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Review

Newborn Screening for Krabbe Disease and Other Lysosomal Storage Disorders: Broad Lessons Learned

Joseph J. Orsini * and Michele Caggana

Newborn Screening Program, Wadsworth Center, New York State Department of Health, Albany, 12201-2002 NY, USA; Michele.Caggana@health.ny.gov
* Correspondence: Joseph.Orsini@health.ny.gov; Tel.: +1-518-473-8366

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Abstract: Newborn screening (NBS) for Krabbe disease (KD) began in New York (NY) in August 2006. In summary, after eight years of screening there were five infants identified with early-onset Krabbe disease. Four underwent transplant, two are surviving with moderate to severe handicaps, and two died from transplant-related complications. An additional forty-six asymptomatic infants were found to be at moderate or high risk for disease. Screening for KD is both analytically and medically challenging; since screening for KD possesses both of these challenges, and many more, the lessons learned thus far could be used to predict the challenges that may be faced when screening for other lysosomal storage disorders (LSDs). This paper briefly reviews reports of NBS for LSDs from varied world programs. The challenges encountered in screening for KD in NY will be highlighted, and this experience, combined with hindsight, will inform what may be expected in the future as screening for LSDs expands.

Keywords: newborn screening; lysosomal storage disorders; Krabbe disease

1. Introduction

Newborn screening (NBS) for Krabbe disease (KD) began in New York (NY) in August 2006. Two reports on the first eight years of screening and subsequent follow-up activities have been published [1,2]. In summary, after eight years of screening, there were five infants identified with early-onset Krabbe disease. Four underwent transplant, two are surviving with moderate to severe handicaps, and two died from transplant-related complications. An additional forty-six asymptomatic infants were found to be at moderate or high risk for disease. A recent review on NBS for KD highlights many of the challenges and lessons learned over the course of 10+ years of live screening for this challenging disorder [3].

In 2004, when work began with scaling up the then-published research assay of Li and coworkers, KD was one of five screenable lysosomal storage disorders (LSDs) [4]. The other diseases were Pompe, Fabry, Gaucher, and Niemann Pick A/B. Of these five LSDs, only KD was known to have a predominant infantile form with 80%–90% of cases being diagnosed in the first year of life [5]. Hence, it could be argued that from this perspective KD fit the traditional paradigm for newborn screening. However the treatment, a hematopoietic stem cell transplant (HSCT), was not traditional; it is risky and only effective if initiated prior to the onset of symptoms [6]. Further, evaluation of the research method showed that screening for KD would be the most analytically challenging of the five LSDs because the GALC activity was the lowest of the five enzymes. Thus, screening for KD was not only medically challenging, it was also analytically challenging. Since screening for KD possesses both of these challenges, and many more, the lessons learned thus far could be used to predict the challenges that may be faced when screening for other LSDs. This paper briefly reviews reports of NBS for LSDs from varied world programs. The challenges encountered in screening for KD in NY will be

highlighted, and this experience, combined with hindsight, will inform what may be expected in the future as screening for LSDs expands.

2. Background

Lysosomal storage disorders (LSDs) are inherited single-gene defects. Gene mutations lead to the production of a non-functional or non-existent lysosomal enzyme. Lysosomes are subcellular organelles present in nearly all mammalian cells. Lysosomal enzymes control the intracellular digestion of a wide range of macromolecules and a variety of other functions. The deficiencies lead to the storage of substrates and eventual disease. Disease symptoms vary widely and affect a range of tissues and organs. There are up to 50 known LSDs currently, and the majority are not treatable [7]. Treatment has been available for over two decades for some of them [8]. With most LSDs early treatment is essential for it to be effective; hence, the widespread interest in newborn screening.

3. Therapies

Currently there are two primary therapeutic approaches for treating patients with LSDs: enzyme replacement therapy (ERT) or hematopoietic stem cell transplantation (HSCT) [9]. Both of these rely on uptake of functional enzymes by the lysosomes of host cells to correct the deficiency. With HSCT the active enzyme is produced by the engrafted donor leukocytes, and with ERT the active enzyme is introduced directly by intravenous infusion. ERT was first demonstrated as an effective treatment for Gaucher disease by Brady and coworkers [10]. Treatment was dependent on the use of a functional version of β-glucocerebrosidase, the enzyme deficient in Gaucher disease, which was purified from human placentae and injected intravenously into two patients. The therapy resulted in a decrease in glucocerebroside levels in liver tissue. These results were promising, but issues with production, safety, and efficacy delayed the widespread use of ERT until the early 1990s. Today, ERT for six LSDs is approved by the U.S. Food and Drug Administration, and clinical trials with recombinant human enzymes are ongoing for several others [11]. ERT is administered by intravenous infusion, at dosages based on the patient's body weight. ERT is usually administered weekly or every other week and the therapy is lifelong.

