Review
doi.org/10.1002/ansa.202100010
Chemistry Europe
European Chemical Societies Publishing

Received: 6 February 2021
Revised: 5 April 2021
Accepted: 8 May 2021

Applications of quantitative metabolomics to revolutionize early diagnosis of inborn errors of metabolism in India

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Abstract

Inborn errors of metabolism (IEMs) are a group of disorders caused by disruption of metabolic pathways, which leads to accumulation, decreased circulating levels, or increased excretion of metabolites as a consequence of the underlying genetic defects. These heterogeneous groups of disorders cause significant neonatal and infant mortality across the whole world and it is of utmost concern for developing countries like India owing to lack of awareness and standard preventive strategies like newborn screening (NBS). Though the predictive cumulative incidence of IEMs is said to be ~1:800 newborns, data pertaining to the true prevalence of individual IEMs is not available in the context of Indian population. There is a need for a large population-based study to get a clear picture of the prevalence of different IEMs. One of the best ways to screen for IEMs is by applying advanced liquid chromatography-mass spectrometry (LC-MS) technology using a quantitative metabolomics approaches such as selected or multiple reaction monitoring (SRM or MRM). Recent developments in LC-MS/MRM based quantification of marker metabolites in newborns have opened a novel opportunity to screen multiple disorders simultaneously from a minuscule volume of biological fluids. In this review article, we have highlighted how LC-MS/MRM based metabolomics approach with its high sensitivity and diagnostic capability can make an impact on the nation's public health through NBS programs.

KEYWORDS
inborn errors of metabolism, liquid chromatography-mass spectrometry, metabolomics, newborn screening, quantification

1 | INTRODUCTION

Birth without any defects is a precious gift from nature. Ensuring healthy life, which starts from preconception to prenatal care, and through all the developmental stages of childhood and adolescence, should be of supreme importance for better future on this globe. Over the years, the drastic environmental perturbations like climate change, stratospheric ozone depletion, changes in ecosystems due to loss of biodiversity, changes in hydrological systems and the supplies of freshwater, land degradation, environmental pollution, urbanization, stresses on food-producing systems (food contaminants and adulterants), and so on have led to an increased rate of birth defects.[1] Further, factors like consanguineous marriages and high birth rate are well known to lead to an increase in inborn errors of metabolism (IEMs) among the newborns worldwide.[2,3] IEMs constitute a heterogeneous group of genetic disorders resulting from the absence

or dysfunction of an enzyme or its cofactor, leading to the accumulation or deficiency of the corresponding metabolites.[4] Ever since the realization of IEMs as manifestations of biochemical individuality by Garrod,[5] there is great progress in our understanding of these rare disorders. The list of IEMs has grown substantially in recent decades, amounting to ≥7000 individual conditions.[6] The cumulative incidence of the widely studied ~1000 disorders was estimated to be ~1:800.[7,8]

As a standard preventive strategy, newborn screening (NBS) programs have been instituted in developed countries like the USA, Canada, Australia, Japan, New Zealand, and a few European countries.[9–12] Under the NBS program, newborns have been mandatorily screened for the most prevalent IEMs based on the geographical area by collecting dried blood spots (DBS) within the initial 48-72 h of birth.[13] NBS is considered to be one of the most successful public health programs in the United States and represents a successful implementation of population genetic screening program. These programs have helped to improve the quality of life of thousands of children providing them with a disability-free life.[14] Recognizing the importance of NBS, other developing countries like China, Philippines, Taiwan, and Thailand have also adopted the screening programs and implemented them successfully.[11] The detailed report of worldwide NBS status has been shown in one of the review articles by Therrell et al., 2015.[9]

India, as a developing country, is going through a demographic transition from communicable to non-communicable diseases. As a consequence, there is an emerging threat of undiscovered genetic disorders and congenital malformations in the Indian population, associated with significant morbidity and mortality among children.[15,16] So, dilution of gene pool becomes crucial to avoid or reduce IEMs in future generations. A large population-based data on the IEMs and their prevalence representative of the pan-Indian scenario is unavailable. However, the published pilot studies for a few known disorders showed that these diseases are not rare in India. Hence, attention should be given for routine screening of newborns. A handful of academic/private referral labs in India focus on this problem by screening only a few known disorders. NBS is yet to be a public health priority in our country and only < 3% of the newborns undergo screening for 3 to 4 conditions [Glucose-6-phosphate dehydrogenase (G6PD) deficiency, Congenital Hypothyroidism (CH), and Congenital Adrenal Hyperplasia (CAH)]. This is mainly due to lack of awareness, lack of NBS facility throughout the country, the cost factor, and limited number of academic/private labs developing new methods for routine use.

The recent advancements in the bio-analytical field, especially liquid chromatography and mass spectrometry (LC-MS) showed a greater promise worldwide to develop disease-specific markers. Separation of biomolecules in the LC, converting those separated metabolites into ions (+ve or –ve) in the ion source followed by their detection in the MS-based on mass/charge (m/z) ratio has revolutionized in the field of biomarker discovery in the recent past. The LC-MS based quantification of metabolites with its capability to screen multiple analytes simultaneously to cover a wide range of disorders in a single method from a minuscule volume of biological fluids has become the benchmark tool in NBS.[17] Today, this LC-MS based quantitative metabolomics using selected or multiple reaction monitoring modes (SRM/MRM) is considered as the gold standard method for absolute quantification of bio-molecules and is also applied for the screening of a wide range of IEMs.[18,19] In case of SRM method, particular parent ion will be selected in the first quadrupole and it will be pushed to the second quadrupole for producing collision-induced product ions and one of the highest intense product ions will be selected in the third quadrupole for quantification. Whereas in the case of MRM, more than one product ions will be selected in the third quadrupole for quantification.[18,20] With increased awareness and knowledge about IEMs in India, the medical community has been asking the government for a mandatory NBS program. Even though it is recognized as essential in the healthcare system and mentioned in the national health policies, NBS is yet to achieve its full potential in India. Indeed, it is imperative to implement such an advanced LC-MS/MRM based expanded NBS (ENBS) program in India. In the present review, we have highlighted the important events in the IEM screening in developed countries, the challenges tackled by the developing countries in the Asia-Pacific region for implementing NBS, and have attempted to put forth the probable strategies to be adopted for the successful implementation of an NBS program in India in the near future.

1.1 | The important events in IEM/NBS research

In 1897, Sir Archibald Garrod's observation of black colored urine in a child set the foundation of research on IEMs.[21,22] Garrod found that the black color is due to alkapton, the oxidized form of homogentisic acid, excreted through urine as a result of deficiency of homogentisate 1,2-dioxygenase – an enzyme that splits the aromatic ring of homogentisic acid in the tyrosine metabolism.[23] It was revealed after further investigation that these are inherited disorders, which occur in infants born from consanguineous marriages stressing on the recessive Mendelian inheritance. Garrod suggested that there can be many such aberrations and he classified them under the new term "Inborn Errors of Metabolism." In 1934, Asbjörn Følling, a Norvegian biochemist cum physician, observed the presence of phenylpyruvic acid in the urine of two intellectually disabled siblings using the ferric chloride test, which showed green color upon reaction.[24,25] On screening several children with intellectual disability, the presence of phenylpyruvic acid in urine in this group became more evident. Later in 1937, Penrose illustrated the autosomal recessive nature of the disease and named it phenylketonuria (PKU).[26] In 1953, George Jervis, outlined the pathogenesis of PKU to be a consequence of either deficiency or absence of the enzyme phenylalanine hydroxylase.[27] The function of this enzyme is the conversion of phenylalanine to tyrosine and its absence/deficiency leads to the accumulation of phenylalanine and its derivatives. This leads to intellectual disability through various mechanisms such as myelin sheath abnormalities, disruption of amino acid transport across the blood-brain barrier, neurotransmitter deficiencies including reduced dopamine levels.[28]

The excess phenyl alanine is excreted as phenylpyruvate through urine.[27,29] The knowledge on the molecular mechanism of PKU led to the development of successful treatment strategies by Bickle et al.[30] They reported that administration of phenylalanine restricted diet significantly improved the mental condition of a 2-year-old over a few months.[30]

