This commentary discusses the importance of conducting newborn screening pilot studies in North Carolina and the lessons learned from performing three pilots for severe combined immunodeficiency (SCID), mucopolysaccharidosis type I (MPS I), and X-linked adrenoleukodystrophy (X-ALD).

Pilot or implementation studies can aid a newborn screening program with early phase additions to the newborn screening panel by examining the testing of laboratory methods, follow-up protocols, and communication with families and health care providers. The Newborn Screening Saves Lives Reauthorization Act of 2014 encourages pilot studies to be conducted on new conditions that the Advisory Committee on Heritable Disorders in Newborns and Children (ACHDNC) has recommended be added to the Recommended Uniform Screening Panel (RUSP) to ensure that screening is ready for nationwide implementation. Over the past few years, there have been many funding opportunities to implement pilot studies for new conditions in newborn screening. RTI International, the North Carolina State Laboratory of Public Health (NCSLPH), the University of North Carolina at Chapel Hill (UNC), and Duke University have formed a consortium to apply for these opportunities. This team has newborn screening laboratory expertise to perform a statewide pilot, a state laboratory with a strong history of newborn screening innovation, and follow-up and clinical care expertise required to monitor and treat patients identified with these conditions.

This partnership started with an administrative agreement between RTI and NCSLPH and subsequently developed into a business associate agreement. With these agreements, RTI and NCSLPH received a cooperative agreement through the Centers for Disease Control and Prevention (CDC) (U88EH001312-01) to develop the laboratory capacity for conducting severe combined immunodeficiency (SCID) screening. In addition, the Eunice Kennedy Shriver National Institute of Child Health and Human Development (NICHD) awarded an Indefinite Delivery Indefinite Quantity (IDIQ) contract to the North Carolina consortium to rapidly set up pilot studies for screening a large number of specimens and following up with screen-positive infants. Under this contract, two task orders have been awarded to screen for mucopolysaccharidosis type I (MPS I) and X-linked adrenoleukodystrophy (X-ALD).

This commentary will discuss the three pilot studies conducted under this collaboration, including the successes and lessons learned.

**SCID Pilot**

SCID, also known as “bubble boy disease,” is an inherited condition in which babies appear normal at birth but are unable to fight off serious and life-threatening infections. Newborn screening from multiple states has shown that SCID affects one in 58,000 infants [1]; it can be deadly but is treatable if detected early in life.

Currently, several states are using the T-cell receptor excision circles (TREC) assay to screen for SCID and other T-cell abnormalities. TREC are extra-chromosomal DNA that are formed when T-cells are produced in the thymus, making it a suitable biomarker for T-cell production that can be measured using real-time polymerase chain reaction (PCR) [2].

Prospective screening for SCID started on April 24, 2017 and was added to the newborn screening report on
December 4, 2017. Figure 1 shows the population distribution of the TREC levels for 90,817 specimens tested during the pilot period. To date, no infant has been confirmed positive for SCID, but several other conditions with known association with T-cell impairment have been detected, such as 22q11 deletion syndrome, trisomy 21, and heart defects. Although no infants have been identified with SCID, the testing method has shown that other infants with severely compromised immune systems were identified, and it is important to follow these cases as well.

The importance of regular communication with specialists and providing regular education to hospitals were two important lessons from this pilot. During the first few months of the pilot, 13 infants were referred to follow-up due to abnormal SCID screening results, but they all showed normal lymphocyte counts and normal evaluations. After further investigation, all specimens were collected from the same hospital using heparinized capillary tubes, causing several specimens to be classified as abnormal or unsatisfactory/inconclusive. The hospital was instructed not to use capillary tubes and shown the Clinical & Laboratory Standards Institute (CLSI) guidelines on proper specimen collection.

MPS I Pilot

MPS I is caused by a deficiency of an enzyme (α-L-iduronidase [IDUA]) involved in the breakdown and recycling of sugars called glycosaminoglycans (GAG) [3]. These sugars slowly accumulate in lysosomes, which can lead to a progressive physical disease, shortened life span, and neurologic involvement with intellectual disability. Early diagnosis of MPS I is needed to initiate appropriate treatment. Hematopoietic stem cell transplantation (HSCT) before age 1 to 2 is the recommended treatment for patients with the
most severe form of MPS I to prevent premature death and cognitive impairment [4]. For those children with the attenuated or milder form of MPS I (normal cognitive function), early initiation of enzyme replacement therapy (ERT) can prevent or reduce disease progression.

In 2016, NCSLPH used a flow injection analysis-tandem mass spectrometry (FIA-MS/MS) assay to detect infants at risk for MPS I. In this assay, a dried blood spot sample was incubated with a synthetic substrate, and the IDUA enzyme cleaved the substrate to produce a product that was detected using FIA-MS/MS. Specimens from MPS I patients had no functional enzyme, so no product was detected, and those samples were considered screen-positive. Due to the unknown stability of the enzyme, the percentage of the daily mean activity (%DMA) was calculated for each sample and a specific %DMA was used to classify samples as screen-positive. Screen-positive samples were sent to the Duke Clinical Molecular Diagnosis Laboratory for DNA sequencing of the IDUA gene to identify any mutations or variants in the coding region of this gene.

A total of 62,734 identifiable specimens were screened at the laboratory and 19 babies screened positive and were reported to the genetic counselor at UNC for follow-up coordination. One infant was diagnosed with the severe form of MPS I and two infants were carriers. All other infants had pseudodeficiency variants only or a combination of variants of unknown significance (VUS) with normal confirmatory test results. The positive predictive value was 33.3% and the false-positive rate was 0.003%.

