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Analysis of genomics implementation in newborn screening for inherited metabolic disorders: an IRDiRC initiative

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Abstract

Since its inception in 1963, newborn screening (NBS) has played a pivotal role in early detection and the establishment of appropriate care for infants and children afflicted with inherited metabolic disorders (IMDs). Despite significant advancements in biomarker identification and metabolomics, current NBS protocols only cover a fraction of known IMDs. The integration of genomics holds promise for expanding the scope of standard NBS, albeit presenting additional challenges. Drawing from the experiences of the authors across three European countries, this article reviews the current landscape of conventional NBS for IMDs and explores the potential integration of genomic tools as a primary screening tier. Recommendations are provided for the seamless transition to genomic NBS, considering factors such as regional birth prevalence differentials, treatability of conditions, and technological capabilities.

Keywords: Newborn screening, inborn errors of metabolism, inherited metabolic disorders, rare diseases, biomarkers, genomics, genome sequencing

INTRODUCTION

The International Rare Diseases Research Consortium (IRDiRC) is a global collaborative initiative on rare diseases research launched in 2011 by the European Commission (EU) and the United States of America National Institutes of Health (NIH) to accomplish the vision to enable all people living with a rare disease to receive an accurate diagnosis, care, and available therapy within one year of coming to medical attention. The Newborn Screening (NBS) Initiative was launched in June 2022 to tackle the increasing interest in NBS for identifying rare diseases at an early stage, but also to highlight the challenges that exist in implementing the screening programs across different regions of the world. While next-generation sequencing (NGS) offers the opportunity to screen for genetic disorders through a single test, there are still challenges in terms of overall cost and accessibility to complex and appropriate equipment.

The remarkable advancements in NGS technology have facilitated the discovery of novel inherited metabolic disorders (IMDs) or inborn errors of metabolism, irrespective of the presence of conventional blood, urine, or cerebrospinal fluid markers. Indeed, the latest International Classification of IMDs (ICIMD) aims to include any primary genetic condition in which alteration of a biochemical pathway is intrinsic to specific biochemical, clinical, and pathophysiological features, regardless of whether laboratory biochemical tests are currently available[1,2]. Rare genetic diseases may go unrecognized for weeks, months, or even years until clinical manifestations are evident, and often when it is too late to establish an adequate outcome with optimal treatment[3]. However, the increasing use of techniques such as metabolomics with tandem mass spectrometry (MS/MS)[4] and NGS has expanded the possibilities of diagnosing patients[5,6]. Still, the complex clinical picture of IMDs, in combination with their rarity, makes the early clinical recognition of these rare conditions challenging[7,8].

The establishment of an IMD diagnosis is generally supported by clinical suspicion and biochemical investigations. Currently, NGS technology has grown as an essential tool for rapid and effective diagnostics even prior to complex functional studies (i.e., enzyme activities). Targeted NGS approaches are currently being implemented in clinical practice, and a clinical exome strategy has facilitated the simultaneous assessment of different IMD phenotypes and the study of undiagnosed clinical problems for which a genetic disease is considered[9,10].

NBS represents a vital public health preventative intervention that allows the early diagnosis of a broad spectrum of genetic diseases. The primary goal of NBS programs is to screen for genetic diseases with the purpose of promptly diagnosing pediatric diseases for which specific effective therapeutic interventions are available. For those IMDs with available treatment (e.g., nutritional, pharmacologic, organ transplant, genetic), pre-symptomatic identification is very beneficial, and this is the main reason for newborn screening. NBS, based on metabolic biomarkers, was initially started with a single amino acid, phenylalanine, for phenylketonuria[11,12]. The resounding success of this first approach resulted in a rapid expansion of the biochemical NBS in dried blood spots (DBS), which has been increasingly introduced in the last few years in many countries as a public health program[13,14]. However, there is a group of IMDs without reliable biochemical markers in DBS (e.g., some urea cycle disorders or citrin deficiency), while other IMDs may need different methods than MS/MS (e.g., galactosemia, biotinidase deficiency), such as

enzyme activities[15]. For the screening of some diseases such as GALT deficiency and lysosomal disorders, the enzyme activities may be measured by MS/MS, but the workflow must be adapted to the individual NBS center[16]. Moreover, in some disorders such as cystinuria and orotic aciduria, the specific biomarker is more accurately analyzed in urine samples, sometimes used to complement DBS. This is further complicated in X-linked conditions, such as Fabry disease, Hunter syndrome, and X-linked adrenoleukodystrophy[17], in which the differentiation between an affected female carrier and a healthy individual may be challenging.

Adding a new disease that needs additional methodology to a national or regional NBS program not only increases cost compared to using current methods, but also uses more of the already limited material of the DBS sample. The actual NBS panorama in Europe is very heterogeneous[18], varying from country to country and even within the same country, such as the case of Spain, where a minimum core of diseases to screen is imposed by the Ministry of Health of the Central Government[19-21]. Still, the 17 autonomous regions, with 20 NBS centers, are free to develop further local implementations of NBS. This situation brings a problem of inequity where, depending on the place of birth, one can have, for example, a positive detection of a fatty acid oxidation disorder or not. Of course, there are justified reasons for the NBS heterogeneity among different European regions depending on the varied frequency of some conditions, for example, in northern countries compared to the Mediterranean ones, but at the same time, it may give rise to inequity of care and may present difficulties with immigrants where it might not always be clear what NBS has been done in a neonate[22-25].