The screening of IEMs gained momentum after the development of simple bacterial inhibition assay-based screening test by Robert Guthrie in the 1960s that could detect high levels of phenylalanine in blood collected on a filter paper shortly after a baby was born.[31] The bacterial inhibition assay was carried out by growing the bacteria, *Bacillus subtilis* in a gel coated with β-2-thienylalanine and placing the DBS samples onto that gel. The amino acid β-2-thienylalanine inhibits the growth of *B. subtilis* while phenylalanine reverses the inhibition. Hence if the blood sample contained phenylalanine, there would be bacterial growth surrounding the blood spot. It became a life-saving test for thousands of newborns in the USA as the early detection and administration of phenylalanine-restricted diet could help to prevent the intellectual disability. This diagnostic advancement led to the first United States NBS program for PKU.[7,32] When the concept of population-based screening programs came to limelight, the need for articulation of the criteria for the inclusion of a condition in the screening panel became apparent. In 1968, Wilson and Jungner came up with their "10-point principle" that became the basic criteria of public screening programs.[33,34] As a result of all these developments, the majority of states in the USA started offering screening for PKU by the end of the 1960s.[35] In the 1980s, gas chromatography coupled to mass spectrometry (GC-MS) was used to extend the initial IEM screening beyond bacterial inhibition assay tests.[36,37] The screening of diseases involving the organic acid metabolites as markers were amenable to GC-MS.[37] By 1984, John B Fenn invented electrospray ionization (ESI) which enabled the coupling of liquid chromatography to mass spectrometry[38] and the ultimate application of LC-MS for NBS came into picture.[39] Millington et al demonstrated that a large number of inborn errors of metabolism could be screened simultaneously using the simple and cost-effective LC-MS/MS method.[40] LC-MS/MS brought about a paradigm shift in clinical diagnosis by changing the one disorder-one test concept to one test for many disorders, with its ability to screen many disorders simultaneously. As a result, there was a massive expansion of testing panels and the number of conditions screened. The ENBS was started in the United States in the late 1990s and several classes of metabolites which can serve as markers for many disease conditions, such as amino acids, organic acids, and acylcarnitines were screened from DBS using LC-MS/MS with high sensitivity, specificity, and selectivity.[40–42] These technological advancements led to revisit the original Wilson and Jungner criteria to incorporate more disorders that can be screened.[43]

In order to achieve the goal of a unified NBS program throughout the country, the American College of Medical Genetics (ACMG) was entrusted to develop a uniform screening panel.[34] They came up with recommendations for inclusion of 29 core conditions (along with 25 secondary conditions) for universal NBS in 2005. In 2007, Advisory Committee on Heritable Disorders in Newborns and Children recommended these 29 core conditions to be included in the Recommended Uniform Screening Panel (RUSP).[34] The latest RUSP consists of 35 core conditions and 26 secondary conditions, out of which 45 can be screened using LC-MS/MS (Table 1). There are more conditions in the pipeline yet to be added in the near future. The important events in the history of IEM screening are shown in Figure 1.

1.2 | IEM screening and NBS- International status

After the establishment of PKU screening in the United States, Guthrie had traveled worldwide extensively advocating for NBS as well as his blood spot procedure and to make other countries aware of its importance.[11] Canada, Australia, New Zealand, Japan, and a few countries in Europe took interest in his venture and started their NBS program in the mid- to late 1960s.[12,47–52] The details of the NBS programs started in different countries for different disorders and the transition period from one disorder-one assay to multi disorder-one assay using LC-MS/MS based screening is shown in Table 2.

The developed countries that started NBS in the early 1960s, covered almost 100% for few disorders as shown in Table 2. It took more than 30 years for those countries to achieve a high percentage of coverage for certain disorders. Some of the developing countries like China, Philippines, and Thailand also showed remarkable achievements in the overall coverage in NBS for selected disorders.[11] All those countries are now moving towards the ENBS by using the advance LC-MS/MS based technology to cover more number of disorders from DBS compared to the previous screening programs. India started an NBS program in the early 1980s,[60] however, it has not progressed to the same extent as in other Asian countries. Our neighboring and more populous nation, China, has achieved great progress in NBS with an overall coverage of > 96% (mainly for CH and PKU) as per 2017 and is now ready to expand the NBS panel.[11] Even though the birthrate of India and Philippines are similar, the coverage for NBS is >75% in Philippines[11] whereas in India it is still <3%.[61] It is important to note what China and Philippines have achieved despite their hurdles as developing countries. It is an inspiration for a country like India and is worth a special mention in the present review.

NBS is a public health program in China leading to the prevention of intellectual disability and serious developmental delay in tens of thousands of newborns. They started their first screening program in Shanghai, in 1981, with assistance from Robert Guthrie (USA) and Hiroshi Naruse (Japan).[11] It was followed by extended pilot projects in seven major cities sponsored by WHO and Ministry of Public Health during 1992–1993.[62] The Law of the People's Republic of China on Maternal and Infant Healthcare, enforced in 1994, mandated that the provincial healthcare departments should start services like NBS, to ensure the welfare of the neonates. As a consequence, CH and PKU screening became mandatory throughout China.[63] Shanghai, Beijing, Tianjin, and Guangzhou provinces systematically progressed in screening and increased their coverage to 80–90% by 1996 for these two disorders.[11] In addition, some provinces offer optional screening for galactosemia

doi.org/10.1002/ansa.202100010

TABLE 1 The core and secondary conditions in the Recommended Uniform Screening Panel (RUSP) with corresponding metabolite markers and ranges

	Metabolic Disorders		
	Organic Acid Conditions	**Fatty Acid Oxidation Disorders**	**Amino Acid Disorders**
Primary Conditions	Propionic Acidemia (PROP)* **C3**↑ (4.7-5.5)	Carnitine Uptake Defect/Carnitine Transport Defect (CUD)* **C0**↓ (7.5-12)	Argininosuccinic Aciduria (ASA)* **Cit**↑ (28-40), **Asa**↑ (0.66-0.90)
	Methylmalonic Acidemia (methylmalonyl-CoA mutase) (MUT)* **C3**↑ (4.7-5.5)	Medium-chain Acyl-CoA Dehydrogenase Deficiency (MCAD)* **C2**↓ (7.0-10), **C8**↑ (0.21-0.71), **C6**↑ (0.18-0.24), **C10**↑ (0.26-0.30), **C10:1**↑ (0.17-0.25)	Citrullinemia, Type I (CIT)* **Cit**↑ (28-40), **Cit/Arg**↑ (4.9-6.0)
	Methylmalonic Acidemia (Cobalamin disorders) (Cbl-A, B)* **C3**↑ (4.7-5.5)	Very Long-chain Acyl-CoA Dehydrogenase Deficiency (VLCAD)* **C14**↑ (0.50-0.80), **C14:1**↑ (0.37-0.71), **C14:2**↑ (0.09-0.15)	Maple Syrup Urine Disease (MSUD)* **Xle**↑ (235-260), **Val**↑ (180-220)
	Isovaleric Acidemia (IVA)* **C5**↑ (0.39-0.47)	Long-chain L-3 Hydroxyacyl-CoA Dehydrogenase Deficiency (LCHAD)* **C16-OH**↑ (0.08-0.19), **C18:1-OH**↑ (0.07-0.08)	Homocystinuria (HCY)* **Met**↑ (44-48)
	3-Methylcrotonyl-CoA Carboxylase Deficiency (3-MCC)* **C5-OH**↑ (0.45-0.69)	Trifunctional Protein Deficiency (TFP)* **C16-OH**↑ (0.08-0.19), **C18:1-OH**↑ (0.07-0.08)	Classic Phenylketonuria (PKU)* **Phe**↑ (97-135)
	3-Hydroxy-3-Methyglutaric Aciduria (HMG)* **C5-OH**↑ (0.45-0.69), **C6-DC**↑ (0.10-0.12)		Tyrosinemia, Type I (TYR-1)* **Suac**↑ (1.4-7.5), **Tyr**↑ (207-226)
	Holocarboxylase Synthase Deficiency (MCD)* **C5-OH**↑ (0.45-0.69), **C3**↑ (4.7-5.5)		
	β-Ketothiolase Deficiency (βKT)* **C5-OH**↑ (0.45-0.69), **C5:1**↑ (0.08-0.24)		
	Glutaric Acidemia Type I (GA-1)* **C5-DC**↑ (0.10-0.18)		
Secondary Conditions	Methylmalonic acidemia with homocystinuria (Cbl-C, D)* **C3**↑ (4.7-5.5)	Short-chain acyl-CoA dehydrogenase deficiency (SCHAD)* **C4**↑ (0.75-1.1)	Argininemia (ARG)* **Arg**↑ (32-40)
	Malonic acidemia (MAL)* **C3-DC**↑ (0.33-0.69)	Medium/short-chain L-3-hydroxyacyl-CoA dehydrogenase deficiency (M/SCHAD)* **C4-OH**↑ (0.33-0.69)	Citrullinemia, type II (CIT-II)* **Cit**↑ (28-40), **Cit/Arg**↑ (4.9-6.0)
	Isobutyrylglycinuria (IBG)* **C4**↑ (0.75-1.1)	Glutaric acidemia type II (GA2)* **C4**↑ (0.75-1.1), **C5**↑ (0.39-0.47), **C8**↑ (0.21-0.71), **C12**↑ (0.41-0.80), **C14**↑ (0.50-0.80), **C14:1**↑ (0.37-0.71), **C14:1/C16**↑ (1.3-1.8)	Hypermethioninemia (MET)* **Met**↑ (44-48)
	2-Methylbutyrylglycinuria (2MBG)* **C5**↑ (0.39-0.47), **C5/C0**↑ (0.017-0.031)	Medium-chain ketoacyl-CoA thiolase deficiency (MCAT)* **C12-16-DC**↑	Benign hyperphenylalaninemia (H-PHE)* **Phe**↑ (97-135), **Phe/Tyr**↑ (1.6-2.5)
	3-Methylglutaconic aciduria (3MGA)* **C5-OH**↑ (0.45-0.69), **C5-OH/C8**↑ (8.2-10)	2,4 Dienoyl-CoA reductase deficiency (DE-RED)* **C10:2**↑ (0.08-0.12)	Biopterin defect in cofactor biosynthesis (BIOPT-BS)* **Phe**↑ (97-135), **Phe/Tyr**↑ (1.6-2.5)
	2-Methyl-3-hydroxybutyric aciduria (2M3HBA)* **C5-OH**↑ (0.45-0.69)	Carnitine palmitoyltransferase type I deficiency (CPT-IA)* **C0**↑ (59-60), **C16**↓ (<0.80), **C18**↓ (<0.31)	Biopterin defect in cofactor regeneration (BIOPT-Reg)* **Phe**↑ (97-135), **Phe /Tyr**↑ (1.6-2.5)
		Carnitine palmitoyltransferase type II deficiency (CPT-II)* **C16**↓ (<0.80), **C18**↓ (<0.31), **C18:1**↓ (<0.49)	Tyrosinemia, type II (TYR-II)* **Tyr**↑ (207-226)
		Carnitine acylcarnitine translocase deficiency (CACT)* **C16**↓ (<0.80), **C18**↓ (<0.31), **C18:1**↓ (<0.49)	Tyrosinemia, type III (TYR-III)* **Tyr**↑ (207-226)

