA, urine organic acids.

follow-up techniques are summarized below (Table 4) [3, 19, 85].

Confirmatory follow-up testing procedures for amino acid analysis

Heparinized plasma from fasting patients is the ideal specimen type because it can be processed immediately. Serum can be used, but is not ideal due to the time required for clot formation at room temperature which may lead to artifacts. Cerebrospinal fluid (CSF) and urine amino acid analysis can be performed for some special disorders. Deproteinization is required before analysis. Methanol is used for LC-MS/MS and sulfosalicylic acid is used for ion exchange chromatography. Calibration is performed with a series of external standards. Butylation for derivatization is used to enhance ionization and analytic specificity. Chromatography is required for MS/MS to separate isobaric amino acids (e.g., alloisoleucine, isoleucine, leucine, and hydroxyproline). Integrated peak areas are calculated using MS/MS in MRM mode. Ion-exchange techniques use a retention time internal standard. O-phthalaldehyde, phenylisothiocyanate, and ninhydrin can be used for derivatization. Post-column detection with ninhydrin is used commonly which provides consistency. Ninhydrin derivatives are analyzed at two wavelengths (570 and 440 nm) to assess peak purity [3].

Confirmatory follow-up testing procedures for acylcarnitine analysis

Plasma is the preferred specimen type for acylcarnitine analysis. Methanol extraction is commonly used for sample deproteinization. Butylation for derivatization is used to enhance ionization and analytic specificity. Full external calibration is not possible due to the unavailability of pure forms of many carnitine esters. Signal intensity of a specific ester is calculated in comparison to an appropriate deuterated internal standard with the nearest appropriate chain length. Carnitine esters should be analyzed by ESI MS/MS in either MRM or precursor ion scanning mode, or both. In the presence of interfering substances, chromatographic separation of acylcarnitines or the use of other complementary techniques might be employed to augment accuracy. Linearity, imprecision, and limits of detection must be frequently validated under the conditions used [3].

Confirmatory follow-up testing procedures for organic acid analysis

Organic acid are concentrated in urine, and their analysis in random urine is easier for detection as opposed to blood. Analysis may be performed quantitatively or semi-quantitatively. Results should be normalized to urine creatinine concentration. Extraction of organic acids with ethyl acetate and derivatization with trimethylsilyl should be formed before capillary GC-MS analysis. Abundant molecular species are detected by full ion scan from m/z 50 to 550 and less abundant species are detected by selected ion monitoring (SIM). Application of stable isotopes improves detection and reduces inter-laboratory variability in organic acid analysis. Organic acids are identified by retention time and mass spectra [3]. Urine organic acids analyzed by GC/MS yield diagnostic profiles that may need confirmation by direct enzymatic or metabolic pathway flux studies [19].

Follow-up programs

NBS should be integrated with follow-up and clinical management. A well-organized follow-up program must be in place to respond appropriately and in a timely fashion for screen positive results [3, 15, 20]. Guidelines describing in detail a well-functioning and designed NBS and Follow-Up Program were published by NACB [3, 15].

Future trends

The current NBS programs including LSDs are expected to grow and more widely implemented in the next years as the technology and options for therapies increase. Public health policy development must keep pace with the scientific developments. The ultimate goal of NBS and follow-up programs is to reduce morbidity and mortality from the disorders. The early identification and treatment have changed the lives of many children and families [3, 86]. However, NBS programs is only the beginning of the progress to health and identification of an affected infant must be extended to the appropriate confirmatory testing and treatment [87].

