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Tandem mass spectrometry in newborn screening for inherited metabolic diseases: A comprehensive review

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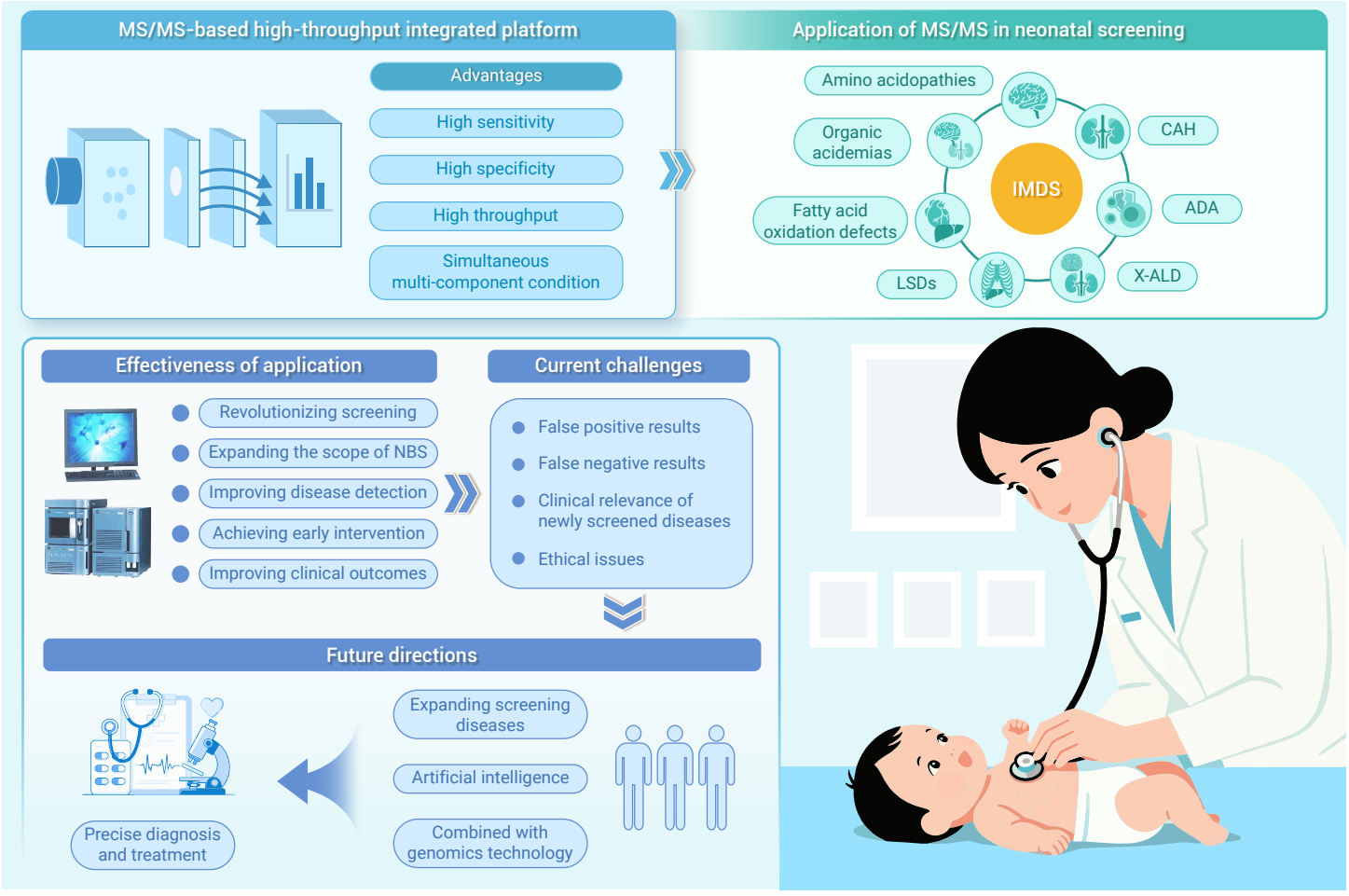
GRAPHICAL ABSTRACT



PUBLIC SUMMARY

- Newborn screening is a key public health strategy for preventing childhood diseases and deaths.
- MS/MS has revolutionized screening by detecting dozens of metabolic disorders in a single run.
- This review outlines MS/MS principles and evidence of improved detection, early treatment, and outcomes.
- Challenges remain, including false-positive results, clinical relevance confirmation, and ethical concerns.
- Future directions include AI, expanded panels, and genomics for precise diagnosis and personalized care.

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Newborn screening represents a critical public health strategy for preventing childhood morbidity and mortality. The introduction of tandem mass spectrometry (MS/MS) has revolutionized the screening landscape for inherited metabolic diseases, shifting the paradigm from single-disease assays to a high-throughput, integrated platform capable of detecting dozens of disorders in a single run. This review systematically outlines the applications and challenges of MS/MS in newborn screening. It begins by outlining the technical principles underlying MS/MS, highlighting its unique capacity to simultaneously quantify multiple amino acids, organic acids, and fatty acid oxidation metabolites. Then, it summarizes global implementation experiences and overviews evidence to demonstrate that MS/MS significantly enhances disease detection rates, enables early intervention, and substantially improves clinical outcomes. However, its widespread adoption introduces new challenges, such as the interpretation of false-positive results, confirmation of clinical relevance for newly screened conditions, and accompanying ethical concerns. Future directions include leveraging artificial intelligence to refine interpretive workflows, expanding the screening panel, and integrating genomic technologies with MS/MS to achieve more precise diagnosis and personalized management. This review aims to offer public health policymakers, clinicians, and laboratory specialists a comprehensive interdisciplinary overview to advance the next developmental phase of newborn screening systems.

