

Integrated Metabolomics and Second-Tier Genomic Analysis for Expanded Newborn Screening of Inborn Errors of Metabolism: Protocol for a Multicentric Prospective Cohort Study in India

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Abstract

Background

Inborn errors of metabolism (IEM) cause substantial neonatal morbidity and mortality. India's large birth cohort, genetic diversity, and consanguinity in some communities heighten risk, yet expanded newborn screening (NBS) remains fragmented. This multicentre prospective cohort study evaluates integrated omics-based expanded NBS combining Liquid chromatography-tandem mass spectrometry (LC–MS/MS) metabolomics with targeted next-generation sequencing (NGS) for early IEM detection.

Methods

At least 10,000 neonates born at or admitted to Neonatal Intensive Care Units across nine centres will be recruited over 30 months. Dried blood spots collected at 48–72 hours of life will undergo first-tier LC–MS/MS quantification of amino acids and acylcarnitines, targeting aminoacidopathies, organic acidaemias, and fatty acid oxidation disorders. Abnormal profiles will trigger second-tier targeted NGS for confirmatory variant analysis. Confirmed cases will receive structured clinical evaluation, condition-specific treatment, and longitudinal follow-up to assess outcomes.

Results

The study will produce India's first multicentre, population-level estimates of IEM birth prevalence across diverse geographic and genetic settings. Diagnostic performance—sensitivity, specificity, and positive and negative predictive values—will be evaluated for the integrated algorithm. Second-tier NGS is expected to reduce false-positive recalls substantially compared with metabolomics alone, while offering preliminary evidence on clinical benefits of early treatment.

Conclusions

This protocol establishes a reproducible and scalable evidence base for expanding newborn screening for IEM within India's public health system, and provides a structured framework to inform the development of a National Newborn Screening Programme for inherited metabolic disorders.

Introduction

Inborn errors of metabolism (IEM) are a broad group of individually rare but collectively common genetic disorders caused by inherited defects in enzymes, transporters, or cofactors essential to normal metabolic function (Ferreira et al., 2019). In cases of IEM, if left diagnosed and treated, it can lead to severe consequences such as intellectual disability, organ failure, recurring metabolic crises, and even death (Mak et al., 2013; Saudubray & Garcia-Cazorla, 2018). The collective birth prevalence of IEM is estimated at 1 in 800 to 1 in 2500 live births globally, with considerable variation across populations and regions (Blau et al., 2010; Pampols, 2010). In India, limited data from tertiary care centres and regional screening programmes suggest

that IEM may be significantly under recognised, primarily because of the absence of a universal, government-mandated expanded newborn screening (NBS) programme (Chikkalingaiah et al., 2024; Raveendran et al., 2022).

Tandem mass spectrometry (LC-MS/MS) has revolutionized NBS in the last 3 decades, allowing for the simultaneous measurements of dozens of metabolites, including amino acids, acylcarnitines, and succinylacetone, from a single dried blood spot (DBS) within hours (Millington, 2024). This approach, called "expanded NBS," is now offered as a universal government-funded program in US, EU, Australia, and many other developed countries, screening for up to 29–50 + different conditions (Millington, 2024; Therrell et al., 2024). However, first-tier metabolomics-based NBS is associated with up to 0.2–0.5% false-positive rates, causing significant parental distress, unnecessary recall, and re-testing (Schulze et al., 2003); the addition of a second tier molecular genetic (genomic) step to the same original DBS has been shown to significantly reduce false-positives while providing increased diagnostic certainty (Gandotra et al., 2023; Malvagia et al., 2020).

Next-generation sequencing (NGS), encompassing gene-panel sequencing, whole-exome sequencing (WES), and whole-genome sequencing (WGS), has transformed the molecular diagnosis of IEM (Nolan & Carlson, 2016; Rabbani et al., 2014). The implementation of targeted NGS panels as a second-tier screen for neonates with abnormal first-tier LC-MS/MS results is now being explored by several leading NBS programmes worldwide (Chan et al., 2024; Therrell et al., 2024). This integrated "omics" approach – metabo-genomics – represents the state-of-the-art strategy for NBS in the current era (Xie et al., 2025).

India presents unique epidemiological and genetic challenges for IEM screening. A large population of approximately 1.4 billion, 25 million births annually, marked genetic diversity across more than 4,000 distinct communities and tribes, high rates of consanguinity in specific subpopulations, and wide geographic variability all contribute to a complex landscape with potentially high IEM burden (Bhushan et al., 2024; Sivasubbu & Scaria, 2019). Specific metabolic disorders prevalent in Indian populations include methylmalonicacidaemia (MMA), propionic acidaemia, maple syrup urine disease (MSUD), phenylketonuria (PKU), glutaricaciduria type 1 (GA-1), isovalericacidaemia (IVA), and multiple acyl-CoA dehydrogenase deficiency (MADD/GA-2) (Chikkalingaiah et al., 2024; Sindgikar et al., 2017). High rates of consanguinity, particularly notable in South India and certain North Indian communities, further elevate the risk of autosomal recessive IEM (Kapoor & Thelma, 2018).

Despite the scale of the problem, comprehensive expanded NBS using LC-MS/MS is currently available only in a handful of private tertiary care centres in India and remains inaccessible to the vast majority of the population, particularly in public sector hospitals and rural areas (Chikkalingaiah et al., 2024). The ICMR has been instrumental in supporting pilot newborn screening initiatives, and the present study represents a significant scale-up effort to generate the scientific evidence needed to inform a National NBS Policy for IEM (Raveendran et al., 2022).

This study aims at developing and validating an integrated, omics-based expanded newborn screening protocol that is applicable to India's heterogeneous public health landscape. Combining first-tier LC–MS/MS metabolomics with second-tier targeted NGS in a prospective multicentre cohort design, the study will generate population-level data on IEM birth prevalence, diagnostic performance metrics, and early clinical outcomes across six geographically distinct regions of India.

