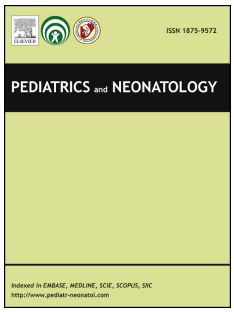

The modern face of newborn screening

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MINIREVIEW

The modern face of newborn screening

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Running title: current NBS

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Abstract

Newborn screening (NBS) has been developed for years to identify newborns with severe but treatable conditions. Taiwan's NBS system, after the initial setup for a total coverage of newborns in 1990s, was later optimized to ensure the timely return of results in infants with abnormal results. Advancements in techniques such as Tandem mass spectrometry enable the screening into a multiplex format and increase the conditions to be screened. Furthermore, advances in therapies, such as enzyme replacement therapy, stem cell transplantation, and gene therapy, significantly expand the needs for newborn screening. Advances in genomics and biomarkers discovery improve the test accuracy with the assistance of second-tier tests, and have the potential to be the first-tier test in the future. Therefore, challenge of NBS now is the knowledge gap, including the evidence of the long-term clinical benefits in large cohorts especially in conditions with new therapies, phenotypic variations and the corresponding management of some screened diseases, and cost-effectiveness of extended NBS programs. A short-term and a long-term follow-up program should be implemented to gather those outcomes better especially in the genomic era. Ethical and psychosocial issues are also potentially encountered frequently. Essential education and better informed consent should be considered fundamental to parallel those new tests into future NBS.

Keywords:

Tandem mass spectrometry, second-tier tests, NGS

Introduction of newborn screening (NBS) and evolution

Newborn screening (NBS) offers the potential for the early detection of severe or life-threatening conditions, best in presymptomatic stages, and proper management to ensure the most benefit to the newborns. Pioneered in the 1960s by Dr. Robert Guthrie, NBS using dried blood spot testing has since been widely adopted worldwide. Many countries now screen for at least one disorder shortly after birth, and more than 30,000 children each year benefit from this life-changing intervention. While these achievements have been recognized as a significant public health success, challenges and opportunities lie ahead. The recommended screening panels may vary among countries. Even in the same country, the equity for diagnosis and intervention resources, especially following the screening, needs improvement. The recent extensive use of genetic screening or diagnosis has begun to affect NBS, serving as an adjunct to traditional biochemical means of detection or even the potential to be the first-tier screening method. Given the time-sensitive conditions, other sampling/testing methods, such as point-of-care testing, are also emerging. Nevertheless, the most essential component of the screening, not limited only to the screening methods, are the treatment, follow-up, long-term outcomes, and effect on the families and society.

Introduction of Taiwan NBS

Taiwan has started pilot NBS for metabolic disorders using dried blood spots for six conditions since 1981[1]. The official NBS program began in 1985 with two screening laboratories, including National Taiwan University Hospital (NTUH) Newborn Screening Center. The initial screening included phenylketonuria (PKU) and congenital hypothyroidism (CH), but homocystinuria, galactosemia, and glucose-6-phosphate dehydrogenase deficiency were soon added. Moreover, screening coverage rose exponentially and exceeded 94% in 1992 and 98% since 1996. A new screening condition, i.e., congenital adrenal hyperplasia (CAH), and a new technique, i.e., tandem mass spectrometry (MS/MS), were first added to the NTUH program in 2001[2] and 2002[3], respectively, and soon were opened to the whole population. The feasibility of screening for more than one condition using a single technology platform dramatically increased the number of conditions amenable to NBS. Therefore, in 2008, the government adopted 11 conditions using five assays, including MS/MS[4], as the recommended panel. In 2019, 10 additional conditions, also generated by MS/MS, were added to the official recommended panel. Specifically, MS/MS alone is known to have high false negatives in screening for *citrin* deficiency[5], carnitine palmitoyltransferase type 2 (CPT2) deficiency, and early-onset

glutaric acidemia type 2 (GA II)[6]. Therefore, second-tier molecular tests, including hotpots analysis of the *SLC25A13* gene[7] and next-generation sequencing (NGS) for genes associated with very long-chain acyl-CoA dehydrogenase deficiency (VLCAD), carnitine palmitoyltransferase type 1 deficiency, CPT2 deficiency, and early-onset GA II, were included in the recommended assays to avoid false negatives caused by the normalization of the markers in the second samples[6]. Newborns who were found to have equivocal results in the first screening will proceed with the second-tier testing, and further confirmation will be only needed for those with positive molecular results.