HSCT initially utilized bone marrow and was first performed in 1980 on a patient with Hurler syndrome [12]. In that report, the one year old was transplanted with bone marrow from his mother in an attempt to replace the deficient enzyme, alpha-1-iduronidase. Thirteen months after transplant, alpha-1-iduronidase activity was confirmed in his serum and his urine contained a considerable amount of glycosaminoglycan metabolites. Additionally, his symptoms improved: hepatosplenomegaly disappeared, corneal clouding cleared, and further deterioration of the child's development was arrested.

Unfortunately, although both ERT and HSCT have been greatly improved, complications remain. HSCT effectively treats neuronopathic LSDs because the active enzyme can cross the blood–brain barrier, but the treatment itself can be life-threatening. ERT is safer than HSCT, but expensive, requires lifelong periodic infusions, and does not effectively treat LSDs with neuronopathic symptoms. Hence, other therapies are under evaluation [9,13,14]. These include gene enhancement, gene therapy [15], chaperone therapy, and substrate reduction therapy [16]. More recently, combination therapies, such as HSCT/ERT or HSCT/gene therapy, have been under investigation [13,14]. Since newborn screening offers an opportunity for early diagnosis, the cohort of newborns affected with LSDs detected by screening will expand and the development of better therapies will be accelerated.

4. Newborn Screening

There was early recognition that treatment is most effective when administered prior to the onset of symptoms; hence, assays to detect LSDs in newborns using dried blood spot samples were developed shortly after the development of therapies [17–19]. The first assays were introduced in the late 1990s, the earliest approach was an immunoassay to detect elevated lysosome-associated

membrane proteins (LAMP1 and LAMP2). LAMP1/LAMP2 is elevated in many, but not all, LSDs so a two tiered approach was developed to detect newborns with an LSD. In the first tier, elevated LAMP levels would lead to second tier tandem mass spectrometry analysis to detect an elevated substrate. The elevated specific substrate would point to the LSD. Later, an assay targeting specific LSDs was reported by Chamoles and coworkers, where screening for eight lysosomal enzymes in dried blood spots on filter paper permitted the detection of infants at risk for 12 LSDs with a Hurler-like phenotype [20].

In 2004 two papers were published that would be the starting points for the first ongoing live newborn screening of LSDs. In one paper, Chamoles and co-workers published a fluorescent-based assay to screen for Pompe disease [21]. This dried blood spot assay was developed shortly after it was shown that acarbose could be used to inhibit the activity of maltase glucoamylase (MGA); an isoenzyme that interfered with activity measurements of acid α-glucosidase, the deficient enzyme in Pompe disease. In the second paper, Li and coworkers provided a tandem mass spectrometry (MS/MS) assay enabling multiplex screening for five lysosomal storage disorders; Krabbe disease, Pompe disease, Fabry disease, Gaucher disease, and Niemann Pick A/B disease [22]. In 2006, the implementation of screening for LSDs began nearly simultaneously in New York state and the Taiwan newborn screening programs [1,23].

The Taiwan newborn screening program (NSP), which is comprised of three centers, began screening for Pompe disease with an adaptation of the Chamoles fluorescent substrate-based assay [23]. At one of the centers, the fluorescent assay was discontinued and a tandem mass spectrometry assay was utilized; the results of this study were published in 2014 [24]. A paper published in 2011 indicated that 19 of 344,056 infants screened in the years 2005–2009 were confirmed to have Pompe disease [25]. Six of these had infantile Pompe disease with neonatal hypertrophic cardiomyopathy and the other 13 newborns without hypertrophic cardiomyopathy were classified as having later-onset Pompe disease.

4.1. Krabbe Disease

In New York, newborn screening for Krabbe disease was initiated in August 2006 using a modified version of Li and co-workers' method [1,2] in conjunction with a second tier molecular test to detect the two common deletion mutations in parallel with full DNA sequence analysis of the GALC gene. Now, after 10 years of testing, 2.4 million infants have been screened and 444 infants were referred for follow-up diagnostic evaluation. However, only five infants have been determined to have infantile Krabbe disease. There are an additional 14 infants that have been categorized at "high risk" and 52 more categorized as being at "moderate risk" for KD. Since none of these "at risk" individuals have developed the disease to date, they are still at risk for the late-onset forms of KD.

4.2. Pompe Disease

Using a similar two-tiered assay, New York initiated screening for Pompe disease on 1 October 2014. As of August 2016, 460,000 infants have been screened. Four babies have been diagnosed with infantile PD and 18 possible late-onset cases have been identified (unreported data). Possible late-onset cases are defined as infants having low GAA activity in leukocytes and two mutations in the GAA gene (which may include known pathogenic variants or variants of unknown significance). To date, none of these 18 children have displayed symptoms.