Methods

Study Design and Participating Centres

This is a multicentric, prospective observational cohort study designed in accordance with the Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) guidelines (Cuschieri, 2019). The study was developed as an ICMR Ad-hoc Research Project and will be conducted across nine participating centres representing diverse geographic regions and genetic populations within India. The study encompasses four sequential phases: (1) site preparation and capacity building; (2) active neonatal recruitment and DBS sample collection; (3) laboratory processing (LC-MS/MS primary screen and NGS second-tier confirmatory analysis); and (4) clinical evaluation, intervention, follow-up, and data analysis.

Maulana Azad Medical College and the associated Lok Nayak Hospital, New Delhi (MAMC-LNH), will serve as the central coordinating centre and principal investigator (PI) site, responsible for protocol oversight, central laboratory coordination, and data management. All India Institute of Medical Sciences, New Delhi (AIIMS-Delhi), will serve as the co-PI site. Both institutions are high-volume urban tertiary care referral hospitals in North India with established neonatal intensive care units. Additional North Indian centres include the Government Medical College and Hospital, Sector 32, Chandigarh (GMCH-Chandigarh), and the Institute of Medical Sciences, Banaras Hindu University, Varanasi, Uttar Pradesh (IMS-BHU), the latter serving as a major academic referral centre for the densely populated Gangetic plain.

Central India will be represented by the All India Institute of Medical Sciences, Bhopal, Madhya Pradesh (AIIMS-Bhopal), a national institute providing advanced tertiary care services to central Indian populations, and the Mahatma Gandhi Institute of Medical Sciences, Sewagram, Maharashtra (MGIMS-Sewagram), which provides a geographically and demographically distinct rural and semi-urban healthcare perspective. The Rajendra Institute of Medical Sciences, Ranchi, Jharkhand (RIMS-Ranchi), represents an unexplored population in the north-central region and serves tribal and economically marginalised communities in an aspirational district.

Southern India will be represented by the SAT Hospital, Government Medical College, Thiruvananthapuram, Kerala (GMC-Thiruvananthapuram), a high-volume tertiary centre with a well-established neonatal care infrastructure. The North-Eastern region will be represented by Assam Medical College and Hospital, Dibrugarh, Assam (AMCH-Dibrugarh), a major tertiary referral centre serving the geographically and ethnically distinct populations of North-East India.

Collectively, these nine centres encompass diverse ethnic groups, genetic backgrounds, and healthcare delivery settings, providing a representative cross-section of the Indian population for evaluating the burden and spectrum of IEM. MAMC-LNH will function as the central reference laboratory for LC-MS/MS-based metabolomic screening. Second-tier confirmatory genomic analysis by targeted NGS will be centralised at a designated accredited genomics facility operating under the study framework.

Study Population

The study population will include 10,000 new born delivered at or admitted to the neonatal intensive care units (NICUs) of the nine participating centres. [Figure 1].

Eligibility Criteria

Inclusion and Exclusion Criteria

Neonates eligible for inclusion in the study will comprise all intramural neonates from birth to 28 days of life admitted to the neonatal intensive care units (NICUs) of the participating centres, as well as extramural neonates referred to these tertiary care hospitals during the neonatal period. Both term and preterm neonates will be eligible for screening. For preterm neonates, the first dried blood spot (DBS) sample will be collected at approximately 24 hours of life, with a repeat sample obtained at two weeks postnatal age or at discharge, whichever occurs later. Neonates receiving total parenteral nutrition (TPN) or antibiotic therapy will not be excluded; however, their metabolic screening results will be analysed and interpreted separately. For neonates who have received blood transfusion, sample collection will be performed at least seven days after transfusion. Neonates with a family history suggestive of inborn errors of metabolism (IEM) in a previous sibling, as well as those presenting with congenital anomalies potentially associated with metabolic disorders, will also be included. Such anomalies may include cataract (suggestive of galactosemia or peroxisomal disorders), structural brain abnormalities such as agenesis of the corpus callosum or cystic brain changes (associated with disorders such as non-ketotichyperglycinemia or sulphite oxidase deficiency), and renal, cardiac, or skeletal malformations observed in peroxisomal disorders, dystroglycanopathies, or congenital disorders of glycosylation.

Neonates will be excluded if they present with conditions that can be clearly explained by non-metabolic causes, such as neonatal jaundice attributable to ABO or Rh incompatibility, or major congenital malformations not typically associated with inborn errors of metabolism, including neural tube defects, abdominal wall defects, tracheoesophageal fistula, and anal atresia. In addition, neonates with skeletal dysplasias unrelated to metabolic disorders, such as thanatophoric dysplasia, osteogenesis imperfecta type II, perinatal hypophosphatasia, or ciliopathies, will be excluded from the study.

Sample Size

The study aims to screen a minimum of 10,000 neonates across all nine participating centres. Estimate prevalence of combined IEM with sufficient precision: Assuming an estimated aggregate IEM prevalence of 1 in 1,000 live births (conservative estimate for India) (Latheef, 2010), a sample of 10,000 would be expected to identify approximately 10 confirmed IEM cases, providing a 95% confidence interval of approximately 0.05–0.18% (Alexander, 2018), which is adequate for initial prevalence estimation purposes.

Study Procedures

Pre-Study Preparation and Capacity Building

Prior to the initiation of participant recruitment, a series of preparatory and capacity-building activities will be conducted at all participating centres to ensure standardized implementation of the study protocol. Orientations will be conducted among obstetricians, neonatologists, pediatricians, and nurses about the study, its objectives, research protocols, and the importance of newborn screening (NBS). Selected study staff and research assistants will be trained on sample collection, labeling, and transportation of dried blood spot (DBS) as per standard operating procedures (SOP). Necessary consumables, including DBS collection cards

(PerkinElmer 226 or equivalent, compliant with CLSI LA4-A6 standards), sterile heel-prick lancets, gloves, and sample transport materials, will be procured and distributed to participating sites. In addition, logistical arrangements for maintaining appropriate sample transport conditions, including cold-chain mechanisms from peripheral sites to the central reference laboratory at Maulana Azad Medical College (MAMC), New Delhi, will be established. An electronic data capture (EDC) system will be developed and pilot-tested to ensure secure and standardized data collection across centres. Furthermore, Institutional Ethics Committee (IEC) approvals will be obtained from all participating institutions prior to the commencement of study recruitment.