The benchmarks for the timelines of NBS, which are composed of the collection, transportation, testing, and reporting of the results, were set in 2009 to ensure the overall efficiency of NBS. The recommended screening timelines for high risk babies is to report within 6–8 days of life, including blood sampling at day 3, shipping to the laboratory in 2 days, and reporting in 3 days. In 2008, only 91.5% of newborns met the age requirements for blood sampling, only 65% of the samples met the delivery requirement as 2 days, whereas nearly 100% of reports were available in 3 days. Since 2011, 96% of the newborns had blood sampling 3 days after birth, and 96% of the samples arrived in the labs in 2 days. As a result, >95% of the samples arrived in the labs by 5 days of age, and >98% of the babies had reports by 8 days of age.

After the development of MS/MS[3], the number of screened conditions has increased in Taiwan (Fig. 1). Treatment, including enzyme replacement, stem cell transplant, gene therapy, and genotype-specific therapies, leads to screening for more conditions for effective treatment early in life that could prevent or significantly reduce morbidity and mortality. In addition, advances in screening technology, such as quantitative polymerase chain reaction (qPCR) and NGS, lead to the increase in the number of conditions that are candidates for NBS. Other screening platforms, such as stool card screening for biliary atresia (BA), hearing tests for congenital hearing loss, and pulse oximeters for critical congenital heart disease, also expand the conditions amenable for NBS. In addition, multiplex screening methods further improve the screening accuracy.

Taiwan's experience in NBS contributes to several new conditions to be considered or included in NBS worldwide. Pompe disease, first piloted in 2005 by NTUH NBS center, demonstrates that enzyme screening using Dried blood spot (DBS) in a population is feasible[8]. The following revised algorithms have solved the initial problem of high false-positive rates[9-11]. Babies identified through NBS and had early initiation of therapy gain the most benefits in survival and motor function[12]. In 2013, after the second nomination, Pompe disease was added to RUSP in the United States. Spinal muscular atrophy (SMA), with its first pilot in 2014, demonstrated the

robustness of SMN1 exon7 homozygous deletion as a screening tool[13]. More importantly, the study confirmed the presymptomatic status in babies with SMA[14, 15]. SMA was included in RUSP in 2018 and in many countries[16].

Taiwan also follows or foresees the importance of certain diseases and has initiated screening before or immediately after the addition into RUSP. For example, severe combined immunodeficiency (SCID) was added to RUSP in 2010, when the live nationwide NBS followed pilot screening[17] in Taiwan. In a later report, seven cases of typical SCID, with an incidence of 1 in 131,485 newborns, were identified through screening, and hematopoietic stem cell transplantation was performed in six patients before overt infection occurred, with a survival rate of 100% [17].

Currently, Pompe disease[18, 19], SCID [17], SMA[13], mucopolysaccharidosis (MPS) type I and type II[20-22], X-linked adrenal leukodystrophy (X-ALD)[23], and Gaucher disease[19, 20] are implemented nationwide. However, the methods used and the screening algorithms vary among screening programs[19]. Other conditions such as Fabry disease[20], MPS-IV A[20], MPS-VI[22], Duchene muscular atrophy (DMD)[14], and aromatic L-amino-acid decarboxylase deficiency deficiency[24] are not part of the current process or implemented only partially. Parents of newborns consented and paid for the tests as additional add-on items to the original NBS service.

Current trends in screening methods

Second-tier tests: biomarkers and genomic analysis

A disease-screening performance comes with a tradeoff in cutoffs. Although we are eager to have a screening measure that maximizes sensitivity and specificity for conditions included in the NBS, we regard-favoring sensitivity (e.g., minimizing false negatives) over specificity (e.g., minimizing false positives) as a good strategy. On top of the choice of cutoffs, adding another test helps differentiate true positives from false positives. Furthermore, if the test could be performed on the same samples for the initial screening, the add-on test may markedly reduce the false-positive results and the associated costs and anxiety.

