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Newborn Screening for Krabbe's Disease

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Abstract

Live newborn screening for Krabbe's disease (KD) was initiated in New York on August 7, 2006, and started in Missouri in August, 2012. As of August 7, 2015, nearly 2.5 million infants had been screened, and 443 (0.018%) infants had been referred for followup clinical evaluation; only five infants had been determined to have KD. As of August, 2015, the combined incidence of infantile KD in New York and Missouri is ~1 per 500,000; however, patients who develop later-onset forms of KD may still emerge. This Review provides an overview of the processes used to develop the screening and followup algorithms. It also includes updated results from screening and discussion of observations, lessons learned, and suggested areas for improvement that will reduce referral rates and the number of infants defined as at risk for later-onset forms of KD. Although current treatment options for infants with early-infantile Krabbe's disease are not curative, over time treatment options should improve; in the meantime, it is essential to evaluate the lessons learned and to ensure that screening is completed in the best possible manner until these improvements can be realized.

Keywords

GALC; galactocerebrosidase; tandem mass spectrometry; lysosomal storage disorder

OVERVIEW OF KRABBE'S DISEASE

Newborn screening (NBS) for Krabbe's disease (KD) was initiated in New York, New York, on August 7, 2006 (Orsini et al., 2016). KD is both a leukodystrophy, affecting white matter of the central and peripheral nervous systems, and a lysosomal storage disorder (LSD). The

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CONFLICT OF INTEREST STATEMENT

J.J. Orsini, C.A. Saavedra-Matiz, and M. Caggana declare that they have no conflicts of interest with regard to publication of this article. M.H. Gelb is a consultant with PerkinElmer and is also working with PerkinElmer to develop assay reagents and kits for newborn screening of LSDs.

ROLE OF AUTHORS

JJO, CAS-M, and MC had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. Study concept and design: JJO, CAS-M, MC, MHG. Acquisition of data: JJO, CAS-M, MC. Analysis and interpretation of data: JJO, CAS-M, MC. Drafting of the manuscript: JJO, MHG. Critical revision of the Review for important intellectual content: MC, MHG. Study supervision: JJO, CAS-M, MC.

disease is caused by a deficiency of galactosylceramidase (GALC), and it primarily affects babies in their first year of life, with approximately 80–90% of patients having the severe infantile form of the disease (Duffner et al., 2009a; Wenger et al., 2013). Later-onset forms are thought to be less common, making up approximately 10–20% of the diagnosed cases; however, later-onset forms of disease may be underdiagnosed (Orsini et al., 2016). The adult forms of the disorder progress slowly; the oldest patient reported to have been diagnosed was in her seventh decade of life (Hedley-Whyte et al., 1998; Wenger et al., 2013). Although rare, there are cases of KD with symptoms appearing in early childhood, followed by rapid progression, leading to death within a year of the first symptoms (Duffner et al., 2012a). The most recent estimate of incidence of KD in the United States is 1 in 250,000 births (Barczykowski et al., 2012). Prior to NBS, incidence rates in the United States were estimated to be 1 in 100,000 in individuals with northern European ancestry (Wenger et al., 2013). Based on combined NBS results for New York and Missouri, the incidence of the early-onset forms of KD appears to be a much lower 1 in 500,000.

Infants with early-infantile KD (EIKD) appear healthy at birth. However, pronounced irritability and twitching may appear soon thereafter; vomiting, feeding difficulties, and gastroesophageal reflux can also be present, starting within the first few weeks of life (Wenger et al., 2013). Often, at this point, parents will seek primary medical care, and, frequently, diagnosis is delayed because symptoms are not pathognomonic and, thus, often are not initially attributed to KD. As the disease progresses and more serious symptoms appear, such as worsening feeding difficulties, extreme irritability, and unexplained crying, the primary care physician will refer the family to a specialist, which eventually leads to a diagnosis. Regrettably, after a diagnosis of KD is confirmed, it may be too late for the currently available treatment, hematopoietic stem cell transplantation (HSCT), to reverse the neurologic damage caused by the disease because this treatment must be performed prior to the onset of clinical symptoms to be effective (Escobar et al., 2005). Generally the untreated child will not live past 2 years of age. Without a family history, NBS offers the only chance of identifying infants with KD prior to the onset of symptoms and is the only hope for a life-saving treatment for the affected infant. However, even in the setting of NBS, infants with EIKD require immediate referral to a transplant center for evaluation and treatment for transplantation to be effective.

SIGNIFICANCE

This Review includes updates related to newborn screening for Krabbe's disease (KD) in New York and Missouri. It includes a compilation of important observations, lessons learned, and recommendations for improvements to the screening and followup algorithms, which should reduce the number of screen-positive infants and the number of infants defined as at risk for later-onset forms of KD for future screening.

NBS FOR KD

In 2002, the Maternal and Child Health Bureau of the Health Resources and Services Administration of the U. S. Department of Health and Human Services commissioned the

American College of Medical Genetics (ACMG) to conduct an analysis of the scientific literature on the effectiveness of NBS. A major goal of this effort was to develop a system to evaluate disorders and then determine which would be best suited for NBS. The ACMG accomplished this by convening a group of experts from various areas of subspecialty and primary care medicine, health policy, law, public health, and consumers who worked with a steering committee and several expert work groups. They developed a two-tiered approach to assess and rank conditions. The first step was to develop a set of principles to guide the analysis and criteria by which conditions could be evaluated. The criteria to assess each condition were 1) the availability and characteristics of the screening test, 2) the availability and complexity of diagnostic services, and 3) the availability and efficacy of treatments related to the conditions.

The second step was to use the criteria to assess the appropriateness of screening for a list of 84 conditions with a scoring system. At the completion of the process, 29 primary disorders and 25 secondary disorders were recommended for screening, and the results were published in 2006 (Watson et al., 2006). At that time, KD was not recommended for screening because neither a screening test nor a treatment was available. In the intervening period between condition review and the publication of the condition list, two articles were published. Li and coworkers (2004a) described a tandem mass spectrometry (MS/MS) method that could be used to screen for KD and four other LSDs. Furthermore, a long-anticipated report on the efficacy of cord blood transplant for the treatment of EIKD was published (Escolar et al., 2005). These works fulfilled two of the above-mentioned criteria required to implement NBS for KD. In January, 2005, then-New-York-Governor George Pataki announced in his State of the State address that New York would initiate NBS for KD in New York state, and on August 7, 2006, NBS began.

