Evidence-Based Path to Newborn Screening for Duchenne Muscular Dystrophy

Jerry R. Mendell, MD,1 Chris Shilling, MS,1 Nancy D. Leslie, MD,2 Kevin M. Flanigan, MD,1 Roula al-Dahhak, MD,1 Julie Gastier-Foster, PhD,1,3 Kelley Kneile, BS,3 Diane M. Dunn, BS,4 Brett Duval, BS,4 Alexander Aoyagi, BS,4 Cindy Hamil, AS,4 Maha Mahmoud, BS,4 Kandice Roush, RN,1 Lauren Bird, RN,1 Chelsea Rankin, BS,1 Heather Lilly, BS,5 Natalie Street, MS, CGC,6 Ram Chandrasekar, PhD,5 and Robert B. Weiss, PhD4

Objective: Creatine kinase (CK) levels are increased on dried blood spots in newborns related to the birthing process. As a marker for newborn screening, CK in Duchenne muscular dystrophy (DMD) results in false-positive testing. In this report, we introduce a 2-tier system using the dried blood spot to first assess CK with follow-up DMD gene testing.

Methods: A fluorometric assay based upon the enzymatic transphosphorylation of adenosine diphosphate to adenosine triphosphate was used to measure CK activity. Preliminary studies established a population-based range of CK in newborns using 30,547 deidentified anonymous dried blood spot samples. Mutation analysis used genomic DNA extracted from the dried blood spot followed by whole genome amplification with assessment of single-/multiexon deletions/duplications in the DMD gene using multiplex ligation-dependent probe amplification.

Results: DMD gene mutations (all exonic deletions) were found in 6 of 37,649 newborn male subjects, all of whom had CK levels >2,000U/l. In 3 newborns with CK >2,000U/l in whom DMD gene abnormalities were not found, we identified limb-girdle muscular dystrophy gene mutations affecting DYSF, SGCB, and FKRP.

Interpretation: A 2-tier system of analysis for newborn screening for DMD has been established. This path for newborn screening fits our health care system, minimizes false-positive testing, and uses predetermined levels of CK on dried blood spots to predict DMD gene mutations.

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Over the past 3 decades, creatine kinase (CK) testing on dried blood spots has been attempted as a method of newborn screening (NBS) for Duchenne muscular dystrophy (DMD),1–9 because CK is elevated at birth in individuals with this condition.10–12 CK elevation is then validated by retesting of venous blood at 4 to 6 weeks of age with subsequent DMD gene analysis employed to establish a definitive diagnosis. Presently, this approach survives only in Antwerp, Belgium9 (NBS was stopped in Wales on November 30, 2011). It has been difficult for programs to justify NBS for DMD because of the lack of evidence that early treatment improves the outcome of affected newborns.13,14 In addition, the Wales/Antwerp DMD NBS model, requiring extensive follow-up through retesting of venous blood several weeks after birth for CK with subsequent DNA testing, is impractical to implement in the United States.

Nevertheless, recent advances in diagnostic testing methods and promising molecular-based therapies for DMD have rekindled interest in establishing a pathway...
for NBS for DMD.\textsuperscript{15–19} Especially relevant is clinical improvement following the systemic administration of anti-sense oligonucleotide (AON) PRO051 to induce skipping of exon 51 during premessenger RNA splicing of the \textit{DMD} gene.\textsuperscript{15} Patients enrolled in this exon-skipping trial upregulated dystrophin at the sarcolemma and also showed functional improvement in the 6-minute walk distance. In another study, using a morpholino-based AON to skip exon 51 (AVI-4658; eteplisen), dystrophin expression was increased in a dose-dependent manner at the sarcolemma.\textsuperscript{16,17} Additionally, long-term evidence suggests that glucocorticoid treatment prolongs ambulation, reduces the need for spinal surgery, and increases both survival and quality of life.\textsuperscript{20–27} The current recommendation for initiation of corticosteroids is to start treatment when boys with DMD have stopped gaining motor skills and not to wait until motor skills have begun to decline.\textsuperscript{28} Considering the mean age of DMD diagnosis to be 5 years of age,\textsuperscript{29} and that loss of function can begin before this time, an earlier diagnosis could result in an earlier corticosteroid start time, resulting in potential long-term benefits.