4.3. Missouri LSD Screening

In January 2013, Missouri became the first state to screen for four LSDs (Pompe, Fabry, and Gaucher diseases, and Hurler syndrome (mucopolysaccharidosis I (MPS1))), using a newly-introduced digital microfluidics-based fluorescent assay [26]. Within the first six months, 43,701 specimens were screened and 27 babies were reported to have "a confirmed diagnosis of an LSD genotype" (eight with Pompe disease, one with Gaucher disease, 15 with Fabry disease, and three with

MPS1). Of these 27 cases, 14 were reported to be cases of early-onset disease, seven were reported to be late-onset cases, and six were of unknown phenotype. All diagnoses were based on low diagnostic laboratory enzymatic activities and genotype information. These numbers correspond to detection rates of 1:5,463 for Pompe disease, 1:43,701 for Gaucher disease, 1:2,913 for Fabry disease, and 1:14,567 for Hurler syndrome. All of these are higher than reported incidence rates and are calculated assuming that all late-onset cases and cases with an unknown phenotype will eventually develop disease.

The detection rate of Fabry disease in the context of newborn screening in Missouri is more than 13 times the expected frequency. However, this trend is not unprecedented. An Italian prospective pilot study for Fabry disease screened 37,104 consecutive Italian male neonates and found 12 boys with deficient α-galactosidase A activity [27]. A more recent study in Taiwan found an unexpectedly high prevalence of the cardiac variant Fabry mutation IVS4+919G3A among both newborns (1 in 1600 males) and patients with idiopathic hypertrophic cardiomyopathy in the Taiwan Chinese population [28].

In the Italian study one of the 12 male neonates had a splice site mutation observed in patients with classic Fabry disease and the other 11 had mutations indicative of late-onset Fabry disease. The authors suggest that the later-onset phenotype of Fabry disease is underdiagnosed among males and that older individuals with cardiac, cerebrovascular, and/or renal disease may have undiagnosed late-onset Fabry disease. They also state that the earlier identification of these patients via newborn screening would permit family screening and earlier therapeutic intervention. Additionally, they noted that the higher incidence of the later-onset phenotype in patients raises ethical issues related to the timing of screening; they questioned whether screening should be in the neonatal period or later in life with screening for other treatable adult-onset disorders.

In addition to live screening, there have been multiple reports of retrospective pilot studies to screen for LSDs [29–31]. In these studies, samples that screen positive, biochemically, for a given LSD are genotyped. This combination of tests 'rules in' the diagnosis if pathogenic variants are detected and they were used to estimate the incidence of the studied LSD in the screened population. However, these estimates assume all individuals with both biochemical and molecular findings will develop disease. Based on current experience, with the exception of NBS for infantile KD in NY, the incidence rates are higher than expected for the LSDs, particularly when later onset forms of the LSDs are considered.

5. Krabbe Disease Screening in NY—Analytical Challenges

5.1. Analytical Challenges in LSD Screening

In 2004, when feasibility studies began, it was evident that screening for KD would likely be the most analytically challenging of the five LSDs published by Li et al [22]. GALC activity was the lowest of the five enzymes and there was a reported inhibitor effect with GALC. The publication indicated that all enzyme activities could be measured simultaneously on a MS/MS but, to the contrary, each enzyme reaction had to be set up individually, so multiplexing was not straightforward. At the start of screening, we considered using a reference enzyme to measure along with GALC, but we thought a single reference enzyme would not suffice as all of the LSD enzymes had a broad range of activities when measured in dried blood spots and, thus, we did not think it was prudent to rely on results of one reference enzyme. As a result, the KD screening algorithm in NY included measurement of only the GALC enzyme activity combined with second-tier DNA testing. The latter reduces the number of referrals but, even with second-tier DNA testing, the false positive rate was still very high [1].

Screening for low LSD enzyme activity differs from screening for amino acids and acylcarnitines in expanded newborn screening. In that screening, the concentration of the accumulated marker in the dried blood spot is directly measured. In LSD enzyme screening however, the activity is a measure of how much enzymatic product is produced from the enzyme reacting with added substrate. This reaction is dependent on the amount of available enzyme. Since lysosomal enzymes are primarily present only in the white blood cells, the measured activities are, therefore, dependent on the

infants' leukocyte counts, which are not accounted for in screening assays, and can normally vary by a factor of six from 5000–30,000 leukocytes/mm^3 (newborns to two-week old infants) [32]. In addition, secondary effects from exposure of the specimens to environmental effects of heat and humidity are more pronounced. Moreover, specific to Krabbe NBS, there is the added variable of hematocrit, which seems to act as an inhibitor of the GALC reaction [22], so higher hematocrits contribute to low measured activities. These variables all impact enzyme activities and accounting for them in dried blood spots is not trivial, although attempts have been made to quantify them [3,33]. Since GALC activity, measured in dried blood spots, is already quite low due to the relatively low initial activity of this enzyme, contributions from the factors described above can lead to a misperception of a disease and a false positive screen result. A surrogate for assessment of leukocyte levels and enzyme stability is the measurement of multiple LSD enzymes' activities on each sample. Since the screening assay has been vastly simplified (six-plex assay/GELB, and other references), it is now practical to perform multiplex screening for six LSDs.