INTRODUCTION

Inherited Metabolic Diseases (IMDs), also known as Inborn Errors of Metabolism (IEM), refer to a group of monogenic genetic diseases caused by gene mutations that lead to defects in enzymes, carrier proteins, or membrane functions, resulting in abnormalities in biochemical pathways. The majority of these disorders are autosomal recessive, with a very small number being autosomal dominant or X-linked. There are thousands of types of IMDs, among which about 500–600 are relatively common. Although individual diseases have a low prevalence and fall into the category of rare diseases, the overall incidence should not be overlooked—approximately 50.9 per 100 000 live births.[1]

Newborn screening (NBS) is critical in preventive medicine. It aims to achieve early detection, early diagnosis, and early intervention to avoid or mitigate irreversible organ damage, thereby reducing neonatal mortality and disability rates. Traditional screening methods, such as biochemical immunoassays, can only detect a limited number of analytes at a time.[2-4] These methods are often time-consuming, costly, and prone to interference from newborns' physiological conditions, which moreover increases the risks of both underdiagnosis and misdiagnosis. The emergence of Tandem Mass Spectrometry (MS/MS) has revolutionized the landscape of newborn metabolic screening. In 1990, Dr. Millington and colleagues in the United States first proposed the application of MS/MS for screening inherited metabolic disorders.[5] This technology enables qualitative and quantitative detection of dozens of small molecule metabolites within just several minutes, significantly improving both the accuracy and efficiency of screening.[6] In 2004, the U.S. Food and Drug Administration (FDA) issued guidelines for the use of MS/MS in analyzing amino acids, free carnitine, and acylcarnitines in newborns. By 2006, the American College of Medical Genetics (ACMG) recommended using MS/MS technology to screen for 20 out of 29 primary target diseases and 22 out of 25 secondary target diseases.[7] This review aims to summarize the current applications and future prospects of MS/MS technology in NBS for IMDs.

Core principles and key advantages of MS/MS technology

In the field of NBS, MS/MS currently relies primarily on electrospray ionization to ionize analytes extracted from dried blood spot (DBS) and introduce them into the MS/MS system. Heel-prick blood is typically collected between 48 hours and 7 days after birth and then prepared as DBS samples. After the sample is introduced into the mass spectrometer, the first quadrupole mass analyzer (Q1) can be set to scan ions within a predefined mass range or selectively detect multiple target ions based on their predicted masses. Following ion fragmentation in the second quadrupole collision cell (Q2), the third quadrupole mass analyzer (Q3) detects one or more analyte-specific product ions, thereby allowing the identification and abundance measurement of specific ions derived from the intact target analytes. For most biomarkers, the combination of precursor and product ion pairs provides sufficient specificity for the target analytes, facilitating rapid and interference-free detection of multiple markers in a single analysis. To ensure accurate quantification, internal standards are added to the DBS extract. These internal standards are stable isotope-labeled analogs of the target analytes with identical chemical properties. Quantification is achieved by comparing the signal response ratio between the analytes and the internal standards in the MS/MS analysis.

MS/MS technology is renowned for its high sensitivity, specificity, throughput, and capability for simultaneous multi-component detection. Compared to conventional methods, MS/MS enables the simultaneous detection of over 30 IMDs involving amino acids, fatty acids, and organic acids.[8,9] Moreover, the number of metabolites that can be analyzed in a single assay has been steadily increasing,[9-11] shifting the paradigm from "one test for one disease" to "one test for multiple diseases." Furthermore, this technique offers additional advantages including a wide linear range, rapid detection (typically completing an analysis within minutes), minimal sample requirement (only a single dried blood spot), and relatively low cost (with decreasing per-test cost as the number of detected items increases).

Main applications of tandem mass spectrometry in newborn screening for inherited metabolic diseases

Technological progress and evolving public health demands have driven the expansion of MS/MS screening programs in accordance with WHO

disease selection principles, incorporating an increasing spectrum of disorders. First and second-tier screening for IMDs now routinely employs MS/MS technologies.[12] This study adopts the core disorders listed in the Recommended Uniform Screening Panel (RUSP) — issued by the U.S. Health Resources and Services Administration (HRSA) — as its analytical framework. It systematically summarizes screening strategies for major diseases, the biomarkers utilized, along with the strengths and limitations of different screening methodologies (Table 1).[7,13,14] The first-tier involves MS/MS analysis of neonatal DBS, detecting amino acidopathies, organic acidemias, fatty acid oxidation defects, and related conditions, with extended applications now including lysosomal storage disorders (LSDs), X-linked adrenoleukodystrophy, and combined immunodeficiencies (Table 1). Second-tier screening, which is conducted on initially positive specimens using the original DBS sample, is a critical confirmatory step that substantially reduces false-positive rates and prevents missed diagnoses from the initial screening. Second-tier screening primarily relies on liquid chromatography-tandem mass spectrometry (LC-MS/MS), enabling retesting for over 30 IMDs.[15] Representative conditions include congenital adrenal hyperplasia (CAH), methylmalonic acidemia, and propionic acidemia, as well as isovaleric acidemia, homocystinuria, and LSDs, among others (Table 1).[16]

Application of tandem mass spectrometry in screening for amino acid disorders, organic acidemias, and fatty acid oxidation disorders in newborns. MS/MS as a revolutionary tool in the field of NBS for IMDs has been widely applied in the early detection of amino acid disorders, organic acidemias, and fatty acid oxidation disorders.[16,17] Amino acidopathies originate from enzymatic deficiencies within amino acid metabolic pathways, resulting in aberrant plasma amino acid concentrations and accumulation of alternative metabolites that induce multisystem damage. While traditional screening approaches are labor-intensive and lack precision, MS/MS enables accurate quantification of amino acid levels, thereby revolutionizing diagnostic accuracy. Organic acidemias arise from defective enzymes in organic acid metabolism, leading to toxic compound accumulation. These metabolites undergo partial renal excretion or conjugate with free carnitine to form diverse acylcarnitine species. Given their diverse and potentially life-threatening clinical manifestations, early detection is imperative. MS/MS fulfills a critical diagnostic role by identifying pathological acylcarnitine profiles for rapid assessment. Fatty acid oxidation disorders stem from impaired mitochondrial import pathways or beta-oxidation enzymatic defects. Free carnitine and