In 2004, a workshop sponsored by the Centers for Disease Control and Prevention (CDC) was held to review experiences, benefits, and risks in conducting NBS for DMD. The workshop participants concluded that there was inadequate evidence showing medical benefit from early identification, but that early diagnosis of DMD could have other advantages for the family (http://www.cdc.gov/ncbddd/duchenne/documents/nbs_lay_report.pdf). Following the CDC workshop, funds were made available to explore the potential for establishing a model for DMD NBS in the United States and to address ethical issues identified by the workgroup. Through this funding, we implemented a voluntary DMD NBS program in Ohio, initially through a pilot study in several birthing hospitals in Columbus and Cincinnati, followed by expansion to birthing hospitals throughout the state. Here we describe a 2-tiered method for conducting DMD NBS with initial screening for CK followed by DNA isolation and \textit{DMD} gene analysis on the same dried blood spot. The addition of DNA analysis provides information useful in reducing the number of false positives based on CK alone and a path for initial follow-up of newborns with positive CK screening results. This study provides a model for conducting newborn screening for DMD.

### Subjects and Methods

#### Study Populations

This study included 4 phases. Phase 1 efforts were devoted to creating a population-based range of CK to serve as the first tier of analysis, establishing a threshold that would trigger second-tier \textit{DMD} gene mutation analysis. These studies were carried out at the Ohio Department of Health (ODH) using anonymous dried blood spots from male and female newborns. In phase 2 of this study, parents of newborn males were invited to participate in a pilot NBS study conducted at 1 of 4 major birthing hospitals in Columbus and Cincinnati, Ohio between March 2007 and September 2008. Newborn male infants born at any of the participating hospitals were eligible for the study. In phase 3, the DMD NBS program was expanded to include a total of 43 hospitals throughout the state of Ohio, with recruitment starting in October 2008 and extending through September 2010. In phase 4, the final phase of the study, deidentified blood spots from the newborn screening cards of males and females were again anonymously screened through the ODH (June 2010–January 2011). The goal in the final phase was to increase the number of male samples and to include females. We included both genders, not with the expectation of identifying carriers of an X-linked disease like DMD, but with the specific intent of enhancing our chances of identifying mutations of autosomal genes validating the 2-tier method of screening (CK on dried blood spot followed by DNA testing) to address the issue of elevated CK levels in subjects not found to have \textit{DMD} gene mutations. Considering that false-positive CK elevation is a potential concern of NBS, we wanted to determine if mutations in other muscular dystrophy genes could be found in cases without \textit{DMD} mutations.

#### Study Design

**POPULATION-BASED CK ANALYSIS ON ANONYMOUS DRIED BLOOD SPOTS.** In phase 1 of the study, a population-based range of CK was built upon CK testing of 30,547 consecutive anonymous dried blood spot samples (Table 1, Fig 1). Previous concerns had been raised regarding variability related to gender, neonatal weight, and age at time of sample collection. The current study is important in establishing future guidelines for CK, because we found no significant difference in the mean values between males (251.52 ± 113.85U/L) and females (246.39 ± 113.86U/L), with a minimal effect related to birth weight. Another point of interest is that although our target group for NBS focuses on dried blood spots collected within the first 48 hours, concerns have also been raised that CK activity diminishes over time. The data we collected show little effect out to 5 days (>120 hours). From this database, we were able to design our protocol for the 2-tier testing protocol. Initially, we chose to launch DNA testing for the \textit{DMD} gene at a CK level of ≥600U/L, 3 standard deviations from the mean (0.75 % of screened population; see Table 1, Fig 1).