Although conventional NBS is a successful program, it has several limitations, some resulting from the lack of a reliable neonatal biochemical marker, such as Wilson disease, while others being of organizational/administrative, economic, or methodological reasons (e.g., homocysteine for classical homocystinuria is not commonly measured as a first tier despite being the best marker). The goal of any NBS program is to deliver rapid results to enable the initiation of a timely therapeutic strategy that would avoid the appearance of irreversible disease complications. The availability of a rapid NBS result, indeed, depends on various organizational factors, such as the established hours of life for the sample collection, the model of transport to the reference NBS laboratory, and the coordination in the reference center until management by the clinical metabolic team of the newborn tested positive is fully in place. The conventional NBS for metabolic diseases, mainly of the intermediary metabolism (amino acids, organic acids, carbohydrates, and fatty acids), was considered to require that the newborn had taken some food for at least 24 h to avoid false negatives, but this consideration has been found not to be true for every biomarker (especially if ratios are being used)[26,27]. Other known limitations of conventional NBS include preterm infants, parenteral nutrition, transfusions, or metabolic decompensation due to various causes. In some centers, the collection of DBS samples is performed in two steps, across two different days, and even including the collection of a dried urine sample[28].

Since IMDs, by definition, have a genetic origin, there is an ongoing discussion to add other treatable IMDs into NBS, using NGS as the screening test. Genomic testing as a first tier for IMDs has already been introduced in the clinical setting for the rapid diagnosis of severe pediatric conditions in neonates and older children in intensive care units[29], and some pilot studies are underway for the use of genome sequencing techniques for NBS (gNBS)[30-32]. While gNBS may provide a more extensive disease identification[33], and independence from the blood collection timing, there are also inherent specific challenges and controversies, encompassing technical, interpretative, social, ethical, and economic aspects, as well as implications at the healthcare level[34]. Although well known in a diagnostic context (symptomatic newborns), the analytical and clinical validity, sensitivity, and specificity of genome sequencing have not been extensively examined in a screening context, mainly concerning healthy newborns[35]. Furthermore,

even in a well-defined context of conditions selected for the screening, there is the possibility of identifying incidental findings, late-onset conditions, and non-treatable diseases, thereby affecting individuals' autonomy in deciding whether to be informed or in self-determining their future life. In addition, the ownership, custody, and protection of genetic data are still a matter of controversy. Altogether, it raises ethical questions that are difficult to address[8]. All these and other open questions must be carefully pondered before national genomic screening programs are implemented. The most prudent approach to implement, particularly when considering cost containment while evaluating analytical and clinical validity, is using targeted gene panels that screen for treatable conditions[36,37].

This report presents an analysis of the present situation of NBS for IMDs in parts of Europe, including a potential set of treatable IMDs not screened today for the reasons described before. Moreover, this report describes the various NGS approaches, such as targeted gene panels, whole-exome sequencing (WES), and whole-genome sequencing (WGS) with virtual panels, the challenges of including such methods in NBS, and the possible solutions. For practical reasons, we consider WES and WGS, such as "genome sequencing with short reads" (GS), because the concerns regarding these two comprehensive techniques are, to a large degree, similar.

The current situation with conventional biomarker-based NBS (BIO-NBS)

NBS is a public health program that includes a multidisciplinary organization responsible for overseeing the entire screening process, from taking the sample to diagnostic confirmation and referral to appropriate clinical care pathways, improving the quality of the total process based on short and long-term data. Together, this requires a tuned coordination of a flexible and sustainable entire NBS system.

Table 1 lists the conditions included in the conventional NBS programs of three European countries, Spain, Netherlands, and Italy, based on the authors' expertise. As anticipated, the emerging panorama is exceptionally heterogeneous for the list of diseases tested and for technical aspects, such as timing of blood sampling, quantity of blood, and cut-offs used. For example, the age at which the sample is taken may influence the cut-off levels used to classify a sample as abnormal[18].

The purpose of Table 1 is to provide an inventory of IMDs that have at least one informative biomarker to be screened in a population program. Most of these diseases are concurrently screened using tandem MS, referred to as expanded newborn screening. The informative biomarkers commonly used for each disease, along with the second-tier tests that are useful to improve the specificity of the initial screening and the appropriate confirmation methods, are extensively described in the literature and clinical guidelines[26].

In Spain, the official recommendation is to obtain the DBS sample at 24-72 h of life and transport the sample to the NBS laboratory at 3-4 days; the lab result should be ready in < 4 days, so the result of a first sample should be optimally ready in < 10 days after the obtention of the DBS sample, and < 20 days in case of inconclusive results and a second sample is needed for verification[20].

In Netherlands, the time of blood sampling varies from 72 to 168 h after birth, usually after 96 h in combination with the hearing test. Samples are afterward dispatched via regular post to 5 NBS laboratories. For most IMDs, except MMA, PA, MPS1, and ALD, the sample from the child should arrive at the metabolic clinic by day 10 at most.