Table 1. Summary of screening strategies for major disorders: Tandem Mass Spectrometry (first or second-tier screening), GC-MS, and genetic testing.[13,31,36,48,56]

	Disorder(s)	Screening strategies			
		First-tier test marker(s)	Second-tier test marker(s)	GC-MS test marker(s)	Genetic test marker(s)
Amino acid disorders	Hyperphenylalaninemia	phenylalanine, phenylalanine/tyrosine ratio		phenylacetic acid, phenyllactic acid, phenylpyruvic acid	PAH, PTPS, QDPR, GTPCH, PCD, DNAJC12
	Maple syrup urine disease	total 'leucine, isoleucine, alloisoleucine' and valine	allo-isoleucine, isoleucine, leucine, valine, hydroxyproline	2-ketoisovaleric acid, 2-hydroxyisovaleric acid, 2-ketoisocaproic acid, 2-hydroxy-3-methylvaleric acid	BCKDHA, BCKDHB, DBT
	Tyrosinemia, type I	tyrosine, succinylacetone		4-hydroxyphenyllactic acid, 4-hydroxyphenylpyruvic acid, succinylacetone	FAH
	Argininosuccinic aciduria	citrulline		orotic acid, uracil, argininosuccinic acid	ASL
	Citrullinemia, type I	citrulline		orotic acid, uracil	ASS1
	Homocystinuria	methionine	methylmalonic acid, methylcitric acid, total homocysteine, 3-hydroxypropionic acid	total homocysteine	CBS, MTHFR
Organic acid disorders	Methylmalonic acidemia	C3, C3/C2, C3/C0	methylmalonic acid, methylcitric acid, total homocysteine, 3-hydroxypropionic acid	methylmalonic acid, methylcitric acid	MMAA, MMAB, MMACHC, MMADHC, LMBRD1, MUT, TCN1, TCN2, MTR, MTRR, PRDX1 ABCD4, HCFC1
	Propionic acidemia	C3, C3/C2, C3/C0	methylmalonic acid, methylcitric acid, total homocysteine, 3-hydroxypropionic acid	3-hydroxypropionic acid, methylcitric acid	PCCA, PCCB
	Isovaleric acidemia	C5		isovalerylglycine	IVD
	Glutaric acidemia type I	C5DC		glutaric acid, 2-hydroxyglutaric acid	GCDH
	3-Hydroxy-3-methylglutaric acidemia	C4DC+C5OH		3-hydroxy-3-methylglutaric acid	HMGCL
	3-Methylcrotonyl-CoA carboxylase deficiency	C4DC+C5OH		3-methylcrotonylglycine, 3-hydroxyisovaleric acid	MCCA, MCCB
	Holocarboxylase synthetase deficiency	C4DC+C5OH		3-methylcrotonylglycine, 3-hydroxypropionic acid, methylcitric acid, pyruvic acid	HLCS
	ß-Ketothiolase deficiency	C4OH, C5OH,C5:1		2-methyl-3-hydroxybutyric acid, Methylcrotonylglycine, 3-Hydroxybutyric acid	ACAT1

Table 1. *(Continued)*

	Disorder(s)	Screening strategies			
		First-tier test marker(s)	Second-tier test marker(s)	GC-MS test marker(s)	Genetic test marker(s)
Fatty acid oxidation disorders	Primary carnitine deficiency	C0			SLC22A5
	Long-chain 3-hydroxyacyl-CoA dehydrogenase deficiency	C14OH, C16OH, C18OH		dicarboxylic acid	HADHA
	Very long chain acyl-CoA dehydrogenase deficiency	C14:1		dicarboxylic acid	ACADVL
	Medium chain acyl-CoA dehydrogenase deficiency	C6, C8, C10, C8/C8:1, C8/C10		dicarboxylic acid	ACADM
Endocrine disorder	Congenital adrenal hyperplasia		17-hydroxyprogesterone, androstenedione, 11-deoxycortisol, 21-deoxycortisol, cortisol		CYP21A2
Other disorders	Mucopolysaccharidosis-I	α-iduronidase activity	glycosaminoglycans		IDUA
	Mucopolysaccharidosis-II	iduronate-2-sulfatase activity	glycosaminoglycans		IDS
	Krabbe disease	galactosylcerebrosidase activity	psychosine		GALC
	Pompe disease	acid α-glucosidase activity			GAA
	Fabry disease	α-galactosidase A activity	globotriaosylsphingosine		GLA
	Niemann-Pick A/B	acid sphingomyelinase activity	lysosphingomyelin, N-palmitoyl-ophosphocholineserine (lyso-SM-509		SMPD1
	Gaucher	β-glucocerebrosidase activity	glucosylsphingosine		GBA
	X-linked adrenoleukodystrophy	C26-lysophosphatidylcholine	C26-lysophosphatidylcholine		ABCD1
	Neuronal ceroid lipofuscinosis 2	tripeptidyl protease 1 activity			TPP1
	Cerebrotendinous xanthomatosis	cholestanetetrol glucuronide	7-α-hydroxy-4- cholesten-3-one;7-α,12 α-dihydroxycholest-4-en-3-one		CYP27A1
	Metachromatic leukodystrophy	C16:0-sulfatide	arylsulfatase A activity		ARSA
Advantages		High sensitivity, specificity, throughput, broad spectrum, rapid detection, minimal sample requirement, and relatively low cost.	High specificity and sensitivity allow for precise quantification of initially positive samples, effectively ruling out false positives, differentiating disease subtypes, and guiding clinical decisions.	Enables broad-spectrum qualitative analysis and is the gold standard for urine organic acid testing. It can non-targetedly screen and identify hundreds of metabolites, which is key to diagnosing organic acidemias and detecting novel metabolites.	It offers high specificity and a proactive approach, providing molecular-level confirmation, enabling pre-symptomatic screening, and facilitating targeted management for certain conditions.
Limitations		The issue of false positives, incomplete coverage of the disease spectrum, the limited diagnostic validity of initial screening results.	This targeted biochemical method is limited to known biomarkers. For clinically heterogeneous or atypical cases, definitive diagnosis may require supplementary molecular techniques like genetic sequencing. It also demands stringent instrumentation, laboratory controls, and expert interpretation.	The complex sample preparation (requiring derivatization), low analytical throughput, and long reporting cycle.	Complexity in result interpretation, inability to fully predict the clinical phenotype, relatively high cost, and the associated challenges of genetic counseling and psychosocial ethics.