(Continues)

TABLE 1 (Continued)

	Endocrine Disorders	Haemoglobin Disorders	Other Disorders
Primary Conditions	Primary Congenital Hypothyroidism (CH)* **T4** ↓ (51-77 nmol/L)[44]	S, S Disease (Sickle Cell Anemia) (Hb SS)	Biotinidase Deficiency (BIOT)
	Congenital Adrenal Hyperplasia (CAH)* **17-OHP**↑ (54 nmol/L)[45]	S, β-Thalassemia (HB S/βTh)	Critical Congenital Heart Disease (CCHD)
		S, C Disease (Hb S/C)	Cystic Fibrosis (CF)
			Classic Galactosemia (GALT)
			Glycogen Storage Disease Type II (Pompe) (GSD II)
			Hearing Loss (HEAR)
			Severe Combined Immunodeficiencies (SCID)
			Mucopolysaccharidosis Type 1 (MPS I)
			X-linked Adrenoleukodystrophy (X-ALD)*, **C26:0-LPC** ↑ (>0.30)[46]
			Spinal Muscular Atrophy due to homozygous deletion of exon 7 in SMN1 (SMA)
Secondary Conditions		Various other hemoglobinopathies (Var Hb)	Galactose epimerase deficiency (GALE) Galactokinase deficiency (GALK) T-cell related lymphocyte deficiencies

Note:- Each entry is represented in the following format: Condition (Abbreviation), Marker (high/low cut-off range)
*The diseases screened by LC-MS/MS
The up and down arrows represent the increase and decrease of the corresponding metabolites from normal ranges. The cut-off ranges are given in μ**mol/L** unless stated otherwise; the upper cut off ranges were given for the regulated and the lower cut off ranges for the down regulated.
Ranges: The ranges in the table are from the R4 S project results (except for CH, CAH and X-ALD) where they have compiled data from around 46 countries and covered a sample size of 25–30 million babies. In the actual practice, it is advisable the laboratory establish the reference interval by analyzing the positive and negative cases in a population.
Abbreviations: C0, free carnitine; **C2**, acetylcarnitine; **C3**, propionylcarnitine, **C3-DC**, malonylcarnitine; **C4**, butyryl-/isobutyrylcarnitine; **C4-OH**, hydroxy butyrylcarnitine; **C5**, isovaleryl-/2-methylbutyrylcarnitine; **C5-OH**, hydroxy isovalerylcarnitine; **C5-DC**, glutarylcarnitine; **C6**, hexanoylcarnitine, **C6-OH**, hydroxy hexanoylcarnitine; **C6-DC**, methylglutarylcarnitine; **C8**, octanoylcarnitine; **C10**, decanoylcarnitine; **C10:1**, decenoylcarnitine; **C10:2**, decadienoyl-carnitine; **C12**, dodecanoylcarnitine; **C14**, tetradecanoylcarnitine; **C14:1**, tetradecenoylcarnitine; **C14:2**, tetradecadienoylcarnitine; **C16**, palmitoylcarnitine; **C16-OH**, hydroxy palmitoylcarnitine; **C18**, stearylcarnitine; **C18:1**, oleylcarnitine; **C18-OH**, hydroxy stearylcarnitine; **C26:0-LPC**, 1-hexacosanoyl-2-lyso-sn-3-glycero-phosphorylcholine; **T4**, thyroxine; **17-OHP**, 17-hydroxyprogesterone; **Arg**, arginine; **Asa**, argininosuccinic acid; **Cit**, citrulline; **Galt**, galactose; **Met**, methionine; **Phe**, phenylalanine; **Suac**, succinylacetone;**Tyr**, tyrosine; **Val**, valine; **Xle**, leucine/isoleucine

(GALT) and histidinemia (HIS) according to the local prevalence.[62] To ensure the quality of the screening program in China, the National Center for Clinical Laboratory was authorized for quality control and to monitor the activities of the laboratory since 1998.[64]

The screening panel was expanded by the addition of CAH and G6PD deficiency based on the pilot studies in 2002.[65] After the huge success of the NBS program for screening CH and PKU, many provinces in China wanted to expand the screening panel using LC-MS/MS. The pilot study using LC-MS/MS was started in 2003 in Shanghai by screening for 6 conditions, the collective prevalence of which was found to be 1:5800.[62] Recognizing the advantage of early detection in the prevention of IEMs, expanded screening of newborns was launched as a national program in Suzhou in 2014, targeting 27 different IEMs.[66] This program has been running successfully with a 100% coverage rate among the Suzhou population. The patients who are screened positive for a particular IEM are subjected to genetic analysis using next-generation sequencing (NGS) for confirmation.[66] There are more studies recently from Xinxiang and Eastern China using LC-MS/MS, which have helped to expand the number of disorders falling under amino acid (AA), organic acid (OA), fatty acid oxidation (FAO), and urea cycle disorders, and established the actual prevalence in those regions.[67,68] Currently, there are at least 100 laboratories in China providing LC-MS/MS based expanded screening. Around 5 million babies have been screened using LC-MS/MS, in 2017. By 2018, there are 238 dedicated centers in China for NBS and more than 15 million neonates have been screened for a minimum of three disorders. When translated into a percentage of coverage, it is a remarkable 97.5%. They are making the system more efficient using state of the art technology to cover the whole spectrum of IEMs prevalent in the country.[65]

Another success story from Asia-Pacific region comes from Philippines. It started under the dedicated and strong leadership of Dr. Carmencita Padilla, a pediatrician turned geneticist. They started the pilot project in 1996, to establish the prevalence of 5 inborn errors (CH, CAH, GALT, PKU, homocystinuria (HCY)), and subsequent

