Genomics in Newborn Screening

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“For in much wisdom is much vexation, and he who increases knowledge increases sorrow”

-Ecclesiastes 1:18

Newborn screening has substantially changed the genetic-metabolic world and greatly expanded the concept of preventive medicine. This expansion has been marked by two major milestones in the 50-year history of newborn screening: the first, pre-tandem mass spectrometry, included the early detection of phenylketonuria (PKU), galactosemia, homocystinuria, maple syrup urine disease, congenital hypothyroidism, congenital adrenal hyperplasia, sickle cell disease, and biotinidase deficiency; the second, tandem mass spectrometry-based, has seen an explosive increase in information, often instrumental for diagnosis, prevention, and appropriate management of many additional metabolic disorders including the organic acidemias and fatty acid oxidation defects not previously covered. The latter era, however, has also had its share of shortcomings and pitfalls, much of which related to inconclusive diagnosis and incomplete knowledge of natural history.1-3 Determining the precise disorder in the identified infant is critical to his/her proper clinical care and treatment as well as to providing accurate information and genetic counseling to the family.

There are several possibilities for making a definitive diagnosis. In some diseases, such as tyrosinemia type I, there is a specific analyte, succinylacetone, which defines the disorder. In other disorders, represented most frequently by PKU, the metabolite profile is so abnormal and so characteristic that there is virtually no doubt as to the diagnosis. Many other disorders now included in newborn screening, however, require a determination of clearly reduced activity of the relevant enzyme or finding two pathogenic mutations in the gene that encodes the enzyme to unequivocally establish the diagnosis.4 Proving reduced enzyme activity can be considered the gold standard but enzyme assays may require tissue not readily accessible or assays not widely available or that sometimes yield equivocal results. Determining the mutations (genotyping) is more widely available and easier to perform but its role has not been clearly formulated. The limited genotyping for 1 or only a very few mutations known to be frequent in a disorder has been implemented in the newborn screening laboratory as a second-tier test for 2 or perhaps 3 disorders. In even fewer instances, second-tier testing might allow immediate confirmation of a disorder and suggest its likely clinical effect before the identified infant receives medical evaluation.4 Even among these few disorders, however, second-tier testing covers only the most frequent mutation(s), so evaluating the identified infant may require further examination of the gene of interest.

Although genotyping in newborn screening is currently confined to confirming the disorder suggested by the newborn screen, serious consideration is being given to examining the potential application of genomic sequencing to newborn screening itself. This consideration has emerged because of a new technology referred to as next-generation sequencing (NGS) whereby the entire sequence or a significant portion of the sequence of DNA in a newborn screening specimen could be determined. NGS could dramatically increase the number of disorders identified by newborn screening as well as identify genetic variations that indicate risk of the infant (and, by extension, family members) for subsequent development of many more disorders. Sequencing of this nature could also enhance the reliability of the confirmatory process. Although these could be great advantages, NGS in newborn screening raises many questions and very serious potential problems.

In this review we will discuss the current status of genomics in newborn screening, including the new DNA sequencing technology, its applications and limitations, as well as the inevitable clinical, ethical, and psychosocial challenges it poses when applied to newborn screening.

Current Newborn Screening

Until 2006, newborn screening varied widely among states, thus depriving many families of its benefits. Concern about this inequality led the American College of Medical Genetics (ACMG), with a commission from the Maternal and Child Health Bureau of Health Resources and Services Administration, to recommend a uniform panel of conditions for inclusion in state newborn screening programs. Although the newborn screening panel is selected by the state rather than nationally, the recommended uniform panel is now

ACMG American College of Medical Genetics
NGS Next-generation sequencing
PCR Polymerase chain reaction
PKU Phenylketonuria
VLCAD Very long chain acyl-CoA dehydrogenase
VUS Variants of unknown significance
WES Whole exome sequencing
WGS Whole genome sequencing

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employed throughout the US. It includes 29 mandatory conditions and an additional 25 conditions, which are part of the differential diagnosis of a condition in the core panel, or represent incidental findings for which there is potential clinical significance. These conditions are listed in the ACMG website and described elsewhere.