acylcarnitines—essential components and intermediates in fatty acid metabolism—demonstrate characteristic alterations in affected individuals. Quantitative MS/MS analysis of fatty acids, free carnitine, and acylcarnitines facilitates early disease identification, significantly enhancing clinical outcomes through timely therapeutic intervention.

MS/MS Data interpretation begins with instrument-specific software analysis to quantify target metabolite concentrations. This is followed by metabolic profiling that evaluates patterns of amino acids and acylcarnitines to differentiate between normal and aberrant metabolic states. Subsequent disorder-specific pattern recognition, based on pathognomonic metabolic signatures, leads to definitive positive or negative screening classifications. Crucially, this interpretive process requires an integrated assessment that incorporates demographic variables, analytical methodologies, and corroborating clinical findings.

Application of tandem mass spectrometry in screening for lysosomal storage disease in newborns. Lysosomal storage disorders (LSDs) are a group of inherited metabolic diseases caused by deficiencies in lysosomal enzymes, resulting in the accumulation of undigested or partially metabolized substrates within lysosomes.[18,19] These disorders are characterized by their incurable nature and clinical heterogeneity, affecting multiple organ systems such as the skeleton, skin, heart, spleen, and central nervous system.[19] Based on the nature of accumulated substrates and associated pathogenic genes, LSDs are classified into six major categories: (1) mucopolysaccharidoses, (2) sphingolipidoses, (3) oligosaccharidoses, (4) neuronal ceroid lipofuscinoses, (5) lysosomal membrane transport defects, and (6) other lysosomal disorders.[20,21] The combined estimated incidence of LSDs ranges between 1 in 4,000 and 1 in 9,000 live births.[22] Early diagnosis and timely intervention are associated with improved clinical outcomes, making NBS for LSDs a crucial initial step. Current NBS strategies for LSDs generally involve the assessment of lysosomal enzyme activity, detection of relevant biomarkers, and analysis of genes encoding lysosomal proteins.

The measurement of lysosomal enzyme activities in DBS samples serves as a key approach for the early screening of LSDs, primarily through fluorometric assays and MS/MS.[23-27] However, fluorometric methods are generally limited to measuring one enzyme at a time, allowing screening for only a single disorder, which hinders their applicability to broader, multi-disease LSD screening panels. In contrast, MS/MS allows the simultaneous quantification of six lysosomal enzyme activities—acid-β-glucocerebrosidase (ABG), acid sphingomyelinase (ASM), galactosylceramidase (GALC), α-L-iduronidase (IDUA), α-galactosidase (GLA), and acid α-glucosidase (GAA)—from a single DBS sample. This capability supports large-scale screening for Gaucher disease, Niemann–Pick disease types A/B, Krabbe disease, mucopolysaccharidosis type I, Fabry disease, and Pompe disease.[27-29] The method enables multiplex enzyme detection without cross-reactivity, providing high specificity and rapid analysis, and is currently considered the preferred technique for LSD newborn screening. For sample preparation, the MS/MS assay involves incubating six enzyme-specific substrates along with internal standards in extracts from 3.2 mm DBS punches. The mixtures are subsequently purified via solid-phase extraction or liquid–liquid extraction, dried under a nitrogen stream, and reconstituted for instrumental analysis.[27-29] This approach omits chromatographic separation; samples are introduced directly into the mass spectrometer, where qualitative and quantitative data are obtained based on the mass-to-charge ratios of target ions. NBS for LSDs was initiated in 2003, when Italy launched a screening program for Fabry disease involving 37,000 newborns.[30] Subsequently, the U.S. Public Health Service approved the inclusion of glycogen storage disease type II (Pompe disease), mucopolysaccharidosis type I, and mucopolysaccharidosis type II into the Recommended Uniform Screening Panel (RUSP). Pilot screening programs have also been implemented in several other countries, including Japan,[31-33] Brazil,[34] China,[27] and Hungary.[35]

Samples exhibiting enzyme activity below the established cut-off value are classified as positive in the initial screening and undergo repeat testing using the original blood sample.[26,27] If enzyme activity remains below the threshold upon retest, the newborn is recalled for further confirmatory evaluation, including repeated enzyme activity assays, biomarker analysis, genetic testing, and clinical assessment to establish a diagnosis.[27] Appropriate cut-off setting is critical for accurate screening. Although lysosomal enzyme activity in DBS remains relatively stable, it is still subject to significant variation due to factors such as storage time, seasonal effects, batch-to-batch experimental differences, and population-specific characteristics. To mitigate these influences and improve inter-laboratory comparability, the American Association of Public Health Laboratories has recommended using multiples of the daily median enzyme activity as the cut-off value for LSD screening.[26] This approach minimizes the impact of environmental and procedural variations and facilitates consistent result interpretation across different testing platforms. In practice, most laboratories adopt a cut-off value set at 15% to 20% of the median enzyme activity observed within the same batch of specimens.[20,23,27]

