The North Carolina Experience with Mucopolysaccharidosis Type I Newborn Screening

Jennifer L. Taylor, PhD[1], Kristin Clinard, MS, CGC[2], Cynthia M. Powell, MD[2], Catherine Rehder, PhD[3], Sarah P. Young, PhD[3], Deeksha Bali, PhD[3], Sara E. Beckloff, PhD[4], Lisa M. Gehtland, MD[1], Alex R. Kemper, MD, MPH, MS[5], Stacey Lee, PhD[1], David Millington, PhD[3], Hari S. Patel, MS[4], Scott M. Shone, PhD[1], Carol Woodell, BSPH[1], Scott J. Zimmerman, DrPH[4], Donald B. Bailey, Jr, PhD[1], and Joseph Muenzer, MD, PhD[2]

Objective To evaluate the performance of a 2-tiered newborn screening method for mucopolysaccharidosis type I (MPS I) in North Carolina.

Study design The screening algorithm included a flow injection analysis-tandem mass spectrometry assay as a first-tier screening method to measure α-L-iduronidase (IDUA) enzyme activity and Sanger sequencing of the *IDUA* gene on dried blood spots as a second-tier assay. The screening algorithm was revised to incorporate the Collaborative Laboratory Integrated Reports, an analytical interpretive tool, to reduce the false-positive rate. A medical history, physical examination, IDUA activity, and urinary glycosaminoglycan (GAG) analysis were obtained on all screen-positive infants.

Results A total of 62 734 specimens were screened with 54 screen-positive samples using a cut-off of 15% of daily mean IDUA activity. The implementation of Collaborative Laboratory Integrated Reports reduced the number of specimens that screened positive to 19 infants. Of the infants identified as screen-positive, 1 had elevated urinary GAGs and a homozygous pathogenic variant associated with the severe form of MPS I. All other screen-positive infants had normal urinary GAG analysis; 13 newborns had pseudodeficiency alleles, 3 newborns had variants of unknown significance, and 2 had heterozygous pathogenic variants.

Conclusions An infant with severe MPS I was identified and referred for a hematopoietic stem cell transplant. Newborn IDUA enzyme deficiency is common in North Carolina, but most are due to pseudodeficiency alleles in infants with normal urinary GAG analysis and no evidence of disease. The pilot study confirmed the need for second-tier testing to reduce the follow-up burden. *(J Pediatr 2019;211:193-200)*.

Mucopolysaccharidosis type I (MPS I, OMIM 607014) is a rare, autosomal recessive disorder caused by a deficiency of the lysosomal enzyme α-L-iduronidase (IDUA, EC 3.2.1.76) encoded by the *IDUA* gene.[1] The IDUA enzyme is required for the catabolism of the glycosaminoglycans (GAGs), heparan sulfate, and dermatan sulfate. The accumulation of GAGs leads to a chronic and highly variable multisystem disease. MPS I is a pan-ethnic disorder with an estimated incidence of 1 in 100 000 live births. It has a wide spectrum of severity with 2 phenotypes now recognized: severe (Hurler syndrome) and attenuated (Hurler-Scheie and Scheie syndromes). All patients with MPS I have a broad range of clinical manifestations, including skeletal disease, hepatosplenomegaly, cardiac valve dysfunction, recurrent otitis media with hearing loss, obstructive airway disease, and corneal clouding. The hallmark of patients with severe MPS I is the development of progressive cognitive impairment with death in childhood if untreated, whereas the attenuated patients have mild to absent central nervous system disease and a longer life span.

Treatment options for MPS I include weekly intravenous enzyme replacement therapy and hematopoietic stem cell transplantation (HSCT). HSCT is the recommended treatment for the severe form of MPS I, if less than 2 years of age, and enzyme

CLIR	Collaborative Laboratory Integrated Reports	MPS I	Mucopolysaccharidosis type I
DBS	Dried blood spot	MS/MS	Tandem mass spectrometry
DMA	Daily mean activity	NBS	Newborn screening
DST	Dual scatter plot tool	NCSLPH	North Carolina State Laboratory of Public Health
DUHS	Duke University Health System	PCP	Primary care provider
IDUA	α-L-iduronidase	UNC-CH	University of North Carolina at Chapel Hill
GAG	Glycosaminoglycan	VUS	Variants of uncertain significance
GAA	Acid alpha-glucosidase	S/IS	Substrate and internal standard
GBA	Glucosidase, β, acid		
HSCT	Hematopoietic stem cell transplantation		

From the [1]RTI International, Research Triangle Park; [2]University of North Carolina at Chapel Hill, Chapel Hill; [3]Duke University School of Medicine, Durham; [4]North Carolina State Laboratory of Public Health, Raleigh, NC; and [5]Nationwide Children's Hospital, Division of Ambulatory Pediatrics, Columbus, OH