Recruitment and Informed Consent

Parents or legal guardians of eligible neonates will be approached by trained members of the study team following delivery, during the routine postnatal hospital stay, or at the time of admission to the neonatal intensive care unit (NICU). The objectives, procedures, potential risks, and anticipated benefits of the study will be explained clearly in a language understood by the parents or guardians. This information will be provided through the approved Participant Information Sheet (PIS) available in English and relevant local languages, including Hindi, Malayalam, Punjabi, and Assamese, as applicable at each participating centre. Adequate time will be provided for questions and clarification. Written informed consent will be obtained from the parent or legal guardian prior to the initiation of any study-related procedures.

Dried Blood Spot (DBS) Sample Collection

Dried blood spot (DBS) samples will be collected from all enrolled neonates following the Clinical and Laboratory Standards Institute (CLSI) guideline LA4-A6 (2012) for newborn screening. For term neonates, sample collection will be performed 48–72 hours after birth, preferably after adequate protein feeding, whereas for preterm or clinically ill neonates, samples will be obtained at approximately 48 hours of life irrespective of feeding status. Prior to collection, the infant's heel will be gently warmed using a warm heel pad for 3–5 minutes to enhance peripheral blood flow. The lateral aspect of the heel will then be cleansed with 70% isopropyl alcohol and allowed to air-dry before puncture using a sterile disposable lancet. The first drop of blood will be discarded, and subsequent drops will be applied to the pre-marked circles on the DBS filter paper card, ensuring that at least three circles (≥ 8 mm diameter) are completely filled from one side without excessive pressure.

Following collection, DBS cards will be air-dried horizontally at ambient temperature for a minimum of three hours, protected from direct sunlight, heat, and humidity. Each card will be labelled with a unique study identification number, date of birth, date and time of sample collection, gestational age, and birth weight. After drying, the cards will be individually sealed in zip-lock polythene bags containing desiccant sachets, stored at 4°C at the respective study site, and transported to the central LC–MS/MS reference laboratory at Maulana Azad Medical College (MAMC), New Delhi, through a validated cold-chain logistics system within seven days of collection. In addition, a second set of DBS cards will be preserved at – 20°C at the collection site as a backup sample for repeat or confirmatory testing if required.

Primary Screening: LC-MS/MS Metabolomics

First-tier metabolic screening will be conducted on all dried blood spot (DBS) samples at the central LC–MS/MS reference laboratory at Maulana Azad Medical College (MAMC), New Delhi, using a validated tandem mass spectrometry–based metabolomics platform. Metabolite analysis will be performed using the NeoBase™ 2 Non-derivatized MS/MS kit (PerkinElmer, Waltham, MA, USA) or an equivalent validated non-derivatized analytical method. Quantification of amino acids, acylcarnitines, and other relevant metabolites will be carried out using a certified tandem mass spectrometer, such as the AB SCIEX 3200 QTRAP System, following the manufacturer’s protocol and established quality control procedures. This first-tier screening approach enables the simultaneous detection of a wide spectrum of metabolic abnormalities associated with amino acid disorders, organic acidemias, and fatty acid oxidation disorders [Table 1].

Table 1

Inborn errors of metabolism detectable by tandem mass spectrometry (MS/MS) in dried blood spots collected from newborns aged 24–120 hours (1–5 days)

Disorder category	Metabolic disorder	Primary marker(s)	Informative ratio(s) / secondary marker(s)	Gene(s) for second-tier analysis
Amino acid disorders	Phenylketonuria / hyperphenylalaninaemia	↑ Phe	Phe/Tyr	<i>PAH; PTS, QDPR, GCH1, PCBD1</i> (BH4 defects)
	Maple syrup urine disease	↑ Leu + Ile (Xle), ↑ Val	Xle/Phe, Xle/Ala; allo-isoleucine (second tier)	<i>BCKDHA, BCKDHB, DBT, DLD</i>
	Classical homocystinuria (cystathionine β-synthase deficiency)	↑ Met	Met/Phe; total homocysteine (second tier)	<i>CBS</i>
	Hypermethioninaemia (MAT I/III deficiency)	↑ Met	Met/Phe	<i>MAT1A</i>
	Citrullinaemia type I	↑ Cit	Cit/Arg	<i>ASS1</i>
	Argininosuccinic aciduria	↑ Cit; ↑ argininosuccinate (ASA)	Cit/Arg	<i>ASL</i>
	Tyrosinaemia type I	↑ Tyr	Succinylacetone (second tier); Tyr/Phe	<i>FAH</i>
Fatty acid oxidation disorders	Medium-chain acyl-CoA dehydrogenase deficiency (MCAD)	↑ C8, C6, C10, C10:1	C8/C10, C8/C2	<i>ACADM</i>
	Very-long-chain acyl-CoA dehydrogenase deficiency (VLCAD)	↑ C14:1, C14, C14:2	C14:1/C2, C14:1/C16	<i>ACADVL</i>
	Short-chain acyl-CoA dehydrogenase deficiency (SCAD)	↑ C4	C4/C2, C4/C3	<i>ACADS</i>
	Multiple acyl-CoA dehydrogenase deficiency	↑ C4, C5, C6, C8, C10, C12, C14, C16, C5DC	Multiple chain-length pattern;	<i>ETFA, ETFB, ETFDH</i>

Note. Markers listed are indicative rather than exhaustive and reflect analytes and ratios in routine use in MS/MS-based newborn screening programmes. Screening performance depends on age at sampling, feeding status, gestational age, birth weight and laboratory-specific cutoffs. Several markers are shared across disorders – for example, C3 in propionic and methylmalonic acidaemias, C5-OH in 3-methylcrotonyl-CoA carboxylase, HMG-CoA lyase and multiple carboxylase deficiencies, and C4 in short-chain acyl-CoA dehydrogenase and isobutyryl-CoA dehydrogenase deficiencies – and therefore cannot alone assign a diagnosis. MS/MS does not resolve leucine, isoleucine, allo-isoleucine and hydroxyproline, which are reported as a single isobaric peak (Xle). Certain conditions are incompletely detected in specimens collected before 48 hours of life, notably classical homocystinuria and tyrosinaemia type I, for which tyrosine may remain within reference limits; succinylacetone is applied as a second-tier analyte for the latter (Table 2). Analyte ratios, second-tier biochemical assays and confirmatory molecular testing are therefore applied sequentially, as detailed in the Methods.