In 2008, the Newborn Screening Saves Lives Act established the Secretary's Advisory Committee on Heritable Diseases of Newborns and Children (SACHDNC) to take over the work of condition review. This committee comprises physicians, public health workers, and liaisons from other areas related to child health. The committee was charged with evidence review and making formal recommendations on NBS and children's health to the Secretary of Health and Human Services. In 2008, the Hunter's Hope Foundation nominated KD for inclusion in the recommended uniform screening panel (RUSP). An external evidence review was initiated, and, following discussion of the work group's findings at a September, 2009, meeting, the SACHDNC voted not to recommend KD for inclusion on the RUSP because of a lack of data with respect to the case definition of EIKD, the establishment of an appropriate screening algorithm, and the long-term efficacy of treatment. (Kemper et al., 2010).

DEVELOPMENT OF NBS TESTS FOR KD

More than 10 years ago, a mass spectrometry-based assay was developed to measure GALC activity with a 2-mm punch of a dried blood spot (DBS) as the enzyme source (Li et al., 2004a). The natural substrates for GALC are galactosylceramides with long-chain fatty acids (typically 16 or 18 carbons) as part of the ceramide moiety. The substrate used in the mass spectrometry assay is a close synthetic analog, containing an eight-carbon fatty acyl chain.

The ceramide generated by the action of GALC on this artificial substrate is not present in the biological sample and allows for determination of enzyme activity. One advantage of using a substrate that is close in structure to the natural substrate is that the use of highly artificial substrates sometimes leads to misdiagnosis (Wenger et al., 2013; Ghomashchi et al., 2015). Early studies with the mass spectrometry assay for GALC showed that testing of DBS specimens readily distinguished patients with KD from individuals without the disease (Li et al., 2004a). At about the same time, it was shown that the GALC assay could be combined with the assay of four other lysosomal enzymes to detect Pompe's, Fabry's, Niemann-Pick-A/B, and Gaucher's diseases with a multiplex assay (Li et al., 2004b). For this first-generation mass spectrometry assay, two sample preparation methods were described. In the first method, each enzyme was incubated with its individual substrate/internal standard pair in a pH-optimized buffer solution with a separate 2-mm DBS punch. In the second method, a single 2-mm DBS punch was eluted into a common buffer, aliquots of the solution were distributed to separate wells, and a cocktail specific for each LSD was added to each well. After incubation, all mixtures were combined for liquid-liquid extraction with chloroform/water and solid-phase extraction through silica gel. The final mixture was infused into the triple-quadrupole, tandem mass spectrometer by flow injection for quantification of all enzymatic products and internal standards. The activity was calculated from the ratio of the enzymatic product to internal standard based on the known amount of internal standard initially spiked onto the DBS punch.

Investigators at Genzyme Corporation modified the Li et al. multiplex assay by making it more appropriate for NBS laboratories (by using a 3-mm punch, eliminating use of chloroform, combining substrates/internal standard pairs, and reducing solvent volumes). They also completed studies of enzyme stability in DBS (Zhang et al., 2008). A modified version of this assay was used to initiate screening for KD in New York on August 7, 2006, and was in use until May, 2015, for statewide NBS (Orsini et al., 2009). Initially, commercially available reagents were used for the assay. Shortly thereafter, the reagents developed by Gelb and coworkers were prepared by Genzyme Corporation and made available to NBS laboratories by the Centers for Disease Control and Prevention (CDC). The CDC also made available DBS quality control samples (De Jesus et al., 2009).

A few years later it became desirable to simplify and streamline the multiplex mass spectrometry assay because multiple buffers and plates had been used to perform the assay. This was first accomplished by combining all substrate/internal standard pairs into a common buffer and eliminating the requirement to extract the DBS and distribute aliquots to each individual substrate/internal standard pair. Screening for Pompe's disease, Fabry's disease, and Hurler syndrome (MPS-I) was developed (Scott et al., 2013). Orsini and coworkers extended this approach (Orsini, et al., 2010). They used buffer and pH conditions that were optimized for KD and used a single DBS punch to screen for four enzymes causing Gaucher's, Fabry's, Krabbe's, and Pompe's diseases, and a second punch was used for Niemann-Pick-A/B. This so-called 4 +1-plex assay still required liquid-liquid and solid-phase extraction cleanup of the combined solutions after incubation. After cleanup, a single solution could be infused into the mass spectrometer to test all five enzymes. In a second approach, the liquid-liquid and solid-phase extraction steps were eliminated, the sample was quenched with organic solvent, and, after centrifugation, the soluble component was

analyzed by ultrahigh-performance liquid chromatography (UHPLC) combined with MS/MS (Metz et al., 2011; Spacil et al., 2013).

After multiple discussions with key personnel from several NBS laboratories in the United States, it became clear that elimination of UHPLC and the solid-phase extraction step with silica gel were desirable to minimize instrumentation complexity and to simplify preassay spectrometry sample preparation. The latest screening assay, developed in a collaboration between the University of Washington and PerkinElmer, tests for Gaucher's, Krabbe's, Niemann-Pick-A/B, Pompe's, and Fabry's diseases and MPS-I. The assay, UW/PE 6-plex, uses a single 3-mm DBS punch, which is incubated in a single-assay cocktail with all substrates and internal standards. After incubation and a liquid-liquid extraction, samples are analyzed by flow injection MS/MS. All internal standards are chemically identical to the corresponding enzymatically generated products but isotopically differentiated with deuteration. In this way, all enzymatic products are quantified via a chemically identical internal standard that experiences the exact same fate as the products (losses to downstream enzymes, adherence to surfaces, suppression in the electrospray ionization source of the mass spectrometer, and others). The UW/PE 6-plex is currently being piloted in the Washington State NBS laboratory, with results for ~50,000 DBS expected for publication in early 2016. The new 6-plex will be commercially available outside of the United States in 2016, and submission for FDA approval for use in the United States is in progress. New York is currently performing full population screening for KD and Pompe's disease using a subset of these reagents.

NEW YORK PILOT SCREENING

Prior to live screening, New York developed and implemented a high-volume assay to validate screening for KD (Orsini et al., 2009). An important step in the validation was testing samples from patients with KD. In total 16 DBS specimens from KD patients and 56 specimens from obligate carriers were obtained via a sample collection drive held at the Hunter's Hope Family and Medical Symposium in 2005. The samples from the KD patients had percentage of daily mean activity values (%DMV) ranging from 1.9% to 10.9% (the 10.9% value was an outlier among four measurements from the same positive control; all other measurements were <6%). The carrier cohort had %DMV values ranging from 6.4% to 60%. In addition to these controls, a DBS was taken from the cord blood of an infant diagnosed with KD in utero who, prior to transplantation, had an average %DMV of 8.3%, which was higher than values from all other patients. Based on these results, assay cutoffs and a screening algorithm were established. The screening algorithm called for testing all infants for GALC activity and converting each sample activity to %DMV. A fail-safe cutoff %DMV that required retesting in duplicate all samples that were ≥ 20 %DMV was set. After retesting, those samples with an average %DMV $\geq 10\%$ (subsequently changed to 12%; see below) were subjected to in-house second-tier DNA testing. Second-tier DNA sequencing was implemented to reduce the number of infants referred for followup diagnostic testing because a large amount of nondisease-causing activity reducing polymorphisms has been reported for the GALC gene. This testing consisted of Sanger sequencing of all exons and all exon/intron boundaries of the GALC gene; Gap-PCR analysis was used to detect the common 30-kb and 7.4-kb deletions (del), and PCR-fluorescent resonance energy transfer