In addition to screening all infants for Krabbe and Pompe, NY has been pilot screening a subset of newborns for four additional LSDs (Fabry, Hurler, Gaucher, and Niemann Pick A/B). Thus, pilot samples are tested for six LSDs. Not surprisingly, evaluation of five other reference enzymes along with GALC or GAA, is invaluable for reducing false positive rates. In general, specimens from infants with low GALC or GAA activity also exhibit reduced activities for all of the other enzymes. Table 1 shows the pilot cohort sorted by those infants with GALC daily mean activity <20% and those with GAA <20% of the daily mean activity. Data were sorted by this value because <20% daily mean activity is the preliminary cut-off for retesting in duplicate for both enzymes. The data shows that in specimens with low GALC, the average GLA activity (Fabry disease) is 57.8% and averages for all of the other enzymes are below 100%. The averages of all the reference enzyme activities for samples with GALC <20% is 67% and for GAA it is 59.3%. Thus, this bias to overall lower activities influences referral rates if only a one enzyme activity is examined. The full impact of this effect on the number of false positives was not known when NY began testing for one LSD enzyme activity (GALC) and referred all infants with one or two mutations (2006–2014).

Table 1. Pilot cohort showing the averaged percent of mean activity values for each enzyme; for those specimens with either GALC or GAA that is below 20% of the daily mean.

<20% Initial Analyte	Average 6-Plex %DMA						Avg of Extra Enzymes
	GALC	IDUA	GLA	ABG	ASM	GAA	
GALC	18.0	64.1	57.8	58.3	86.0	69.0	67.0
GAA	61.7	47.0	69.2	50.7	67.8	17.5	59.3
Population Average	100	100	100	100	100	100	

These data, and earlier work in NY and that of other programs [26,34], showed multiplex LSD testing could reduce false positives; yet there were concerns with the potential to identify individuals with other LSD disorders not mandated on the screening panel if multiplex testing was performed on all infants. The Biochemical Genetics Laboratory (BGL) at the Mayo Clinic has developed a novel approach to eliminate this concern. The results of the multiplex assay are used to calculate permutations of all possible ratios. The measured activities and ratios are adjusted for relevant covariates [35] and interpreted by post-analytical interpretive tools [36,37] based on a multivariate pattern recognition software (CLIR, Collaborative Laboratory Interpretive Reports; https://clir.mayo.edu) which does not use absolute measurement of cutoff values. As no tools are created for non-mandated conditions, any low enzyme activities remain blinded and secondary calculated ratio abnormalities for the target conditions are dismissed by rules (if the activity of a target enzyme is not reduced then any abnormal ratios are ignored). This system has been applied prospectively to the selective screening of Krabbe disease, Pompe disease, and MPS-I at BGL for the state of Kentucky since February 2016 (Dr. Piero Rinaldo, personal communication). Although testing each sample for six enzymes can

reduce retest and referral rates, it is expensive to test every sample for six enzymes when they are not part of the screening panel. Currently, in NY, only samples that initially screen positive for GALC and/or GAA are retested for all six enzymes, thereby reducing the cost, and minimizing the possibility of detecting an infant with an LSD other than Krabbe or Pompe disease. NY plans to use the CLIR tool in the future. There are two additional variables that impact enzyme activity and are related to factors described above. First, low birthweight infants are generally premature and have lower hematocrits than full term infants, since hematocrit acts as an inhibitor of GALC enzyme activity, higher activities are measured in low birthweight infants. The second factor is the infant's age at the time of collection. In general, white cell counts decrease with age following birth. A higher white cell count leads to higher activities. Hence, use of these two variables (birthweight and patient age at time of sample collection) in the CLIR data assessment further reduces false positives, while protecting against false negative results.

It is customary for NBS programs to use a conservative approach to prevent false negative results, in establishing the initial screening criteria, i.e., "cutoffs" to categorize infants as high risk or low risk. Over time, the feedback obtained from the medical evaluation of screen positive newborns is used to fine tune the criteria for subsequent screening. Even under the best of circumstances, it is challenging to set the criteria for new conditions. True positive dried blood specimens from newborns may be difficult to obtain. Older specimens used in method validation are less reliable than ones obtained prospectively, as marker concentrations or enzymes' activities may change with time and/or storage conditions. Lastly, blood from clinically-diagnosed children or adults may have different marker concentrations or enzyme activities due to age or treatment. Therefore, with only a limited number of positive samples available, newborn screening programs almost always err on the side of preventing false negatives, which means initially setting the criteria at a level that detects more false positive infants. This has been the case in NY, where only second-tier DNA testing was initially used to reduce false positives. However, the majority of the samples that screen positive for the enzyme will have one or more mutations, so second-tier DNA testing has not substantially reduced the false positive rate.