In Italy, DBS samples are collected at 48-72 h of life and sent to the regional NBS laboratory for the first-tier test via a dedicated transport service that ensures delivery of the samples within 24/48 h of collection and,

Table 1. Different numbers of IMDs included in the conventional NBS in three selected European countries (Spain, Netherlands, and Italy)

Condition	Gene/s	Treatments approved by the European Medicines Agency (not necessarily implying that the treatment is available in a specific country)	NBS-(Andalucía/ Spain)	NBS-Netherlands	NBS-Italy
Biotinidase deficiency	*BTD*	Biotin		X	X
Multiple carboxylase (MCD) Holocarboxylase synthetase deficiency	*HLCS*	Biotin	X	X	X
Classic galactosemia (GALT) deficiency	*GALT*	Galactose free diet		X	X
Galactokinase (GALK) deficiency	*GALK1*	Galactose/lactose-restricted diet		X	
Hyperphenylalaninemia (HPA)	*PAH*	Low phenylalanine diet, tetrahydrobiopterin	X	X	X
Phenylketonuria (PKU)	*PAH*	low phenylalanine diet, pegvaliase, tetrahydrobiopterin	X	X	X
Tetrahydrobiopterin (BH4) deficiency	*GCH1*, *PCBD1*, *PTS*	Tetrahydrobiopterin, levodopa combined with a decarboxylase inhibitor, 5-hydroxytryptophan	X	Tested only if phenylalanine is increased	X
Dihydropterin reductase (DHPR) deficiency	*QDPR*	Tetrahydrobiopterin, levodopa combined with a decarboxylase inhibitor, 5-hydroxytryptophan, low phenylalanine diet	X	X	X
Maple syrup urine disease (MSUD)	*BCKDHA*, *BCKDHB*, *DBT*	Dietary management, thiamine	X	X	X
Branched-chain ketoacid dehydrogenase kinase (BCKDK) deficiency	*BCKDK*	Dietary management, branch chain amino acid supplementation	X		
Tyrosinemia type 1	*FAH*	Nitisinone, low-phenylalanine-tyrosine diet	X	X	X
Tyrosinemia type 2, and type 3	*TAT*	Low-phenylalanine-tyrosine diet	X		X
Argininemia deficiency (ARG1D)	*ARG1*	Protein restriction, liver transplant, sodium benzoate, phenylbutyrate (sodium, glycerol)	X		X
Argininosuccinic aciduria (ASA)	*ASL*	Protein restriction, liver transplant, sodium benzoate, phenylbutyrate (sodium, glycerol)	X		X
Citrullinemia type 1	*ASS1*	Protein restriction, sodium phenylbutyrate, glycerol phenylbutyrate, L-carnitine, liver transplantation	X		X
Citrullinemia type 2	*SLC25A13*	MCT milk and lactose-free milk, lipid-soluble vitamins, and ursodeoxycholic acid. Liver transplantation	X		X
Methionine adenosyltransferase (MAT) deficiency	*MAT1A*	Low methionine diet (some patients)	X		X
Classic Homocystinuria (CBS) deficiency	*CBS*	Vitamin B6 (pyridoxine), methionine-restricted diet, folate, vitamin B12, betaine	X		X
Remethylation defects	*MTHFR*	Betaine, 5-methyltetrahydrofolate	X		X
Glutaric aciduria type 1 (GA1)	*GCDH*	avoid fasting, carnitine, protein-restricted diet, restrict lysine, hydroxylysine, and tryptophan	X	X	X
Isovaleric acidemia (IVA)	*IVD*	Low protein diet, l-carnitine, glycine	X	X	X
3-methylglutaconic acidemia (primary 3-MGA)	*AUH*, *HMGCL*, *CLPB*, *SERAC1*	Low-protein diet, carnitine	X		X
2-methyl butyryl glycinuria (SBCAD)	*ACADSB*	Carnitine (avoidance of valproate)	X		
Methylmalonic acidemia (Mutase deficiency, CblA, CblB)	*MMUT*, *MMAA*, *MMAB*	IM hydroxycobalamin, carnitine, diet, N-carbamylglutamate, liver transplant	X	Intended to find only MMUT	X
Methylmalonic acidemia with homocystinuria (CblC, CblD)	*MMADHC*, *MMACHC*	IM hydroxycobalamin, carnitine, diet, betaine, N-carbamylglutamate, liver transplant	X		X
3-OH-3-methylglutaryl-CoA lyase deficiency (HMG)	*HMGCL*	IV glucose during acute episodes, avoid fasting, carnitine, protein-restricted diet	X	X	X
Beta-ketothiolase deficiency (BKT) Mitochondrial acetoacetyl CoA	*ACAT1*	Avoid fasting, carnitine, riboflavin, protein-restricted diet	X		X