**IMPLEMENTATION OF CK TESTING ON NEWBORNS.** For phases 2 and 3, NBS was voluntary and required signed consent by a parent or guardian based on approved protocols by the institutional review board (IRB) at Nationwide Children’s Hospital, Cincinnati Children’s Hospital Medical Center, the ODH, University of Utah, the CDC, and every
prior to the start of recruitment for phases 2 and 3, we provided an in-service teaching session to the staff of the delivery suites so that they could appropriately obtain consent from parents of newborn males. Within 48 hours of birth, trained staff members approached parents of newborn boys about participation in the study. A brochure describing the NBS was provided describing benefits and risks. Consenters then proceeded to answer questions and obtain consent from parents who wanted to participate. Ohio currently mandates testing (http://www.odh.ohio.gov/odhprograms/phl/newbrn/nbrn1.aspx) of all newborns for 35 disorders through dried blood spots obtained 24 to 48 hours after delivery (extenuating circumstances can permit testing up to 5 days of age). The blood spot cards supplied by the ODH include demographic information, the date and time of collection, and five 100μl blood samples obtained by heel stick imprinted in separate circles on a piece of filter paper attached to the top of the collection card. Two of the circles are used for the mandated

### TABLE 1: Population-Based Creatine Kinase in Anonymous Newborns

<table>
<thead>
<tr>
<th>Group</th>
<th>Count (n)</th>
<th>Mean, U/l</th>
<th>SD</th>
<th>Mean + 1 SD</th>
<th>Mean + 2 SD</th>
<th>Mean + 3 SD</th>
<th>Mean + 4 SD</th>
<th>Mean + 5 SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>All infants</td>
<td>30,547</td>
<td>247.92</td>
<td>109.40</td>
<td>357.36</td>
<td>471.21</td>
<td>585.06</td>
<td>698.91</td>
<td>812.76</td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>15,446</td>
<td>251.52</td>
<td>113.85</td>
<td>365.37</td>
<td>479.22</td>
<td>593.07</td>
<td>706.92</td>
<td>820.77</td>
</tr>
<tr>
<td>Female</td>
<td>14,983</td>
<td>246.39</td>
<td>113.56</td>
<td>359.95</td>
<td>473.51</td>
<td>587.07</td>
<td>700.63</td>
<td>814.19</td>
</tr>
<tr>
<td>Weight</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&gt;2,500g</td>
<td>27,506</td>
<td>250.61</td>
<td>115.99</td>
<td>366.60</td>
<td>480.16</td>
<td>593.72</td>
<td>707.28</td>
<td>820.84</td>
</tr>
<tr>
<td>&gt;2,000 to 2,500g</td>
<td>1,555</td>
<td>231.68</td>
<td>87.78</td>
<td>319.46</td>
<td>433.02</td>
<td>586.58</td>
<td>660.14</td>
<td>773.70</td>
</tr>
<tr>
<td>1,500 to 2,000g</td>
<td>538</td>
<td>210.41</td>
<td>71.01</td>
<td>281.41</td>
<td>394.97</td>
<td>508.53</td>
<td>622.09</td>
<td>735.65</td>
</tr>
<tr>
<td>&lt;1,500g</td>
<td>573</td>
<td>226.36</td>
<td>75.88</td>
<td>302.24</td>
<td>415.80</td>
<td>529.36</td>
<td>642.92</td>
<td>756.48</td>
</tr>
<tr>
<td>Total</td>
<td>30,172</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Data are from creatine kinase testing of dried blood spots of 30,547 deidentified newborn samples (broken down by gender, weight, and age at sample collection).

SD = standard deviation.