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replacement therapy using laronidase (recombinant IDUA) is used to treat the non-neurologic manifestations of MPS I.[2] Because MPS I is a rare heterogeneous disorder, most patients are only diagnosed after significant clinical involvement and/or developmental delays. Consistent evidence from multiple peer-reviewed studies indicates that the earlier initiation of treatment for children with the severe form of MPS I (Hurler syndrome) is associated with markedly better cognitive functioning overall.[3]

The Advisory Committee for Heritable Disorders in Newborns and Children reviewed evidence demonstrating the existence of effective laboratory technologies available for MPS I newborn screening (NBS) and the benefits of early identification and treatment on cognitive outcomes. Based on this evidence, the Advisory Committee for Heritable Disorders in Newborns and Children recommended the addition of MPS I to the Recommended Uniform Screening Panel in March 2015, and the Secretary of the US Department of Health and Human Services accepted the recommendation and added MPS I to the Recommended Uniform Screening Panel on February 2016.[4]

NBS for MPS I in the US has been slow to be implemented, with only 2 states screening for MPS I at the start of the pilot, and currently only 15 states screening for MPS I as of October 2018.[5] To help increase implementation of NBS for MPS I, a contract was awarded in 2016 to a partnership led by RTI International and including the University of North Carolina at Chapel Hill (UNC-CH), Duke University, and the North Carolina State Laboratory of Public Health (NCSLPH) to conduct a prospective NBS pilot study for MPS I, by the *Eunice Kennedy Shriver* National Institute of Child Health and Human Development, National Institutes of Health, Department of Health and Human Services.

Methods

MPS I NBS was performed on samples received in NCSLPH from August 15, 2016, through March 10, 2017, during which 62 734 prospective identifiable consecutive specimens were screened.

Laboratory Test

The IDUA enzyme activity was measured using a modified version of the Spacil et al method.[6] Enzyme substrate, buffer solution, and quality control materials were purchased from PerkinElmer (Waltham, Massachusetts). A buffer solution containing synthetic IDUA substrate and internal standard (S/IS) was dispensed onto a 3.2 mm dried blood spot (DBS) punch in a 96-well plate. The resulting mixture was incubated for 19 hours at 37°C. The mixture was subsequently quenched with a 50/50 mixture of methanol and ethyl acetate to stop the enzymatic reaction. The product and internal standard were separated from the buffer salts and surfactant via liquid-liquid extraction using water and ethyl acetate. An aliquot of the ethyl acetate phase, which contained the analyte of interest, was transferred to a clean microtiter plate. The plates were dried and reconstituted in a water-methanol ammonium formate solution. Samples were analyzed using 99% methanol: 1% deionized water solution as the mobile phase and flow injection analysis-tandem mass spectrometry on an Acquity-TQD system (Waters, Milford, Massachusetts). In the single-plex assay the IDUA S/IS products were detected by selected reaction monitoring for the transitions: m/z 431.3 to 322.2 (IDUA-IS) and m/z 426.2 to 317.2 (IDUA-S). The enzyme activity was calculated in μmol/L of blood/hour. The percentage of the daily mean activity (DMA) was also calculated for each sample by dividing the sample IDUA activity by the mean IDUA activity of all samples run that day multiplied by 100. Samples that were retested used the same methodology as described above but were incubated with a 3-plex assay solution, which included internal standards and reagents for the analysis of glucosidase, β, acid (GBA) and acid α-glucosidase (GAA) activities, in addition to IDUA. Only 2 additional lysosomal enzymes were chosen for the retested samples to minimize the risk of identifying infants with other disorders. The selected reaction monitoring transitions for GBA and GAA internal standards and reaction products were m/z 391.4 to 271.3 (GBA-IS), m/z 384.4-264.3 (GBA-S), m/z 503.5-403.3 (GAA-IS), and m/z 498.3-398.3 (GAA-S).

A second-tier sequencing assay was performed on screen-positive samples at the Duke University Health System (DUHS) Clinical Molecular Diagnostics Laboratory. The coding and flanking intronic sequences of exons 1 through 14 of the *IDUA* gene were amplified by polymerase chain reaction from purified genomic DNA isolated from two 3.2 mm DBS punches. Sanger sequencing was performed in the forward and reverse directions, and the sequences were compared with the reference DNA sequence (NM_000203.3). Variants were classified as pathogenic, likely pathogenic, variants of uncertain significance, likely benign, and benign, according to American College of Medical Genetics and Genomics criteria or categorized as a suspected pseudodeficiency variant.[7] Evidence used to classify variants included the allelic frequency as documented in the Exome Aggregation Consortium database of population variation, the ClinVar database of clinically curated variation, the Leiden locus-specific database, previous reports in the scientific literature, and in silico prediction tools (eg, polyphen-2, MutationTaster). Variants were confirmed on screen-positive cases through sequencing of the specific exon regions from DNA extracted from whole blood obtained from the infant during the follow-up visit.