Biomarkers play a valuable role in confirming or excluding positive cases identified during initial screening. Using the original DBS samples, disease-specific biomarkers are quantified via MS/MS for the following disorders: Niemann–Pick disease types A/B (sphingomyelin), Fabry disease (globotriaosylsphingosine), Gaucher disease (glucosylsphingosine), globoid cell leukodystrophy (psychosine), and mucopolysaccharidosis type I (dermatan sulfate and heparan sulfate).[36] For Pompe disease, no reliable DBS biomarker is currently available; in such cases, urine glucose tetrasaccharide may be analyzed as an alternative reference marker upon patient recall.[37] In samples with low enzyme activity, biomarker testing performed prior to recall serves as an effective second-tier screening step, helping to reduce false-positive rates and enhance positive predictive value. Furthermore, the combination of enzyme activity measurements with biomarker profiling, genetic testing, and clinical evaluation aids in confirming clinical subtypes in pediatric cases and improves the detection rate of late-onset forms through screening.

Application of tandem mass spectrometry in screening for peroxisomal disorders in newborns. Peroxisomal disorders comprise a group of rare inherited conditions resulting from genetic defects that impair peroxisomal functions, classified within the broader category of inborn errors of peroxisomal metabolism, often involving abnormalities of very long-chain fatty acid (VLCFA) metabolism. To date, at least 15 distinct types have been identified, with an overall estimated incidence of approximately 1 in 5,000, though prevalence varies across ethnic populations. With the exception of X-linked adrenoleukodystrophy (X-ALD), all peroxisomal disorders follow an autosomal recessive pattern of inheritance. Although these disorders are rare, advances in MS/MS and molecular diagnostics have significantly enhanced their detection. The quantification of VLCFAs in blood and urine, combined with next-generation sequencing and correlation with clinical and imaging findings, now allows accurate diagnosis in a growing number of cases. Genetic counseling further aids in reducing recurrence risks in high-risk families. Globally, the application of MS/MS and molecular diagnostic techniques in population-wide newborn screening is gaining momentum.

X-ALD represents the most prevalent peroxisomal disorder, caused by mutations in the X-chromosomal gene encoding ATP-binding cassette subfamily D member 1 (ABCD1). The incidence in males is approximately 1 in 42,000, while the carrier rate in females is about 1 in 28,000. These genetic defects result in structural and functional impairments of the peroxisomal membrane protein, disrupting the active transport of saturated VLCFAs into peroxisomes for β-oxidation. Consequently, VLCFAs accumulate abnormally in various cells, tissues, and body fluids—particularly within the central nervous system, adrenal glands, and retina. This pathological accumulation leads to progressive demyelination of the central nervous system and adrenal insufficiency. The clinical presentation and age of onset vary across different forms of X-ALD, and the disease phenotype cannot be reliably predicted based on VLCFA levels, ABCD1 mutation profile, or family history. However, early diagnosis prior to symptom onset—coupled with dietary interventions, steroid replacement therapy, and hematopoietic stem cell transplantation—can help prevent intellectual, cognitive, and motor developmental delays, as well as adrenal cortical dysfunction in pediatric patients.[38] The critical question remains: how can we effectively utilize this narrow "window of opportunity" to initiate timely treatment? NBS provides access to this essential and limited therapeutic window.[39] In February 2016, the ACMG evaluated and added X-ALD to the core conditions list of the RUSP.[40]

The diagnosis of X-ALD is based on a combination of clinical manifestations, biochemical marker assays, imaging studies, and genetic mutation analysis. VLCFAs serve as crucial biochemical indicators for the disease.[41] GC-

MS and LC-MS/MS are the primary laboratory techniques used to quantitatively analyze fatty acids—such as behenic acid (C22:0), tetracosanoic acid (C24:0), and hexacosanoic acid (C26:0)—in serum or plasma. Elevated levels of C24:0, C26:0, and the ratios C24:0/C22:0 and C26:0/C22:0 support diagnosis, with persistently high C26:0 being a key diagnostic indicator. However, sample preparation requires 3–4 hours, and each sample takes over 30 minutes for mass spectrometry analysis, leading to low throughput and rendering the method unsuitable for large-scale population screening. Additionally, measuring serum VLCFAs alone fails to detect approximately 15% of female carriers, resulting in false negatives.[42] Non-fasting blood collection, hemolysis, and ketogenic diets may also cause false-positive outcomes. Moreover, VLCFAs levels can be elevated in healthy newborns, further increasing the risk of false-positives. In 2006, Hubbard et al.[43] reported that C26:0 lysophosphatidylcholine (C26:0-LPC) in DBS from newborn males could serve as a biomarker for diagnosing X-ALD and other peroxisomal disorders. More recently, in 2020, Jasper et al.[44] demonstrated that C26:0-LPC levels in DBS and plasma—measured via high-performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS)—were elevated in all affected males and over 99% of females, even when VLCFA levels were normal. This indicates that C26:0-LPC is a more reliable biochemical marker for X-ALD diagnosis than VLCFAs profiling.