assay was used to detect polymorphisms and mutations thought to be common at the time screening began. Initially, samples with a %DMV of $\geq 8\%$ were referred without respect to the molecular results. Samples with %DMV between 8.1% and 10.0% and one or more disease-causing variants or a variant of unknown significance (VOUS) were referred for followup diagnostic testing and evaluation. Note that streamlining of the molecular test allowed for elimination of the immediate referral of infants with $\geq 8\%$ activity. Furthermore, five infants with $\geq 8\%$ activity carried only activity-reducing polymorphisms and were determined to be at low risk ($n = 1$) or not at risk ($n = 4$) for KD after the diagnostic testing laboratory measured GALC activities. Hence, low GALC activity by the screening assay was not definitely indicative of a serious KD case.

DIAGNOSTIC TESTING, RISK ASSESSMENT, AND FOLLOWUP RECOMMENDATIONS

Before live screening began, a group consisting of the directors of the state's eight Inherited Metabolic Disease Specialty Care Centers (IMDSCCs), the scientists involved in NBS at the Wadsworth Center in Albany, and others met in New York City to discuss the imminent start of the program. The pilot screening data and testing algorithms were presented, and a plan for diagnosing screen-positive patients was discussed. It soon became clear that, although the NBS testing procedures and algorithm had been well worked out, the clinical evaluation and followup of screen-positive children required further development. To meet this requirement, the Krabbe's Consortium of New York State (Consortium) was formed. Child neurologists, neuroradiologists, transplant physicians, neurodevelopmental pediatricians, and others were added to the original group (Duffner et al., 2009b). The Consortium was cognizant that screening would identify infants with low diagnostic laboratory GALC activity and variants in the *GALC* gene that did not warrant bone marrow transplantation. The Consortium wanted to ensure absolutely that none of these infants was subjected to an unnecessary HSCT, so all evaluation criteria were set conservatively.

Under the leadership of Dr. Patricia Duffner, the Consortium developed a risk assessment and evaluation protocol and schedule. The risk assessment was based solely on diagnostic laboratory GALC enzyme activities; infants with activities ≤ 0.15 nmol/hr/mg protein were classified as at high risk for KD, infants with activities 0.16–0.29 nmol/hr/mg protein were classified as at moderate risk, and infants with activities 0.3–0.5 nmol/hr/mg protein were classified as at low risk. The low-risk category was eliminated in 2012 after reassessment by the Consortium (Wasserstein et al., 2016). A rating scale for transplant urgency was created that combined results from a series of neurodiagnostic tests, a physical examination, and a genotype (presence/absence of the 30-kb del) with the goal of differentiating newborns with infantile KD from those that may develop later-onset forms of the disease (Duffner et al., 2009b). Referred infants were first seen by IMDSCC directors as part of the diagnostic workup, and blood was drawn from the referred infant, which was sent to the lysosomal diseases testing laboratory at Sidney Kimmel Medical College, Thomas Jefferson University, where GALC enzyme activity was measured in leukocytes.

The following describes the evaluation protocol. All screen-positive infants found to have a diagnostic laboratory activity in any of the risk categories described above were recommended to have a neurodiagnostic workup. This included a physical examination and tests, such as brain magnetic resonance imaging (MRI), cerebral spinal fluid protein concentration determination, visual-evoked response, and other neurodiagnostic tests. Each test had an assigned point value, and all abnormal results were added together. If the score was 4 or higher, then treatment via hematopoietic transplant was recommended. For infants with scores below 4, the Consortium recommended that a followup evaluation be scheduled based on the assigned risk, with more frequent and extensive followup to be performed on infants in the high-risk category (Duffner et al., 2009b). Infants that were homozygous for the 30-kb del were assigned a baseline score of 4 that increased based on the results of the other tests.

LIVE SCREENING: NEW YORK (AUGUST, 2006, TO AUGUST, 2015)

Live NBS for KD was initiated in New York on August 7, 2006, after a declaration by then-Governor George Pataki. Thorough reports on the first 8 years (August, 2006, to August, 2014) of screening (Orsini et al., 2016) and outcomes have been published (Wasserstein et al., 2016). As of August 7, 2015, the results and outcomes have remained largely unchanged. Specimens (2,352,249) from 2,203,360 infants have been screened with the New York State KD algorithm. Within this time frame, more than 99.9% of all specimens tested were screen negative. A total of 712 infants had enzyme activities $\geq 12\%$; after molecular testing, 393 infants screened positive (0.18/ 1,000) and were referred for clinical evaluation. An additional 319 infants (41.3% of all sequenced samples) had low activity and, after molecular analysis, had only nondisease-causing polymorphisms in the GALC gene and were considered screen negative. In the 9 years of screening, 15 infants (one identified in July, 2015) were classified as at high-risk for KD, and five were determined to have EIKD. An additional 45 infants were determined to be at moderate risk for KD. As of December 10, 2015, none of the 55 high- or moderate-risk patients has developed findings of KD other than the five infantile cases.

FULL POPULATION PILOT SCREENING: MISSOURI (AUGUST, 2012, to JULY, 2015)

Missouri initiated mandated screening for KD in August, 2012. This screening was originally performed in New York with New York methodology and continued for nearly 3 years (through July 31, 2015). On August 1, 2015, after nearly 4 months of parallel testing with New York, Missouri began performing its own screening with a plate-based fluorescence assay. During the 3-year period in which New York performed the screening, 266,629 samples from ~230,700 infants were screened, and none was determined to have KD based on results from followup diagnostic testing and clinical evaluations (unpublished data). The Missouri screen-positive rate was similar to that observed in New York, with 54 infants referred for followup diagnostic testing (screen-positive rate = 0.020% compared with 0.018% for the New York population). An additional 53 infants (49.5% of all sequenced samples) had only nondisease-causing polymorphisms in the GALC gene.

Missouri did not use the same confirmatory testing laboratory as New York, so the risk assessment was different. As of December 16, 2015, all of the screen-positive infants detected in Missouri screening remain asymptomatic. Screening data from the Missouri fluorescence assay have not been reported.

OVERALL SCREENING RESULTS

After 9 years of screening in New York and nearly 3 years of screening in Missouri, more than 2.5 million infants have been tested. Four hundred forty-three (0.018%) infants have been referred for followup clinical evaluation, and only five infants, two of whom were siblings, have been determined to have KD, with an additional nine infants categorized as at high risk (New York). The combined incidence of infantile KD in New York and Missouri to date is ~1 per 500,000; patients who develop later-onset forms of KD may still emerge.