Screening Algorithm

For the initial screening algorithm (**Figure 1**, A), newborn DBS specimens were tested at NCSLPH using the single-plex reagents that only contained the IDUA S/IS. At the start of the pilot study, newborns determined to have IDUA activity below 2.27 μmol/L/hour (DMA was less than 41.9%) were retested in duplicate using a 3-plex assay. The 3-plex assay used the same method as measuring IDUA alone but also included the reagents for measuring the activity of 2 reference enzymes, GAA and GBA. Data

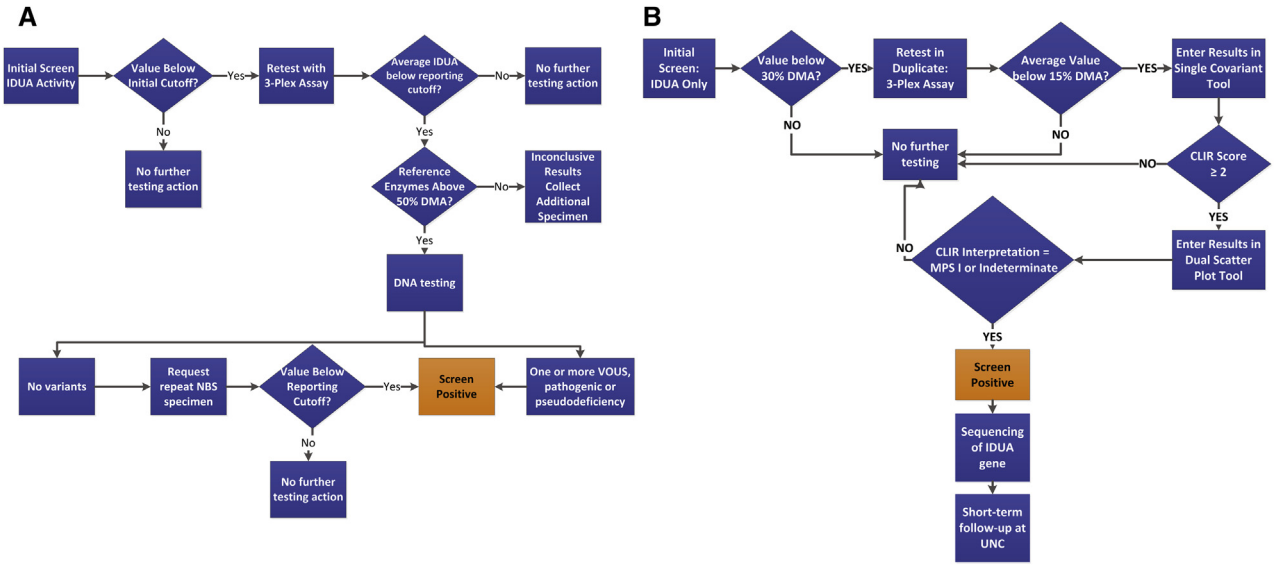

Figure 1. **A,** The initial screening algorithm and **B,** the modified screening algorithm.

for only 2 reference enzymes were analyzed to minimize the risk of identifying infants with other lysosomal storage disorders. Upon retesting, if 2 out of the 3 IDUA activity values were below 1.353 μmol/L/hour, or the DMA value was less than 25%, the reference enzymes were analyzed. If the reference enzymes were above 50% mean activity, the specimen was sent to DUHS Clinical Molecular Diagnostic Laboratory for sequencing, the infant was referred for clinical follow-up, and the UNC-CH medical team was notified. If the average reference enzymes were below 50% mean activity, the result was considered inconclusive, and an additional specimen was requested for reanalysis.

The screening algorithm was modified on February 14, 2017 because of the high number of screen-positive specimens (Figure 1, B). The cut-off for the initial test result was lowered to a DMA value below 30%, and samples with an activity below this level were retested in duplicate with the 3-plex assay. If 2 out of the 3 IDUA DMA values from the initial and retest assays were less than 15%, a .csv file containing the mean activity of IDUA, GAA, and GBA, and 2 covariates (age in hours at the time of collection and birth weight in grams) as well as sex were analyzed using site-specific postanalytical interpretive tools created using a past version of the software Collaborative Laboratory Integrated Reports (CLIR, v 2.07; Mayo Clinic, Rochester, Minnesota). CLIR is a web application that maintains an interactive database of laboratory results from multiple sites and provides on demand clinical decision support for their integrated interpretation. CLIR replace conventional reference intervals with continuous, covariate-adjusted moving percentiles for primary marker(s) of a given condition (for MPS I, the IDUA activity), and all possible permutations of ratios (in this study, the ratios IDUA/GAA and IDUA/GBA). It also replaces analyte decision limits (ie, cut-off values, DMA) with a condition-specific degree of overlap between reference and disease ranges. A single condition postanalytical interpretive tool (SCT) was used to combine all results into an integrated score.[8] Such a score was deemed to be informative when equal to or greater than a threshold set below the lowest score rendered for a known case affected with the target condition. Comparison of each score to all confirmed cases also provides a percentile ranking as a measure of likelihood of disease. A score below the 10th percentile, between the 10th percentile and the 25th percentile, and greater than the 25th percentile are classified as possibly informative, likely, and very likely, respectively. The outcome of the SCT is verified by a dual scatter plot tool (DST), a tool for to the differential diagnosis between MPS I and false positive cases, which include screening results from carrier and pseudodeficiency status.[9]