5.2. Second-Tier Molecular Testing

Low enzyme activities are not only a result of mutations in the GALC gene, but can also result from the other factors described above. While second-tier DNA testing does reduce referrals, the DNA testing also identifies gene variants, and while some of the variants will be known disease-causing mutations, others may be non-disease-causing variants or variants of unknown significance. In fact, in NBS for KD, DNA analysis has detected multiple variants in almost every sample that was sequenced, with benign variants found in >40% of the cases. Early in screening we found a number of infants with very low dried blood spot (DBS) GALC activity (<8% of the daily mean) who only had benign variants; in all likelihood these infants also had reduced GALC activity because of low white cell counts, and/or high hematocrit, or other factors. Another significant proportion of referred infants (~75%) had only one identified mutation and were likely only carriers, but there was concern that some had deletions or other rearrangements that would be missed by DNA sequence analysis, so they were referred for clinical evaluation that included deletion/duplication analysis. This additional analysis is now under development in NY screening and the use of this test would permit reporting these infants as screen-negative because they would be "true carriers". The other variables that can lead to pronounced measured enzyme deficiencies in dried blood spot samples would be assessed by the multiplex enzyme testing.

In retrospect, the conservative approach taken for Krabbe screening provided invaluable information on the correlation between enzyme activity measured in the diagnostic laboratory and genotypes, and the population-based ascertaining of common genotypes in NY. Over time we have been able to use these data to predict to some degree the diagnostic enzyme activities returned after the evaluation of the infants. While our conservative approach resulted in a higher false positive rate, important genotype information was collected for NY and for other programs performing NBS for

KD. For example, in NY, we initially considered the p.Thr112Ala (legacy: p.Thr96Ala) mutation to be mildly pathogenic and 85 infants who carried the p.Thr112Ala variant were referred. However, we came to understand that infants with a common genotype containing the p.Thr112Ala mutation without p.Ile562Thr (legacy: p.Ile546Thr) in cis were not at risk for KD [38], and as of November, 2015 infants carrying this GALC variant were no longer referred [3]. Similarly, DNA testing for GAA mutations has identified a high number of cases with the p.Val222Met variant and this variant was reported at a fairly high frequency in retrospective screening of dried blood spots for Pompe disease in Hungary [29], yet there is no knowledge that this variant is disease-causing.

Initially, in NBS for KD, genotyping was the most useful tool for prediction of early-onset KD but, as discussed before, unknown genotypes were detected. Clearly if two known or highly likely deleterious mutations are detected, severe disease can be predicted, but the prediction of disease severity becomes more difficult, if not impossible, as one considers other potential phenotypes. Reports of late-onset cases for a given LSD are generally based on a combination of symptoms, enzyme activity and, sometimes, genotype. However, when there are only a few reported or known cases of late-onset disease associated with a particular genotype, the following questions remain: do these individuals have a rare genotype that leads to disease? Is this a prevalent genotype in KD but patients harboring it have later-onset disease and remain undiagnosed, misdiagnosed, or unreported? Are those with it asymptomatic? A recent report by Musumeci and coworkers concluded that the most possible explanation for a low frequency of a homozygous c.32-13T>G genotype in Caucasian Pompe patients was reduced or incomplete penetrance, based on the fact that these patients were phenotypically similar to those reported for patients who were compound heterozygous for their c.32-13T>G mutation and another mutation [39]. There are multiple hypotheses for reduced penetrance and efforts are underway to better understand these observations [40]. It seems highly likely that there are many more variables contributing to late-onset disease prediction and manifestation, whereas with severe disease, the presence of two severe mutations in a single gene are most likely to be the sole driving force.

Through screening for Krabbe disease and Pompe disease, we are finding that the literature has little to no information on many of the genotypes detected in infants who are asymptomatic at clinical evaluation. For example, p.Tyr319Cys (legacy: p.Tyr303Cys), a mutation in the GALC gene, has been detected in NY in infants with South Asian ethnicities. Another example, p.Leu634Ser (legacy p.Leu618Ser) is primarily found in infants with Japanese ethnicity. Infants homozygous for these variants have been assigned to the high risk category, but in the literature there are only two cases of individuals homozygous for p.Leu634Ser, and both patients were adults when diagnosed with KD [41]. There are no known cases of Krabbe disease homozygous for p.Tyr319Cys. Both variants attenuate activities similarly when expressed in vitro [42]. Genetic studies measuring the overall frequency of these two variants in the relevant sub-populations is needed to establish the true risk for disease. Therefore, allele frequencies in cases can be compared to the frequencies in controls to determine the risk of disease attributed to the variant. Next generation sequencing of a series of genes in individuals with and without disease can be used to determine if there are modifiers that contribute to phenotype. While the most experience has been gained with KD, the same arguments can be made for other LSDs. In the Italian Fabry disease screening study, the authors contend that patients attending clinics for treatment of kidney disease may have late-onset Fabry disease [27].