GENOTYPE DATA

New York Population

Complete genotype data from New York's 8-year cohort revealed 117 *GALC* variants detected among the 348 infants in the referral population, as of August, 2014 (Orsini et al., 2016). The three most common disease-causing variants that were detected include the 30-kb del (48 infants), p.T96A (96 infants), and p.Y303C (39 infants). Both p.T96A and p.Y303C are associated with late-onset disease and are considered mild mutations (Wenger et al., 2013). Other mutations previously reported for infantile and later-onset KD patients were also detected. However, many of the sequence variants detected in the referral population were novel VOUS. Because of the *GALC* gene's high allelic heterogeneity, parental blood specimens are requested for phasing mutations in the infant. The vast majority of our referrals have been phased, so the gene haplotype of alleles (mutations and/or polymorphisms segregating on each chromosome) in the infant are known. It is known that many disease-causing mutations are found in cis with known polymorphisms (Wenger et al., 2014). In the New York referral population, this was also observed. Among 696 alleles, only 40 (5.7%) alleles were found to contain a single mutation (either known disease causing or a VOUS).

Missouri Population

In the 3-year period when New York performed KD screening for Missouri, 54 infants were referred. Among the 54 infants referred, 45 were apparent carriers. Just as with the New York referral population, most of these carriers (35/47) had nonpathogenic enzyme reducing polymorphisms on one allele (p.R168C and p.I546T), and p.T96A was the most common suspected disease-causing variant (20 alleles). The second most common variant was the 30-kb del, which was detected in 16 of the referred infants. The p.Y303C mutation was observed in only one infant. None of the referred infants had two severe disease-causing mutations.

Infantile KD Patient Genotypes

The genotypes of the five infants that were determined to have infantile KD are provided in Table I. Three of the five were homozygous for 30-kb del. The other two referred infants were compound heterozygous for 30-kb del and one additional deleterious mutation.

Infants at High Risk to Develop KD

Table II shows the genotype data for the remaining nine infants classified as at high risk for KD. All children remain asymptomatic as of December, 2015. By the New York risk-assessment test, all of the high-risk cases had very low GALC enzymatic activity in lymphocytes (0.15 nmol/hr/mg). Not surprisingly, sequence analysis showed that all of these infants have at least two *GALC* mutations on opposite chromosomes. However, in four of the cases (cases 6–9), only one allele has a severe mutation; for case 6, the p.R380W variant is reported to be severe, and for cases 7–9 the nonsense mutations are presumed to be severe because of the production of a truncated protein. Case 10 was homozygous for p.L618S, a variant reported in a late-infantile patient in trans with a more severe mutation and in a 51-year-old male homozygote (Xu et al., 2006). The remaining five cases all have two mild variants. The variant p.Y303C (cases 8, 13, and 14) is a mutation that is thought to be disease causing only when found in trans with a severe mutation (Wenger et al., 2014). There have been no reported cases of patients homozygous for p.Y303C. It is important to note that some patients categorized in the high- and moderate-risk categories have identical *GALC* genotypes. Three possible reasons for this are that 1) there is a difference in the quality of the whole-blood samples; 2) the lymphocyte GALC activity assay is not sufficiently accurate, thus giving some sample-to-sample variation; and 3) factors other than mutations in a single gene (*GALC*) could modulate the activity of GALC in cell lysates. Because of this, New York now recommends repeat GALC enzyme testing for high- and moderate-risk patients at a subsequent visit to the specialist.

Infants at Moderate Risk for Developing KD

After 8 years of screening, 37 infants were placed in the moderate-risk category; 32 had confirmatory GALC activity between 0.16 and 0.29 nmol/hr/mg protein, and five had GALC activity in the low-risk range but were classified as moderate because they had two known or potentially pathogenic mutations. Currently, no moderate-risk children, who range in age from 13 months to 9 years, are known to be symptomatic. The genotypes of the moderate-risk cohort were previously reported (Orsini et al., 2016). Fourteen moderate-risk infants have one copy of the p.T96A variant. This variant is carried by more than 25% of referred infants (96/348), representing, by far, the most common potentially pathogenic variant detected. It has been reported in two patients with adult-onset KD (one with p.D171V on the other chromosome [Luzi et al., 1996] and the other with p.P138R [Debs et al., 2013]), although it is not known whether p.T96A is in cis with p.I546T in this patient. Table III shows a subset of the moderate-risk infants with one severe (observed in patients who were symptomatic in early childhood) and one late-onset mutation.

PSYCHOSINE TESTING

The main substrate for GALC is galactosylceramide, and psychosine (galactosylsphingosine) is a secondary GALC substrate that accumulates in patients with KD. The accumulation of psychosine has been proposed to cause death to the oligodendrocytes and Schwann cells, which are responsible for maintaining myelination of the central and peripheral nervous tissue, respectively (Haq et al., 2003). With KD and other LSD screening, MS/MS is used to measure enzymatic products formed from the reaction of the enzymes present in the blood with exogenous artificial substrates. This approach is different from other MS/MS applications in which the MS/MS is used to measure elevated substrates in the blood, such as amino acids and acylcarnitines. These substrates are elevated in newborns with enzyme deficiencies associated with many other inherited metabolic disorders. With the advent of highly sensitive MS/MS, we had hypothesized that psychosine may be elevated in DBS samples of newborns with KD. Results from a small study showed this to be correct (Chuang et al., 2013). The original DBS specimens from the first four infantile KD cases identified through NBS had very elevated psychosine concentrations, whereas the psychosine levels of all of the asymptomatic high- and moderate-risk infants were only slightly elevated compared with DBS from infants with normal GALC activities. Turgeon and coworkers (2015) extended this work. They obtained consent to test the original NBS DBS samples from KD patients born in states other than New York. All of these DBS samples had elevated psychosine concentrations, further supporting the use of psychosine as a biomarker in ascertaining newborns most likely to develop EIKD. Currently, data are insufficient to determine whether infants with late-infantile, juvenile, or other late-onset forms of KD would be detected by using psychosine as a biomarker. Prospective studies of asymptomatic but at-risk late-onset patients are required to determine whether psychosine can be used to predict disease onset.