thiolase					
3-methyl crotonyl CoA carboxylase (MCC)	*MCCC1/MCC2*	IV glucose during acute episodes, avoid fasting, carnitine, protein-restricted diet	X	X	X
Propionic acidemia (PA)	*PCCA, PCCB*	Diet, carnitine, biotin, metronidazole, liver transplantation, N-carbamylglutamate	X	X	X
MCAD medium-chain acyl-CoA dehydrogenase (MCAD) deficiency	*ACADM*	Avoid fasting	X	X	X
VLCAD very-long-chain acyl-CoA dehydrogenase deficiency (VLCAD)	*ACADVL*	Avoid fasting, carnitine, restrict LCFA, bezafibrate, triheptanoin	X	X	X
long-chain 3-OH acyl-CoA dehydrogenase deficiency (LCHAD) mitochondrial trifunctional protein (TFP) deficiency	*HADHA, HADHB*	IV glucose during acute episodes, avoid fasting, carnitine, restrict LCFA, bezafibrate, MCT, triheptanoin	X	X	X
carnitine palmitoyl-1 transferase (CPT-1) deficiency	*CPT1A*	Avoid fasting, low-fat and high-carbohydrate diet, MCT, triheptanoin	X	X	X
carnitine palmitoyl-2 transferase (CPT-2) deficiency	*CPT2*	Bezafibrate, high-carbohydrate and low-fat diet, carnitine, MCT, triheptanoin	X		X
carnitine-acylcarnitine translocase (CACT) deficiency	*SLC25A20*	High-carbohydrate and low-fat diet, MCT, frequent feeds, carnitine, avoid fasting, triheptanoin	X		X
short-chain acyl-CoA dehydrogenase (SCAD) deficiency	*ACADS*	As most individuals are asymptomatic, there is no need for treatment but careful follow-up	X		X
Isobutyrylglycinuria	*ACAD8*	As most individuals are asymptomatic, there is no need for treatment but careful follow-up	X		
multiple acyl-CoA dehydrogenase deficiency (MADD)	*ETFDH*	Riboflavin, carnitine, glycine, Coenzyme Q10 supplementation, fat restriction, avoidance of fasting, and a diet rich in carbohydrates	X		X
CUD/CTD (carnitine uptake/carnitine transporter deficiency)	*SLC25A20*	High-carbohydrate and low-fat diet, medium chain triglycerides, frequent feeds, carnitine, avoid fasting, triheptanoin	X		X
X-linked Adrenoleukodystrophy (X-ALD)	*ABCD1*	HSCT, BMT, gene therapy (elivaldogene)	Pilot	X	Pilot
Cystic Fibrosis (CF)	*CFTR*	Ivacaftor, tezacaftor, lumacaftor, pancreatic enzyme, inhaled antibiotics, dornase alfa	X	X	X
Mucopolysaccharidosis type 1 (MPS1)	*IDUA*	ERT: laronidase, BMT, HSCT		X	Regional
Pompe disease (GSD II)	*GAA*	ERTs: avalglucosidase alfa and alglucosidase alfa			Regional
Fabry disease	*GLA*	ERTs: agalsidase alfa, agalsidase beta, pegunigalsidase alpha PCT: migalastat			Regional
Gaucher disease	*GBA*	ERTs: imiglucerase; velaglucerase alfa; and taliglucerase alfa. SRT: miglustat, eliglustat. PCT: Ambroxol			Regional
Metachromatic leukodystrophy (MLD)	*ARSA*	HSCT - BMT, atidarsagene autotemcel (Libmeldy)			Regional

NBS in Spain is very heterogeneous among the autonomous communities and cities. In this table, the case of Andalucía, one of the most populated regions of Spain and the site where one of the authors (RY) is responsible for the NBS, is presented as representative of the different communities of Spain. X: condition included. For Italy, X means performed in all regions of the country. Regional: only in some regions of the country. Pilot: still under evaluation. National NBS in Italy includes 48 conditions by law; national NBS in Spain includes a minimum core of 7 diseases, but a variable expanded NBS is in place in most of the regions, such as Andalucía (35 conditions). In Netherlands, the NBS screening program is standardized across the whole country. ERT: Enzyme replacement therapy; SRT: substrate reduction therapy; PCT: pharmacological chaperone therapy; BMT: bone marrow transplant; HSCT: hematopoietic stem cell transplantation; MCT: medium-chain triglycerides; LCFA: long-chain fatty acids; IV: intravenous; IM: intramuscular.

only in exceptional circumstances, within 72 h, while in other countries, the regular post or regular post with extra care and velocity, called medical post, is being used.

In general, the reference NBS laboratory carries out the first-tier test mainly using the MS/MS method. Minimizing the number of "false positives", i.e., those subjects who are shown positive for the screening test but who are not ill, is relevant in terms of containing healthcare costs and reducing the social impact of a positive test that can have emotional implications for parents. For this purpose, second-tier tests have been developed, always carried out on the same DBS and with a higher specificity, for an initial evaluation of the first screening tests, either positive or negative. Usually, when a result is obtained, a re-test is performed with the same sample to establish its reference range, and if there is divergence, the same test is repeated using another DBS of the same screening sample. The selected second tier test may be performed within the rest of DBS from the original screening test (i.e., amino acids, succinylacetone, total homocysteine, genomic sequencing), or it may be necessary to collect urine (i.e., organic acids, orotic acid, acylglycines) sometimes with a confirmatory second screening sample. Most of the time, the referral is organized after the re-test or the triplicate sample without waiting for the result of the second tier. However, in some cases of vitamin B12 deficiency, the increased level of C3 may not be sufficiently discriminative and it might be wise to wait for the second tier for MMA/PA before referral takes place. In some countries, genetic diagnostic confirmation tests are performed after the second tier or as an alternative to these for disorders such as fatty acid beta-oxidation defects and some organic acidurias. The blood spot is taken from all live births, including live births with subsequent death between 48 and 72 h of life, for which the sampling is carried out peri-mortem, communicating this circumstance to the regional NBS laboratory.

As already described in the introduction, a wide range of IMDs cannot or cannot easily be identified due to a lack of clear biochemical markers in the DBS. Table 2 presents examples of treatable IMDs without a sufficiently robust biochemical marker where NGS is used as a confirmatory test or where NGS as first-tier could be the best option for the screening. The number of diseases in this category heavily depends on several factors, and the complete list is over the scope of this report. Indeed, the list of IMDs beyond the Wilson & Jungner criteria (1968) is influenced by the discussion on treatability versus actionability of the disease, knowledge on the natural course of the disease, ease to differentiate patients presenting early from individuals that may have a later presentation, in which sometimes the definition of "later presentation" is unclear (e.g., presentation after one year, ten years, or as late as adulthood), and also by several other factors.