5.3. Screening for LSDs with Late Onset Forms

If a disease has an early age of onset, presenting in infancy or early childhood, presumably there would be more support for screening. However, if onset is not until late childhood and into adulthood, would screening of newborns still be desired? Researchers in the Netherlands surveyed their population to determine the overall acceptance of newborn screening for Pompe disease [43]. One component of the survey was aimed at measuring the acceptance of screening for late-onset PD. The authors reported 87% of the general population supported Pompe disease screening even if it might detect individuals with late-onset disease. However, the questionnaire did not specifically ask

if they supported screening if some proportion of babies who screen positive may not ever or never develop disease. It would be interesting to see how this question impacted the results of the survey. Since it is possible that newborns who screen positive for a condition with a late-onset component will never develop symptoms, newborn screening programs have difficulty segregating true from false positives, and this conundrum persists for many years. This challenge to NBS, although not new, is certainly exacerbated when programs screen for diseases known to have a late-onset phenotype and families cannot be offered any prognostic predictions. We currently have an ongoing consented pilot study that is addressing the ethical, legal, and social implications of newborn screening for late onset LSDs.

The feedback NBS programs receive from the medical community regarding the clinical status of their screen-positive infants is an important means for setting cut-off criteria for continuous quality improvement. If programs wish to capture all cases, including those that are late-onset, then controls from patients with a biochemical and molecular pictures indicative of late-onset disease must be used in the evaluations. If an infant harboring a mild variant is detected in a population, programs must consider the risk versus benefit of detecting and referring the infant to medical attention.

To illustrate this point, in NY, we have been screening for Pompe disease since October 2014. Infants with GAA activities that are <15% of the daily mean are sent for second-tier DNA testing. In this subset of infants with low GAA activity, we are finding that the activities striate. Infants referred and diagnosed with the infantile form of Pompe disease have lower activity ranges than those determined to be possible late-onset cases. Interestingly, when NY set up Pompe screening, the Missouri newborn screening program provided blinded samples containing infantile, asymptomatic late-onset, and other referred infants for NY to evaluate. The NY cutoffs were conservatively set to ensure all PD patients, early and late, would be picked up via screening. A lower cutoff could have been set to ensure only the early onset cases would be detected, but this would mean potentially missing the late-onset cases. Thus, with higher cutoffs come higher referral rates and a higher number of possible late-onset cases.

Programs cannot overwhelm the medical community with referrals, nor should programs subject affected families to unnecessary evaluation and long-term follow-up required to determine if their child is indeed at risk for late-onset Pompe disease if it is not necessary. Of course, it is newborn screening that allows geneticists to better understand the relationship between the biochemical and molecular data in these seemingly well children. Adding to this complexity, it is thought that patients with later-onset forms of disease benefit the most from ERT and HCST, while those with the most severe forms of disease may have substantial complications from the treatment. In NBS for KD, outcomes for the latter cohort have not been good [2]. However, no children have presented with mild disease, so none of these children have been transplanted. Consequently, outcomes in this group remain unknown. Perhaps in screening for PD, we will see the efficacy of treating the late onset cases sooner.

5.4. Referred Infants and the Challenges of Diagnostic Testing

At the time KD screening began in NY, it was anticipated that 1–3 cases of KD would be detected based on a birthrate of 250,000/year and a reported incidence of 1:100,000 births with most cases having the infantile form of disease. Yet, thus far, NY newborn screening has detected a larger than anticipated number of "possible" late-onset Krabbe cases. Infants are defined as high or moderate risk for late-onset KD if GALC activity measured in leukocytes is low. As of August 2016, five of ~2.4 million infants screened had infantile KD; an additional nine infants are categorized at high risk, and 40 are at moderate risk for late-onset KD. These risk categories were defined by the New York State Krabbe Disease Consortium and are currently being re-evaluated. The actual number of children truly at risk for KD will likely be revised and these numbers are expected to be reduced. However, since KD is a disease primarily diagnosed in infants, detecting a high number of possible late-onset patients was not expected. With other LSDs, such as Pompe, a higher rate of late-onset cases is expected and this will likely lead to an even larger cohort of possible late-onset cases for any LSDs with a predominant late-onset form.

Diagnostic laboratories are realizing the same challenges as newborn screening laboratories in that they may not have large cohorts of known late-onset patients to set reference ranges for these types of cases and use screening results as a surrogate to assist with categorization. Assays are designed to provide a rule-in of a diagnosis in the context of clinical symptoms, but referred infants identified via NBS are almost always asymptomatic. For example, in the initial Taiwan report on NBS for Pompe disease, 117 of 132,538 infants screened who were referred for follow-up diagnostic testing had activities above the reference range set by the clinical laboratory [23]. These individuals reportedly could have been either false positives or they could be individuals who may develop late-onset Pompe disease. Diagnostic enzyme activity-based tests were not designed to predict which patients will go on to develop late-onset disease. Unfortunately, there is always some level of measureable residual activity, and determining what level is enough to prevent disease is difficult and disease-specific. Thus far, GALC enzyme activity values measured either through screening or the diagnostic lab do not predict disease severity [1]. Infants with two severe mutations have similar activities to those with two mild mutations [3]. In the screening and diagnostic realms, more specific assays are under development and additional markers may be developed for other LSDs. Initially, in NY, the diagnostic lab activity results were considered to be definitive. However, those results were not always consistent with the genotype or the phenotype of the baby. The Krabbe Consortium recommended retesting of all infants with low enzyme activity. Of 24 retested infants 10 had GALC activities that were higher in the second sample and in none was the activity lower on the repeat sample. As a result of this work, one infant was re-categorized from high to moderate risk for KD, three were re-categorized from high to no-risk for KD, and five were re-categorized from moderate to no risk for KD. Having second-tier GALC sequence information is advantageous to recognize incongruent results between genotype and the GALC activity.