TABLE 2 Status of NBS in selected countries

Sl No	Country	NBS Started year	Coverage	Disorders		NBS using LC-MS/MS started year	Birth Rate	References
				Mandatory*	Optional/ Regional			
1	USA	1962	100% by 2005	PKU, CH, GALT, Hb, CAH, BIOT, CF	AA- 6 core and 8 secondary conditions OA- 9 core and 6 secondary conditions FAO- 5 core and 8 secondary conditions LSDs	Late 1990 s	12.5	Therrel et al., 2015 [9], McCandless et al 2020 [34], Mak et al., 2013 [7], Wiley et al., 2019 [53]
2	Canada	1963		PKU, CH, MCAD deficiency	AA- 6 core and 8 secondary conditions OA- 9 core and 6 secondary conditions FAO- 5 core and 8 secondary conditions	1980 s	10.3	Therrel et al., 2015 [9], Mak et al., 2013 [7], Wiley et al., 2019 [53]
3	Australia	1967	100% in 2015	CH, PKU, GALT, MSUD, HYS, CF	ENBS	1998	12.1	Therrel et al., 2015 [9], Padilla et al., 2007 [11], Wiley et al., 2019 [53]
4	Bangladesh	1999	~10%	CH	-	-	18.8	Hasan et al., 2008 [54]
5	China	1981	97.5% in 2018	CH, PKU	GAL, MSUD, CAD,HYS, G6PD	2014	12.3	Therrel et al., 2015 [9], Padilla et al., 2007 [11], Wiley et al., 2019 [53]
6	India	1980	<3%		CH, PKU, GAL, MSUD, CAD,HYS, G6PD	2011	19	Therrel et al., 2015 [9], Padilla et al., 2007 [11], Sahai et al., 2011 [55]
7	Japan	1967	>99% in 2006	CH, PKU, MSUD,CAH, HSY, GALT	ENBS	2004	7.7	Therrel et al., 2015 [9], Padilla et al., 2007 [11], Wiley et al., 2019 [53]
8	South Korea	1991	94% in 2006	CH, PKU, MSUD,CAH, HSY, GALT	ENBS	2008	8.3	Therrel et al., 2015 [9], Padilla et al., 2007 [11], Wiley et al., 2019 [53]
9	Malaysia	1980		CH, G6PD	PKU, MSUD, HYS		19.1	Leong et al., 2014 [56]
10	New Zealand	1969	97.7% in 2007	CH, PKU, MSUD,CAH, HSY, GALT,CF	ENBS	2006	13.2	Ministry of Health, 2019 [57]

(Continues)

TABLE 2 (Continued)

Sl No	Country	NBS Started year	Coverage	Disorders		NBS using LC-MS/MS started year	Birth Rate	References
				Mandatory*	Optional/ Regional			
11	Palau	2009	100%	CH, PKU, MSUD,CAH, GALT,G6PD	-	-	11.3	Therrel et al., 2015 [9], Padilla et al., 2007 [11], Wiley et al., 2019 [53]
12	Philippines	1996	91.6% in 2019	CH, CAH, PKU, GALT, MSUD, G6PD	HYS, CF	2014	23.7	Therrel et al., 2015 [9], Padilla et al, 2020 [58]
13	Singapore	1965	>99% in 2006	CH, PKU, MSUD, HYS G6PD	ENBS	2006	8.6	Therrel et al., 2015 [9], Padilla et al., 2007 [11], Wiley et al., 2019 [53]
14	Taiwan	1985	>99% in 2006	CH, PKU, MSUD,CAH, HSY, GALT, G6PD	ENBS	2001	8.3	Therrel et al., 2015 [9], Padilla et al., 2007 [11], Wiley et al., 2019 [53]
15	Thailand	1992	94% in 2014	CH, PKU	MSUD, CAH, HYS	2013	11	Thiboonboon et al, 2015 [59], Therrel et al., 2015 [9]
16	European Countries[¶]	1964		PKU, CH, CF, BIOT, GALT, CAH, MCAD deficiency	ENBS	2000		Therrel et al., 2015 [9], Wiley et al., 2019 [53], Bodamer et al., 2007 [52]

* Nationwide screening

#Screening restricted to particular provinces or states according the prevalence or availability of facility

‡Available at: https://worldpopulationreview.com/country-rankings/birth-rate-by-country, Accessed on January 13, 2021

¶There is a large variation in the NBS programs throughout Europe. It started in 1964 in Poland and Belgium and expanded NBS was started in Germany in 2000. The diseases given are majorly screened in most of the countries.

Chemistry Europe
European Chemical Societies Publishing

doi.org/10.1002/ansa.202100010

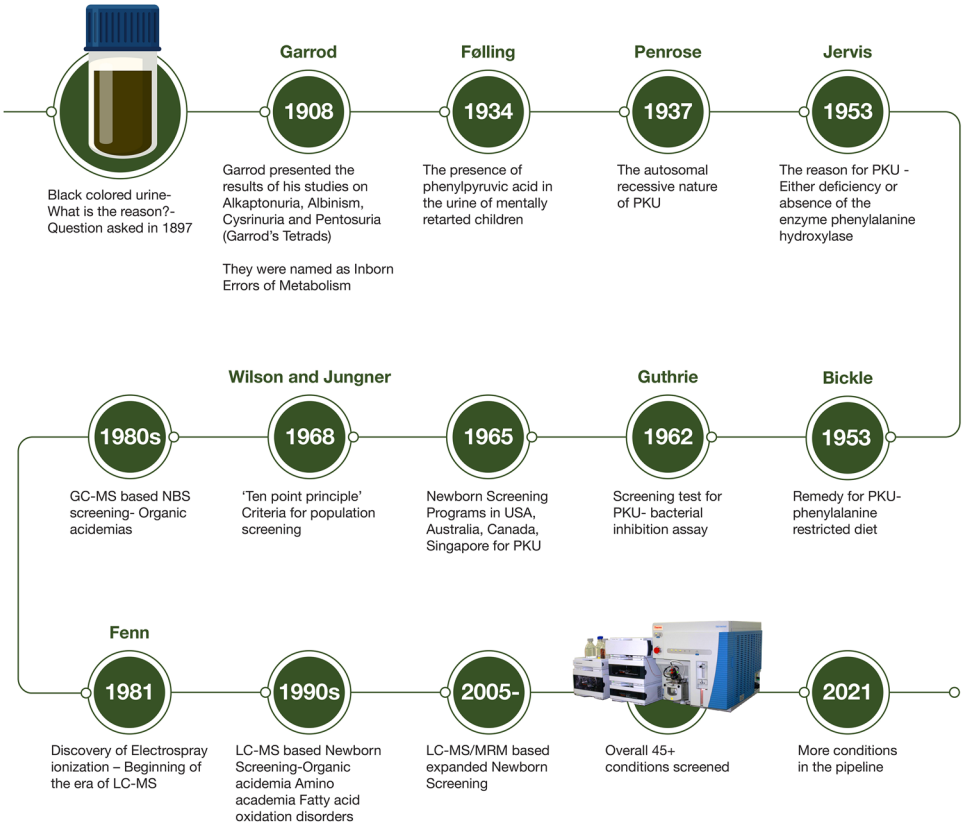

FIGURE 1 Milestones in the history of newborn screening

recommendation for a policy implementation to the government.[69] A group of pediatricians and obstetricians from 24 hospitals in Metro Manila came forward with this initiative. Another project was started in 1998 for screening G6PD deficiency also.[69] These were followed by evaluation of NBS as a national program and its cost-effectiveness. As a result of the streamlined efforts of the NBS Group, the National Newborn Screening act, 2004, mandating NBS throughout the country came into being.[70] It is remarkable that they took only eight years from pilot studies to the enforcement of the policy by law and integration of the NBS program into the public health delivery system.

There have been tremendous improvements in the program over the past five years. They enlarged their facilities by establishing more laboratories for screening and confirmation for hemoglobinopathies and G6PD deficiency. Expanded screening (panel includes more than 29 conditions) was introduced in 2014 with coverage in the national insurance.[58,71] Despite the challenges of high cost of confirmatory tests, home deliveries, laboratory and quality assurance services, and long-term recall management, Philippines has succeeded in achieving a steady increase in the percentage of screening. They covered almost 76% of the newborns by 2015 mainly for six disorders whereas with the introduction of ENBS, they expanded to 29 disorders with an overall coverage of 91.6%. The total coverage of ENBS increased drastically from < 5% in 2015 to almost 70% in 2019. The data clearly showed the advantage of using high-end technology in the NBS program.