The so-call second-tier test on DBS has been widely used in conditions included in the expanded NBS by MS/MS, such as measurement of 3-OH-propionic and methylmalonic acids (MMA) [25], alloisoleucine[26], and isovalerylglycine[27]. Usually, these measurements apply a rapid liquid chromatography-tandem mass spectrometry (LC-MS/MS) method, and the total analysis time was five min per sample[25]. We studied NBS spots from 536 infants with abnormal propionylcarnitine (C3-carnitine), of whom only 6 (1%) truly had elevated MMA and would have been recalled for additional exams and management. The positive predictive value would have been 100% by adding the second-tier test. Therefore, in 2008, we utilized MMA

as the second-tier test for samples with abnormal C3-carnitine.

Such second-tier biomarker applications also have been used in screening for CAH[28], X-ALD[29], Fabry disease[30], and several lysosomal storage diseases[20, 31]. Although the implementation of these biomarker measurements need extra efforts[32], quantifying these biomarkers from DBS not only serves as a diagnostic marker to increase the accuracy of the screening assay but also provides information on the disease severity on top of the genotype[33]. For example, lysosphingolipids[20], psychosine, heparan sulfate/dermatan sulfate, and sulfatides are used to better stratify patients with Gaucher and Fabry disease, Krabbe disease, MPS, and metachromatic leukodystrophy, respectively.

A second-tier test may be also a direct gene analysis, such as in the screening of cystic fibrosis[34] in samples with abnormal immunoreactive trypsinogen. DBS DNA extraction is widely used, particularly after SCID screening, which measures T cell receptor excision circles (TREC) in DBS. We also developed a second-tier mutation scanning for *citrin* deficiency, with a marginal increment of screening cost but vastly improved screening test performance, which improved the detection incidence rate from 1/32,673 to 1/18,006 after the second-tier test was implemented[7]. Second-tier NGS with an amplicon-based targeted gene panel using the same DBS DNA have been introduced in many conditions, such as in SCID[35, 36], X-ALD[23], DMD[14], Pompe disease[37], other lysosomal storage diseases[38], and several long-chain fatty acid oxidation defects. Whole-exome sequencing (WES), such as in the NBSeq[39] project, is also used in NBS for inborn errors of metabolism (IEM). However, owing to the relatively long turnaround time of these DNA tests, the primary purpose of these second-tier molecular tests is to improve the screening accuracy and reduce diagnostic delay. Second-tier molecular tests should not affect urgent referral or management, such as in amino acidopathy or organic aciduria.

NGS as the first-tier test

The NBSeq[39] project also assesses the possibility of WES as the first-tier for NBS. The authors concluded that WES could reduce false-positive results, facilitate timely case resolution, and in some instances even suggest a more appropriate or specific diagnosis than that initially obtained. However, 12% exome false-negative cases were recorded. In another study, the BabySeq Project[40] also reported discordant results between biochemical NBS and WES NBS and suggested gathering complementary information from the biochemical NBS and WES in the newborn period. Whole-genome sequencing has been tested for NBS, with discordant results[41-43] from the initial NBS. These studies have suggested that relying on WES/WGS alone to detect treatable IEMs, where prompt intervention is critical, would not be advised at this

time because of limitations in variant interpretation or sequencing technology. However, owing to the potential to detect many genetic defects in neonates with severe illness, further optimization are undergoing. The NBS-rWGS describes screening for 388 treatable diseases and reveals that rWGS-based interventions have been started on day five after birth[44]. The Dutch group initiated the NGS-first for the NBS (NGSf4NBS) project[45] to screen for 89 treatable IMDs in 4 days.

Other biomarkers as screening targets

It appears inevitable that a functional biomarker is required for screening as a first-tier or even a second-tier test. Proteins, such as thyroid-stimulating hormone or blood immunoreactive trypsinogen (IRT), are measured quantitatively by single-target immunoassays and have been used as targets for NBS. New methods, including LC-MS/MS, multiple reaction monitoring MS (MRM-MS), and antibody array platforms (such as sandwich, quantitative, and biotin label-based platforms) expand the potential to quantify protein and peptides from dried blood spots. For example, screening for Wilson disease[46] has a long history without success. A recent publication by Dr. Hahn and the group demonstrated the method of quantifying ATP7B peptides and subsequently confirmed the efficacy in assisting the diagnosis of Wilson disease[47]. Another unmet need is NBS for BA. Although Taiwan used the stool color card to start the first national BA screening program[48] and improved the age at the Kasai portoenterostomy to 48 days[49], earlier identification in the newborn period[50] may be possibly further enhance the outcomes. New markers for BA, such as serum matrix metallopeptidase-7[51], could be potential screening markers, and further studies are ongoing.