MS/MS enables the detection of very long-chain acylcarnitines (VLCACs) and lysophosphatidylcholines (LPCs) in DBS, serving as effective screening markers for X-ALD. The more sensitive LPCs, particularly C26:0-LPC, have been widely adopted as the core biochemical marker for X-ALD newborn screening. To improve assay reliability, the ratios between different LPC species can be established to minimize pre-analytical variations caused by diet, physiological status, or uneven blood distribution in DBS specimens. Infants identified through NBS—whether males with X-ALD or female carriers—are typically asymptomatic at diagnosis and require ongoing follow-up monitoring and dietary guidance. In addition to X-ALD, NBS using LPC profiling can also detect Zellweger syndrome (ZS), a severe peroxisomal biogenesis disorder with an estimated incidence ranging from approximately 1 in 25,000 to 1 in 500,000 worldwide. Although no curative treatment currently exists for ZS, a definitive diagnosis enables genetic counseling and prenatal diagnosis, thereby helping to reduce the risk of recurrence in future pregnancies.

Tandem mass spectrometry-based second-tier screening for inherited metabolic diseases in newborns

In order to enhance clinical sensitivity, primary NBS often compromises specificity to some extent, which tends to increase the rate of false-positive results. The implementation of second-tier screening can effectively reduce false-positives from the initial screening round, thereby significantly improving clinical specificity.[12] Since this approach does not require recall or additional sample collection, it substantially shortens the overall screening timeline and helps lower the public healthcare burden.[45,46] Owing to its high analytical sensitivity and specificity, as well as its broad analyte compatibility—including both non-targeted and targeted multi-analyte detection—the LC-MS/MS platform is widely utilized in second-tier screening. It not only facilitates effective separation of target analytes from interfering substances and various isomers via (U)HPLC, but also enables simultaneous detection of multiple disease-related biomarkers using MS/MS.[47] The next section will take Congenital Adrenal Hyperplasia (CAH) as an example to introduce the second-tier screening program based on the LC-MS/MS platform.

Second-tier screening for congenital adrenal hyperplasia. Congenital adrenal hyperplasia encompasses a group of autosomal recessive disorders caused by mutations in genes encoding enzymes essential for steroid hormone synthesis. To date, mutations in seven key genes have been implicated in CAH, including those encoding 21-hydroxylase (CYP21A2), 11β-hydroxylase (CYP11B1), type II 3β-hydroxysteroid dehydrogenase (HSD3B2), 17α-hydroxylase/17,20-lyase (CYP17A1), cholesterol side-chain cleavage enzyme (CYP11A1), cytochrome P450 oxidoreductase (POR), and steroidogenic acute regulatory protein (StAR).[48] A congenital deficiency in any of these enzymes impairs cortisol biosynthesis, triggering a compensatory increase in the secretion of corticotropin-releasing hormone (CRH) and adrenocorticotropic hormone (ACTH). This hormonal imbalance subsequently leads to adrenal cortical dysfunction and abnormalities in sexual development.[49,50]

21-hydroxylase deficiency (21-OHD), caused by mutations in the CYP21A2 gene, is the most common type, accounting for 90% to 95% of CAH cases. This mutation impairs the synthesis of 11-deoxycortisol and deoxycorticosterone (DOC) from 17α-hydroxyprogesterone (17-OHP) and progesterone, resulting in insufficient secretion of cortisol and aldosterone, accumulation of 17-OHP and progesterone, and hyperandrogenemia. Based on clinical manifestations, 21-OHD is divided into classic (salt-wasting type, 75%; simple virilizing type, 25%) and non-classic types.[48,51,52]

To enable early identification and treatment of CAH and thereby reduce associated morbidity and mortality, many countries have incorporated CAH into their NBS programs. The concentration of 17-OHP is measured by immunoassay and interpreted against a predetermined positive cut-off value. Since premature and low-birth-weight infants typically exhibit significantly higher 17-OHP levels than full-term newborns,[53] establishing an appropriate 17-OHP cut-off value is critical for effective CAH screening. This value is often stratified based on gestational age and birth weight to improve accuracy.[53,54] When the initial 17-OHP result exceeds the cut-off, a recall is initiated for repeat testing. A persistently positive retest necessitates further biochemical and genetic evaluation. To minimize the risk of missing true cases, the initial screening strategy typically employs a low 17-OHP cut-off value. However, this approach inevitably increases the false-positive rate and reduces the positive predictive value of the screening test.

To reduce recall rates and lower follow-up diagnostic costs, LC-MS/MS is recommended as a second-tier screening method for CAH. This technique enables the simultaneous quantification of multiple steroids in DBS, including 17-OHP, androstenedione, 11-deoxycortisol, 21-deoxycortisol, and cortisol.[53,55,56] Diagnostic accuracy is further enhanced by calculating enzyme reaction substrate-to-product ratios, such as (17-OHP + androstenedione)/cortisol and (17-OHP + 21-deoxycortisol)/cortisol.[53,55] The LC-MS/MS-based approach significantly improves the positive predictive value of screening. Moreover, it facilitates the differential diagnosis of CAH subtypes.

Application of gas chromatography-mass spectrometry in the detection for IMDs in newborns. Gas chromatography-mass spectrometry (GC-MS) is primarily employed for diagnosing organic acidemias in NBS, in addition to confirming certain amino acidopathies and disorders of carbohydrate metabolism. Organic acidemias are a group of metabolic disorders primarily caused by pathogenic mutations in genes encoding specific metabolic enzymes. These mutations lead to impaired enzyme function and the subsequent accumulation of organic acids and their metabolites. Such accumulation can induce metabolic acidosis and result in multi-organ damage, particularly affecting the brain, liver, kidneys, heart, and bone marrow. The clinical onset of organic acidemias may occur at any stage from fetal development to adulthood. Clinical manifestations are highly heterogeneous: some patients present acutely with symptoms such as hypoglycemia, coma, vomiting, and metabolic acidosis, while others exhibit progressive neurological deterioration. Without timely diagnosis and intervention, mortality is markedly increased, and survivors often suffer from severe neurological sequelae. The impact of a mutation on enzymatic function depends on its location and nature. Mutations causing only mild impairment of enzyme activity are generally associated with attenuated clinical symptoms, whereas those that substantially disrupt function often lead to severe disease manifestations. Consequently, even among patients with the same type of organic acidemia, there can be considerable variability in age of onset and disease severity, posing significant challenges for accurate diagnosis and effective clinical management.