Problems of Follow-Up in Newborn Screening

Some children identified in newborn screening do not have a defined diagnosis even after confirmatory testing with standard metabolic assays. In the infant with a fatty acid oxidation disorder, the acylcarnitine profile, which was abnormal in the newborn screening specimen may have normalized or become equivocal when the infant was clinically evaluated. In addition, overlap may occur in the metabolic profiles of several disorders delaying establishment of the correct diagnosis. This uncertainty can have a significant negative impact on the family. In addition, screening programs aim at identifying all affected infants (ie, avoiding missing an infant, a false negative result) while tolerating only an acceptable number of false positive results. This tension is exacerbated by the desire to identify not only infants with the potential for becoming severely affected but even those mildly affected whose newborn screening findings often overlap those of unaffected infants.

An example is very long chain acyl-CoA dehydrogenase (VLCAD) deficiency, a fatty acid oxidation disorder relatively frequent among the disorders identified by expanded newborn screening, which has a severe phenotype characterized by cardiomyopathy, skeletal myopathy, liver disease, and the possibility of sudden death, but also has a very mild, possibly benign phenotype. Because the majority of newborns identified with VLCAD deficiency have remained asymptomatic through their childhood years, it is likely that they have this mild form. However, it can be difficult to differentiate the occasional neonate with the potentially severe phenotype from the neonate who may remain asymptomatic throughout life. This difficulty applies to the spectrum of all disorders detected by newborn screening, not only those metabolic but also to others such as congenital hypothyroidism and cystic fibrosis.

To be as diagnostically precise as possible and develop some information about likely prognosis, several methods are used for medical evaluation of the infant’s screening finding. These include biochemical analyses, enzyme activity measurements, and genotyping. When an enzyme assay is not available or is very difficult to obtain, genotyping is critical for defining the metabolic disorder.

Genotyping for Follow-Up of Newborn Screening

Genotyping Methodologies

Following are brief descriptions of the genotyping methodologies currently or potentially used in relation to newborn screening. These methodologies are summarized in the Table. Detailed descriptions of the techniques are beyond the scope of this article.

Targeted Genotyping in Second-Tier Newborn Screening and Family Studies. When an analyte abnormality is detected in newborn screening, a frequent mutation known to be associated with the disorder may be sought by targeted mutation analysis in the newborn screening laboratory using the newborn screening specimen. This is known as second-tier testing and is most often employed to follow an abnormal initial screening result for cystic fibrosis, galactosemia, and medium chain acyl-CoA dehydrogenase deficiency. The method of choice is polymerase chain reaction (PCR) amplification of the portion of the infant’s respective gene (exon) in which a known mutation resides and then applying a specific mutation detection method or hybridization technique (allele-specific oligonucleotide hybridization) to detect the mutation in the infant. The purpose of the second-tier test is to reduce the frequency of false positive results as well as provide the newborn screening program and the clinician with a sense of the urgency for clinical evaluation when urgency is required. This methodology is also employed for a family study when the proband’s genotype is known.

Sanger Sequencing of Exons and Exon-Flanking Regions of a Gene. When the infant identified by newborn screening is evaluated, confirmatory testing in DNA obtained from a venous blood specimen may include sequencing the gene of interest or a critical portion of the gene. This involves PCR amplification of all exons and exon-flanking regions of the gene associated with the suspected disease and

<table>
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<td>Sequences gene to find mutations</td>
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ASO, allele-specific oligonucleotide.
bidirectional Sanger (dideoxy-chain termination) sequencing of the PCR products. Depending on the gene, this can lead to the detection of up to 95% of the disease-causing mutations (sometimes considerably less), but is laborious with a turnaround time of 4-6 weeks and a cost of up to several thousand dollars per gene.