FIGURE 1: A normal distribution of creatine kinase (CK) on dried blood spots was obtained during a phase 1 population-based study of 30,547 deidentified male and female newborns. Based on this study, we used CK 600U/l as the threshold to trigger DNA testing for phase 2 newborn screening. In phase 3 testing, we increased the threshold to CK 750U/l.
spot card was submerged in 300°C using a previously published methodology.1,2,7,30,31 Dried spots obtained for all 4 phases performed at the ODH laboratory for CK testing. CK testing was performed on the dried blood spots obtained for all 4 phases performed at the ODH laboratory for CK testing. Materials

**CK TESTING.** CK testing was performed on the dried blood spots obtained for all 4 phases performed at the ODH laboratory using a previously published methodology.1,2,7,30,31 Dried blood spots were punched using a Wallac DBS Puncher (Perkin Elmer, Boston, MA) and placed in wells of a filter plate with the addition of diadenosine pentaphosphate (USB Corporation, Cleveland, OH). Following incubation at room temperature to inhibit red blood cell enzyme activity, the supernatant was transferred to a fresh tube containing 600μl of isopropanol, mixed, and placed at −20°C for 30 minutes. DNA was pelleted by centrifugation at 4°C for 10 minutes at 10,000 × g, rinsed with 70% ice-cold ethyl alcohol, air dried, and resuspended in 20μl of TE buffer, pH 7.6. Whole genome amplification (WGA) of this purified DNA from each blood spot was performed with the REPLI-g kit (Qiagen, Valencia, CA; catalog No. 150045). Five microliters of genomic DNA was denatured for 3 minutes at room temperature and neutralized according to the manufacturer’s specifications, and a 50μl final volume reaction containing REPLI-g DNA Polymerase was incubated at 30°C for 16 hours, followed by heating at 65°C for 3 minutes to inactivate the enzyme.

**MUTATIONAL ANALYSIS.** Deletion and sequencing analysis of the DMD gene was performed on WGA template DNA using the 2-step SCAIP method, as described in detail elsewhere.32 This method uses polymerase chain reaction (PCR) amplification and capillary-based fluorescent DNA sequencing to screen for deletions and point mutations in all 79 coding exons and approximately 50 nucleotides of flanking intronic sequences of the major mRNA transcript isoform in muscle plus the 5′ untranslated region (UTR), 3′ UTR, and 6 alternate promoters. PCR was carried out in 10μl reactions using Platinum Taq DNA Polymerase (Invitrogen, Carlsbad, CA). Each PCR reaction contained 0.14μl of WGA template, and 93 total PCR reactions were analyzed per sample. Enzymatic cleanup was performed with the ExoSAP-IT reagent (Affymetrix, Santa Clara, CA), and the treated samples were sequenced using ABI (Applied Biosystems, Foster City, CA) BigDye Terminator v3.1 chemistry. Samples were run on an ABI 3730xl sequencer, and analyzed using the base-calling sequence software described previously.33 Nucleotide positions were determined according to the standard reference DMD sequence used for mutation analysis (GenBank accession number NM_004006.2). Nucleotide numbering reflects cDNA numbering, with +1 corresponding to the A of the ATG translation initiation codon in the reference sequence, according to established guidelines (www.hgvs.org/mutnomen).

All samples were analyzed for single-/multiexon deletions/duplications in the dystrophin gene using multiplex ligation-dependent probe amplification (MLPA) kit P034/P035 DMD/Becker MLPA; MRC-Holland, Amsterdam, the Netherlands) as described.33 One microliter of WGA template in 5μl of TE buffer was fragmented at 98°C for 5 minutes, cooled, and split between 2 separate tubes; 1.5μl of Salsa P034 and P035 primers were added to the tubes, respectively, incubated at 95°C for 1 minute, followed by annealing at 60°C for 20 hours. DNA ligase buffer and enzyme were added to each reaction in a total volume of 20μl and incubated at 54°C for 15 minutes, followed by 5-minute incubation at 98°C. Five microliters was
subsequently used in a 25μl PCR reaction that consisted of 35 cycles: 95°C for 30 minutes, 60°C for 30 minutes, and 72°C for 1 minute, followed by a final incubation at 72°C for 20 minute; 1.5μl of the sample was run on an ABI 3730xl instrument and analyzed for fragment size, peak height, and peak area using GeneMapper software (Applied Biosystems). DMD exon copy number was determined by dosage quotient analysis generated for each MLPA peak by using 10 individual flanking exon copy number was determined by dosage quotient analysis of controls, and the mean of these ratios formed the denominator for each MLPA peak.