In recent years, NBS has been considerably expanded by the availability and improved sensitivity of MS/MS technology which allowed additional analytes to become

Sample type	Amino acids	Acylcarnitines	Organic acids
	Heparinized plasma	Heparinized plasma	Random urine
Pre-analytical collection/processing	Separate plasma promptly; +4°C up to 4 h; −20°C if analysis delayed >4 h	Separate plasma promptly; +4°C up to 4 h; −20°C if analysis delayed >4 h	Store at +4°C up to 24 h; −20°C if analysis delayed >24 h
Analytical technique	LC-MS/MS; Ion exchange chromatography (IEC) with ninhydrin detection	LC-MS/MS	Capillary GC/MS
Sample preparation	Deproteinization required; methanol for LC-MS/MS and sulphosalicylic for IEC	Deproteinization required (methanol)	Ethyl acetate extraction
Derivatization	Butylation for LC-MS/MS and ninhydrin, o-phthalaldehyde, phenylisothiocyanate, for IEC	Butylation	Trimethylsilyl derivatives
Calibration	External calibration for each amino acid	Full external calibration not possible (internal standard of nearest appropriate chain length)	Full external calibration for each organic acid
Internal standard	Ion exchange techniques use a retention time internal standard (aminoethylcystine); array of isotopologs for isotope-dilution quantification in LC/MS/MS protocols	Broad chain length deuterated isotopologs	Array of isotopologs for isotope-dilution quantification
Chromatography	Required to separate isobars (alloisoleucine, isoleucine, leucine, and hydroxyproline).	In case of interfering substance suspected	Fused silica capillary
Quality control	Analysis of normal and abnormal quality control specimens	Analysis of normal and abnormal quality control specimens	Analysis of normal and abnormal quality control specimens
Data acquisition	Integrated peak areas calculated using MS/MS in Multiple Reaction Monitoring (MRM) mode	Precursor ion scan or MRM mode	Full ion scan m/z 50−550 for abundant; Selected ion monitoring (SIM) for less abundant species

Table 4 Recommendations for optimal confirmatory follow-up testing procedures for MS/MS detected newborn metabolic disorders. GC/MS, gas chromatography/mass spectrometry; LC-MS/MS, liquid chromatography/tandem mass spectrometry.

available and be added to the list of disorders being screened for. Improvements in therapy such as enzyme replacement and gene therapy, and new pharmacological drugs have caused some disorders to be considered as potential candidates for NBS. It still remains to be clarified how effectively new tests can be applied, which diseases to screen for, how to confirm diagnoses quickly and accurately and how to follow-up patients identified as screen positive [14, 15].

A primary goal of expanded NBS is to enhance sensitivity, develop more specific markers, minimize the risk of FP results, and improve the PPV of screening results [69]. Multiplexed protein assays using coded microbead immunoassay technology may simplify NBS testing which can be applied to a wide range of proteins as demonstrated for LSDs [14, 88]. A chip with a large array of targeted mutations can detect more disorders than those currently tested [14, 89]. Digital microfluidics may be an alternative way of multiplexing many assays using very small volumes of blood for NBS. Microfluidics

technology utilizes enzyme assays, immunoassays and DNA-based molecular testing [15, 90]. Immunoassays performed either on a microfluidic platform or as the classic immunoassay in a tube still suffer from a high FP rate. Until these problems are eliminated, MS/MS will remain the method of choice for NBS. Pilot studies have shown the feasibility of genome wide scans of DBS and complete genome sequencing of newborns is expected to occur in the near future [14, 91, 92]. The use of next-generation sequencing will enable the detection of a larger number of genetic disorders without substantially increasing the costs of NBS. However, if implemented by state programs, new sequencing technologies may have a number of undesirable effects. Even though the implementation of genomic technology may improve the quality of NBS, premature adoption of these tools could ultimately place children at risk. Before implementing genomic screening, state health departments need to consider available clinical resources for ensuring adequate counseling about genetic test results.

A second area of concern centers on how states would utilize the vast amounts of information generated by use of genomic technologies in NBS. State storage of these data may lead some parents to view genomic evaluation of newborns as a form of research. Third, genomic screening would represent an even greater departure from the core public health aims served by NBS than the extension of these programs. [93]. It is not known if such a large-scale genetic testing is feasible and cost-effective, and will be accepted by the parents, general public and health policy-makers. These opinions are debatable, however, well-designed NBS and follow-up programs have obvious benefits for many disorders and will be used more widely as the cost reduces to levels with acceptable cost-benefit ratio.

Conflict of interest statement

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