The National Comprehensive NBS system of Philippines contains different components such as (a) an NBS Reference Center for national program management, (b) seven NBS Centers for testing, (c) 17 Department of Health Offices for education, implementation, and follow up (regional), (d) 7000 Newborn Screening Facilities for prenatal education, specimen collection, short-term follow up, and management, and (e) 14 NBS Continuity Clinics for monitoring, long-term follow up, and testing support.[58] All these modules are continuously subjected to the monitoring and quality assurance by the Philippines Performance Evaluation and Assessment Scheme, a tool they have developed similar to the U.S. Evaluation and Assessment Scheme.[58] This contributes greatly to the success of their NBS program.

It is important to note that, challenges like large regional and social-cultural differences, higher rate of homebirths were also hurdles for both Philippines and China. However, they made it possible by strong leadership of government, extensive collaboration between different stakeholders and involvement of the society. Most importantly, they made it mandatory as per law. The screening cost for the core panel is covered either by the central or the provincial government or the national insurance companies. Only a few provinces offer free ENBS, and the rest depend on the charity organizations and companies like Perkin Elmer.[65] India needs strong policies, similar to China and Philippines, by the central and state governments in order to achieve this goal.

TABLE 3 Recommendations for mandatory screening under NBS in three different conditions according to National Neonatology Forum, India

Group	Category	Disorders screened
A	All newborns	Congenital hypothyroidism (CH)
		Congenital adrenal hyperplasia (CAH)
		G6PD deficiency
B	High risk population ○ Newborns with symptoms/ signs of IEM ○ Critically ill neonates ○ Previous children with unexplained intellectual disability ○ Seizure disorders ○ Previous unexplained siblings death suggestive of IEM	Classic Phenylketonuria (PKU)
		Tyrosinemia (TYR)
		Homocystinuria (HCY)
		Alkaptonuria (AKU)
		Galactosemia (GALT)
		Maple syrup urine disease (MSUD)
		Medium chain acyl CoA dehydrogenase deficiency (MCAD)
		Fatty acid oxidation (FAO) defects
		Biotinidase deficiency (BIOT)
		Cystic fibrosis (CF)
		Sickle cell anemia and other hemoglobinopathies
C	Screening in resource rich settings	Expanded newborn screening (ENBS) for 30 to 40 IEM disorders done by LC-MS/MS

1.3 | IEM screening and NBS: The Indian scenario

Provisioning basic medical needs is a daunting task for a densely populated country spread across various socio-economic strata like India. High birth rates, rigid religious and caste system, and the prevalence of consanguineous marriages within specific communities make India highly vulnerable to IEMs.[72] This may lead to distinct patterns of disease susceptibility within different ethnic and linguistic communities.[73,74] Also, epidemiologic transition of the developing countries comes with an increasingly high burden of genetic diseases.[75] Even though the significance of IEMs is well recognized by the medical practitioners in India, there is a lack of reliable data on the incidence of IEMs representative of the diverse Indian population. It is good that some initiatives on screening and preventing birth defects and IEMs have been taken by the government of India, however, the percentage of NBS is still <3% as of 2019.[61]

Scientists have recognized the importance of genetic disorders by the early 1960s in India. There were pilot studies from all over India to find out the prevalence of G6PD deficiency, the most common inherited enzymopathy. Baxi et al published the results of their pilot studies in 1961 and 1963, revealing the high prevalence (13-15%) of G6PD deficiency in the Parsi community in Maharashtra.[76,77] Several studies on G6PD screening available from different parts of India have indicated an overall frequency of 8.5%.[77] The neonatal screening for IEMs has been started by Rao et al from Indian Institute of Science with a large-scale pilot study to screen the neonates for amino acidemias.[78] They screened 98256 neonates from Bangalore and Mysore in the state of Karnataka for a period of 8 years and detected hyperphenylalaninemia, tyrosinemia, glycinemia, histidinemia, branched-chain academia, and general aminoacidemia with a cumulative incidence of approximately 1:847. It was followed by several studies on CH, CAH, and G6PD deficiency screening in various parts of India. All the publications in this area till 2018 have been reviewed by Kaur et al and Kapoor et al.[60,79] From the review, it is clear that the frequencies of CH, CAH, and G6PD deficiency varies greatly from one study to another (CH- 1:22 to 1:3400, CAH- 1:200 to 1:6813, G6PD deficiency – 0.8% to 17.5%).[60] A recent multicenter ICMR study covering 104066 neonates from Delhi, Mumbai, Kolkata, Chennai, and Hyderabad showed the prevalence of 1:1130 and 1:5762 for CH and CAH, respectively.[80] All these studies seem to be random and greatly vary in sample size and the technologies used for the analysis. However, it showed that there is a higher prevalence of CH, CAH, and G6PD deficiency in India with high regional variations. It is to be noted that majority of the studies focus on screening these three disorders and there are only very limited number of studies on AA, OA and FAO.[81–84] Even though the frequency of these disorders varied greatly in these studies (AA- 1.7-6.1%; OA- 1.3-8.7%, FAO- 0.1-0.2%), their cumulative prevalence was found to be high in Indian population.[60]

Based on the literature and the experiences from the clinical practice, the National Neonatology Forum provided a set of recommendations for NBS in India in the Evidence-Based Clinical Practice Guidelines published in October 2010.[85] The foundation proposed a group-wise screening strategy (Table 3). CH, CAH, and G6PD deficiency (Group A) were strongly recommended to be screened because of their high incidence, availability of definitive diagnosis and treatment, and high chance of being undetected at birth. If the infant falls in the high-risk population, the disorders in the Group B should be screened. They recommended ENBS in urban setting where the facilities are available.[85]

The available data suggest the need for a universal NBS program in India. However, the fact that nearly 27 million babies are born in India annually makes its implementation a herculean task. Despite this,

there are certain Indian states/union territories like Kerala, Chandigarh, and Goa, setting examples for implementing NBS programs. It is to be noted that all these states are performing well in public health sector and have significantly reduced child mortality in comparison with the national figures. Thus there should not be major problems in implementing similar approaches in other states also. It is crucial now to have collaboration between academic and clinical labs to encourage translational research among the students to tackle these kinds of future challenges.

1.4 | NBS programs in the public and private sectors in India

Chandigarh, Goa, and Kerala are the three states/union territory in India with a public NBS program running for more than five years. None of these programs were started based on any prior pilot studies and the subsequent realization of urgent need of screening; instead, they were started to improve neonatal care or to study the prevalence of only certain diseases in that particular state or territory. The program started in 2007 in the union territory Chandigarh, involving four urban government hospitals.[60,61] All these laboratories participate in the Newborn Screening Quality Assurance Program offered by the Center for Disease Control and Prevention, USA. The three major disorders, such as CH, CAH, and G6PD deficiency were screened, at a rate of 15000 births per year.[60] Goa 1.0 NBS program (2008-2013) started with public-private partnership, covered a comprehensive panel of more than 50 disorders. Even though it was terminated in 2013 due to political reasons, it served as a model for a successful NBS program for all other states. Learning from the shortcomings of Goa 1.0 NBS, Goa 2.0 started in 2018 with a reduced testing panel for six disorders, viz. CH, CAH, G6PD deficiency, GALT, biotinidase deficiency (BIOT), and cystic fibrosis (CF).[61] NBS program in Kerala started in 2012 and the testing panel comprises CH, CAH, G6PD, and GALT.[61] It screens all births taking place in over 90 government hospitals in the state free of cost. The successful running of NBS in these states gives hope to the rest of the country that such a program can be initiated and sustained all over India.

There are certain private sector organizations offering an expanded NBS facility using the state of the art techniques like LC-MS/MS. Dr. Lal Path Labs in New Delhi,[86] Navigene in Mumbai,[87] NeoGen Labs in Bangalore,[88] LifeCell International Pvt. Ltd in Chennai,[89] Sandor Life Sciences in Hyderabad,[90] and Perkin Elmer India Ltd[91] are few of the service providers in NBS. There is a huge opportunity for the private sectors in collaboration with academic labs in India to be a part of tackling this kind of public health-related issues. Goa government collaborates with NeoGen Labs for the expanded screening in the case of high-risk deliveries.[92,93] These organizations offer screening of a comprehensive panel of disorders resembling the RUSP. National Institute of Mental Health and Neurosciences (NIMHANS) under the government of India has been offering NBS since 2007.[61] However, the expanded screening still remains a luxury in India where large proportions are living in poverty, without access to the basic healthcare amenities. A grassroots level change in our healthcare system and policies is necessary for the successful implementation of an NBS program in the country.