Additionally, genotypes do not always explain all of the variability in the enzyme activities, especially when patients with the same genotype have different enzyme activities (see above). There are some things programs can do to aid in the interpretation of results. Genotypes can be studied by in vitro methods designed to assess the potential impact of the genotype on activity [42]. These are time-consuming and not available to most laboratories. In addition, having diagnostic lab results from the same laboratory is useful as inter-lab variability is eliminated. However, biological variables and variables in sample handling may still impact the in vitro enzyme activities measured at diagnostic laboratories. For example, in NY after 10 years of screening for Krabbe disease we have referred 490 infants, 45 of which had the same genotype with one allele containing p.Arg184Cys (legacy: p.Arg168Cys) (a common non-disease-causing polymorphism) and g.30Kb-Del (the most common severe disease causing variant) and the second allele containing the two non-disease-causing variants Arg184Cys and p.Ile562Thr. Diagnostic laboratory activities for this cohort of infants ranged from 0.20–0.70 nmol/mg/h, with six of the 45 infants categorized at moderate risk for KD based on the activity value, even though only one disease-causing mutation was detected in the GALC gene. Certainly sample quality issues could explain the low diagnostic laboratory activity values, however it is more difficult to explain the higher activity values detected in other samples. Sample quality was only questioned by the diagnostic laboratory in one of the 45 samples, however it is difficult to quantitatively measure the quality of a white cell pellet isolated from whole blood. Contamination from red cell lysate can contribute to low bias in the results and it can be readily visualized. There may be cellular level variables, such as leukocyte profile differences, variation in lysosome number in the leukocytes, co-enzyme and inhibitor effects, etc., that all affect the in vitro enzyme activities. Lastly, next-generation sequencing and/or MS/MS biochemical profiling of this cohort of 30 kb carriers could possibly reveal the existence and influences of modifier genes on activities.

5.5. Psychosine Testing

The inability to predict KD phenotype from enzyme activity and genotype led to the development of another biomarker on dried blood spots [44,45]. The compound psychosine is thought to be

a substrate of GALC, and babies with infantile KD have been shown to have very elevated psychosine concentrations in their initial dried blood spot specimens. Therefore, elevated psychosine levels in dried blood spot samples has thus far proven to be predictive of infantile KD [45]. However, currently no data exists to determine if and when later-onset phenotypes of KD will develop. Long-term follow up and prospective studies examining the psychosine concentrations over time in the high-risk population are needed. Unfortunately, not all families are willing to be involved in these studies. Studies are under way to re-evaluate psychosine concentrations in dried blood spot samples from infants in the high risk cohort as initial testing of these samples on sensitive mass spectrometers showed a positive correlation between psychosine concentration and genotype (unpublished data). Additionally, the development of more sensitive MS/MS GALC assays with lower limits of detection are underway. This work should improve the precision of residual enzyme activity measurements. It is likely the use of these two tests will reduce the number of infants that require follow-up for possible late-onset KD [46].

5.6. Diagnostic Evaluations

Once an infant with a positive screen is referred for evaluation for a LSD, the family is called so the infant can be evaluated clinically. Blood is collected for diagnostic enzyme testing and, most of the time, at least with KD, the diagnostic laboratory result provides evidence of a false positive screen. Infants with very low diagnostic enzyme results and two mild or otherwise private mutations in the GALC gene have posed a major challenge to the metabolic centers [2]. In practice, a diagnosis of most autosomal recessive genetic disorders in the absence of symptoms relies on a combination of information from three tests: enzyme activity, measured substrate level, and the detection of two mutations. If any two are abnormal and the clinical evaluation shows subtle abnormalities, then the disease is considered confirmed. Prior to the addition of psychosine testing, the diagnosis of infantile KD in newborns with low GALC activity and two mutations with unknown severity relied on a clinical examination and results from a panel of neurodiagnostic tests. The neurodiagnostic test panel included lumbar puncture to measure protein levels in the CSF, brain MRI, brainstem auditory evoked response, and nerve conduction studies [2]. Results from these tests are difficult to interpret in newborns. Hence, a scoring system was developed to quantify the results from this battery of tests; but the metabolic centers in NY still found this system difficult to use. This was complicated by the fact testing had to be conducted quickly to allow parents to opt into treatment with the highest chance of efficacy. Now that psychosine levels can be determined, this can greatly enhance the diagnosis of infantile Krabbe disease. There is a need for a similar biomarker to aid in the diagnosis of Pompe disease, and there are reports of biomarkers that can be used in the diagnosis of other LSDs [47–49]. There is also a need for more quantitative diagnostic tests and standardized methods for clinical evaluations and follow up. The diagnostic tests should include age appropriate reference ranges, and clinical evaluations need to be more quantifiable in order to distinguish the various phenotypes. These more quantitative test results and clinical findings can be used to better establish the phenotypes prior to administering therapy. This will be important for long-term follow-up, as there needs to be a better understanding of the phenotypes of patients prior to treatment in order to better understand the true efficacy of the therapies.