PATIENT OUTCOMES

EIKD Patients

Fourteen infants were identified as at high risk for KD based on GALC enzyme activity in lymphocytes. All of these infants had followup neurodiagnostic evaluations, and the details of the results have been published (Wasserstein et al., 2016). Abnormal results were scored and are shown in Table IV, and infants who received scores of 4 or less were considered candidates for transplantation. Among the 14 high-risk infants, seven had scores of 4 or less. Additional evaluation showed that five of these seven infants had EIKD. Among this group, three died, two from HSCT-related complications and one from untreated EIKD. Two EIKD children who received HSCT have moderate to severe motor delays. Two other infants had initial scores of 4 and 5 (cases 6 and 9, Table II) and were considered candidates for HSCT. In both cases, the myelination pattern on initial MRI was interpreted as abnormal by two independent neuroradiologists. The two families refused HSCT but agreed to have the infants undergo another evaluation and in both cases, an MRI several weeks later showed improvements in myelination, so they were no longer considered candidates for HSCT.

Asymptomatic High- and Moderate-Risk Patients

Ideally, the recommended evaluation schedule would have been followed for the at-risk infants. However, for a variety of reasons, compliance was poor, so a more pragmatic approach for following these infants was introduced (Duffner et al., 2012b). In this study, consenting families of at-risk infants were interviewed by telephone at infant ages of 4, 8, 12, 18, and 24 months. Various tools were used to assess development, including the ages and stages questionnaires, the clinical linguistic and auditory milestone scale, the gross motor quotient, and the Bayley scales of infant and toddler development (Bayley III). Seventeen patients were enrolled, and scores were within the normal range on all tests of developmental and functional status, with the exception of expressive language. Only seven patients completed the Bayley III assessments; all their scores were in the normal range. The authors concluded that this telephone-based technique allowed for close and less invasive monitoring of the developmental and functional status of the at-risk children, and compliance was improved compared with the prior recommended formal neuropsychological testing.

OBSERVATIONS, LESSONS LEARNED AND FURTHER STUDIES

Below is a summary of lessons learned and observations from 9 years of NBS for KD, some of which have been partially discussed in this Review and may be applicable to other LSD screening.

Disease Positive Controls

During the pilot study prior to live screening, the initial %DMV cutoff values for the screening algorithm were established primarily from fresh DBS samples collected from older KD patients. Early in the course of NBS, we learned that DBS from presymptomatic KD newborns have activities that are approximately two- to fourfold higher than DBS from older patients. The increased activity most likely is explained by the difference in white cell counts of newborns compared with those of older patients (Ebel et al., 2000). Hence, residual DBS specimens from the original newborn screen should be used as controls when evaluating LSD enzyme cutoffs.

Screening Laboratory Activities, Sample Collection, Repeat Samples, and Diagnostic Laboratory Activities

GALC in premature infants—Premature newborns have measured GALC activities that are approximately threefold higher than full-term infants (Orsini et al., 2016). From this observation, it is possible for premature infants with KD to have activities above the cutoff and, thus, not be detected. Although we have not identified any premature infants with KD, to avoid false-negative results, we consider samples taken from premature infants as unsuitable. However, if the measured GALC is below the cutoff, then the test is considered screen positive, and second-tier molecular testing is performed. A possible explanation for the higher measured activities is that premature infants have lower hematocrits than full-term infants (Ebel et al., 2000). Li and coworkers (2004b) reported that GALC activity decreases with increasing blood volume and attributed this to an inhibitor effect. This effect was observed in New York screening (unpublished data). The markedly higher activities

observed in premature infants may be explained by the lower hematocrit from the premature infants.

Sample collection—It is universally recommended that NBS samples be taken via a heel stick (Hannon et al., 2013). Sometimes, especially with premature infants, it is essential to collect venous samples. As part of this sampling method, the blood is often collected into a tube and then pipetted or applied onto a filter paper blood collection form. Our observation is that samples taken in this way are less reliable for GALC testing because there is high variability in results when punches are taken from different areas of the same circle or other circles (unpublished data). Although more work is required, we speculate that clotting or possibly the aggregation of white cells in the plasma is the cause of this decreased precision. Hence, if the blood is allowed to settle and the white cells are allowed form a buffy coat between the plasma and the red cells, the aggregated white cells may not fully homogenize with stirring; this may explain the variability in GALC activities observed on such spotted specimens. If venous sampling must be performed, it is important to apply the collected blood to the filter paper as quickly as possible, preferably via a butterfly valve, to minimize the effects of clotting. Anticoagulants should not be used because these can interfere with other NBS tests.

GALC activities measured in DBS—GALC activities measured from newborn DBS show much more variability than GALC activities measured from leukocytes. Scatterplotting screen laboratory activities vs. diagnostic laboratory activities shows poor correlation (unpublished data). However, when isolated leukocytes (provided to screening laboratory from diagnostic laboratory) from infants who had screened positive were tested with the MS/MS screening assay, the measured activities showed a linear correlation with results from the radio-metric assay. This indicates that variables present in the DBS contribute to the lack of correlation in the screen and diagnostic laboratory results (unpublished data). Variables include but are not limited to the hematocrit and white cell counts (hence protein levels) at the time of collection. In an effort to reduce false positives with the newborn screen, we developed assays to estimate the hematocrit and white cell counts of DBS specimens. Hematocrit can be estimated by analysis of HPLC data collected for hemoglobinopathy screening (Orsini et al., 2010). We determined that the total area under the hemoglobin peaks in ion chromatographs obtained for hemoglobinopathy screening was proportional to the hematocrit of the sample. If the estimate of hematocrit is found to be very low or very high, then GALC activities (and other marker concentrations) are affected, with GALC activities being lower in infants with higher hematocrits (unpublished results). More work is required to determine whether the estimated hematocrits can be used in decision making for samples near established cutoffs. To estimate the white cell count on a DBS, Oli-Green, a dye that fluoresces with increasing intensity that is proportional to the amount of DNA in solution, was used to estimate the DNA concentration of DNA extracts obtained from the DBS. Because DNA is present at a constant amount in white cells (~6.5 pg DNA/cell), the number of white cells can then be calculated from the measured DNA concentration. The white cell counts measured with this approach compared favorably with those obtained via flow cytometry. Hence, it would be possible to normalize DBS GALC activities to the white cell counts from this method. This may improve the correlation of

screen activities with values measured from leukocytes at the diagnostic laboratories and reduce false positives. More work is required to determine whether this approach would be beneficial.

Requirement to repeat diagnostic enzyme analysis—Diagnostic laboratories determine GALC activity on leukocytes, and the results are normalized to the amount of white cell protein, so results are less dependent on sample quality. However, even leukocyte-based enzyme assays can vary depending on sample quality, handling, variability of the test, and other factors. Enzyme testing should be repeated with infants determined to be at risk for KD when the values are not consistent with those measured on prior referred infants with the same genotype. We had four high-risk infants who were recategorized to low or moderate risk after repeating the leukocyte test.