1.5 | The need for large-scale epidemiological studies using LC-MS/MS based metabolomics

The status of IEM screening in India suggests that there should be large-scale epidemiological studies using state-of-the-art technologies to create the background information for a successful NBS program. LC-MS/MS has been proved to be comprehensive, versatile, and effective for mass screening. It has been reported that the false positive rate of PKU detection using MS/MS is 10-fold lower than the best method available earlier.[94] The ability to analyze multiple metabolites for the detection of numerous IEMs in a single run makes it a desirable choice in the case of NBS.[95] In India where there is a need to establish the incidence of various diseases, adopting the LC-MS/MS technology would be highly beneficial. It is the time for us to act, to fill the gap of 30 years in terms of IEM/NBS research making use of the state-of-the-art technologies such as the LC-MS/MS.

The LC-MS/MS based metabolomics approach is suitable for the unambiguous identification and quantification of small molecular weight metabolites produced by metabolic processes.[96] This enables the laboratories to analyze the chemical fingerprints of multiple metabolites in blood, body tissues, and fluids including dried blood spot.[94,97,98] Though the development and validation of a method satisfying all bioanalytical and clinical parameters is highly challenging, the final method has the potential to apply it for the quantification of a wide range of metabolites. These methods reduce the demand for previously used, time-consuming, or less accurate measurements. Due to its sensitivity at the pico- to femto- gram range, this technology is used in almost all fields where quantification of biomolecules is required.[97] Another added advantage in the LC-MS based quantitative metabolomics approach is its capability of absolute quantification of metabolites with spiking of stable isotope analogs (^{13}C-, ^{15}N-, ^{2}H-, ^{18}O-, ^{34}S-) as an internal standard in the beginning of the sample preparation, which not only acts as an essential control for throughout the analysis, but also it helps in absolute quantification of particular metabolites of interest[99] (Figure 2). This kind of stable isotope dilution method is the best way to achieve the accuracies of ≥95%. The method of absolute quantification by plotting the ratio of the peak areas of the standard and internal standard (light/heavy) versus the concentration of standard is well recognized in the bio-analytical field.[99] This becomes the most suitable method for many clinical applications today, ranging from drug monitoring, disease diagnosis, and analysis of steroids and biogenic amines.[19,100,101] Its best utility is for the NBS where the quantification of amino acids, acylcarnitines, and organic acids allows screening of different disorders.[40,41,102,103]

Both targeted (selected known metabolites)[104] and untargeted (wide range of known/unknown metabolite)[105] LC-MS-based metabolomics approaches have been used to expand the range of disease-associated metabolites (Figure 2). The targeted analysis is

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doi.org/10.1002/ansa.202100010
Chemistry Europe
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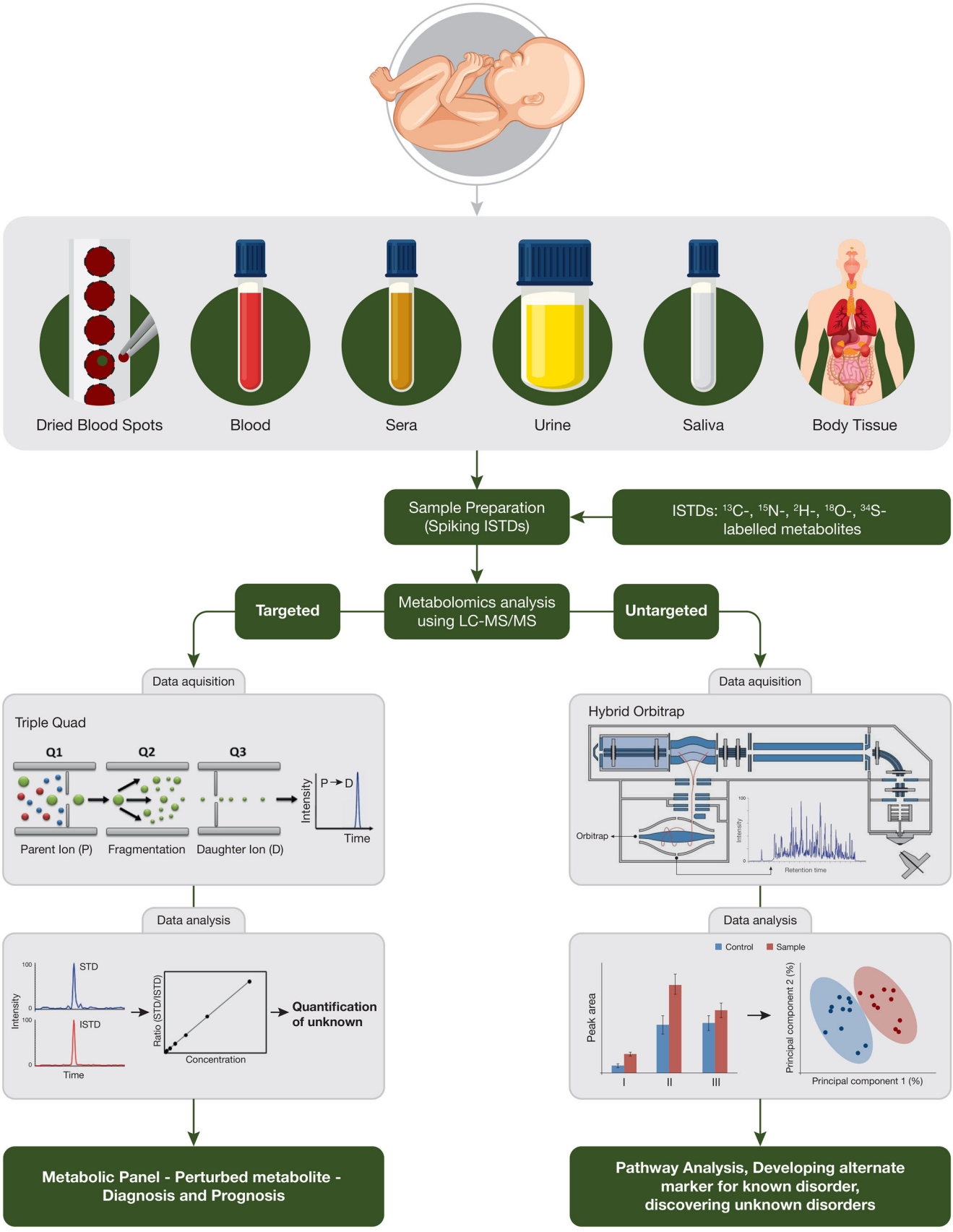

FIGURE 2 Flow chart for metabolite analysis from biofluids using targeted and untargeted approaches

specific and can be performed in two different ways: (a) method is developed for specific metabolites which are known to be associated with particular IEMs, (b) method is developed for a wide range of known metabolites (such as amino acids, organic acids, fatty acids, etc.) so that it can be applied to screen for all possible IEMs.[104,106,107] Untargeted analysis is highly descriptive as the method is developed in such a way that it detects as many metabolites as possible. Hence, untargeted analysis offers the provision to look for both known and unknown IEMs in the biological fluids.[105,108] Untargeted approach and its applications in finding alternative markers for known IEMs have also been reviewed recently by Ismail et al.[109] As per the recent nosology, there are around 1015 well characterized IEMs causing alterations in the specific metabolic pathways.[110] There is always a scope for the detection of new IEMs and their inclusion in the screening panel.

Even though the applicability of LC-MS/MS in screening of multiple disorders simultaneously is undisputed now, there were speculations regarding the scientific basis of uniform screening panels and consequences of poor performance/false positive values at the early stages. To overcome that, researchers from all over the world came together to create evidences necessary for the clinical validation of cut-off values for most of the metabolite markers. The project known as Region 4 Stork (R4 S) / Collaborative Laboratory Integrated Report (CLIR) has participants from 46 countries with data from millions of newborns.[111] They give the high/low cut-off target ranges for the discriminatory markers and ratios of markers for several disease conditions including the diseases listed in RUSP. The high cut-off target range for a particular marker is the interval between the cumulative 99th percentile of the normal population and the lowest 5th percentile of the diseased population. Whereas the low cut-off range is the interval between the 1st percentile of the normal population and 99th percentile of the diseased population.[111] Table 1 gives the metabolites markers and their corresponding high/low cut-off ranges (from the seminal paper by R4 S team, published in 2011,[111] except for CH, CAH, and X-adrenoleukodystrophy) for those diseases in RUSP which can be screened by LC-MSMS. The CLIR database continues to be refined which may lead to unambiguous reference ranges and the inclusion of new conditions and biomarkers in the future.