**NGS.** NGS is the newest method of DNA sequencing. It is currently used to sequence large gene panels, the exome (the sum of all exons in the human genome whole exome sequencing [WES]) or even the entire genome (whole genome sequencing [WGS]). In contrast to Sanger sequencing, where genes are sequenced one at a time, NGS is massive parallel sequencing in which all of the exons of large panels of genes or even much more of the genome is sequenced in a single procedure. For this purpose, it is faster and more efficient than sequencing each of the genes individually. It is widely used in research laboratories for a variety of investigational purposes and is increasingly being adopted by clinical laboratories for genetic diagnosis. It may one day be added as a detection method to the newborn screening panel and is already in limited use for confirmation of a disorder.

One question will be whether to use WES or WGS. WES examines only about 2% of a genome but covers the known coding regions (the exome) (ie, the sequences that are expressed as proteins), and their adjacent sequences. Although the exome is also covered by WGS, WES provides better sequencing coverage of the coding regions and is superior to WGS in finding DNA changes of known medical significance.18 By covering the genome, however, WGS identifies not only variations in the coding regions but also sequence variations in non-coding regions that may alter the expression of a gene, substantially increasing the likelihood of genetic diagnosis. In addition, WGS requires less upfront laboratory work because, unlike WES, it does not require capture and enrichment of the exons, but the greater amount of raw data generated adds time for analysis. In both sequencing approaches, however, as in all sequencing approaches, differentiating disorder-related mutations from insignificant variations is a major problem even in the known coding regions of genes, and in WGS represents an even greater challenge in the non-coding parts of the genome where function is not yet clearly delineated. Thus, the clinical significance of the majority of the over 3 million genome variants and 50,000 exome variants that may be detected by NGS cannot presently be interpreted with certainty.

In time, technological and interpretive progress along with a dramatic reduction in cost may result in WGS being added to the arsenal of tests available for making a genetic diagnosis. WGS seems to ultimately promise a better opportunity for DNA diagnosis, wherein a single laboratory test can focus on a single variant, single gene, panel of genes, or the exome, and also expand the analysis as needed to cover the entire genome once all of the analytical challenges have been resolved.

**Importance of Genotyping for Follow-Up of Newborn Screening**

Sequencing the respective gene as part of the confirmatory process can be critical in establishing the presence of a disorder. Identifying the mutations may also yield important information concerning severity of the defect and likely prognosis.19,20 Notable among these disorders are PKU and other causes of hyperphenylalaninemia,21 VLCAD deficiency,14 isovaleric acidemia,19 and combined methylmalonic acidemia and homocystinuria attributable to cobalamin C disorder.20 In all of these disorders, gene mutations have been identified that correlate with severe forms or with mild or benign variants of the disorders. Even in cystic fibrosis, certain mutations are associated with a definite and likely severe disease (eg, F508del) and others with milder disease.16 Consequently, it is important that genotyping for this purpose is widely available, reliable, and affordable. Centralization of genotyping in a few laboratories would address these needs.

**Limitations of Genotype Interpretation**

Although the sensitivity of gene sequencing can be over 95% for detection of nucleotide changes and small deletions or insertions in a specific gene, and can be further enhanced by array comparative genomic hybridization analysis (often referred to as “microarray”) to detect deletions or duplications, some inherent analytical and post-analytical limitations are inevitable. Current analytical limitations include amplification-resistant gene regions and pathogenic changes in parts of the gene not included in standard gene sequencing, such as the promoter region, deep intronic regions (ie, not in flanking intron sequences), or other non-coding regions.22 Beyond sequencing, interpretation of the results is critical. Although genotyping does yield definite pathogenic mutations, the functional significance of many sequence variants that are found when comparing a sequence with reference sequences is not clear and requires much more sequencing experience in association with clinical follow-up. Patients often have private or novel mutations (a mutation not previously observed in an affected individual), which lead to uncertainty about the effect that the mutation might have on the function of the respective protein and, therefore, the phenotype. These changes are reported as variants of unknown significance (VUS). They may be either benign sequence variations or functional mutations and a number of algorithms are used by DNA diagnostic labs to decide between the two possibilities but often a decision cannot be reached with certainty. Finally, even though genotype–phenotype correlation may be very helpful in prognosis and management of the infant (as discussed above), it is critically important to recognize that in many diseases the genotype may not correlate with a particular phenotype.3