Currently, NBS for multiple IMDs relies predominantly on LC-MS/MS technology.[57] However, this approach has inherent limitations: an abnormality in a single biomarker—whether elevated or reduced—often indicates several possible IMDs, complicating definitive diagnosis. In contrast, GC-MS enables quantitative analysis of over 130 organic acids in a single urine assay. This comprehensive profiling capability makes it a valuable complementary tool to MS/MS-based screening, effectively aiding clinicians in the differential diagnosis of IMDs and guiding the development of individualized treatment strategies. Since its initial application by Tanaka in 1966 for identifying organic acidemias, GC-MS has been progressively adopted worldwide for

urinary organic acid detection. To date, more than 250 disease-associated abnormal organic acid markers have been identified, and over 30 distinct types of organic acidemias have been characterized. Although the incidence of any single disorder is relatively low—ranging from approximately 1 in 10,000 to 1 in 100,000—the collective prevalence is considerable due to the number of distinct conditions involved. Common types include methylmalonic acidemia, glutaric acidemia type I, maple syrup urine disease (MSUD), 3-methylcrotonyl-CoA carboxylase deficiency, and propionic acidemia (Table 1).

Currently, the use of GC-MS for detecting urinary organic acids and diagnosing organic acidemias based on characteristic metabolite profiles has become a well-established clinical procedure. This methodology demonstrates considerable promise and substantial clinical utility, particularly in the analysis of organic acids in neonatal urine. With continuous technological innovation and refinement, GC-MS is expected to offer increasingly robust support for the screening and diagnosis of IMDs, ultimately contributing to improved patient outcomes.

Future research directions and challenges

NBS programs are expected to be increasingly implemented in the coming years as the technology and options for therapies increase. Public health policy development must keep pace with scientific progress. The ultimate goal of NBS and follow-up programs is to reduce the incidence and mortality of related disorders. Early diagnosis and intervention have significantly improved the lives of many children and their families.[58,59] However, NBS represents only the starting point of the healthcare intervention process. The identification of screen-positive infants must be extended to include subsequent confirmatory testing and systematic treatment.[60] In recent years, the widespread adoption of MS/MS and improvements in its detection sensitivity have significantly expanded the scope of NBS. This technology has enabled the inclusion of more biomarkers into the screening panel. At the same time, continuous advances in treatment approaches—such as enzyme replacement therapy and gene therapy—along with the emergence of new pharmacological treatments, have led to the gradual inclusion of additional conditions as potential candidates for NBS.

Despite its considerable advantages, MS/MS still cannot completely replace traditional screening methods. Its application in NBS faces several key challenges. First, the substantial initial investment and ongoing operational costs of MS/MS systems pose financial constraints for many screening programs. Second, test results can be influenced by multiple factors—such as gestational age, birth weight, postnatal age, maternal and fetal nutritional status, and medication exposure; therefore, establishing stratified cutoff values is necessary to control the false-positive rate. Furthermore, aspects such as the efficacy of new screening technologies, selection of diseases to include, strategies for rapid and accurate diagnosis, and systematic follow-up management of screen-positive cases still require further clarification and refinement.[61,62]

Integration of mass spectrometry with artificial intelligence tools. With the deepening understanding of IMDs and the continuous advancement of MS/MS technology, the screening spectrum based on MS/MS is expected to expand significantly, thereby covering a broader range of metabolic disorders. However, the extensive metabolite detection involved in MS/MS screening also has its limitations, particularly in the potentially severe consequences of false-positive and false-negative results.[63,64] Not only do these errors often subject families to unnecessary psychological stress and economic burden but may also delay critical treatment opportunities.

Driven by advances in science and technology, computational and machine learning (ML) methods provide promising approaches for analyzing high-dimensional data.[65-68] In recent years, ML technology has been gradually applied in the field of NBS, aiming to enhance the sensitivity and specificity of IMD diagnosis. However, most current studies focus on the analysis of single or limited categories of diseases.[69] For example, Zaunseder et al. used ML techniques to optimize the specificity of isovaleric acidemia (IVA) detection in newborn screening;[70] Zhu et al. developed an ML logistic regression model to improve the diagnostic accuracy for phenylketonuria;[71] and Peng et al. conducted an in-depth evaluation of the performance of the Random Forest algorithm in diagnosing glutaric acidemia type I, methylmalonic acidemia, ornithine transcarbamylase deficiency, and very long-chain acyl-CoA dehydrogenase deficiency.[72]

In contrast, Haibo Li et al. recently adopted a holistic strategy, integrating nine different algorithms to construct an ML system model capable of extensively predicting and analyzing 31 different IMDs.[73] This model significantly reduced the screening positive rate from 1.17% to 0.33%, greatly minimizing unnecessary patient recalls, while demonstrating superior diagnostic performance: achieving a sensitivity of 93.42% and a specificity of 78.57%, identifying 142 true positive cases, outperforming traditional cutoff methods. However, in the continuous pursuit of improving diagnostic accuracy and efficiency, it is essential to acknowledge false negatives as a key issue inherent to any diagnostic system. To further investigate the reasons, researchers conducted a comprehensive analysis of the genetic test results and metabolite concentrations of the missed cases.[73] They found that the model had a higher tendency for misclassification of heterozygous carriers, revealing the complexity of accurately identifying carriers within the diagnostic framework. To address this challenge, further research is urgently needed to optimize ML algorithms, with a focus on enhancing their ability to accurately identify carriers. Additionally, some IMDs may not exhibit significant metabolite changes in MS/MS screening during the early stages, leading to false negative results. As such cases accumulate, the capability of ML systems to identify asymptomatic cases is expected to become increasingly prominent.