Present situation with NGS as a first-tier test in NBS

A wide range of pilot studies based on NGS are ongoing or planned around the world to demonstrate the technical feasibility of NGS as a first-tier test for NBS[27,32]. The majority of these studies addresses only a single or a few aspects related to the use of genomic methodologies in NBS, such as technical, interpretative, social, ethical, and economic challenges, to name a few[38,39].

If NGS will be used as the first tier, there is a need to build a database with the NGS data and their biomarkers -if performed - and the conclusion of whether it is a 4-5 genetic variant, or still a VUS or (likely) benign. This will require solutions for long-term storage of data. At present, biomarker-based NBS (Bio-NBS) samples are stored for a minimum of 5 years (following established European ISO regulations). They can be revised in various situations, e.g., when looking for causes of an unexpected death in childhood. In this case, parents or the individuals themselves (if the related data or collected samples are stored for patients older than 16 years of age) should give their consent for data sharing and storage. This process would encourage the construction of large data libraries with NGS information but should be limited to the genetic diseases agreed on before, and it must comply with the bio-bank storage regulations and informed consent specifications for genomic samples.

Table 2. IMDs without a sufficiently robust biochemical marker where NGS may be used as a first-tier test

Condition	Gene/s	Treatment
N-acetylglutamate synthase, NAGS deficiency	*NAGS*	N-carbamylglutamate
Carbamoyl-phosphate synthetase I, CPS1 deficiency	*CPS1*	Protein restriction, citrulline, sodium benzoate, phenylbutyrate, liver transplantation, N-carbamylglutamate
Ornithine transcarbamylase, OTC deficiency	*OTC*	Protein restriction, citrulline, sodium benzoate, phenylbutyrate, glycerol phenylbutyrate, liver transplantation
Carbonic anhydrase VA, CAVA deficiency	*CA5A*	N-carbamylglutamate, limit protein with illness, ensure caloric intake
Delta-1-pyrroline-5-carboxylate dehydrogenase deficiency, Hyperprolinemia type II	*ALDH4A1*	Pyridoxine
Mitochondrial Ornithine transporter 1 (ORNT1) deficiency, HHH syndrome	*SLC25A15*	Low-protein diet, citrulline, arginine
Mitochondrial aspartate glutamate carrier 2, Citrin deficiency	*SLC25A13*	MCT milk and lactose-free milk, lipid-soluble vitamins, and ursodeoxycholic acid. Liver transplantation
Lysinuric protein intolerance (LPI)	*SLC7A7*	Citrulline
Hyperinsulinism-hyperammonemia syndrome	*GLUD1*	Diazoxide, somatostatin analogs, nifedipine, glucagon, IGF-1, glucocorticoids, growth hormone, pancreatic resection, mTOR inhibitors, GLP-1 receptor antagonists, sirolimus
Thiamine transporter 1	*SLC19A2*	Thiamine (vitamin B1), insulin
Biotin-thiamine responsive basal ganglia disease (thiamine transporter 2)	*SLC19A3*	Thiamine (vitamin B1) and biotin
Thiamine metabolism dysfunction syndrome 5	*TPK1*	Thiamine (vitamin B1)
Transcobalamin II deficiency	*TCN2*	Hydroxocobalamin
Brown-Vialetto-van Laere syndrome 1, 2 (Riboflavin transporter deficiency)	*SLC52A3* *SLC52A2*	Riboflavin (vitamin B2)
Manganese transporters	*SLC30A10*, *SLC39A14*	Manganese chelation therapy with EDTA-CaNa2
Magnesium transporters	*SLC41A1*, **ABCC6*, ***ABCG5/ABCG8*	*Etidronate, anti-hypertensive, calcitriol and oral phosphate supplements **Diet low in shellfish sterols and plant sterols, ezetimibe, cholestyramine
Epithelial magnesium transporter deficiency, Hypomagnesemia with secondary hypocalcemia	*TRPM6*	Magnesium
Hypomagnesemia-hypercalciuria-nephrocalcinosis	*CLDN16*, *CLDN19*	Magnesium, thiazide, renal transplant
Bartter syndrome type 1, 2	*SLC12A1*, *KCNJ1*	Sodium chloride, potassium chloride, indomethacin
Calcium transporters and receptors: Autosomal dominant hypocalcemia* Neonatal hyperparathyroidism and familial hypocalciuric hypercalcemia type I**	*SLC25A24*, *SLC25A13*, *SLC8A1*, *SLC8B1*, *SLC30A10*, *SLC24A2*, *CASR*	Thiazide diuretics, calcium, calcitriol* Bisphosphonate, parathyroidectomy, cinacalcet**
Hyperoxaluria type 1 (hepato-renal), type II, type III	*AGXT*, *GRHPR*, *HOGA1*	Lumasiran, pyridoxine, drinking large volumes, alkalinization of urine, pyrophosphate-containing solutions, liver-kidney transplant
Congenital Defects of Glycosylation (CDG)	*ALG1-CDG*, *MPI-CDG*, *PMM2-CDG*, *PGM1-CDG*	Mannose, D-galactose, fucose
mtDNA disorders: Thymidine kinase deficiency	*TK2*	Deoxycytidine (dC) and deoxythymidine (dT)
Molybdenum cofactor deficiency	*MOCS1*, *MOCS2*, *GEPH*	Cyclic pyranopterin monophosphate (*MOCS1*)
Pyrimidine disorders: orotic aciduria, early infantile epileptic encephalopathy-50	*UMPS*, *CAD*	Uridine, triacetyluridine
Wilson disease	*ATP7B*	Zinc, D-penicillamine, trientine
Menkes disease	*ATP7A*	Subcutaneous injections of copper histidine or copper chloride
Cerebral creatine deficiency syndrome 2	*GAMT*	Creatine monohydrate and ornithine supplementation. Arginine restriction
Cerebral creatine deficiency syndrome 3	*GATM*	Creatine monohydrate
RPE65 associated Leber congenital amaurosis, early-onset severe retinal dystrophy	*RPE65*	Gene therapy (Luxturna)