Results

Newborn Screening CK Studies: Phase 2 and Phase 3 Analyses

A phase 2 pilot study screening 6,928 newborns was done at the major birthing hospitals in Columbus and Cincinnati, Ohio. In this phase of the study, we tracked the number who declined consent and found it to be 6.0% (n = 478) of those authorized to give approval. We found that 110 subjects exceeded the CK >2,000U/l testing threshold, requiring DNA analyses. Only the 2 subjects with CK >2,000U/l (2,461 and 2,675) were found to have proven DMD gene mutations. The false-positive rate for this phase was 1.6% (108 of 6,926).

The pilot study provided the impetus to move the CK threshold for the statewide, phase 3 program to >750U/l. The CK data collected from enrollment of an additional 10,937 newborn males led to the identification of 58 with elevated CK. One newborn was found to harbor a DMD mutation, and his CK was again >2,000U/l (2,003U/l). The false-positive rate for phase 3 was 0.52% (57 of 10,936). Increasing the CK threshold from 600U/l to 750U/l reduced the number of newborn males requiring DNA testing by 68%. The number declining enrollment in this phase of the study was not accurately tracked. Forty-three individual birthing sites were responsible for obtaining consent in the statewide program, exceeding our tracking capabilities.

Of additional interest regarding a frequently expressed concern of CK testing in the newborn period is the potential contribution of enzyme elevation from trauma as the neonate progresses through the birth canal.35–37 We have examined this by checking CK levels on follow-up venous blood samples obtained through the primary care physician for participants in phase 2 and phase 3 studies. We were able to obtain samples for only 43 of 165 subjects who were negative for DMD gene mutations and in whom CK was elevated on dried blood spots (distributed as follows: 35 between 600 and 999U/l; 6 between 1,000 and 1,499U/l; and 2 between 1,500 and 1,999U/l). In most cases, the follow-up venous CK was lower compared to the blood spot-derived CK (Fig 2). Of particular note, the highest of the non-DMD group was 1,700U/l yet the repeat venous blood showed a CK level of 46U/l. In only 2 cases, the venous CK remained slightly elevated >500U/l on follow-up (888 reduced to 672, 809 reduced to 656). This confirms that CK elevation on dried blood spots can be attributed to birth trauma and accounts for most values above normal, findings similar to previous reports.35–37 A point of interest is that 1 of the infants with a documented DMD gene mutation, whose dried blood spot CK was 2,462U/l, had a repeat venous blood sample at 6 weeks showing a dramatic elevation to 8,888U/l.

Phase 4 Newborn Screening CK Study

In the fourth and final phase of this study, to increase the sample size to further validate the 2-tier approach for DMD identification in the newborn period, we screened a large cohort of deidentified newborn samples anonymously. This increased our sample size by 19,884 newborn males (total 37,649). Based on results of phase 2 and phase 3 studies, we limited DNA screening on dried blood spots to those males with CK >500U/l. Of additional interest regarding a frequently expressed concern of CK testing in the newborn period is the potential contribution of enzyme elevation from trauma as the neonate progresses through the birth canal.35–37
DNA Analysis on Dried Blood Spots

Among a total of 37,649 newborn males screened for DMD (phase 2, 3, 4), 6 males were found to have DMD gene mutations. All were single-exon or multiexon deletions, 5 out-of-frame and 1 in-frame mutation (Table 2); no point mutations or duplications were found. These exon deletion mutations followed a typical distribution seen from large cohorts, although the in-frame deletion, exons 5–41, has been reported only once previously (http://www.leiden.nl), and was associated with DMD, likely due to a deletion that encompasses critical actin-binding domains.