It is worth noting that the performance of ML algorithms typically benefits from large-scale datasets; theoretically, larger datasets lead to stronger predictive power. With the continuous advancement of related research and data accumulation, the application and refinement of ML algorithms in the field of NBS are expected to become increasingly widespread, thereby further improving the predictive accuracy and computational efficiency of IMD risk assessment models.

Integration of mass spectrometry with genomic technologies. Currently, the application of DNA sequencing in NBS is largely confined to second-tier testing. With the declining cost of DNA sequencing, improved ability to interpret genomic data, and advances in therapeutic approaches, whether and how to incorporate first-tier targeted sequencing, exome sequencing, or genome sequencing into NBS to expand screening for diseases lacking biomarkers has become an important topic.[74,75] The use of genome sequencing for NBS also raises numerous concerns regarding acceptability, equity, and scalability, with anticipated challenges including potential adverse psychosocial impacts, testing costs, limitations in specialized human resources, patient privacy concerns, and difficulties in variant interpretation across diverse ancestral groups.[76] To date, several studies involving cohorts of over 100 children have evaluated the use of sequencing for NBS.[74,77-84] While these studies support the potential of genome sequencing as a supplement to traditional NBS, most are limited in sample size, disease coverage, or ancestral diversity of the populations studied. Discussions continue regarding the costs, turnaround time, feasibility within public health systems, and how to ensure high sensitivity and specificity compared to biochemical screening when implementing genome sequencing within the NBS framework. Furthermore, universal genome sequencing for NBS may raise public perception concerns. Therefore, NBS programs need to develop specific policies to maintain public trust by strictly protecting the confidentiality of genomic data.

A large-scale study named "Genomic Uniform-screening Against Rare Disease in All Newborns" demonstrated the feasibility of targeted interpretation of a predefined gene set via genome sequencing in a newborn population of diverse racial and ethnic backgrounds.[85] This study, based on large-scale population data, provides important insights for evaluating the scalability of targeted genome sequencing in NBS. This scalability depends on the number of infants screened, the scope of genes included, variant reporting criteria, and the degree of automation in the analytical pipeline. Notably, targeted genome sequencing yields a higher positive rate than traditional screening, necessitating significant resources for follow-up confirmatory testing and clinical management. To achieve large-scale newborn screening, further automation of the process is essential. There is an urgent need to establish scalable and cost-effective solutions covering DNA extraction, library preparation, sequencing, accurate variant interpretation, and follow-up for screen-positive newborns. Promoting regional collaboration in testing and

analysis can help alleviate the infrastructure, personnel, and funding pressures associated with implementing first-tier genetic analysis in NBS projects, while reducing reporting time will also be a key breakthrough.

DNA sequencing provides an important complementary tool for NBS: it can enhance screening efficacy for already-covered diseases (such as Severe Combined Immunodeficiency) and extend screening to treatable diseases that are difficult to detect due to the lack of biomarkers in dried blood spots. It is important to note that biochemical testing helps reduce false-positive and false-negative results generated by next-generation sequencing-based NBS. However, for highly time-sensitive conditions where treatment must be initiated or planned within the first two weeks of life, the longer turnaround time required for sequencing alone must be significantly reduced. Therefore, for the foreseeable future, MS/MS is likely to remain the preferred analytical platform for effective and efficient expansion of NBS. Even if next-generation sequencing is used for first-tier screening of some or many conditions, biochemical testing based on MS/MS remains indispensable for interpreting positive results associated with variants of uncertain significance.

Furthermore, integrating MS/MS metabolomic data with genome sequencing data for comprehensive analysis can not only drive targeted genetic analysis through metabolic phenotypes and accelerate the determination of pathogenicity for variants of uncertain significance but also support the construction of a "metabolism-genetics" dual-dimensional decision tree in clinical practice, providing evidence-based support for precise diagnosis and personalized management. Such technological synergy holds the potential to advance newborn screening from "index detection" to "mechanistic analysis," driving the newborn disease prevention and control system toward predictive, preventive, and personalized development in a leapfrog manner.

MS/MS technology is being applied rapidly and extensively in NBS in China. Efforts are currently underway to establish national technical standards for MS/MS screening and to develop unified clinical management guidelines for diagnosed conditions. Future efforts will focus on expanding the screened disease portfolio and implementing second-tier testing through the incorporation of more specific biomarkers, to reduce false-positive rates and increase positive predictive value.

CONCLUSION

MS/MS has emerged as a transformative tool and the gold standard analytical platform in NBS for IMDs, owing to its unique technical advantages. It significantly improves screening efficiency and facilitates early detection, diagnosis, and intervention for a range of disorders, thereby effectively reducing neonatal mortality and disability rates. Beyond its application in NBS, MS/MS also demonstrates potential for prenatal diagnosis through the analysis of amino acids and acylcarnitines in amniotic fluid. By enabling timely management of these disorders, MS/MS also helps alleviate the economic and emotional burden on affected families and broader society. Although challenges such as high equipment costs, technical complexity, and lack of standardization persists, continuous technological advancements and expanding applications are expected to further establish MS/MS as a cornerstone in the prevention and control of IMDs. Its ongoing integration into healthcare systems will substantially contribute to improving population health outcomes and strengthening public health infrastructure.

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AUTHOR CONTRIBUTIONS

All authors contributed to and approved the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

DATA AND CODE AVAILABILITY

No new data were created or analyzed in this study.