A .csv file containing the mean activity of IDUA, GAA and GBA, age at collection, birth weight, and sex for each specimen was uploaded to the SCT. A score of 1 meant the results were noninformative and the likelihood that this specimen was from an infant with MPS I was very low. If the score was 2 or higher, the same data elements were run through the CLIR DST.[9] If the DST interpreted the result as "false positive," the specimen was considered screen negative and no further action was taken. If the tool interpreted the result as MPS I or indeterminate, the specimen was considered screen positive and the follow-up team at UNC-CH was notified. All prospective screen-positive samples were sent to DUHS Clinical Molecular Diagnostic Laboratory for Sanger sequencing, and sequencing results were shared with the follow-up team at UNC-CH. The CLIR tools were also retrospectively used on samples classified as screen-positive before February 14. It was determined not to follow-up with patients that CLIR classified as false-positive and that the risk for MPS I was low.

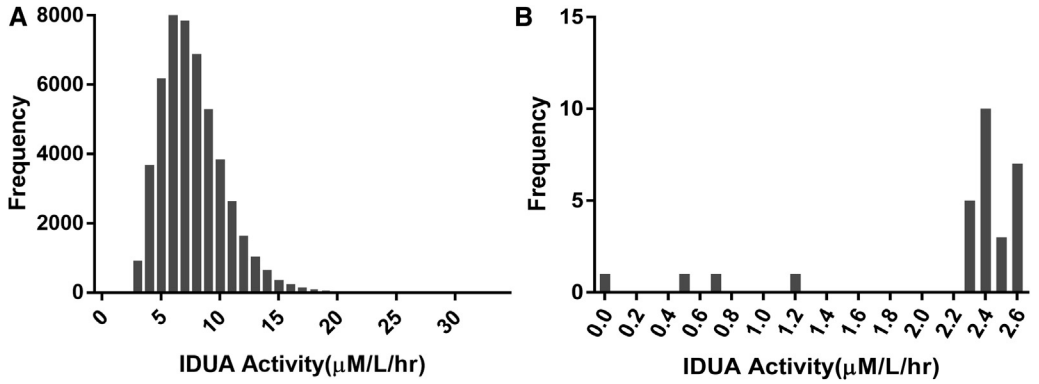

Figure 2. **A,** The North Carolina IDUA activity population distribution and **B,** the frequency of the IDUA activity below 3 μM/L/hour.

Follow-Up and Confirmatory Testing of Screen-Positive Specimens

The genetic counselor contacted the infant's primary care provider (PCP) by telephone to inform him/her of a positive newborn screen for MPS I, and results were faxed to the PCP's office for review. The PCP initially notified the family of positive screening results and connected the family with the genetic counselor to provide additional information. One family was contacted by the genetic counselor directly, as a PCP for that infant was unable to be identified. The family met with a genetic counselor and a clinical biochemical geneticist at UNC-CH who specialized in the care of patients with MPS I. The family received extensive genetic counseling services, including a review of the NBS results, basic genetic concepts, inheritance risks, natural history, and treatment and management of MPS I. In addition, pertinent medical and family history was obtained, and the infant received a complete physical evaluation to assess for early signs and features that may be suggestive of MPS I. Blood and urine samples were collected and sent to the DUHS Biochemical Genetics Laboratory for IDUA enzyme activity and urinary GAG confirmatory testing. Once results were available, the family was contacted by telephone. Infants with deficient enzyme activity, but normal urine GAG levels, were offered a 12-month follow-up appointment; only 1 family was interested in returning.

Urinary dermatan sulfate (DS) and heparan sulfate (HS) concentrations were determined using a tandem mass spectrometric-based method, as previously described.[10] IDUA activity was also confirmed in whole blood samples spotted onto filter paper using a fluorometric method with 4-methylumbelliferyl α-L-Idopyranosiduronic as the artificial fluorogenic substrate.[11,12]

Results

IDUA Activity in Newborn DBSs

The mean IDUA activity in the North Carolina population was 7.61 ± 2.79 μmol/L/hour, and the median activity was 7.26 μmol/L/hour (n = 62 734). **Figure 2** shows the distribution of enzyme activity for the study population (**Figure 2**, A) and the low frequency of specimens with activity less than 2.80 μM/L/hour (**Figure 2**, B). The demographic data for this population are described in **Table I** (available at www.jpeds.com). Because MPS I screening was the last condition to be tested after completion of the standard NBS tests, 928 specimens were classified as unsatisfactory for the MPS I pilot study only because of quantity or quality of the residual DBS specimen. In these cases, reports were sent to providers requesting that an additional specimen be collected. An additional specimen was obtained for approximately 60% of specimens that were initially unsatisfactory. The results for all repeat specimens were normal IDUA enzyme activity.