Disorder category	Metabolic disorder	Primary marker(s)	Informative ratio(s) / secondary marker(s)	Gene(s) for second-tier analysis
	(MADD; glutaric acidaemia type II)		C8/C10 not discriminatory	
	Carnitine palmitoyltransferase I deficiency (CPT-I)	↑ C0; ↓ C16, ↓ C18:1	C0/(C16 + C18)	<i>CPT1A</i>
	Carnitine palmitoyltransferase II deficiency (CPT-II)	↑ C16, C18:1, C18	(C16 + C18:1)/C2	<i>CPT2</i>
	Carnitine–acylcarnitine translocase deficiency	↑ C16, C18:1, C18; ↓ C0	(C16 + C18:1)/C2	<i>SLC25A20</i>
	Long-chain 3-hydroxyacyl-CoA dehydrogenase deficiency (LCHAD)	↑ C16-OH, C18:1-OH, C18-OH	C16-OH/C16	<i>HADHA</i>
	Mitochondrial trifunctional protein deficiency	↑ C16-OH, C18:1-OH, C18-OH	C16-OH/C16	<i>HADHA, HADHB</i>
Organic acid disorders	Glutaric acidaemia type I	↑ C5DC	C5DC/C8, C5DC/C16	<i>GCDH</i>
	Propionic acidaemia	↑ C3	C3/C2, C3/C16, C3/Met	<i>PCCA, PCCB</i>
	Methylmalonic acidaemia (including cobalamin-responsive forms)	↑ C3	C3/C2, C3/C16; methylmalonic acid ± homocysteine (second tier)	<i>MMUT, MMAA, MMAB, MMADHC, MMACHC, MCEE</i>
	Isovaleric acidaemia	↑ C5	C5/C2, C5/C3	<i>IVD</i>
	3-Hydroxy-3-methylglutaryl-CoA lyase deficiency	↑ C5-OH, ↑ C6DC	C5-OH/C8, C5-OH/C3	<i>HMGCL</i>
	3-Methylcrotonyl-CoA carboxylase deficiency	↑ C5-OH	C5-OH/C8, C5-OH/C0	<i>MCCC1, MCCC2</i>
Note. Markers listed are indicative rather than exhaustive and reflect analytes and ratios in routine use in MS/MS-based newborn screening programmes. Screening performance depends on age at sampling, feeding status, gestational age, birth weight and laboratory-specific cutoffs. Several markers are shared across disorders – for example, C3 in propionic and methylmalonic acidaemias, C5-OH in 3-methylcrotonyl-CoA carboxylase, HMG-CoA lyase and multiple carboxylase deficiencies, and C4 in short-chain acyl-CoA dehydrogenase and isobutyryl-CoA dehydrogenase deficiencies – and therefore cannot alone assign a diagnosis. MS/MS does not resolve leucine, isoleucine, allo-isoleucine and hydroxyproline, which are reported as a single isobaric peak (Xle). Certain conditions are incompletely detected in specimens collected before 48 hours of life, notably classical homocystinuria and tyrosinaemia type I, for which tyrosine may remain within reference limits; succinylacetone is applied as a second-tier analyte for the latter (Table 2). Analyte ratios, second-tier biochemical assays and confirmatory molecular testing are therefore applied sequentially, as detailed in the Methods.				

Abbreviations: Ala, alanine; Arg, arginine; ASA, argininosuccinic acid; BH4, tetrahydrobiopterin; Cit, citrulline; DBS, dried blood spot; Ile, isoleucine; Leu, leucine; Met, methionine; MS/MS, tandem mass spectrometry; Phe,

phenylalanine; Tyr, tyrosine; Val, valine; Xle, combined leucine + isoleucine peak. Acylcarnitines are designated by carbon chain length (C0, free carnitine; C3, propionylcarnitine; C4, butyryl/isobutyrylcarnitine; C5, isovaleryl/2-methylbutyrylcarnitine; C5-OH, 3-hydroxyisovaleryl carnitine; C5DC, glutaryl carnitine; C6DC, methylglutaryl carnitine; C16-OH, 3-hydroxypalmitoylcarnitine); ↑ denotes elevation and ↓ reduction relative to laboratory reference intervals.

Cut-off Values: Age- and gestational-age-specific reference ranges and cut-off values will be established during the initial 6-month set-up phase using healthy neonatal DBS samples collected at each site. Samples with analyte values exceeding established cut-offs (or in defined ratios, e.g., Phe/Tyr, C3/C2, C5DC/C8) will be classified as screen-positive and referred for second-tier genomic analysis.

Quality Control: Second-tier confirmatory QC using certified reference materials (CRM) from NIST (SRM 1951c for amino acids and acylcarnitines) will be performed with every analytical run. Internal controls at three concentration levels (low, medium, high) and external proficiency testing (via ERNDIM or College of American Pathologists [CAP] Newborn Screening Survey) will be conducted twice yearly.

Second-Tier Screening: Targeted NGS Genomics and Enzyme Assays

All neonates with an abnormal first-tier LC-MS/MS result will undergo second-tier testing [Table 2].