IMPROVEMENT OPPORTUNITIES

Overview

New York was the first state to implement KD testing, and it was imperative to begin conservatively. As a consequence, the positive predictive value of the assay was low (high false-positive rate). Additionally, the enzyme activity-based risk assessment was conservative; this contributed to the high number of moderate- and high-risk patients identified. Changes have recently been implemented, newer changes have been proposed, and areas in which more work is required to improve the entire process of KD screening have been identified. Much has been learned through the course of screening, and several additional improvements are under consideration. This past summer (July, 2015) at the Hunter's Hope medical symposium, an afternoon was dedicated to reviewing all NBS for KD processes. However, more work was required, so a second meeting was planned. This second meeting was held in Buffalo, New York (October 29–30, 2015). At this meeting, a new NBS for a KD task force was sanctioned. This group has representatives from states in which NBS for KD is currently performed or will be performed in the near future; it also includes experts in KD diagnosis, followup, and treatment. The group's mission is to continue to evaluate each step of the screening process and to recommend improvements. Some of the ideas below emerged from this discussion, and others were already under consideration.

Multienzyme Testing

One major reason for the high New York referral rate was that only the GALC enzyme activity was measured for most of the years of the screening (New York recently added screening for Pompe's disease in October, 2014). As discussed previously, GALC activity is dependent primarily on the newborn leukocyte counts at the time of sampling. When measuring only GALC, as was the case in New York for the first 8 years of screening, when the GALC value was below cutoff, the only option was to move forward with DNA sequencing and refer when mutations were detected. With multienzyme testing, it is possible to detect samples with low leukocyte counts based on the other measured enzyme activities. New York and other states performing multi-LSD enzyme testing have noted that multiple enzymes often have low normal activity in DBS samples. In these cases, it may be possible

to consider the samples from infants with multiple low-enzyme activities as screen negative. This approach is being explored with postanalytical interpretive tools based on multivariate pattern recognition software developed at the Mayo Clinic (collaborative laboratory interpretive reports [CLIR], <https://clir.mayo.edu>; Marquardt et al., 2012; Hall et al., 2014; Mørkrid et al., 2015). In New York, we plan to perform repeat testing (on the in-house sample) of samples that test low for GALC with a six-enzyme multiplex assay. If, after testing, there are multiple enzymes with low activities, this would indicate that the sample possibly has low white cell counts or that there are other quality issues with the sample. Then it would be reasonable to report normal results for GALC based on computation of ratios to other enzyme activity results. This change is expected to reduce dramatically the number of samples that are referred to second-tier molecular testing and also reduce the number of infants referred for followup.

Psychosine Testing

From the two small psychosine studies described above, all NBS specimens from patients with EIKD also had elevated psychosine levels. Although the number of NBS specimens tested from symptomatic EIKD patients was small ($n = 10$), all had very elevated psychosine levels compared with normal controls. These data are very promising. Implementation of psychosine testing as part of followup diagnostic testing or the use of psychosine testing as a second-tier test in NBS laboratories could dramatically benefit NBS for KD.

If psychosine testing were added as a supplemental followup diagnostic test to be used when the diagnostic-laboratory-measured activity is in an affected patients range, infants most likely to have EIKD would be identified. This approach would not reduce the number of infants referred; however, it could be used in states in which in-house genotyping or psychosine testing is not available to identify infants with EIKD. The benefits of this approach are that genotyping would not be required. Genotyping can be difficult for followup centers to complete because of insurance issues. However, because not much is known about the psychosine levels in DBS in newborns that will eventually develop later-onset forms KD, later-onset KD cases may be missed.

Another approach would be to perform second-tier psychosine testing in conjunction with second-tier DNA testing. The results of this combination would provide an additional tool for risk assessment of referred newborns and allow for the continued genotype data collection.

Finally, NBS programs could choose to add psycho-sine testing as a second-tier test to be used in place of second-tier DNA testing. With this approach, the number of infants referred for followup diagnosis would likely be significantly reduced. Thus far, all NBS DBS from infants known to have EIKD have had elevated psychosine. However, the number of infants who have been tested is small, so it may be risky to implement this approach. One possibility for reducing the risk of not detecting a true EIKD newborn is to have an activity level cutoff whereby very-low-activity infants would still be referred for followup diagnostic testing without respect to the psycho-sine test results.

In referring back to the outcomes data previously discussed, it is worth noting that, for the two asymptomatic high-risk infants with initial abnormal MRI results (that normalized in later testing), the NBS DBS psychosine levels were normal (data not previously reported). This further supports the value of psychosine testing for infants expected to be at risk for KD. At the time of analysis, the use of psychosine as a marker was experimental, so the results could not be considered in the evaluation. It is also of interest to note that an NBS DBS specimen obtained from a KD patient who presented with disease at approximately 2 years of age had psychosine levels in the abnormal range. Additional prospective studies are required to determine whether psychosine can be used to identify other late-onset cases of KD.

Unfortunately, at this point, testing for psychosine requires an MS/MS instrument that is more sensitive than that typically available in NBS laboratories. Furthermore, UHPLC is required for this MS/MS analysis. Thus, it is unlikely that most NBS programs will be able to implement in-house psychosine testing in the near future. At the moment, samples for psychosine analysis can be sent to only a handful of laboratories in the United States.

Genotype Interpretation

As discussed earlier, there are several haplotypes that have been observed repeatedly in the New York referral cohort. The most common haplotype observed is p.A5P + p.T96A + p.D232N (96/348), where p.T96A is considered the disease-causing mutation. This haplotype has been observed 47 times in trans with the p.R168C + p.I546T haplotype. A 2014 report indicated that a symptomatic KD patient had p.T96A in cis with the polymorphism p.I546T. A second patient with late-onset KD has been reported with p.T96A and p.I546T (Debs et al., 2013), although it is unknown whether the variants are in cis or in trans for this patient. To date, none of the patients in the New York referral population has had these two variants on the same allele. We have referred two New York and two Missouri infants with the p.A5P + p.T96A + p.D232N haplotype in trans with a 30-kb del allele; both had activities in the moderate-risk range but higher GALC activity than any known affected patients (0.29 and 0.27 nmol/hr/mg). We also identified eight infants who were homozygous for the p.A5P + p.T96A + p.D232N haplotypes; all had diagnostic laboratory activities that were 0.3 nmol/hr/mg. These data support the notion that p.A5P + p.T96A + p.D232N does not cause disease. Hence, in November, 2015, the Consortium agreed to stop referring infants with the p.A5P + p.T96A + p.D232N when in trans with either p.R168C + p.I546T or p.I546T alone. Although it is possible to phase p.T96A and p.I546T in an infant only if the parental DNA is collected, the Consortium concluded that the risk of an infant being affected with KD is very low when no other disease-causing variants are detected and heterozygosity is observed at the amino acid 546 position (Ile/Thr). Reassignment of p.T96A carriers from referral status to polymorphisms reduces the number of referrals only from 348 to 283 in our 8-year cohort. We may be able to use this logic with other common variants to reduce the number of referrals further while still detecting high-risk infants. GALC expression studies showed that the p.A5P + p.T96A + p.D232N allele had 34% residual activity compared with wild type, which was reduced to 7% when in cis with p.I546T (Saavedra-Matiz et al., 2016).