There is a general notion that the cost of the analysis is high while using the high-end instruments like LC-MS for routine screening. It is true that the instrument costs approximately Rs. 1.5 crore and the LC-MS/MS-based screening of IEMs costs Rs. 3000–4000 per sample depending on the number of disorders screened. There are evidences that if the number of samples and the diseases screened per analysis are high, the cost per sample can be further reduced drastically.[85,112] Considering the reduced burden of mortality and morbidity on the family and society, the monetary benefits of ENBS outweighs the cost of its implementation. According to the National Neonatology Forum, if the customs duty on the instrument and consumables is reduced by the government and if a mass spectrometer analyses 1000 samples per month, the cost can be reduced to Rs. 500 per sample for the ENBS in India.[85] Allocation of funds to the academic labs with a focus on developing new validated methods for IEM screening that can be directly applied in the clinical setup will also be useful. Implementing an ENBS in the country would be a giant leap in the public health sector in India with far-reaching future benefits.

1.6 | NGS in IEM screening

Even though genomic analysis has the capability to reveal all possible perturbations in our genetic code, analysis typically reaches a diagnosis in just 35% of cases, with a diagnostic gap arising due to limitations in prioritization and interpretation of detected variants.[113] Based on these considerations, IEMs can be arbitrarily classified into three categories based on the metabolic profile of the disease and the nature of their diagnostic biomarkers. The first category of IEMs are those with specific diagnostic biomarkers and do not require any genetic analysis for confirmation of diagnosis. The second category of IEMs has nonspecific biomarkers that limit differential diagnosis as the markers are not distinctive enough to perform a definite diagnosis. Targeted NGS covering an appropriate panel of genes can be recommended as a confirmatory test for this group. The third category consists of those metabolic disorders without known biomarkers. The whole-exome sequencing (WES) is the most appropriate and cost-effective solution in this case.

However, WES was found to have an overall sensitivity of 88% and specificity of 98.4%, compared to 99.0% and 99.8%, respectively for MS/MS, although effectiveness varied among individual IEMs. Thus, WES alone is insufficiently sensitive or specific to be a primary screening test for most of IEMs. As a secondary test for infants with abnormal MS/MS screening results, WES could reduce false-positive results, facilitate timely case resolution or suggest more appropriate or specific diagnosis.[114] Hence, NGS have a potential advantage as a second-tier screening method to verify the primary biochemical testing results. Nonetheless, suitability of WES or whole-genome sequencing (WGS) must be evaluated for each disorder. As a form of screening, sequencing would require weighing of benefits versus costs and societal implications. Genetic diagnosis can affect a patient's outcome through the changes in treatment and provide measures for prenatal diagnosis in patent's family.[115]

Thus, it is important to note that the results of WES/WGS should be appraised carefully. Genetic testing is a useful method to help verify newborns positive for an IEM and guide specific biochemical confirmatory testing. In order to improve the current standard of care, it would be important to advocate second-tier gene testing for newborns with a primary screen positive IEM. By this approach, clinicians will be more empowered with informative genetic and biochemical results, allowing them to reach a definitive clinical diagnosis and, where appropriate, administer early treatments to the newborn to manage the disease symptoms over a lifetime.

1.7 | Future perspectives

The importance of NBS/IEMs screening for newborns in the early days of life is well understood worldwide and it is mandatory in most of

the developed and few of the developing countries. In the developed world, millions of newborn babies are screened within 48 to 72 hours after birth to diagnose any rare disorders that can be identified to avoid any future health complications. In contrast, in India where NBS is not yet mandated, the screening percentage is very low and it has to be improved in the upcoming years to ensure a healthy future for the generations to come. There are challenges that exist in front of us to execute NBS/IEMs screening program for newborns.

According to a report by National Family Health Survey (NFHS-4) in 2017, there has been a tremendous increase in the percentage of institutional births in India from 38.7% in 2005–06 to 78.9% in 2015–16.[116] This can be attributed to increased awareness amongst the pregnant women about proper antenatal care, safe delivery practices and postnatal health check for mothers and newborns. This has been possible because of the integration of the mother and child health to the existing health infrastructure. A similar model needs to be implemented for NBS. With the rise in the institutional deliveries, the percentage of newborns receiving postnatal check within 48 h of birth offers immense potential to look for signs of IEM at an earlier stage.

Early diagnosis of treatable IEM is critical in many instances for timely intervention before the onset of irreversible damage and/or premature mortality.[117] Even though there are no definitive treatment for certain disorders, detecting them by using the advanced LC-MS/MS based technology will generate the epidemiological data for that particular disorder. This also provides insight to healthcare practitioners and researchers alike to develop therapeutic and nutritional strategies for these disorders. Certain disorders are treatable (esp. CH, BIOT) and few of them are manageable (e.g., hyperphenylalaninemia) if the diagnosis is done in the early stages.[118] The main aim of therapy is to restore the metabolic balance by following dietary restrictions with nutritional supplements. The unavailability of a special diet for the treatment of the children with certain IEMs is a serious concern in India. Recently FSSAI has given approval to import most of the special dietary formula for IEMs and food industries are taking effort to make it available in India.[119] Now there are chances for effective treatments for number of disorders if it is diagnosed accurately in the early stage.

There is a great need for strengthening our health system towards a mandatory screening program in India. Initially, it can be implemented in the major cities and slowly that can be expanded all over the nation. The success stories of NBS from various countries emphasize the need of a strong leadership to advocate and work for the implementation of the program. Based on the strategies other countries followed to implement NBS programs and taking in into account the developments in the healthcare sector in India, we suggest (figure 3) a detailed workflow for implementing NBS. It can be done in two different phases, in phase I it is imperative to find a good leadership with a team of physicians and scientists to tackle the challenges in executing the program as in the case of Philippines.[69] In India there is already a collective conscience formed among the neonatologists, physicians, and researchers on the relevance of IEM screening and hence they can act as leaders in this field. The team can work closely with the government bodies to implement or amend policies. It is to be noted that the Indian government has started giving priority to the health sector and there are several new policies and programs launched in recent years. The national child health policy has already recognized NBS as a necessity, however, it does not give thrust to the screening of IEMs. Further, it is to be noted that there is already an infrastructure in our country suitable for launching such a program. To cite some examples, the National Health Mission constituted in 2013 aims to cater to the healthcare needs of the marginalized sectors in the rural and urban population. There are services like Rashtriya Bal Swasthya Karyakram (RBSK) and Janani Shishu Swasthya Karyakram (JSSK) to provide screening facility for diseases in the childhood free of cost.[120] Also, there is a screening panel of birth defects and deficiencies under the RBSK program in which CH, sickle cell anemia, and β-thalassemia have been included for optional screening. To implement and sustain a successful NBS program, it is important to have a firm collaboration of government with private sectors and different stakeholders including NGOs, to make a strategic plan for setting up advanced LC-MS/MS centers across the country. These centers can focus initially on IEM/NBS screening to generate adequate dataset to get an idea about the actual prevalence of different disorders. The advanced LC-MS/MS-based laboratories thus set up in different parts of the country can perform the screening and establish the prevalence of the various IEMs at a regional level. These centers can also focus on developing new improved methods using DBS or other biological fluids. The results can be analysed by an advisory committee deployed by the government, and come up with a region-specific screening panel. This panel can be integrated with the RBSK screening panel. The strategic planning for the initial funding and finance management should also be done at this stage. Once the NBS is launched, there should be a close collaboration between neonatologists, researchers, counsellors, and social workers for its successful running. The LC-MS/MS laboratories can be converted to NBS centers or referral labs where constant research and training activities can take place. By proper training, we can create a skilled workforce for carrying out the sample analysis, data management, follow up and recall.

In phase II, it is important to focus on the major six pillars as suggested in the previous review,[34] which can help in successfully running the program in the future. It starts from educating the family members and clinicians about the NBS and its necessity to check in the early hours of life. Sample collection (within 48-72 h post birth) and testing (within 7 days post birth) should be done in a time-bound manner without fail. It is important to redo the analysis for the positive cases to confirm results. In certain cases, further confirmation with genetic screening is also important to understand the kind of disorder. To achieve this, the close collaboration of genetic and metabolic laboratories under one roof is mandated. Implementation of these centers is always challenging, but it is doable. There should be an understanding between the clinicians, NGS labs, and the NBS centers to evaluate the results obtained. The NBS program can be integrated to these existing endeavors. In addition, there is a network of health-related service providers throughout the country consisting of Anganwadi workers and ASHA (Accredited Social Health Activists) workers who are working at the grassroots level.[61] They can be deployed for recall and providing the required drug or special diet formula to the infants diagnosed with IEM.