Evaluation of Specimens with Low IDUA Activity

Out of the 62 734 specimens screened for MPS I, 1289 (2.05%) were retested using the 3-plex assay. Of the retested specimens, 189 specimens had IDUA activities ≤25% of the DMA (the original cut-off value), 93 specimens had activities ≤20% of the DMA, and 54 specimens had activity ≤15% of the DMA (the final cut-off value). Once data were entered into the CLIR tool, 20 of the 54 specimens were classified as MPS I or indeterminate, including 2 specimens from the same infant. Thus, utilization of the CLIR tools to assist with the interpretation of first-tier tandem mass spectrometry (MS/MS) results reduced the number of specimens sent for sequencing by 64% and increased the positive predictive value for the first-tier MS/MS assay to 5.3% with a false-positive rate of 0.03%.

Second-Tier Testing by Gene Sequencing

Table II shows the pseudodeficiency variants identified in 33 of the 37 specimens sent for sequencing and their population frequencies. Eighteen specimens with activities between 25% and 15% of the DMA were sequenced prior to modification of the screening algorithm. It was determined that follow-up was not needed for these cases, as the CLIR score indicated a noninformative or false-positive result. Of these 18 specimens, 2 had variants of uncertain significance (VUS),

Table II. Pseudodeficiency alleles

dbSNP ID	DNA change	Protein change	Allele frequency*
rs58037052	c.235G>A	p.A79T	4.05%
rs147490060	c.296C>T	p.T99I	3.35%
rs183347428	c.667G>A	p.D223N	0.83%
rs148775298	c.246C>G	p.H82Q	0.55%
rs76722191	c. 965T>A	p.V322E	0.71%
rs11934801	c.1225C>T	p.G409R	7.66%

*Allele frequency for the African population from the Exome Aggregation Consortium, except for p.H82Q which is for European (non-Finnish) population as of December 22, 2018.

10 had only pseudodeficiency variants, 4 had both pseudodeficiency variants and VUS, and 2 were found to be heterozygous for a single pathogenic variant and pseudodeficiency variants. As previously reported by the Illinois NBS program, p.A79T was the most common sequence change observed.[13] Most of the specimens had multiple pseudodeficiency variants, and p.A79T commonly appeared with p.T99I. The IDUA enzyme activity for these specimens ranged from 0.42 to 1.42 μmol/L/hour, and the DMA ranged from 5.6% to 20.3%.

Cases Referred for Follow-Up

All 54 specimens with activity ≤15% of the DMA were entered into CLIR, and only 19 babies had a CLIR result of indeterminate or MPS I and were sent for follow-up after sequencing. **Table III** shows the screening results, sequencing results, and confirmatory testing results of all infants that were followed up. The urinary GAG results for the screen-positive patients in comparison with the normal controls are also shown in **Figure 3** (available at www.jpeds.com). One case of confirmed MPS I was identified with deficient IDUA enzyme activity in plasma, elevated urinary GAGs and homozygosity for a known pathogenic variation (p.Q70*), consistent with the severe form of MPS I (Hurler syndrome). On initial examination at 4 1/2 months of age, the infant had mild corneal clouding, decreased joint range of motion, lumbar kyphosis, a history of multiple failed hearing tests, chronic serous otitis media, and pressure equalizer tube placement. This patient was referred to the Duke University Medical Center for HSCT evaluation. At 7 months of age, the patient received a cord blood transplantation without complications.

All other infants who were screen-positive had normal clinical examinations and normal urinary GAGs (**Table III**), except for 1 newborn (sample 68) who had abnormal examination findings consistent with his prematurity. Two newborns (samples 5 and 63) were identified as MPS I carriers, each carrying a heterozygous pathogenic variant. In addition to the pathogenic variant, 1 infant had 2 pseudodeficiency variants (sample 5), and 1 infant had a VUS and a pseudodeficiency variant (sample 63). Thirteen newborns had only pseudodeficiency variant(s) in the *IDUA* gene, and all reported some African American ethnicity. Three infants had at least one pseudodeficiency variant and 1 VUS. The enzyme activity from the screen-positive patients ranged from 0.16 to 0.80 μmol/L/hour, and the DMA ranged from 2.1% to 7.7%. One patient was lost to follow-up, but this infant is unlikely to have MPS I because the confirmatory enzyme activity was within normal reference limits, and only pseudodeficiency variants were identified.