Table 2. Second-tier biochemical analytes and corresponding genomic targets applied to abnormal primary newborn screening results

Primary screening finding	Second-tier biochemical analyte(s)	Disorder(s) differentiated or confirmed	Gene(s) interrogated on second-tier genomic panel
↑ Phe; ↑ Phe/Tyr	Blood and urine pterins (neopterin, biopterin); dihydropteridine reductase activity in DBS	Phenylketonuria / hyperphenylalaninaemia vs. defects of BH4 synthesis or recycling	<i>PAH; PTS, QDPR, GCH1, PCBD1</i>
↑ or ↓ branched-chain amino acids (Xle, Val)	Allo-isoleucine, resolved from leucine, isoleucine, valine and hydroxyproline by LC-MS/MS	Maple syrup urine disease (elevated); BCKDK deficiency (reduced BCAA)	<i>BCKDHA, BCKDHB, DBT, DLD; BCKDK</i>
↑ Met; ↑ Met/Phe	Total homocysteine; methionine quantitation	Classical homocystinuria (CBS deficiency) vs. MAT I/III, GNMT and AHCY deficiencies	<i>CBS; MAT1A, GNMT, AHCY</i>
↑ Tyr	Succinylacetone in DBS	Tyrosinaemia type I vs. transient neonatal tyrosinaemia and tyrosinaemia types II and III	<i>FAH; TAT, HPD</i>
↑ C3; ↑ C3/C2; abnormal methionine	Methylmalonic acid, methylcitric acid, total homocysteine	Propionic acidemia; isolated methylmalonic acidemia; combined methylmalonic acidemia with homocystinuria; maternal or infant vitamin B12 deficiency	<i>PCCA, PCCB; MMUT, MMAA, MMAB, MMADHC, MMACHC, MCEE</i>
↑ C5DC	3-Hydroxyglutaric acid, glutaric acid	Glutaric acidemia type I; glutaric acidemia type II (MADD)	<i>GCDH; ETFA, ETFB, ETFDH</i>
↑ C4	Ethylmalonic acid, methylsuccinic acid, butyrylglycine	SCAD deficiency; isobutyryl-CoA dehydrogenase deficiency; ethylmalonic encephalopathy	<i>ACADS; ACAD8; ETHE1</i>
↑ C5	Isovalerylglycine, 2-methylbutyrylglycine	Isovaleric acidemia vs. 2-methylbutyryl-CoA dehydrogenase deficiency; excludes pivalate-containing drug exposure	<i>IVD; ACADSB</i>
↑ C5-OH	3-Hydroxyisovaleric acid, 3-methylcrotonylglycine, methylcitric acid	3-Methylcrotonyl-CoA carboxylase deficiency (infant or maternal); HMG-CoA lyase deficiency; holocarboxylase synthetase deficiency;	<i>MCCC1, MCCC2; HMGCL; HLCS; ACAT1</i>

Primary screening finding	Second-tier biochemical analyte(s)	Disorder(s) differentiated or confirmed	Gene(s) interrogated on second-tier genomic panel
		β -ketothiolase deficiency	
↓ C0 with globally reduced acylcarnitines	Free and total carnitine in plasma; maternal carnitine assessment	Primary carnitine deficiency (OCTN2 deficiency), including maternal carnitine deficiency	<i>SLC22A5</i>
Abnormal C26:0-lysophosphatidylcholine	C20–C26 lysophosphatidylcholine species; plasma very-long-chain fatty acids	X-linked adrenoleukodystrophy; peroxisomal biogenesis disorders	<i>ABCD1</i> ; <i>PEX</i> genes
↑ 17OHP on first-tier immunoassay	LC-MS/MS steroid profile: 17OHP, androstenedione, cortisol, 11-deoxycortisol, 21-deoxycortisol; (17OHP+androstenedione)/cortisol ratio	Congenital adrenal hyperplasia due to 21-hydroxylase deficiency and related steroidogenic defects	<i>CYP21A2</i> ; <i>CYP11B1</i> , <i>HSD3B2</i>

Note. Second-tier testing is performed on the original dried blood spot specimen, avoiding a repeat collection and reducing the number of infants referred for diagnostic evaluation on the basis of a false-positive primary result. Second-tier biochemical analysis and targeted genomic analysis are applied in parallel or sequentially according to the analyte flagged and the turnaround time required; where a disorder carries a risk of acute neonatal decompensation, clinical referral is not deferred pending second-tier results. The final two rows are included for completeness of the screening programme but are not detected on the amino acid and acylcarnitine MS/MS panel described in Table 1: C26:0-lysophosphatidylcholine requires a dedicated flow-injection MS/MS assay, and 17-hydroxyprogesterone is measured by first-tier immunoassay with LC-MS/MS steroid profiling as the second tier. Gene lists are not exhaustive; copy-number and deep-intronic variants, and the pseudogene-mediated rearrangements affecting *CYP21A2*, may require orthogonal methods.

Abbreviations: BCAA, branched-chain amino acids; BCKDK, branched-chain ketoacid dehydrogenase kinase; BH4, tetrahydrobiopterin; CBS, cystathionine β -synthase; DBS, dried blood spot; HMG-CoA, 3-hydroxy-3-methylglutaryl-coenzyme A; LC-MS/MS, liquid chromatography–tandem mass spectrometry; MADD, multiple acyl-CoA dehydrogenase deficiency; MAT, methionine adenosyltransferase; OCTN2, organic cation transporter novel type 2; 17OHP, 17-hydroxyprogesterone; SCAD, short-chain acyl-CoA dehydrogenase. Acylcarnitine nomenclature as in Table 1.

Targeted Next-Generation Sequencing (NGS)

Genomic DNA will be extracted from residual dried blood spot (DBS) samples or, where available, from freshly collected peripheral blood samples in EDTA tubes using the QIAamp DNA Micro Kit (Qiagen, Hilden, Germany) or an equivalent validated extraction protocol. Targeted genomic analysis will be performed using a “virtual panel” strategy, in which sequencing data generated from a comprehensive inborn errors of metabolism (IEM) gene panel or clinical exome/whole-exome sequencing (WES) will be bioinformatically filtered based on a

curated gene list relevant to the specific metabolic abnormality identified during the primary screening. This targeted filtering approach enables focused analysis of genes associated with the biochemical phenotype detected through metabolomic screening, thereby improving diagnostic yield and facilitating accurate genotype–phenotype correlation.