Glut-1 deficiency	*SLC2A1*	Ketogenic diet
Cystinosis (Infantile Nephropathic)	*CTNS*	Cysteamine, Cysteamine slow release, cysteamine eye drops, Carnitine, GH, vit D, phosphate, citrate, kidney transplant
Mucopolysaccharidosis type 1 (MPS1)[#]	*IDUA*[#]	Laronidase ERT, BMT, HSCT[#]
Mucopolysaccharidosis type 2 (MPS2)[#]	*IDS*[#]	Idursulfase ERT, BMT, HSCT[#]
Mucopolysaccharidosis type IVA[#]	*GALNS*[#]	Elosulfase alpha ERT, HSCT[#]
Mucopolysaccharidosis type VI[#]	*ARSB*[#]	Galsulfase ERT, HSCT[#]
Mucopolysaccharidosis type VII[#]	*GUSB*[#]	Mepsevii ERT, HSCT[#]
Pompe disease (GSD type 2)[#]	*GAA*[#]	Avalglucosidase alfa and alglucosidase alfa ERT[#]
Fabry disease[#]	*GLA*[#]	Agalsidase alfa and beta ERT, pegunigalsidase ERT, migalastat PCT[#]
Gaucher disease[#]	*GBA*[#]	ERT: imiglucerase; velaglucerase alfa; and taliglucerase alfa; SRT: miglustat, eliglustat; PCT: Ambroxol[#]
Acid sphingomyelinase deficiency -ASMD1[#]	*SMPD1*[#]	Recombinant human acid sphingomyelinase, ERT: olipudase alfa[#]
Niemann-Pick type C[#]	*NPC1*[#]	SRT: miglustat[#]
Neuronal Ceroid lipofuscinosis (CLN2)[#]	*TPP1*[#]	Cerliponase alfa ERT[#]
Lysosomal Acid lipase deficiency (LALD)[#]	*LIPA*[#]	Sebelipase alfa ERT[#]
Metachromatic leukodystrophy (MLD)[#]	*ARSA*[#]	HSCT, BMT, atidarsagene autotemcel (Libmeldy)[#]
Krabbe disease[#]	*GALC*[#]	HSCT, BMT[#]
Alpha-mannosidosis[#]	*MAN2B1*[#]	Velmanase alfa ERT[#]
AADC deficiency	*DDC*	Pyridoxine/pyridoxal phosphate, folinic acid, dopamine agonists, MAO B inhibitors Gene therapy eladocagene exuparvovec (Intracerebral Upstaza[TM])
PNP deficiency (SCID)	*PNP*	HSCT
ADA deficiency (SCID)	*ADA*	PEG-ADA ERT, HSCT, Gene therapy Strimvelis[TM]
Abetalipoproteinemia	*MTTP*	Vitamin E, A, D
Adenine phosphoribosyltransferase deficiency	*APRT*	Allopurinol
Barth syndrome	*TAZ (Tafazzin)*	Cardiac transplantation, granulocyte colony-stimulating factor (G-CSF), uncooked cornstarch given prior to bedtime, elamipretide
Cerebrotendinous xanthomatosis	*CYP27A1*	Chenodeoxycholic acid (CDCA), cholic acid
Crigler-Najjar syndrome type I	*UGT1A1*	Intensive phototherapy, albumin infusions, and plasma exchanges, liver transplantation, gene therapy (http://clinicaltrials.gov/show/NCT03466463)
Familial chylomicronemia syndrome	*LPL*, *APOC2*, *LMF1*, *APOA5*, *GPIHBP1*	Low-caloric diet, fibrates
Familial hypercholesterolemia	*APOB*, *LDLR*, *PCSK9*	diet, statin, evinacumab, ezetimibe, apheresis, evolocumab, lomitapide
Fructose-1,6-bisphosphatase deficiency	*FBP1*	Restriction of fructose or sucrose, avoid prolonged fasting, IV glucose during metabolic decompensation
Galactokinase deficiency	*GALK1*	Lactose/galactose-restricted diet
Glycogen storage disease type I	*G6PC1* *SLC37A4*	Restriction of fructose or sucrose, avoid prolonged fasting, IV glucose during metabolic decompensation
Glycogen storage disease type III	*AGL*	High-protein diet with corn starch supplementation
Homocystinuria-megaloblastic anemia	*MTTR*, *MTR*	Vitamin B12
Combined immunodeficiency and megaloblastic anemia with or without hyperhomocysteinemia	*MTHFD1*	Hydroxocobalamin, folinic acid, betaine
Primary coenzyme Q10 deficiency	*COQ4*, *COQ6* *COQ2*	Coenzyme Q10
Pyridoxine-dependent epilepsy	*ALDH7A1*	Pyridoxine, lysine-restricted diet, arginine supplementation
Pyridoxamine 5'- phosphate oxidase deficiency	*PNPO*	Pyridoxal 5'-phosphate (PLP) and pyridoxine
Pyridoxal 5'-phosphate binding protein deficiency	*PNPBP*	Pyridoxine (first line) and Pyridoxal 5'-phosphate