Discussion

The North Carolina Newborn Screening Pilot Study for MPS I disorder screened 62 734 newborn specimens using a first-tier MS/MS assay to measure IDUA enzyme activity in DBS specimens. The pilot study evaluated the sequencing of the *IDUA* gene from DBS as a second-tier test and demonstrated its potential to reduce the screen-positive samples if utilized prospectively by eliminating patients with only pseudodeficiency variants. Of the 19 newborns referred for clinical follow-up, one newborn was confirmed to have MPS I with clinical features and a homozygous pathogenic variant consistent with the severe form of the disease (Hurler syndrome). This method is also expected to identify the attenuated form; however, no newborn screening cases have been identified.

The unexpected high number of screen-positive specimens and subsequent need to adjust the screening algorithm highlights the uncertainty associated with being early adopters for new disorders and reinforces the need for implementation pilot studies for new conditions. In addition, the North Carolina experience demonstrates the value of regular expert evaluation and adjustment of the screening algorithm. The changes made in the screening algorithm throughout the pilot benefit NBS programs by demonstrating how lowering cut-offs and adding CLIR affected the interpretation of the screening results in the North Carolina population.

The North Carolina pilot study found that the CLIR tool reduced the number of newborns who required follow-up and confirmatory testing. The pilot study results demonstrate the value of postanalytic tools such as CLIR. With the use of CLIR, there was a reduction in screen-positive specimens by approximately 64%. One concern with using the CLIR tool in an NBS program is that the continuous adjustment of reference ranges and the resulting impact of covariates on the interpretation categories are driven by the submission of data to the CLIR database. Although this continuous revision of interpretations based on clinical data is a strength of CLIR, it creates a level of uncertainty for the tool's users. For example, the interpretation of a patient's results could change from one day to the next depending on the submission of true-positive data and updates to the CLIR tool. Thus, it is critical for a clinical laboratory to thoroughly evaluate the clinical validity and utility of the CLIR tool before implementation and throughout routine use for interpreting laboratory results. Based on the IDUA enzyme activity and CLIR data from the screen positive population, no specimens had daily mean activity above 10%, and changing this cut-off could reduce the number of false positive results. However, the CLIR tool has the potential of

Table III. Follow-up of infants who were screen positive

Sample unique IDs	IDUA activity μmol/L/h (%DMA)	Confirmatory IDUA enzyme* (pmol/punch/h)	Urine GAG† (g/mol creatinine)	Sequencing results	Phenotype
3	0.163 (2.3)	4.3	DS 128.4 HS 466.6	1 homozygous pathogenic: c.208C>T; p.Q70*	Severe MPS I
4	0.280 (4.2)	2	DS 5.7 HS 3.6	heterozygous VUS: c.1582C>G; p.P528A heterozygous likely benign: c.240C>G homozygous PD: c.235G>A; p.A79T homozygous PD: c.296C>T; p.T99I	Normal examination
5	0.198 (3.0)	9.2	DS 4.6 HS 6.1	heterozygous pathogenic: c.1029C>G; p.Y343* heterozygous PD: c.235G>A; p.A79T heterozygous PD: c.296C>T; p.T99I	Normal examination Carrier
22	0.493 (6.3)	1.8	DS 5.8 HS 6.5	homozygous PD: c.235G>A; p.A79T homozygous PD: c.296C>T; p.T99I	Normal examination
27	0.351 (4.3)	10	DS 4.5 HS 2.9	homozygous PD: c.235G>A; p.A79T homozygous PD: c.296C>T; p.T99I	Normal examination
29	0.623 (7.5)	11.1	DS 3.1 HS 2.4	heterozygous VUS: c.787A>T; p.R263W heterozygous PD: c.246C>G; p.H82Q	Normal examination
31	0.423 (5.1)	7.9	DS 2.7 HS 2.5	heterozygous PD: c.235G>A; p.A79T heterozygous PD: c.296C>T; p.T99I heterozygous PD: c.667G>A; p.D223N	Not examined at UNC-CH
37	0.629 (7.5)	13.9	DS 3.4 HS 2.7	heterozygous PD: c.235G>A; p.A79T heterozygous PD: c.246C>G; p.H82Q	Normal examination
46	0.598 (7.7)	8.1	DS 4.0 HS 4.4	homozygous PD: c.235G>A; p.A79T homozygous PD: c.296C>T; p.T99I	Normal examination
52	0.433 (5.2)	6.5	DS 3.2 HS 2.6	heterozygous PD: c.235G>A; p.A79T heterozygous PD: c.667G>A; p.D223N	Normal examination
53	0.351 (4.2)	6.9	DS 4.6 HS 3.3	heterozygous PD: c.235G>A; p.A79T heterozygous PD: c.667G>A; p.D223N	Normal examination
54	0.309 (3.7)	N/A‡	DS 3.4 HS 2.8	heterozygous PD: c.235G>A; p.A79T heterozygous PD: c.296C>T; p.T99I heterozygous PD: c.667G>A; p.D223N	Not examined at UNC-CH
58	0.413 (5.3)	8.7	N/A§	heterozygous PD: c.235G>A; p.A79T heterozygous PD: c.296C>T; p.T99I heterozygous PD: c.667G>A; p.D223N	Normal examination
59	0.330 (4.3)	7.9	DS 4.5 HS 4.9	homozygous PD: c.235G>A; p.A79T homozygous PD: c.296C>T; p.T99I	Normal examination
63	0.175 (2.1)	7.7	DS 7.4 HS 5.8	heterozygous pathogenic: c.1205G>A; p.W402* heterozygous VUS: c.1417A>T; p.T473S heterozygous PD: c.246C>G; p.H82Q	Normal examination Carrier
67	0.454 (5.9)	8.8	DS 4.2 HS 4.7	homozygous PD: c.235G>A; p.A79T homozygous PD: c.296C>T; p.T99I	Normal examination
68	0.454 (5.9)	7.3	DS 7.2 HS 4.1	homozygous PD: c.235G>A; p.A79T heterozygous PD: c.296C>T; p.T99I	Lung disease of prematurity, inguinal hernia, dolichocephaly
71	0.495 (5.9)	10.8	DS 3.6 HS 3.3	homozygous PD: c.235G>A; p.A79T heterozygous PD: c.296C>T; p.T99I heterozygous PD: c.1225G>C; p.G409R	Normal examination
73	0.248 (3.0)	9.6	DS 3.3 HS 4.9	heterozygous VUS: c.1744G>A; p.E582K heterozygous PD: c.235G>A; p.A79T heterozygous PD: c.296C>T; p.T99I	Normal examination