**Genomic Sequencing for Expansion of Newborn Screening**

On occasion, specific genotyping has been successfully included in routine newborn screening in certain populations with a high frequency of a disorder and of a specific
mutation or a set of specific mutations for the disorder. The development of NGS, however, has presented the possibility of sequencing the exome or the entire genome in the newborn screening specimen. This could be directly applied to the DNA in the newborn screening specimen in the same way that targeted genotyping is now performed in second-tier testing. The most obvious advantage would be the possibility of identifying virtually any metabolic and non-metabolic genetic disorder in the newborn (ie, genetic screening). The additional information generated could extend newborn screening into personalized medicine, detecting genetic variations that would identify the newborns (and by extension family members) who are at risk for later onset disorders such as cancer, Alzheimer disease, Parkinsonism, hypercholesterolemia, diabetes, and many others. The foray of newborn screening into personalized medicine, however, would be extremely contentious and its potential ethical and practical problems would have to be very seriously considered.

Presently, the cost of genomic sequencing is much too high for application to newborn screening but is rapidly decreasing and, in the near future, may approach the point at which it could be used for newborn screening (Figure). Turnaround time for NGS would have to be substantially decreased to meet the 2-3 days required for newborn screening. Currently, it takes weeks to report the results of NGS (Table). In addition, it will have to be shown that NGS can be reliably performed with DNA extracted from the dried blood specimen necessary for newborn screening. Beyond these requirements, a major challenge would be the bioinformatics required, analyzing the vast amount of data in the context of rare or novel nucleotide changes. The number of VUS alone that would likely be detected by WES or WGS even when focusing on known disease genes poses a potentially major interpretive problem. Nevertheless, with the current rapid progress in determining the function of each element in the human genome, the significance of many VUS may soon be determined. Furthermore, the foreseeable flood of genomic information could be addressed by using powerful bioinformatic tools filtering for the top statistically and biologically relevant findings, thus eliminating the laborious time required to analyze such an extensive amount of potentially abnormal findings.

Challenges
Several additional challenges require resolution before NGS could be applied to newborn screening. Even if the bioinformatics and turnaround time challenges were addressed, the very formidable challenge of interpreting the clinical implications of the data would remain. Approximately a decade after sequencing of the human genome, it has become obvious that there is much more to know beyond the raw genetic sequence, and this interpretive limitation is particularly applicable to newborn screening. Although the DNA sequence does not necessarily predict the clinical course, genetic newborn screening would very likely expand the phenotypes (mostly to the milder spectrum) of all the identified genetic diseases. This has occurred throughout newborn screening, particularly with expanded screening using tandem mass spectrometry technology. The explosion in genetic information from newborn screening could be misleading to some extent and potentially even harmful with the likelihood that some medical decisions and interventions would be made based on limited and perhaps even incorrect interpretation. This could be exacerbated by the newborn screening paradigm to treat before the infant becomes symptomatic, so a physician may never know if the disease manifestations were prevented or if treatment was unnecessary. Thus, communicating the information and formulating management plans would be an enormous challenge and not enough genetic professionals are currently being trained to provide the needed services. The lack of clarity in understanding clinical significance could profoundly complicate the process of extending newborn screening to medical management for both the clinical geneticist and the patient’s family. The additional testing and follow-up that would likely be required could add substantial additional costs to the already high cost of healthcare in the US.