X-linked hypophosphatemic rickets	*PHEX*	Phosphate supplementation, active vit D, Burosumab (monoclonal Ab)
Hypophosphatemic rickets with hypercalciuria	*SLC34A3*	Phosphate supplement, active vit D
Hypophosphatasia	*ALPL*	Tissue-nonspecific alkaline phosphatase (TNSALP) ERT - asfotase alfa, avoid bisphosphonates
Congenital serine biosynthesis defects	*PHGDH* *PSAT1* *PSPH*	Serine, glycine
Cerebral folate transport deficiency	*FOLR1*	Folinic acid

This list is not intended to be a comprehensive list of treatable IMDs but just a useful indicator. The degree of evidence of the treatments is variable and may be mutation- or patient-specific. ERT: Enzyme replacement therapy; SRT: substrate reduction therapy; PCT: pharmacological chaperone therapy; BMT: bone marrow transplant; HSCT: hematopoietic stem cell transplantation; MCT: medium-chain triglycerides; IV: intravenous; IM: intramuscular; IGF-I: insulin growth factor-I; GLP-I: glucagon-like peptide-1 receptor; mTOR: mammalian target of rapamycin inhibitors. #indicate list of lysosomal disorders that are presently best screened by MS/MS-based enzyme assay followed by genetic confirmation; */** indicate the relationship of the disease or the gene with the correspondent treatment.

As illustrated in the first part of this review, the analytical and clinical validity, sensitivity, and specificity of genome sequencing have not been extensively examined in a screening context. It is imperative to take into account that the primary beneficiaries of NBS are healthy newborns, thus emphasizing the paramount importance of ensuring the integrity and safety of screening methodologies to safeguard this vulnerable population[40].

Table 3 highlights several practical hurdles that need to be considered and some possibilities to address these challenges.

Two main paths can be envisioned for the evolution and progress of NBS for IMDs: the first could be a progressive and prudent transition, including a consistent period of co-existence and thorough cross-checking of metabolomics and NGS methodologies, from a biochemical profile to genomic confirmation up to therapy, with progressive side-by-side support of conventional NBS and genomics. The traditional Bio-NBS can yield false-positive or false-negative results and is affected by biochemical substrate-level fluctuations. The genomic DNA extracted from dried blood spots can be used for NGS, generating reliable sequencing results, and NGS may function as a second-tier diagnostic test for NBS in samples with abnormal MS/MS results. Most centers use a multigene panel, comprising a library of genes related to the IMDs, for NBS. Genetic testing as the second tier is more or less replacing the present clinical situation toward the screening system. We would like to emphasize that using biochemical and genomic NBS in parallel may increase the sensitivity of the screening and more newborns may be identified, decreasing the number of false positives.

The second path could include genomics as the first-tier test and biochemistry/metabolomics as diagnostic confirmation of the disease before starting treatment. However, gNBS is currently used as the first-tier test only for those disorders not included in the Bio-NBS because of the lack of a reliable biomarker.

The primary objective of NBS is to diagnose pediatric diseases for which effective therapeutic interventions exist, thereby mitigating symptom onset or progression and improving patient prognosis, quality of life, and familial well-being. These interventions aim to avert irreversible damage, including severe physical and cognitive impairments and, in extreme cases, mortality[41-43].

Today, besides endocrine disorders (CH, CAH), hemoglobinopathies, SCID, and Cystic Fibrosis, most NBS programs detect treatable IMDs that are identifiable in the first days of life, mainly with mass

Table 3. Possible concerns (with some possible answers) when introducing NGS as first-tier in NBS

Issue	Biomarker-based NBS (BIO-NBS)	Targeted NGS physical gene panel (tNGS)	WES/WGS (virtual panels of genes)
False negatives	Less and less due to experience with methods +	Will be high if compared to present BIO-NBS, also if compared to WES and WGS. The number of false negatives depends on managing variants of unknown significance (VUS) and the lack of a condition/gene in the panel +++	Will be less high if compared to tNGS but will still be higher (at the start) if compared to present BIO-NBS. The number of false negatives depends on managing VU ++
False positives	Relatively high, especially for some diseases presently included in BIO-NBS Less in NBS labs that perform second-tier testing +/++	Probably fewer if compared to BIO-NBS if reported "only" class 4 and 5 genetic variants +	Depending on the handling of VUS, little if compared to BIO-NBS if reported "only" class 4 and 5 genetic variants, but possibly higher compared to tNGS +/++
Costs	Increasing due to the growing number of diseases included and various methods used +	Higher at this moment if compared to BIO-NBS but decreasing if more diseases are screened for using the same method ++	Still higher if compared to present BIO-NBS, but depending on the number of included genetic diseases, the price per disease or found patients will decrease, as the costs of the method would not be that much different if the number of diseases increases +++/++++
Time to result or turnaround time	For IMDs usually very short (1-2 days after DBS reaches the NBS lab +	Additional 4 days if compared to BIO-NBS ++	Still an additional 4 days if compared to BIO-NBS. When long reads are included, time will decrease to 2-3 days +/++
Need for big data infrastructure	Not that large +	Probably comparable to BIO-NBS +/++	WES: Clearly larger if compared to BIO-NBS and tNGS WGS: Much larger compared to BIO-NBS, tNGS and WES +++/++++

The arguments in the table represent an indicative analysis. A prolonged global experience in gNBS as a first-tier test for IMDs is necessary to give strong evidence to the comments and evaluation presented above. A semiquantitative score (+ to ++++) in support to the text was added (+: light, ++: mild, +++: moderate, ++++: strong).

spectroscopy[44,45]. Other rare diseases with a genetic basis without detectable biochemical markers that can be treated if identified early, before symptoms and irreversible damage, such as spinal muscular atrophy (SMA), are already included in some national NBS programs[46].