Challenges of NBS

Definition of cases and management

To treat or not to treat

As a prototype of NBS, PKU met the screening criteria of improving health outcomes through early identification and intervention. Nevertheless, emerging phenotypes and natural disease history are only recently understood. Through population screening especially using the new technology of MS/MS, mild hyperphenylalaninemia (HPA), beyond the target condition PKU, is discovered. After decades of NBS for PKU, infants identified with HPA are now recognized as not required to restrict their diets as severely affected PKU. However, a better understanding of this disorder is still necessary to more safely and appropriately identify, monitor, and manage children with HPA[52].

Similar scenarios could be extracted from screening for CH, MMA, medium-

chain acyl-CoA dehydrogenase deficiency, VLCAD, Pompe disease, SMA, SCID, X-ALD, and others. All these conditions, either due to the improvement in the test performance or newly introduction, need more evidence and a consensus on these patients' clinical care. Pompe disease, having been classified into infantile Pompe disease (IOPD) and late-onset Pompe disease (LOPD) forms, demonstrates the challenges for decision-making. While patients with IOPD benefit from early screening and treatment[53], only one-fifth of the patients who potentially have LOPD developed symptoms after a follow-up of up to 15 years[54]. The rest "GAA deficiency" individuals may remain asymptomatic lifelong. On the other hand, babies even with transient hypothyroidism should be treated base on current evidence[55]. With more evidence collectively, the guidelines for the diagnosis, treatment and follow-up for a specific condition may change over time.

Wait to be patients

The primary target populations for NBS are newborns who have severe diseases and need urgent treatment or management. In PKU, IQ fell progressively roughly by four points for each 4 weeks' delay in starting treatment[56]. In IOPD, those who initiated treatment immediately after birth have the best motor outcomes in addition to the survival advantage[12, 53]. In SMA, those who received therapy pre-symptomatically have the best gross motor and oromotor functions[57-59]. However, the very challenging situation is whether a wait-and-see strategy is justified in patients such as those with SMA or X-ALD. A consensus in the SMA treatment is made about early treatment initiation in patients with 2 or 3 copies of SMN2[60, 61], with a high risk of developing type I or II SMA. However, no consensus is established yet for patients with four copies of SMN2[62], who present a more diverse disease onset age, from the first 2 years of age[63] to asymptomatic in adulthood. Research is urgently needed to understand the markers better to predict disease onset and favor a proactive approach, i.e., to treat the subgroup of patients before symptoms emerge.

Education and follow-ups

The long-term follow-up project is inevitable needed. There is no consensus about when to start the treatment for LOPD. According to the survey of genetic health care practitioners[64], over half (53.9%) experienced difficulties in providing care to patients at risk of LOPD, including the time to initiate treatments and communication with the families. Parents of children at risk of LOPD expressed uncertainty and fear of the unknown initially[65] but could shift to reassurance with time, education, and vigilance with management[66]. Additional education for specialists and the creation of evidence-based disease guidelines are urgently

needed to provide better care for patients with conditions of variable onset, especially those with potentially late-onset forms that will manifest far beyond the newborn period. Given the era of genomics, an increasing number of "actionable" diseases could be screened, producing increased burden elucidating the scope of management in each condition.

Economic assessments on NBS

Because NBS includes public health and clinical care, another essential question is whether the condition fits "an important determinant of population health," although the criteria have been revised in the 2008 WHO criteria[67]. Recently, economic evaluation (EE), such as cost-effectiveness and cost–benefit analysis, is used for evidence-based decision-making in healthcare resource allocation. PKU and CH are universally prioritized in NBS. Most EE studies, although with variable quality[68, 69], have shown that NBS for PKU and CH provides a net gain, i.e., benefits not only individuals and their families but also financially benefit the government, community, as well as taxpayers[70]. Besides the unification of methodologies and improvement, more importantly, understanding the disease, including the health status and outcomes and the unscreened comparators, is critical to the robustness of the results.