With newborn screening for Pompe disease, diagnosis of the classic infantile form is based on the presence of cardiomyopathy, which is generally apparent by echocardiogram and/or X-ray. However, like Krabbe disease, the diagnosis of the later-onset Pompe disease cases has proven to be challenging; these challenges are likely to extend to other LSDs. The burden to provide answers to parents are real and, in some cases, the clinical exam and other test results will show only subtle signs of disease. The pressure to treat in these cases is high but as in other LSDs treatments, can be risky and expensive. This may be compared to the more traditional disorders detected by newborn screening, such as phenylketonuria or congenital hypothyroidism, in which diagnosis is less complex and treatment far less complex and certainly not life-threatening. Since treatment for these traditional diseases is considered low-risk, it is

more often used, even for the milder forms of disease, although this increase in treatment also has the unintended consequences of a perceived increase in incidence. With LSDs, especially KD, the harms from treatment are well understood so, presumably, physicians are less likely to offer it. Therefore, the need to be very sure of a diagnosis can lead to treatment delays in affected infants.

6. Summary

When newborn screening for KD began in NY, it was not possible to distinguish between the early- and late-onset phenotypes using the DBS enzyme assay. Second-tier DNA testing offered the potential to help with the interpretation, but with some cases uncertainty still remains as even diagnostic enzyme results are not predictive of phenotype. Psychosine testing applied to dried blood spots of infants predicted to have EIKD clinically confirmed the diagnosis in the cohort. Use of this biomarker is likely to reduce the number of cases requiring long-term follow-up. More biomarkers, like psychosine, are needed for the other LSDs since substrate accumulation seems to be less ambiguous than enzymatic product detection in the measurement of enzyme dysfunction.

In the absence of a family history of LSDs, newborn screening is the only system for early detection of these devastating diseases. The conundrum surrounding NBS LSD screening is its goal. Is the goal to detect only infantile and early childhood forms of the disease or are programs obligated to also capture late childhood, juvenile, or adult forms? Once we include these latter examples, phenotype prediction becomes less clear and there is not enough data yet to know where to set the timeframes for short- and long-term follow-up. Since LSDs all have a spectrum of presentations, subsequent diagnostic testing, i.e., enzyme activities, biomarker concentrations, neurodiagnostic tests, and physical exams will occur on a continuum. Unfortunately, while the LSDs have a spectrum of phenotypes, treatment, either HSCT or ERT, is not a continuum. Perhaps reverse thinking is needed by NBS programs when defining cut-offs, i.e., setting them conservatively (lower activity cutoffs) in order to exclude juvenile and adult cases. These decisions on the scope of screening should be made prior to the onset of universal testing.

Possibly, the greatest lesson learned in screening for KD is the importance of frequent face to face meetings to review the screening and outcomes data. These meetings should include reviews of the screen and follow-up diagnostic testing results, methods, and necessity, as well as the corresponding elements and frequency of long-term follow-up. They should also consider changes in knowledge of the disease, as well as improvements in the sensitivity of the analytical methods and the availability of new findings; with a focus on reviewing the analytical and molecular findings from the children detected, but who remain asymptomatic. This is challenging, as frequently the data collected on these children is limited since these cases are often lost to follow up. To hasten the understanding of the significance of the constellation of screening and biochemical, molecular, and clinical findings, more of these kinds of data need to be collected from symptomatic late-onset patients. Additionally, it would be helpful if more targeted genetic studies were undertaken to reveal frequencies and backgrounds of gene variants to determine the penetrance of the late-onset variants. These data would assist in the prediction of the true disease risk from the given molecular findings. Additionally, studies with whole-genome sequencing on diseased populations could reveal if secondary genes influence disease severity. Finally, more studies in metabolic profiling, such as that recently reported by Weinstock and coworkers for Krabbe disease [50], are needed as they may reveal secondary biochemical pathways and other potential biomarkers associated with the disease. Face to face meetings offer the best chance for experts to share data and information that can be used to bridge the gaps in knowledge across the varied disciplines so the benefits of newborn screening can be fully realized.

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