A striking finding in the mutation analysis was that all samples with DMD mutations had CK values ≥2,000U/l. Our attention was therefore drawn to subjects (7 males and 2 females) with CK ≥2,000 in whom we did not find DMD mutations. We therefore extended the study to include analysis of mutations in the most common LGMD genes (DYSF, CAPN3, SGCA, SGCB, SGCC, SGCD, and FKRP). Mutations were found in 1 female (a DYSF point mutation) and 2 males (1 with a point mutation in SGCB, and the other with a point mutation in FKRP; see Table 2). In none of these 3 instances was a second mutant allele detected.

Discussion

CK testing on dried blood spots to identify DMD cases in the newborn period was validated in 1979 and relies on enzyme activity to catalyze the transphosphorylation of ADP to ATP. As initially introduced, a luciferase-based bioluminescence assay established enzyme intensity; later modifications (as used here) utilized an NADH-based fluorometric readout as a measure of CK activity. Table 3 tracks the sequential history of NBS for DMD from its early introduction in New Zealand through programs in Edinburgh, Germany, Canada, France, the USA (western Pennsylvania), Wales, Cyprus, and Belgium. Antwerp is the only program that maintains NBS for DMD to this day. In this program, samples with elevated CK are retested through venous blood samples taken at about 6 weeks after birth. Their nationalized health care system is positioned to accommodate

<table>
<thead>
<tr>
<th>Gender</th>
<th>CK value, U/l</th>
<th>Gene</th>
<th>Mutation</th>
<th>cDNA</th>
<th>Frame</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td>2,462</td>
<td>DMD</td>
<td>Deletion ex50</td>
<td>[c.7201-?_7309+?del]</td>
<td>Out</td>
</tr>
<tr>
<td>Male</td>
<td>2,675</td>
<td>DMD</td>
<td>Deletion ex5–41</td>
<td>[c.265-?_5922+?del]</td>
<td>In</td>
</tr>
<tr>
<td>Male</td>
<td>2,003</td>
<td>DMD</td>
<td>Deletion ex8–9</td>
<td>[c.650-?_960+?del]</td>
<td>Out</td>
</tr>
<tr>
<td>Male</td>
<td>2,466</td>
<td>DMD</td>
<td>Deletion ex45</td>
<td>[c.6439-?_6614+?del]</td>
<td>Out</td>
</tr>
<tr>
<td>Male</td>
<td>2,791</td>
<td>DMD</td>
<td>Deletion ex45–48</td>
<td>[c.6439-?_7095+?del]</td>
<td>Out</td>
</tr>
<tr>
<td>Female</td>
<td>2,731</td>
<td>DYSF</td>
<td>Frameshift ex39</td>
<td>[c.4200dupC]</td>
<td>Out</td>
</tr>
<tr>
<td>Male</td>
<td>2,735</td>
<td>SGCB</td>
<td>3 nt dup, ex1</td>
<td>c.21_23dup</td>
<td>In</td>
</tr>
<tr>
<td>Male</td>
<td>2,984</td>
<td>FKRP</td>
<td>p.R143S missense</td>
<td>c.427C&gt;A</td>
<td>In</td>
</tr>
</tbody>
</table>

CK = creatine kinase.
multiple rounds of testing, concluding with DMD gene analysis if indicated. This approach can be challenging, as evidenced by the recent closure in Wales of the longest-running DMD NBS program in history.