After 8 years of screening for New York and 3 years of screening for Missouri, 402 infants had been referred; among these, 313 were apparent carriers. Because of the limitations of Sanger sequencing to detect large deletions or duplications in the gene that could be disease causing, the apparent carrier infants were referred. Molecular testing to detect deletions and duplications could significantly reduce the number of referrals if NBS programs could release carriers as screen negative. Unfortunately, this testing is currently time consuming and costly.

Risk Based on Genotype

In reviewing all of the referred infants who were determined to be at any risk for KD, the five infants determined actually to have EIKD had two severe mutations, and there were infants from the high- and moderate-risk asymptomatic groups having one severe and one mild mutation. If we assign p.L618S as a severe mutation, then in this group there were five infants who had previously been categorized at high risk and six infants who had previously been categorized at moderate risk (see Tables (II and III)). It is of interest that these infants were in both the high- and the moderate-risk groups, with some having identical genotypes. It is unknown why two infants with identical genotypes ended up in two different risk categories. One possibility is related to the quality of the sample sent to the diagnostic laboratory. All other infants were categorized at high risk, and most who were categorized at moderate risk had two mild mutations in the GALC gene. There are reports of patients with late-onset KD having the mild p.T96A (when in cis with p.I546T) and p.Y303C mutations but only when these are in trans with a second severe mutation (Wenger et al., 2014). Although it is possible that some late-onset KD cases with these variants have been missed because of ambiguous clinical presentation, genotype data could be used in conjunction with expression studies and enzyme and psychosine data in redefining risks of screen-positive infants. If, for example, we consider infants with one severe mutation and one mild mutation to be the only truly at-risk infants, then the number of at-risk patients would be reduced from 47 to 11. These are the types of analyses that will be evaluated by the KD task force.

Diagnostic Enzyme Testing

Diagnostic laboratories performing LSD enzyme testing use clinical information from the provider to identify disease in patients who are already symptomatic for a suspected enzyme deficiency. A low activity consistent with the symptoms in question is generally sufficient for clinical diagnosis. Confirmation of the diagnosis is often further supported after gene sequencing detects disease-causing mutations. Other metabolites can also be used to confirm the diagnosis. The age of onset of disease, severity of disease compared with that reported in the literature, and types of mutations detected may eventually help with genotype/phenotype correlations. In NBS screening for KD, we have children who are asymptomatic at birth and remain asymptomatic yet have low enzyme activity and two mutations in the GALC gene. All five infantile cases detected by NBS had two severe mutations. In hindsight, these five diagnoses seem obvious. However, prediction of phenotype becomes less clear for infants with low enzyme activity and two mutations if one or both are mild mutations.

Workers in the Gelb laboratory are testing the hypothesis that the level of residual GALC activity remains a critical determinant of the severity of KD. Currently, GALC enzymatic

activity in lymphocytes isolated from whole blood is measured with a radiometric assay with tritiated galactosylceramide (Wenger et al., 1991). The analytical range of this assay, that is, the ratio of the assay response for the quality control high sample to assay response resulting from all GALC-independent processes (background noise, contributions from non-GALC enzymes, and others) is modest (six- to eightfold) with the radiometric assay. Workers in the Gelb laboratory are currently developing a UHPLC-MS/MS assay of GALC enzymatic activity with CD3⁺ T cells isolated from whole blood. The analytical range of this assay is ~1,500, or more than two orders of magnitude greater than for the radiometric assay (unpublished data). Over the next 1–2 years, the new assay will be tested to see how well it predicts the severity of KD for infants detected by NBS. If successful, this second-tier method could better assess risk and reduce family anxiety.

Diffusion Tensor Imaging

Standard MRI (conventional T2 weighted) was used to evaluate most of the infants in the high-risk group in New York. Interpreting MRI results from newborns is complicated because the degree of myelination is quite variable in this cohort. Gupta and coworkers (2014) recently showed that diffusion tensor imaging (DTI) with quantitative tractography is an effective tool for evaluating infants with KD identified through NBS. However, this technique has not been tested on asymptomatic, at-risk patients identified by NBS. It is possible that DTI will yield a more objective tool in evaluation of these patients because analysis is more quantitative.

Revised Followup Protocol From New Risk Assessment

New York was an early adopter of NBS for KD. Because there was uncertainty in the prediction risk to develop EIKD, extensive followup testing was recommended (Duffner et al., 2009b). Extra caution was taken to ensure that only presymptomatic, truly affected babies were considered for transplant and that infants with a late-infantile or juvenile form of KD were not missed. An unintended consequence of these extensive followup recommendations was that there was low compliance in the followup testing algorithm. Going forward, the frequency and number of followup tests required should be dependent on the newly revised risk assessment (yet to be determined), with only the highest-risk patients being subjected to the extensive followup testing. More passive followup (than was originally recommended) should be considered for infants in whom late-onset KD is possible; this is a topic under discussion by the KD task force.

The newly formed KD task force is currently working on a revised followup protocol that will take into account the lessons learned from 8 + years of NBS for KD. One thing is abundantly clear; it is imperative that this protocol describe the clinical pathway for a rapid diagnosis of infants with EIKD. Parents of these infants should immediately be made aware of the option of a qualified transplant center because timely treatment is required for the best outcomes.

SUMMARY AND CONCLUSIONS

After 9 years of screening in New York, 3 years of screening in Missouri, and nearly 2.5 million infants screened, 443 infants were referred for a diagnostic workup and five of these infants had severe EIKD. The combined incidence of KD in New York and Missouri (1 in ~500,000) is lower than predicted; prior to NBS, the anticipated incidence was 1:100,000–1:200,000. The incidence will be higher if any of the asymptomatic patients develop late-onset KD. Four of the five EIKD infants received HSCT, and two died from treatment complications. These outcomes are worse than expected; published outcomes are more favorable (Escobar et al., 2005), and anecdotal cases of highly successful transplanted patients are known. In addition to the low incidence and poor outcomes, 47 New York families have been told that their child is at risk of developing KD. Advocates of NBS for KD have direct or indirect experience in having known a baby and family affected by KD; they know that KD is a dreadful disease, and the only hope for life-saving treatment is early detection through NBS. This is the reason why screening was originally mandated in New York and Missouri and is now in the planning stages in Illinois, New Jersey, Pennsylvania, New Mexico, Kentucky, Tennessee, and Ohio.