PD, pseudodeficiency variant.
*IDUA enzyme activity reference range is < 8.0 pmol/punch/hour.
†Dermatan sulfate (DS) and heparan sulfate (HS) reference ranges (2.5th to 97.5th percentiles, n = 136) from 0 to 6 months of age are 1.0-10.7 g/mol creatinine, and 0.6-9.2 g/mol creatinine, respectively.
‡Family coordinated follow-up with PCP and blood sample was not collected.
§Family refused to collect additional urine specimen.

changing the specimen classification and there is significant uncertainty with implementing a cut-off lower than 10%.

At the start of the MPS I NBS pilot study, limited information was available about the genotype and frequency of pseudodeficiency variants and their clinical manifestations. As the pilot study progressed, significantly more infants with low IDUA activity were identified than originally anticipated. It was evident by the end of the study that the frequency of the pseudodeficiency variants in the general population posed a challenge to the NBS system. Second-tier DNA sequencing and follow-up evaluations revealed that all screen-positive samples with pseudodeficiency variant(s) had normal urinary GAG concentrations and no clinical examination findings consistent with MPS I. This included the 5 infants identified with either heterozygous pathogenic or VUS variant(s) in conjunction with pseudodeficiency alleles. The follow-up with families of newborns with pseudodeficiency variants presented significant communication

challenges, and explanation required substantial genetic counseling time. The combination of the relatively high allele frequencies in the normal population (Table II), the normal urinary GAG results, and no clinical findings suggestive of MPS I, supports the assumption that pseudodeficiency alleles, even in the homozygous state, are not disease causing. To date, no clinically affected MPS I infant with only pseudodeficiency alleles has been reported (J. Muenzer, oral communication, October 2018). If second-tier DBS DNA sequencing of the IDUA gene was used prospectively in this study, and all infants identified with pseudodeficiency variants only were excluded from the follow-up protocol, the referral rate could have been reduced from 19 to 6, a 68% reduction.

Although second-tier DBS sequencing of the *IDUA* gene reduces the number of screen-positive infants, other approaches could be used in combination with sequencing to further reduce the false-positive rate. As observed, use of the CLIR tool is one option. The measurement of heparan and dermatan sulfates derived disaccharides in DBS is a validated method that can detect newborns with mucopolysaccharidoses types I, II, and III, as reported by de Ruijter et al, and could be considered another option.[14] These authors reported elevated GAGs in 9 DBS collected in the newborn period from infants who were eventually diagnosed with Hurler syndrome, compared with normal controls. Because urinary GAG levels in attenuated MPS I individuals are typically lower than individuals with the severe form of MPS I, concerns have been raised that an NBS program using a second-tier GAG analysis may miss the attenuated form of MPS I. However, de Ruijter et al reported 2 individuals with attenuated MPS I with elevated heparan and dermatan sulfates derived disaccharides in their newborn DBS,[14] further supporting the concept that second-tier GAG analysis should be considered for MPS I NBS programs. Given the rarity of MPS I, estimated incidence of 1 in 100 000 live births, second-tier GAG analysis should dramatically reduce the false-positive rate. The Mayo Clinic Biochemical Genetics Laboratory, on behalf of the Kentucky NBS program is currently using this second-tier GAG testing approach for MPS I.[9]

In summary, the North Carolina pilot study successfully applied a screening method for MPS I and identified a newborn with the severe form of MPS I, who was referred for HSCT. The pilot also showed that use of the CLIR tool reduced the number of specimens that were screen positive for MPS I. The data from this study demonstrate the utility of DNA sequencing as a second-tier test to reduce the number of newborns referred for follow-up by eliminating specimens that have low IDUA activity but contain only pseudodeficiency variants.