From its inception, our goals included creating a DMD NBS program that would fit the obstetrics practice in the USA, where mother and child are discharged within 24 to 48 hours following uncomplicated deliveries, and developing a method to readily distinguish false and true positives. Fulfillment of this task required a 2-tier system of analysis permitting CK testing followed by DNA analysis on the same dried blood spot. The design introduced has similarities to the NBS program for cystic fibrosis based on 2-tier molecular genetics testing that was first introduced in a pilot program in the state of Wisconsin. Prior to implementing a newborn screening program for DMD, 2 components had to be put in place. A validated method was required for extraction of genomic DNA from a small punch of the dried blood spot, followed by whole genome amplification with analysis of single-/multiexon deletions/duplications in the dystrophin gene using SCAIP combined with MLPA. Preparatory trials provided confidence in the methodology based upon 100% accuracy in the blinded identification of 7 exonic deletions and 6 exonic duplications taken from DMD patients with known mutations (voluntary and IRB approved) placed on newborn screening cards at Nationwide Children's Hospital and sent to the clinical DNA sequencing laboratory at the University of Utah. It was also necessary to establish a population-based range of CK on anonymous dried blood spots. This important undertaking was enabled by the full cooperation of the laboratories of the ODH, facilitating a path forward for newborn screening. Through anonymous CK analysis on >30,000 newborns (see Fig 1), we established a starting point for DNA testing at a CK level 3 standard deviations above the mean. Adding CK testing to the full battery of tests performed on dried blood spots at the ODH was not overly burdensome, and the cost for adding this 1 assay (to 35 others) was minimal (approximately $1.00 of raw materials). For those exceeding the CK threshold requiring

### TABLE 3: History of Newborn Screening

<table>
<thead>
<tr>
<th>Year of Report</th>
<th>Investigators/Country</th>
<th>Observations</th>
<th>Incidence</th>
</tr>
</thead>
<tbody>
<tr>
<td>1975</td>
<td>USA</td>
<td>Introduced CK testing on DBS in normal newborns</td>
<td>Established proof of principle</td>
</tr>
<tr>
<td>1979</td>
<td>New Zealand</td>
<td>10,000 newborns screened; 2 DMD cases identified</td>
<td>1:5,000</td>
</tr>
<tr>
<td>1982</td>
<td>Edinburgh, UK</td>
<td>2,336 newborns screened; no DMD cases identified</td>
<td>0</td>
</tr>
<tr>
<td>1986</td>
<td>West Germany</td>
<td>358,000 screened (10% &lt;4 weeks of age; 65% 4–6 weeks of age; 23% 6 weeks to 6 months; 2% 6 months to 1 year; 78 had DMD)</td>
<td>1:4,589</td>
</tr>
<tr>
<td>1988</td>
<td>Manitoba, Canada</td>
<td>54,000 screened; 10 DMD cases identified</td>
<td>1:5,400</td>
</tr>
<tr>
<td>1989</td>
<td>Lyon, France</td>
<td>37,312 newborns screened; 7 DMD cases identified</td>
<td>1:5,330; an earlier report showed 1:5,929</td>
</tr>
<tr>
<td>1991</td>
<td>Western Pennsylvania, USA</td>
<td>49,000 screened; 10 DMD identified</td>
<td>1:4,900</td>
</tr>
<tr>
<td>1993</td>
<td>Wales, UK</td>
<td>34,219 screened; 9 DMD cases found</td>
<td>1:3,802³</td>
</tr>
<tr>
<td>1998</td>
<td>Cyprus</td>
<td>30,014 screened; 5 DMD cases found</td>
<td>1:6,002</td>
</tr>
<tr>
<td>2006</td>
<td>Antwerp, Belgium</td>
<td>281,214 newborns screened at 4–6 weeks; 51 DMD cases found</td>
<td>1:5,500⁹</td>
</tr>
</tbody>
</table>

³Presentation in London, UK, March 18, 2011, reported 335,045 screened, with an incidence of 1:5,266.
⁹Only newborn screening program that continues to actively screen subjects for DMD.
CK = creatine kinase; DBS = dried blood spot; DMD = Duchenne muscular dystrophy.
DNA testing, the cost at the University of Utah laboratory was an additional $150.00 in raw materials.