Even with all of the issues related to NBS for KD, much has been learned in the past 9 years. The false-positive rate after initial screening for the test is high, largely because of variable leukocyte counts and hematocrits of newborns. It should be possible to adjust activity values for these two variables, which would reduce the false-positive rate. Hematocrit can be readily estimated from the hemoglobinopathy results, but estimating white cell counts in DBS must be refined and simplified for it to be implemented universally. White cell counts could be estimated from the DNA extracts used for severe combined immunodeficiency screening; until these efforts are operationalized, samples with low white cell counts can be more readily detected when other LSD enzyme activities are multiplexed with GALC activity. This approach is currently under study with the CLIR software developed at the Mayo Clinic, and it is expected to reduce the false-positive rate for KD and other LSDs significantly. For those infants who remain screen positive, the genotype/phenotype knowledge gained from NBS second-tier molecular testing, GALC haplotype residual activity from expression studies, and psychosine testing should allow for the rapid identification of EIKD patients and identify infants only truly at risk for later-onset forms of KD. Unfortunately, at this time, it is not possible to determine whether or when these at-risk infants will become symptomatic and require treatment, so additional work is required. Currently, DTI magnetic resonance offers a more quantitative approach to monitoring this cohort of patients. Use of this technology to identify infants most at risk occurred because of NBS. Finally, we are working to develop more sensitive assays of GALC activity in white blood cells to help stratify the highest-risk screen-positive individuals.

Most discouraging is that we have learned that the outcomes in treating the EIKD cases detected through NBS is thus far poor and is arguably worse than was expected based on prior outcomes, although the number of cases is small. Below are three possible explanations for the poor treatment outcomes. First, thus far, only very severe KD cases have been detected, which would negatively affect outcomes. Second, for most of the EIKD infants, there were delays in treatment related to socioeconomic and cultural factors. Third,

not all transplants occurred or can easily occur at experienced treatment centers. All of these are practical problems associated with providing treatment for a very rare disorder. Although we have learned more from screening, other researchers and transplant physicians continue to work on methods to improve the treatment. Experimental combination therapies in the murine model of KD that have demonstrated the potential to advance KD treatment further by synergistically increasing life span of treated mice are under evaluation (Li and Sands, 2014; Rafi et al., 2015; Ungari et al., 2015). Over time, treatment options should improve; in the meantime, it is essential to evaluate the lessons learned and ensure that screening is completed in the best possible manner until these improvements can be realized.

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Abbreviations

DBS	dried blood spots
EIKD	early infantile Krabbe's disease
GALC	galactosylcerebrosidase
IMD	inherited metabolic disease
KD	Krabbe's disease
LSD	lysosomal storage disorder
NBS	newborn screening
RUSP	recommended uniform screening panel
SCC	specialty care center

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TABLE I

Newborn Screening Results, Molecular Analysis, and Diagnostic Testing Results for Five Subjects With Infantile KD

Case No.	Allele 1	Allele 2
1	30-kb del + p.R168C	c. -335G>A + p.D94=+ p.I546T + p.*670Qext42
2	30-kb del + p.R168C	30-kb del + p.R168C
3	30-kb del + p.R168C	30-kb del + p.R168C
4	30-kb del + p.R168C	c. -335G>A + p.G360Dfs*2#
5	30-kb del + p.R168C	30-kb del + p.R168C

TABLE II

Nine High-Risk/Asymptomatic Cases From New York Screening *

Case No.	Allele 1	Allele 2
6	c.-335G>A + p.I546T + p.R380W [‡]	c.128_-123delATCAGC + p.L618S
7	p.A5P + p.D232N + p.Y303C	p.I546T + p.D556fs ^{*1} [‡]
8	p.H375Qfs ^{*3} [‡] + p.I546T	c.-348C>T + p.A5P + p.D232N + p.Y303C
9	p.R63C [§] + p.I546T	p.R111 ^{*‡}
10	c.-128_-123delATCAGC + p.L618S [‡]	p.L618S [‡]
11	p.M101V [§] + c.1786 + 5C>G + p.A625T	p.M309V [§] + p.I546T
12	c.147G>C/p.G49=+ p.I546T	p.K83E [§] + p.I546T
13 [‡]	p.A5P + p.D232N + p.Y303C	p.A5P + p.D232N + p.Y303C
14	p.T452I [§]	p.A5P + p.D232N + p.Y303C

* The variants p.Y303C and p.L618S have been reported as mild (Wenger et al., 2013), although there is one report of a late-infantile case with p.L618S (Xu et al., 2006), so we have conservatively assigned this as severe. c.147G>C/p.G49 = is a splice site variant seen in a late-onset KD patient. c.147G>C is a splice site variant detected in a late-onset patient with KD (De Gasperi et al., 1996). Unless indicated otherwise, all other variants are nondisease-causing polymorphisms.

[‡] Genotype of this case was also observed in multiple cases categorized as at moderate risk for KD (Orsini et al., 2016).

[‡] Variants reported as severe (p.R111*) or suspected of being severe (nonsense mutations).

[§] VOUS.

TABLE III

Newborn Screening Results, Molecular Analysis, and Diagnostic Testing Results for Eight Infants *

Case No.	Allele 1	Allele 2
15	p.D445A# + p.L618S [†]	p.L618S [†]
16	c.-335G>A + p.A247T [‡] + p.I546T	p.A5P + p.D232N + p.Y303C
17	p.A5P + p.D232N + p.Y303C	p.I546T + p.D556fs ^{*1#} [†]
18	c.-511C>T + c.535-1G>A# [†]	p.R53Q [§] + p.I546T
19	c.-128_-123delATCAGC + p.L618S [†]	c.-128_-123delATCAGC + p.L618S [†]
20	c.-128_-123delATCAGC + p.L618S [†]	c.-128_-123delATCAGC + p.L618S [†]
21	p.M101V [§] + p.A625T	p.M101V [§] + p.A625T

* Classified as being at moderate risk (activity 0.16–0.29 nmol/mg/hr) and having either one known severe and one mild mutation (15–17) or two mutations observed in known later-onset patients (p.L618S) or variants of unknown. The variants p.Y303C and p.L618S have been reported as mild (Wenger et al., 2013), although there is one report of a late-infantile case with p.L618S (Xu et al., 2006), so this variant may be moderately severe. Unless indicated otherwise, all others variants are nondisease-causing polymorphisms.

[†] Variants reported as severe or suspected of being severe.

[‡] Variant reported in diagnosed patient with KD with no clinical description (Wenger et al., 2013).

[§] VOUS.

TABLE IV

Scoring System for HSCT Referral *

Parameter	Points
Abnormal neurologic examination	2
Abnormal MRI	2
Elevated CSF protein	2
Abnormal nerve conduction velocity	2
Abnormal brainstem auditory evoked response	1
30-kb homozygous deletion	4

* Transplantation is considered for infants with scores ≥ 4.