Variant classification will adhere to the ACMG/AMP 2015 guidelines (Richards et al., 2015) and their updated gene-specific adaptations. Variants will be classified as Pathogenic (P), Likely Pathogenic (LP), Variant of Uncertain Significance (VUS), Likely Benign (LB), or Benign (B). For clinical action, only P and LP variants in established disease-gene associations will be reported as positive. Bioinformatic analysis will use validated pipelines including BWA-MEM for alignment (GRCh38/hg38 reference), GATK 4.x for variant calling, and Alamut Visual or Varsome for variant interpretation, with annotation using gnomAD, ClinVar, and HGMD databases.

Enzyme Assays

For selected conditions where NGS is inconclusive or where functional confirmation is required (e.g., biotinidase activity for biotinidase deficiency, galactose-1-phosphate uridylyltransferase [GALT] enzyme activity for galactosaemia), specific enzyme activity assays will be performed from DBS or plasma/leucocyte preparations at the central laboratory and Co-PI institute.

Clinical Evaluation and Confirmatory Diagnosis

All neonates identified as positive through the combined multi-omics screening approach—defined as the presence of an abnormal primary metabolomic result together with a pathogenic or likely pathogenic variant detected on second-tier genomic analysis, or those with a screen-positive first-tier result supported by definitive clinical or biochemical confirmation—will be recalled for comprehensive clinical evaluation and confirmatory diagnostic assessment.

The evaluation will include a detailed clinical history with particular emphasis on family history, parental consanguinity, and any previously affected siblings. A complete physical examination will be performed, including standardized anthropometric measurements and a focused neurological assessment.

Confirmatory biochemical investigations will be conducted as appropriate and may include quantitative plasma amino acid analysis, urine organic acid profiling using gas chromatography–mass spectrometry (GC–MS), and plasma acylcarnitine profiling. Neuroimaging with brain magnetic resonance imaging (MRI) will be performed when clinically indicated, particularly in the presence of neurological manifestations. In addition, a comprehensive nutritional assessment and dietetic evaluation will be undertaken to guide metabolic management and therapeutic interventions.

Treatment and Early Intervention

On confirmation of an IEM diagnosis, appropriate evidence-based management will be initiated without delay.

Outcome Measures

The primary and secondary outcome measures of this study will include the estimation of the birth prevalence of inborn errors of metabolism (IEM), both for individual conditions and collectively across the screened study population. The study will also assess the diagnostic confirmation rate of the two-tier screening strategy by determining the proportion of cases identified as screen-positive on first-tier liquid chromatography–tandem mass spectrometry (LC–MS/MS) that are subsequently confirmed as true positives through second-tier next-generation sequencing (NGS), thereby quantifying the reduction in false-positive results attributable to the integrated screening approach. In addition, the time to diagnosis, defined as the interval from birth to confirmed diagnosis of an IEM, and the time to treatment initiation, defined as the interval from confirmed diagnosis to the commencement of the first disease-specific therapeutic intervention, will be evaluated. The study will further characterize the allelic spectrum of pathogenic and likely pathogenic variants in IEM-associated genes across diverse Indian ethnic populations, including both previously reported and novel variants. Finally, the feasibility and cost-effectiveness of implementing an integrated multi-omics newborn screening strategy within the Indian public health setting will be systematically assessed.

Data Collection and Management

All study data will be recorded on pre-designed and validated Case Record Forms (CRFs) and concurrently entered into a secure, password-protected, web-based Electronic Data Capture (EDC) system. The EDC platform will incorporate built-in validation features, including range checks, skip logic, and audit trails, to minimise data entry errors and ensure data integrity. Periodic source data verification will be conducted by the Principal Investigator and/or an independent monitor to ensure accuracy and completeness of the recorded data.

The following variables will be systematically collected for each participant: demographic and birth details, including date of birth, sex, gestational age, birth weight, mode of delivery, and place of birth; maternal information such as age, parity, history of consanguinity, prior affected child, and antenatal complications; and dried blood spot (DBS) collection details, including date and time of collection, age at sampling, and pre-collection feeding status. Laboratory data will include first-tier LC–MS/MS screening results (all measured analyte concentrations and calculated ratios), second-tier NGS findings (identified variant(s), ACMG classification, and zygosity), and enzyme assay results where applicable. Additional variables will include the final confirmed diagnosis, clinical evaluation findings, treatment details and compliance, follow-up assessment outcomes, and any reported adverse events.

All data will be stored on encrypted server with access restricted to authorized study personnel. Physical CRFs will be stored at each site. Patient confidentiality will be maintained in compliance with the ICMR National Ethical Guidelines for Biomedical and Health Research Involving Human Participants (2017).

Statistical Analysis Plan

All statistical analyses will be performed using SPSS version 26 or higher (IBM Corp., Armonk, NY, USA). Descriptive statistics will be used to summarise the study variables. Continuous variables will be presented as mean $\pm$ standard deviation (SD) for normally distributed data or as median with interquartile range (IQR) for non-normally distributed data. Categorical variables will be summarised as frequencies and percentages. Statistical linear modeling will be applied to evaluate associations between metabolomic and genomic

variables, followed by data clustering and population stratification analyses to account for potential genetic heterogeneity within the study population. To further explore the underlying biochemical mechanisms, biochemical pathway modeling and simulation will be performed using the Complex Pathway Simulator (COPASI) as described by Hoops et al. (2006). This approach will support the development of an integrated OMICS-based analytical framework, enabling the combined interpretation of tandem mass spectrometry (TMS)-based metabolomic screening results and next-generation sequencing (NGS)-derived genomic data.