The results of our study support the 2-tier system of analysis for newborn screening for DMD, perhaps in a way even more satisfying than anticipated. Over the course of this program, we screened 37,649 males and found 6 males with DMD gene mutations, an incidence of 1 in 6,291. The comparative incidence of newborn boys with documented DMD is lower than other studies throughout the world, which ranged from 1 in 3,802 to 1 in 6,002 (taking all programs together, 1 in 4,087; see Table 3), and would have to be viewed cautiously based on sample size and location in a single state in the USA. What is particularly notable about our study is that all of our patients with DMD (or dystrophinopathies) had CK levels at birth ≥2,000U/l. This margin between documented cases of DMD and those with elevated CK not found to have a DMD mutation provides reasonable assurance for circumventing false positives, enabling us to raise the threshold for DNA testing in phase 3 of the study to CK ≥750U/l. This reduced the number of newborns requiring DMD gene testing by about 68%, representing a significant cost savings for an NBS program. With additional confirmation of our findings, these initial studies suggest that the threshold for DNA testing could be elevated even higher (eg, CK ≥1,000U/l), improving the potential cost–benefit ratio for NBS.

As our program evolved, we had more confidence in the identification of the great majority with DMD mutations, but we were aware of limitations. Additional experience would be required to confirm that point mutations, present in approximately ¼ of DMD patients and not detected in this study, were the result of mutation detection using WGA from DNA isolated from dried blood spots. However, we are confident that appropriate methodology has been applied in this NBS study based on our previous work demonstrating the detection of 506 point mutations (294 nonsense mutations) in the analysis of 1,111 dystrophinopathies representing 46% of subjects (over-represented in this population because of study design). In addition, the group of dystrophinopathies manifesting predominantly as a cardiomyopathy accompanied by relative sparing of skeletal muscle (ie, X-linked cardiomyopathy) will often be missed in any NBS protocol. It is well recognized that many patients in this group have reduced CK levels in venous blood, some even in the normal or near normal range. We were also aware of newborns on the other end of the spectrum with elevated CKs and no diagnosis of DMD. For this reason, we extended the study to address this potential limitation. In the final phase of this study, we did DNA testing for the most common LGMD genes if CK was ≥2,000U/l in the absence of an identified DMD gene mutation. In this small sample, we found 1 individual with a known single-nucleotide insertion mutation in DYSF, 1 with a known missense mutation in FKRP, and another with a 3-nucleotide duplication in SGCβ of unknown pathogenicity that has been reported in 5 sarcoglycanopathy patients (Leiden Database; see Table 2). These findings demonstrate proof of principle illustrating that LGMD gene mutations can be identified as part of the screening process. Only 1 pathogenic allele was detected in each case, a result that is not uncommon for these genes. Further characterization of these deidentified samples would be required to evaluate copy number changes indicative of a second, undetected large deletion or duplication.

Our completed study was not intended to address the question of whether NBS for DMD should be introduced but rather to provide a pathway for implementation given the recent reports of therapeutic benefit for DMD. The phase 2 DMD NBS program explored ethical issues involved by assessing parent and provider experiences through questionnaires; however, this topic has been reserved for a future article. The program we have introduced differs from past programs and the current Antwerp approach to NBS for DMD that require a 3-step process: (1) CK testing on dried blood spots, followed by (2) confirmation of elevated CK levels by venous blood obtained at the 4- to 6-week time point, with (3) a final step that requires an additional blood draw for DNA testing. The approach we have developed is a 2-tier approach, with all testing done using the original blood obtained from the heel stick within the first 24 to 48 hours. All testing is done from the same dried blood spot card. A threshold level of CK determined if DNA testing is to be done without additional blood obtained from the neonate. The DNA assay utilizes the most sophisticated technology available (and can be periodically modified if necessary). Whether treatment has advanced to the point of justifying newborn screening for DMD requires assessment through state and federal agencies with appropriate jurisdiction. If and when an early therapy that improves the health outcome for individuals with DMD becomes available, our study serves as a model for implementation of newborn screening for DMD. If the development of promising therapies for DMD continues to proceed at its current pace, newborn screening could be on the horizon for this disease, not only in the USA, but also in other countries. If successful therapy for dystrophinopathies is available for newborns, guidelines will need to be established for referral to an appropriate muscle specialist. In addition, a pathway for referral could be built into the program for newborns.
those with CK elevations in the absence of DMD mutations, where there is the potential to identify other causative mutations, as we have demonstrated in this report.

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Potential Conflicts of Interest

References