To complicate the picture further, over the last decade a new concept of complex genetic trait, synergistic heterozygosity, has come to attention. This trait posits that multiple partial enzyme deficiencies may lead to biochemical and clinical consequences. These patients would be heterozygous carriers for mutations in multiple genes for proteins located in functionally related pathways, which synergistically could result in a physiologically relevant insufficiency. Importantly, individuals in general are more likely to be carriers for such
mutations at two or more genes than homozygous for mutations at any one gene.37

**Ethical and Practical Considerations**

Although genetic screening could potentially become a tool for greatly expanding newborn screening into presymptomatic detection of many early onset and perhaps some later onset treatable disorders as well as disorders that may not be treatable but could be important to the family, a number of ethical and practical issues pose major challenges to the process. For instance, it is likely that every newborn sequenced will be a carrier for several severe diseases and be at above-average risk for several other disorders.25 Questions would include how much of this information should be reported and whether this information can be adequately explained to the parents and incorporated into the medical care of the infant. Currently, geneticists do not test asymptomatic minors for genetic conditions to avoid burdening a child with information the child is unable to choose or is not wanted, particularly for conditions with onset in adulthood. Performing genome sequencing on every newborn could burden every child and every family with knowledge about the distant future they may not be ready or willing to face.

In almost all states newborn screening is currently a public health mandate and informed consent is not required. However, genetic testing in a clinical setting usually requires informed consent. Thus, genetic screening of the exome or genome of a newborn would likely require informed consent, necessitating a lengthy and medically complex explanation of its potential benefits and disadvantages. Not only would this inevitably be limited in accuracy, given the rapid changes in our understanding of the link between many genetic variations and risk of disease, but under the best of conditions would be difficult for parents, medical personnel, and even most physicians to comprehend. If genetic screening were to become inextricably linked to newborn screening and some parents refuse consent, newborn screening would become less than universal with the likelihood of tragic consequences to children born with PKU or congenital hypothyroidism or other treatable disorders currently identified. Of course, genetic screening could be offered as a supplement to the current routine newborn screening with an opt out provision as was expanded newborn screening by tandem mass spectrometry in its beginning.38 Informed consent would be required for the supplement but current universal newborn screening would be preserved.

When we try to judge objectively the ethical aspects of a new intervention it is important to consider and apply the four principles of biomedical ethics: beneficence, autonomy, non-maleficence, and justice.39 As mentioned, many difficulties associated with genetic newborn screening bear not only clinical but also ethical significance. Even though newborn screening itself might raise questions about all of these principles, genomic screening would magnify these issues. The introduction of a genomic technology into newborn screening might be considered consistent with beneficence, but the other principles are especially at issue when we consider the carriers, indeterminate results, and the likelihood of over-diagnosis because of clinically insignificant findings that are certain to be generated.15,40 In addition, with genomic screening, there might be the indirect consequences of insurance- and socially-related issues not only to the infant but to other members of the family as well. Despite federal protection through the Genetic Information Nondiscrimination Act, consequences could nevertheless occur. Beyond these complex issues would be the overwhelming burden of medical follow-up and counseling.40,41 Highly selective reporting of the findings would greatly reduce the magnitude of the burden,33 but would likely be an issue in terms of the rights of the family to be fully informed.

**Conclusion**

Genotyping benefits newborn screening, both in the screening program and in medical evaluation and follow-up. The rapid advances in genomic sequencing promise to greatly expand these benefits. These advances could also lead to applying sequencing to the entire genome or at least the exome in newborn screening. Before such an application, however, much more needs to be learned about function within the genome and the relationship between function and phenotype. In addition, procedural and ethical considerations would also need to be addressed and the preservation of newborn screening as a means of preventing devastating disease would have to be assured. Genomic sequencing is clearly not currently applicable to newborn screening and a recent policy statement of the ACMG has opposed its use as a first-tier approach to newborn screening.42 Nevertheless, it might be ready for application to newborn screening in the future.

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