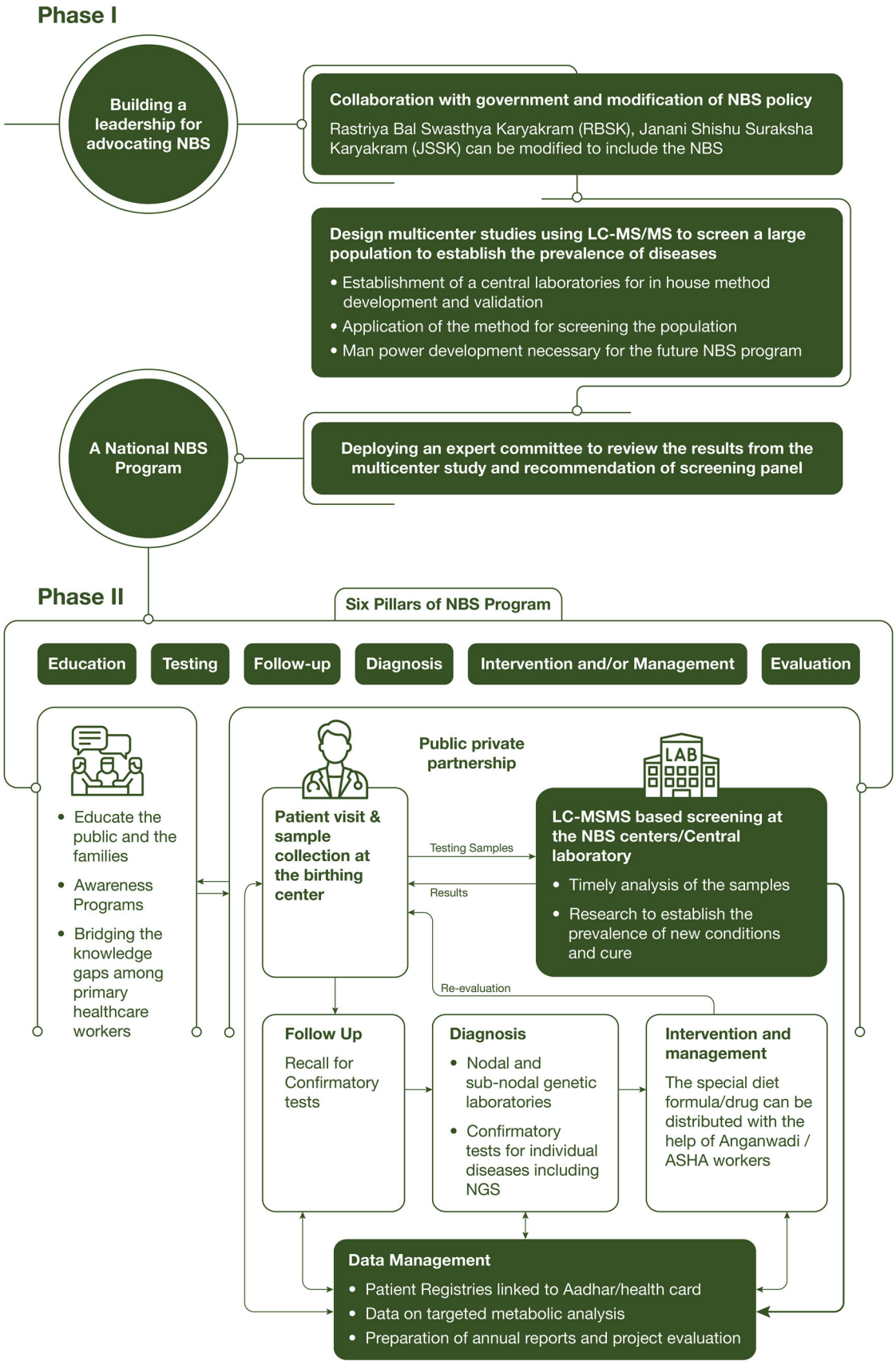

FIGURE 3 Futuristic plan to implement the inborn errors of metabolism screening in India

One of the major hurdles in the management of the IEMs is the difficulties in the follow up and recall. To overcome this issue, a proper online patient registry, which can be accessible by all the NBS centers of the country will be an ideal solution. It will be easier to track if the patient registry is linked to the Aadhaar card/any kind of health card of the patients. This will be maintained by the central laboratory and all the details of the patient and treatment history will be updated in the database in a timely manner. Thus the treatment/intervention can be accessed from any NBS centers across the country. In addition to the patient registry, the central laboratories can build another database/repository for the metabolomics analysis results of all the newborns screened. This will help in the setting up and updating the reference ranges of the metabolite markers and discovery of new IEMs. The proper data management, critical analysis of the data and annual progress reports are important to improve the program in a timely manner.

Accurate diagnosis of any kinds of disease in a timely manner with affordable cost is imperative in the healthcare sectors. As a diagnostic tool, the recent developments in the "omics" approaches, especially "metabolomics" analysis of biological fluids has made remarkable progress in health and medical science worldwide. The ultimate sensitivity, accuracy, and capability of quantifying multiple metabolites in a single method from biological fluids showed greater advantage of using this technology in clinical setup. Screening for disorders among the newborn babies using LC-MS/MS based quantification of metabolites is one of the best examples. The concepts of "individualized therapy" and "precision medicine" are becoming vital in this digitalized world to tackle various health issues and the follow up strategies in the early stage. The accurate diagnosis and treatment will always reduce the economic burden of an individual and will have a positive impact on the economy and growth of the country. LC-MS/MS based screening has clearly showed its positive impact and cost-effectiveness in the public healthcare sector of developing countries like China and Philippines. One can expect similar impact in India where consanguinity leads to an increased incidence of metabolic disorders.

2 | SUMMARY AND OUTLOOK

We have highlighted the important events that happened worldwide in developing a successful NBS program. It started from the developed countries like the United States, Canada, Australia, New Zealand, Japan, Europe, and the UK, and the program was adapted successfully in the developing countries in the Asia-Pacific region. Intriguingly both China and Philippines tackled those challenges that normally exist in the developing nations and implemented the NBS program successfully. All these countries have now started using the advanced LC-MS/MS-based technology to screen a wide range of disorders (~60 disorders) from DBS samples. In India, though it started in the 1980 s, somehow the program has not been implemented all over the nation. Due to that India is lagging behind almost 30 years in terms of NBS program and in detecting the actual prevalence of IEMs in our population. The recent data from ORD (Organization of Rare Diseases) India indicated there is an increase in the prevalence (~1 in 20) of any one of the disorders in the country (https://ordindia.in/about-rd/rare-disease-facts/, Accessed on February 2, 2021). To get a clear picture about the actual prevalence, indeed it is essential to use the recent technology like LC-MS based metabolomics approach, which seems to be the most suitable for metabolite quantification compared to any other existing technologies. It is now imperative to perform multicenter studies to get an idea about the overall IEMs prevalence for a large data set using high-end technology, which can represent the whole nation. On this basis, regional-specific disorders can be grouped and implemented using a mandatory NBS screening program with the support of state and/or central government. Some of the key points that have to be considered to implement the extended NBS program in India have also been highlighted in this review.

For a successful implementation of NBS, a multi-disciplinary approach involving nurses, primary care physicians, pediatricians, biochemical geneticists, and genetic counselors is required. The costs involved should not deter one from implementing this as opposed to the long-term benefits one gains in this field. Over the past decade, a steady decrease in infant mortality rate in India is achieved by implementation of successful public health strategies. These strategies have increased the awareness amongst the public about the benefits of these programs. It is crucial now to solve all possible neonatal problems in the early stage of life, which will have an impact in building a developed India in the near future.

ACKNOWLEDGMENTS

The authors acknowledge Dr. Dinkar Sahal for his critical comments and Mr. Praful Kumar for his kind help in making the high quality figures.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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How to cite this article: Chandran J, Bellad A, Ramarajan MG, Rangiah K. Applications of quantitative metabolomics to revolutionize early diagnosis of inborn errors of metabolism in India. *Anal Sci Adv*. 2021;2:546–563.
https://doi.org/10.1002/ansa.202100010