Recommendations for NBS programs screening for MPS I include the following: Pilot studies are necessary if states are to identify appropriate cutoff values for screen-positive cases. NBS laboratorians and clinical specialists should meet, evaluate, and refine algorithms and protocols to maximize the benefit to their populations; Screening for MPS I should not be done by measuring IDUA activity only. Additional approaches, such as the CLIR tool and/or second-tier testing are required to reduce the false-positive rate and decrease the follow-up burden on genetics clinics and families; Second-tier DNA sequencing using DBS specimens can identify patients with only pseudodeficiency variants and reduce the number of screen-positive infants, however second-tier GAG analysis using DBS has the potential to be a better means to eliminate false-positive screens. ■

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Reprint requests: Jennifer L. Taylor, PhD, RTI International, 3040 Cornwallis Rd, PO Box 12194, Research Triangle Park, NC 27709. E-mail: jtaylor@rti.org

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Appendix

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Table I. Demographic data on the population screened

Identifiable specimens screened	
Number of initial specimens screened	58 365
Number of repeat specimens screened	4369
Total of number of specimens screened	62 734
Sex	
Female	29 898
Male	31 953
Birthweight (g)	
>5000	672
4500-2500	54 274
2500-1501	5650
1500-1000	1136
>1000	843
<500	52
Age at DBS collection (h)	
<24	988
24-48	52 419
49-92	4295
93-145	523
>145	4500

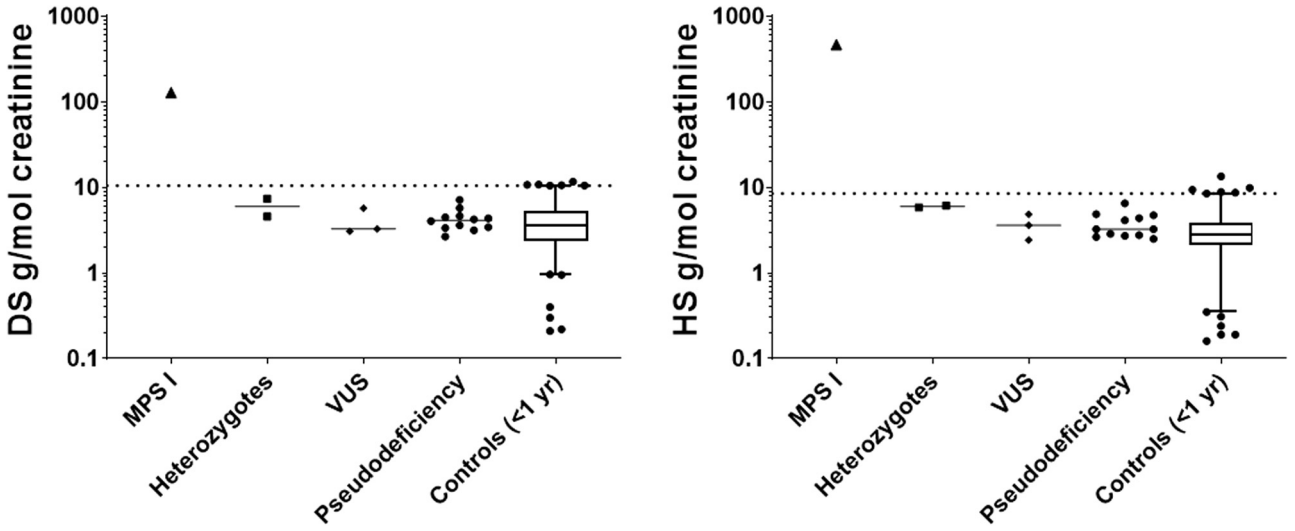

Figure 3. Urinary dermatan sulfate (DS) and heparan sulfate (HS) concentrations in infants evaluated because of a positive newborn screen for MPS I. MPS I: infant with a confirmed diagnosis of MPS I; Heterozygotes: 2 infants heterozygous for a pathogenic variant in the IDUA gene ± 1 or more pseudodeficiency variants; VUS: 3 infants with a VUS + 1 or more pseudodeficiency variants; Pseudodeficiency: 12 infants with 1 or more pseudodeficiency variants (pseudodeficiency) and no other changes; Controls (<1 year): 240 anonymized controls were remnant samples from patients <1 year age referred to DUHS Biochemical Genetics lab for biochemical genetics testing (urine organic acid, amino acid, sulfocysteine and/or creatine, and guanidinoacetate) in which no abnormality was observed. *Box* and *whiskers* show the interquartile ranges and 2.5th to 97.5th percentiles. *Dotted line* shows the upper limit of the reference range.