Within this framework, the concordance and discordance between TMS metabolomic findings and NGS results will be systematically evaluated for each identified genotype. Genotypes will initially be categorized according to mutation type, zygosity, and inheritance pattern, and subsequently aligned with the corresponding metabolomic profiles and association outcomes. Based on the integrated interpretation of these datasets, a molecular diagnostic classification system will be applied to categorize cases as positive, high risk, low risk, or negative for inborn errors of metabolism. This integrative approach is expected to enhance diagnostic accuracy and improve confidence in clinical decision-making through the combined use of metabolomic and genomic data.

Bioinformatic Analysis:

Next-generation sequencing (NGS) data will be processed using a standardized bioinformatic pipeline. Raw sequencing reads (FASTQ files) will undergo quality assessment using FastQC, followed by alignment to the GRCh38 human reference genome using BWA-MEM2. Variant calling will be performed using the Genome Analysis Toolkit (GATK) HaplotypeCaller, and identified variants will be annotated using established tools such as ANNOVAR and the Variant Effect Predictor (VEP). In silico prediction of variant pathogenicity will be conducted using multiple algorithms, including SIFT, PolyPhen-2, Combined Annotation Dependent Depletion (CADD), and REVEL scores. Variants will be filtered based on a minor allele frequency (MAF) threshold of <1% in the gnomAD South Asian reference population, along with additional prioritization criteria as per standard guidelines. The spectrum and distribution of pathogenic and likely pathogenic variants will be summarized using descriptive frequency tables.

Expected Results

This study is expected to generate significant scientific and public health insights. It will provide the first multicentre, population-based estimates of the birth prevalence of inborn errors of metabolism (IEM) across diverse Indian populations, encompassing multiple geographical regions. The study is anticipated to validate the effectiveness of an integrated two-tier omics-based screening approach, combining LC–MS/MS metabolomics with next-generation sequencing (NGS), demonstrating high diagnostic sensitivity and a substantially reduced false-positive rate compared with metabolomics alone. Furthermore, the implementation of early screening is expected to significantly reduce the diagnostic delay, with the majority of screen-positive cases being confirmed within the neonatal period, thereby minimizing the risk of metabolic crises and associated morbidity.

In addition, the study will enable comprehensive characterization of the genomic variant spectrum associated with IEM across ethnically diverse Indian populations, including the identification of potentially novel variants that may contribute to global variant databases. Preliminary data on clinical outcomes and

neurodevelopmental trajectories of early-diagnosed and treated patients will also be generated, providing a foundation for future health economic evaluations. Collectively, the findings are expected to generate robust evidence to inform national health policy, supporting the integration of expanded OMICS-based newborn screening into existing public health programs such as the Rashtriya Bal Swasthya Karyakram (RBSK).

Discussion

Neonatal screening for inborn errors of metabolism (IEM) is widely recognized as one of the most cost-effective preventive health interventions, with evidence from high-income countries demonstrating cost per quality-adjusted life year (QALY) well below accepted willingness-to-pay thresholds (Grosse, 2015). Yet there are multiple challenges to the universal neonatal screening for IEM in India, among them the limited availability of the necessary infrastructure for centralized laboratory testing, limited access to skilled personnel, a lack of reimbursement mechanisms, and the absence of reference ranges relevant to the local populations.

The proposed study aims to address these challenges by implementing a large-scale screening program covering multiple centers and using diverse methodologies. On the one hand, the inclusion of multiple geographic areas and diverse ethnic groups is bound to generate relevant data regarding the prevalence of IEM in these populations, which will serve to inform future treatment and prevention strategies. On the other hand, the proposed two-step approach covers most of the IEMs that may present themselves in the studied population but employs a battery of tests that are not overly resource- and time-intensive. Thus, the study will utilize a first-line metabolomic screening, which is a standard in the industry, and then employ the next-generation sequencing for confirmatory tests only in the cases where the first-line screening has detected IEM.

Moreover, the study will make use of readily available dried blood spot (DBS) samples for second-tier genomic analyses, which will limit the invasiveness of the procedures necessary for the study. Finally, the study will be of great help in addressing the challenges posed by a lack of relevant population-specific reference ranges for routine biochemical tests for the detection of IEM in India, as it will generate such data.

An important contribution of this research is the establishment of Indian neonatal references for the metabolites analyzed by the researchers using LC-MS/MS. The reason for this study is that current references are based on Western populations and might not be entirely suitable for the Indian population due to genetic, nutritional, and perinatal differences. The study will generate a significant long-term benefit by creating a multicentric Indian reference database, which would improve the accuracy of screening and, as a result, decrease false-positive and false-negative rates in the future. The combined approach of clinical work-up, genomic studies, metabolic management, and long-term outcome assessment is another strength of this research. It will allow the researchers to thoroughly analyze the pathway from initial screening to the final outcome and will provide policymakers with the necessary data to support the national implementation of the newborn screening program. The study will be a substantial basis for the analysis of cost-effectiveness and health economics, which is also crucial for the country.

The strengths of this study include the multicentric and multiethnic design, the inclusion of second-tier genomic testing, standardized laboratory and bioinformatics procedures, and the prospective follow-up with

the structured outcome assessment. The main weaknesses and challenges of the research are the complexity of the logistics of sample transportation, the possibility of patient loss during follow-up procedures, and the interpretation of the results of the NGS analysis of the VUS genes. However, the researchers have developed solutions to address these issues. It is assumed that the implementation of the appropriate cold chain technology will ensure the logistics of sample transportation. Particular attention will be paid to the organization of the follow-up process to prevent patients from being lost. The use of counseling for patients and a carefully organized follow-up procedure will reduce the risk of loss. Multidisciplinary discussion of the VUS genes will facilitate the interpretation of the NGS results. Overall, the study will serve as an excellent basis for assessing the diagnostic performance and clinical impact of OMICS-based expanded newborn screening in India, and it will also help the policymakers in the national implementation of the newborn screening program.