The new screening technology and, therefore, the implementation of the NBS system to a new disease are reflected in the increment of screening cost. The EE of NBS for IEM using MS/MS returned a non-robust conclusion, with only cost-saving in PKU and MCAD deficiency combined[71], cost-saving to screening all conditions together[72], to not cost-effective[73]. However, because of the multiplex nature of MS/MS, once a MS/MS screening system is established, adding a test to the existing system only needs a marginal cost and is therefore likely to be cost-saving. A similar assumption was made to WES/WGS. Moreover, the screening cost could be equally divided into many conditions, potentially cost-saving given the accumulated incidence from many rare diseases.

The advances in treatment options, such as enzyme replacement therapy or gene therapies, may introduce economic assessment challenges further. For example, a study of Pompe disease, a disease that needs long-term enzyme replacement therapy, concluded that NBS presents substantial health gains for individuals but with additional costs[74]. On the contrary, NBS for SMA, a disease with available disease-modifying therapies, including gene therapy, versus later symptomatic treatment, is cost-effective use of resources[75, 76]. Timely pre-symptom treatment increases patients' chances for normal motor milestone development, and the strategy appropriately illustrates the value of NBS in improving health outcomes

and cost-saving.

Ethics and Psychosocial Issues

The informed consent process of NBS, if it exists, may be inadequate. In the revised WHO criteria[67], the program should ensure informed choice, confidentiality, and autonomy. Education about NBS is mostly conducted prenatally and perinatally, whereas parents-to-be may not consider seriously these rare diseases. The consent process is usually arranged at the perinatal period when most parents are excited about the new baby. Taking the potential benefit from knowing a severe, urgent, and treatable disease, most parents would consent without fully understanding the disease phenotypes and interpreting the tests for individual conditions. Therefore, parent reaction to NBS results, communication of such results to families, and change in the relationship with their child must be elaborated further and prepared proactively.

Although the overall benefits of screening should outweigh the harm, it needs to point out about the potential harms coupling with screening. Although parents of children with false-positive results and children diagnosed with a condition supported routine NBS[77], they may have a higher degree of anxiety or health-related uncertainty[78], and the parent–child relationship may be affected. Therefore, specifying the test is important to minimize false positives. In addition, educational and psychosocial support are warranted for those families. Studies have also revealed difficulties in informing children of their carrier status and communicating those genetic results to family members[77]. More demands for individualized genetic counseling are expected after genetic NBS, especially WES/WGS.

Furthermore, the overwhelming information from WES/WGS, with many uncertainties, may exaggerate the anxiety and tension of parents and their children. Although all parents anticipate values obtained through genomic screening[79, 80], such concerns may decrease parents' support for genomic NBS[79] and break the health equity. Therefore, those concerns should be carefully regarded and tackled properly, and appropriate solutions, such as informed consent reporting only those actionable variants, should be adapted into the genome sequencing requirement.

In conclusion, advances in genomic, technological, and therapies have contributed to the proliferation of NBS-amenable conditions. We must understand the significant knowledge gap for NBS, such as formal evidence of the long-term clinical benefits in large cohorts especially in conditions with new therapies, phenotypic variations and the corresponding management of some screened diseases, and cost-effectiveness of extended NBS programs. Implementation of short-

term and long-term follow-up programs is important to better gather those outcomes. Ethical and psychosocial issues are also potentially encountered more frequently in the genomic era. Essential education and informed consent should be fundamental to parallel those new tests into future NBS.

Fig. 1. Milestones of Taiwan newborn screening in National Taiwan University Hospital NBS center.

AADC, aromatic L-amino acid decarboxylase deficiency; CAH, congenital adrenal hyperplasia; G6PD, glucose-6-phosphate dehydrogenase; GD, Gaucher disease; MMA, methylmalonic acidemia; MPS, mucopolysaccharidosis; MS/MS, tandem mass spectrometry screening; SCID, severe combined immunodeficiency; SMA-ALD, adrenoleukodystrophy

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