During the planning phase of the pilot study, the Missouri newborn screening program reported screen-positive patients with pseudodeficiency variants and mutations demonstrating reduced or deficient enzyme activity with no phenotypic characteristics of MPS I. Limited information was available about the genotype of pseudodeficiency variants and their clinical manifestations at the time; therefore, the
clinical experts developed a plan to evaluate these children to confirm no clinical symptoms.

One of the challenges faced during the progression of the pilot study was that significantly more infants with pseudodeficiency variants were identified than originally estimated, potentially due to the large African-American population in North Carolina. The high variant frequency found in the state’s population led to modification of the screening algorithm and the question of whether all pseudodeficiency variants should be reported and followed. Table 1 shows the number of specimens screened and the number of specimens under each successive cutoff if the cutoff value were unchanged throughout the pilot. To reduce the screen-positive specimens to a manageable range for the follow-up personnel, we reduced the %DMA from 25% to 15% and the analytical tool Collaborative Laboratory Integrated Reports (CLIR) was implemented. In addition to overwhelming numbers of screen-positive specimens, explaining pseudodeficiencies to patients required significant genetic counseling time, and a letter was developed by the UNC follow-up staff to explain this condition and the confirmatory testing results.

The unexpected number of screen-positive specimens and subsequent need to adjust the screening algorithm highlighted an important lesson that uncertainty is associated with screening for new disorders in the North Carolina population. In addition, the North Carolina experience demonstrates the value of regular expert evaluation and adjustment of the screening algorithm.

**X-ALD Pilot**

X-ALD is a rare genetic disorder that leads to demyelination in the brain or spinal cord and adrenal insufficiency. X-ALD is caused by mutations in the **ABCD1** gene, which encodes a protein that aids in the transportation of very long chain fatty acids (VLCFAs) into the peroxisome for breakdown [5]. **ABCD1** dysfunction leads to a buildup of VLCFAs in cells, primarily affecting the adrenocortex, white matter in the brain, and Leydig cells, leading to Addison’s disease (adrenal insufficiency), adrenomyeloneuropathy (AMN), or cerebral adrenoleukodystrophy (CALD) [6]. The childhood form of CALD (CCALD) can occur between age 2.5 and 10. Early clinical symptoms include attention disorders and hyperactivity [6]. Eventually, patients rapidly decline, and death commonly occurs 2 to 4 years after the onset of symptoms.

The North Carolina team conducted a newborn screening pilot study starting March 5th, 2018, for X-ALD using a high-performance liquid chromatography tandem mass spectrometry (HPLC-MS/MS) assay as a first-tier screening method and Sanger sequencing on dried blood spots that screened positive. The HPLC-MS/MS assay was designed in negative-ion mode to detect two biomarkers: C24:0-lysophosphatidylcholine (C24:0-LPC) and C26:0-lysophosphatidylcholine (C26:0-LPC). Figure 2 shows the C24:0-LPC and C26:0-LPC levels of specimens in the normal range versus specimens classified as borderline or abnormal. Specimens with elevated levels of these biomarkers were sent to the Duke Clinical Molecular Diagnostic Laboratory for **ABCD1** sequencing. A whole-blood specimen was collected for VLCFA analysis at the Kennedy Krieger Institute in Baltimore, Maryland.

A total of 52,301 specimens were screened and 12 infants were identified with screen-positive results and received follow-up services and confirmatory testing. The screen-positive infants included three newborns confirmed with X-ALD, two likely carriers or heterozygous females, two with peroxisome biogenesis disorders, one with Aicardi-Goutières Syndrome, one with possible liver dysfunction, and three false positives. The incidence of X-ALD in the North Carolina population was approximately 1 in 10,000 births. The positive predictive value (PPV) for the screening assay was 83.3%, with a false-positive rate of 0.004%.

Although the testing method identified X-ALD specimens with high certainty, there were several glitches that were uncovered with the HPLC method for high-throughput testing; adjustments in the maintenance procedures were needed for continued screening. In addition, no surveillance protocols were established due to the lack of funding for this activity. Long-term follow-up is an ongoing challenge but is important to close the loop in the newborn screening system. An established long-term follow-up process is particularly important for newborns identified with X-ALD since these children potentially will not develop symptoms for years. There is no correlation between genotypes, phenotypes, or age at onset [7]; therefore, routine clinical monitoring is crucial for the initiation of timely and effective interventions as well as for understanding the clinical presentation of X-ALD cases identified through newborn screening.

**Conclusion**

In conclusion, North Carolina has conducted several pilot studies demonstrating successful identification of infants with rare genetic disorders. Newborn screening is a multi-

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**TABLE 1. Number of Specimens Flagged at Different Cutoff Levels**

| Category | Number of specimens |
|----------|---------------------|
| Total number of specimens screened | 62,734 |
| Number of specimens ≤ 25% DMA | 184 |
| Number of specimens ≤ 20% DMA | 93 |
| Number of specimens ≤ 15% DMA | 54* |
| Number of specimens ≤ 15% DMA and interpreted by CLIR as MPS I or indeterminate | 20** |

Source: RTI International.

*54 specimens from 53 babies

**20 specimens from 19 babies
faceted system and introducing a new condition can lead
to disruption if not implemented appropriately. Pilot studies
give programs the opportunity to add new conditions and
evaluate feasibility and disparities before any disruptions
occur. The North Carolina team has also shared their expe-
rience with other states so implementation can occur with
fewer setbacks.

Jennifer L. Taylor, PhD
research public health analyst, Center for
Newborn Screening, Ethics, and Disability Studies, RTI International,
Research Triangle Park, North Carolina.

Stacey Lee, PhD
laboratory scientist, Center for Newborn Screening,
Ethics, and Disability Studies, RTI International, Research Triangle Park,
North Carolina.

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