Conclusions

This study protocol outlines a scientifically robust, ethically approved, and operationally feasible multicentric cohort study aimed at implementing expanded newborn screening for inborn errors of metabolism (IEM) in India using an integrated OMICS-based approach, combining first-tier metabolomic screening with second-tier genomic analysis. The study is expected to generate the first comprehensive, multiregional estimates of IEM prevalence among Indian neonates, evaluate the diagnostic performance of the combined metabolomics–genomics strategy, and provide preliminary evidence on clinical outcomes following early diagnosis and intervention. Collectively, the findings will establish a critical evidence base to inform the development and scale-up of a national newborn screening programme for metabolic disorders in India, aligned with international best practices and tailored to the country’s diverse population and healthcare landscape.

Declarations

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Institutional Review Board Statement

The study will be conducted in accordance with the Declaration of Helsinki and approved by the Institutional Ethics Committee (IEC) of Maulana Azad Medical College, New Delhi (Reference No.: F.1/EC/AMAMIC/92/04/2022 /No 134 Date: 24/11/2022), and subsequently by the IECs of all co-investigator institutions. Approvals at all sites were obtained prior to commencement of participant recruitment.

Conflicts of Interest

Sunil Kumar Polipalli, Neerja Gupta, Seema Kapoor, Madhulika Kabra, Amit Kumar Gupta, Hemlata, Ashish Jain, Ramesh Aggarwal, Pallavi Mishra, Bhavna Dhingra Bhan, Mahendra Jain, Ankur Singh, Kamlesh

Palandurkar, Deepak Chawla, Anupa Prasad, Manish Jain, Poonam Verma, Anshu, Sankar V. H., Radhika S, Pankaj Kumar Mohanthy, Tapas Kumar Som, Md Faruq

Declares that they have no conflict of interest.

Author Contribution

All authors contributed equally to this work. As this is a protocol paper, all authors jointly conceived and designed the study, developed the integrated metabolomics and second-tier genomic screening framework, contributed to the multicentric cohort methodology, and participated in drafting, critical revision, and final approval of the manuscript. All authors agree to be accountable for all aspects of the work and approve the submitted version.

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Figures

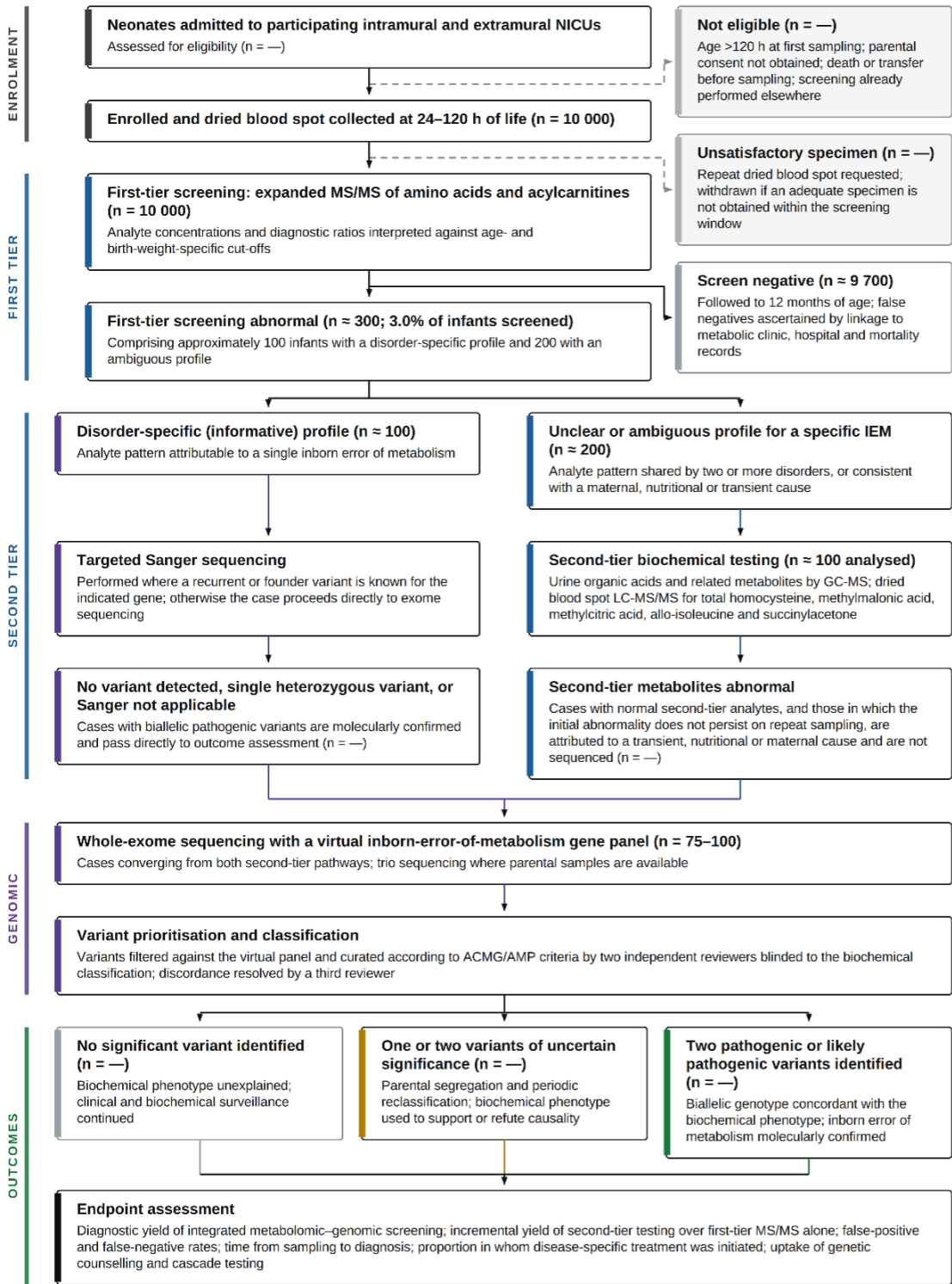


Figure 1

Workflow of the multicentre cohort study for expanded newborn screening integrating metabolomics and genomics with second-tier targeted DNA analysis