NBS are large-scale programs at a population level targeting all newborns, the very most of whom are healthy. The diagnostic context is different since the test may be tailored to the individual patient, considering the clinical manifestations, and numerous clinical and instrumental data may be used to support the diagnosis.

At present, genome sequencing is increasingly used in clinics, especially for diagnosing severely symptomatic pediatric patients hospitalized in intensive care, where the benefits deriving from achieving a timely diagnosis, including the initiation of specific therapies or appropriate clinical management, balance the costs of the test, still high although decreasing steadily[29]. This application of genome sequencing in severely symptomatic newborns for early and timely diagnosis, and the rapid turnaround time of the test has opened the possibility of using these technologies outside of the diagnostic setting in a screening context as a prevention tool. The availability of such powerful genomic tools may shift the concept of "treatability" underlying NBS toward a broader and sometimes more ambiguous concept of "actionability"[47]. This concept introduces some ethical considerations. Screening for diseases that do not have a treatment with

proven efficacy may still bring eventual benefits for patients and their families, for example, decreasing the time to diagnosis and avoiding disease complications. On the other hand, identifying newborns and infants bearing a late-onset condition and predicting a probable later disease manifestation may cause parental anxiety and stigmatize the affected child, but in spite of that, genetic information may help make decisions for the future. The balance between possible future benefits and the psychological hurdle is difficult to achieve[48].

Another aspect to consider is that currently, the interpretation of genetic variants is supported by the deep phenotyping of patients, which is necessary before performing the test, and through reverse phenotyping after the completion of the genomic test, which helps to evaluate the clinical significance of the variants. This essential exchange process between clinic and laboratory, along with the simultaneous analysis of parents (trio), helps to reduce the uncertainties of genomic results (class 3 variant or VUS)[49]. The lack of phenotype in asymptomatic newborns (screening context) makes this exchange process between laboratory and clinic impossible, allowing for reporting only variants with a high probability of pathogenicity (class 4 and 5), increasing the possibility of false negatives in genomic screening. The database will need to be filled with class 4 and 5 variants, but class 3 (VUS) can also be used if they are being controlled with biomarkers (if possible), to help to know whether it can be judged as a class 4 or 5 variant in the future.

This would possibly create confusion even among expert operators in the field, but we want to emphasize the critical importance of careful variant curation, ensuring that only combinations of biallelic variants that are known to be associated with early-onset disease are reported, and that communicating uncertainties or making predictions of a remote future is not necessarily beneficial.

The use of genomic techniques in this target population brings up unprecedented ethical, psychological, and social issues in the field of screening. All these and other open questions must be carefully considered before national genomic screening programs are implemented[30,39,50].

CONCLUSIONS

Recommendations include assessing the accuracy and predictive capacity of gNBS, by cautiously implementing gNBS with a focus on treatable disorders, utilizing second-tier analyses or biomarkers to refine diagnosis and treatment decisions, and establishing protocols for the close monitoring of conditions with uncertain prognostic implications.

A prudent approach would be to start gNBS by including only a combination of (likely) pathogenic DNA variants associated with an established list of treatable disorders. With this approach, some patients will be missed, but it will limit many false positives. Along the way, we will learn how to reclassify the variants from the missed patients who will manifest these disorders.

The evaluation of early-initiated therapies is imperative for determining treatment efficacy, aligning with the criterion of treatability in NBS inclusion criteria. Indeed, one of the classical criteria for including a condition in the NBS is its present treatability, if possible, with strong evidence, to significantly improve the natural history of the disease for most of the patients bearing such genetic variants.

For those conditions with an expected late-onset manifestation or genetic variants without a precise prediction of the disease evolution, we need to carefully discuss whether these should be included and, if included, protocols for a close follow-up of these newborns must be established with the objective of not missing the possibility of initiating a specific treatment before the appearance of irreversible organ/system damage.

Special consideration is necessary for IMDs with a high risk of presenting very early acute symptoms (organic acidurias, urea cycle disorders), even after a few hours of life, so are the sick newborns during the first 3-5 days of life, as they will need a careful clinical evaluation using rapid diagnostics including present metabolic investigations as well as rapid metabolomics and genome sequencing, if possible in a combined effort.

The introduction of gNBS warrants a judicious and phased approach, centralized in experienced reference centers, and characterized by selective gene panels to ensure harmonization and protocolized utilization. Collaborative efforts, including global data sharing, are essential for optimizing screening outcomes and refining screening protocols over time.

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Authors' contributions
Made substantial contributions to the conception and design of the study and performed writing and interpretation: Pintos-Morell G, Iascone M, Casari G, Yahyaoui R, van Karnebeek CDM, van Spronsen FJ
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Conflicts of interest
Pintos-Morell G and van Karnebeek CDM are members of the Diagnostics Scientific Committee of IRDiRC. Tataru EA works at *IRDiRC Scientific Secretariat.* While the other authors have declared that they have no conflicts of interest.

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Not applicable.

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