CLINICAL RESEARCH ARTICLE

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Creatine kinase-MM concentration in dried blood spots from newborns and implications for newborn screening for Duchenne muscular dystrophy

Sunju Park MSc | Breanne Maloney BSc | Michele Caggana ScD, FACMG | Norma P. Tavakoli PhD 🔾

Division of Genetics, Wadsworth Center, New York State Department of Health, David Axelrod Institute, Albany, New York, USA

Correspondence
Norma P. Tavakoli, Division of Genetics, Wadsworth Center, New York State Department of Health, David Axelrod Institute, 120 New Scotland Avenue, Albany, NY 12208, USA.
Email: norma.tavakoli@health.ny.gov

Abstract

Introduction/Aims: Creatine kinase-MM (CK-MM) is a marker of skeletal muscle damage. Detection of elevated levels of CK-MM in newborns can enable an early suspicion of the diagnosis of Duchenne muscular dystrophy (DMD) before symptom onset. Our aim was to investigate CK-MM levels in DMD-affected and unaffected newborns using an immunoassay that measures CK-MM concentration in dried blood spots collected for routine newborn screening.

Methods: To validate the assay in our laboratory, CK-MM measurements and newborn demographic information were collected for 8584 de-identified specimens and 15 confirmed DMD patients. After analyzing validation data, CK-MM normal ranges were determined based on age of newborn at specimen collection. Subsequently, the assay was used to measure CK-MM concentration in 26 135 newborns as part of a consented pilot study to screen for DMD in New York State. Mean and median levels of CK-MM based on age of collection, in addition to the 2.5th, 50th, 97.5th, and 99.5th percentiles, were recalculated using the validation and screening data sets.

Results: Median CK-MM within 1 hour of birth was 109 ng/mL, rose to a high of 499 ng/mL at 25 hours of age, and then declined to 200 ng/mL at 2 days of life. The median continued to decline more slowly and then stabilized at approximately 40 ng/mL at 1 week of life.

Discussion: Because of the marked variability and elevated CK-MM levels observed within the first days of life, it is important to set multiple CK-MM age-related cut-offs when screening for DMD in newborns.

KEYWORDS
age-related cut-offs, creatine kinase, Duchenne muscular dystrophy, newborn screening, normative values

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INTRODUCTION

In December 2019 the US Food and Drug Administration (FDA) approved an assay to aid in the detection of Duchenne muscular dystrophy (DMD) in newborns, paving the way for a consented pilot study for DMD as a first step to demonstrate feasibility for newborn screening.[1] Although there are currently no curative treatments for DMD, research has led to the availability of treatments that slow the decline of muscle strength and there are many more in the pipeline.[2,3] Clinical trials have generally been conducted in patients over 4 years of age, and thus there are few data on the outcomes of early implementation of therapy. Detection of presymptomatic patients in the newborn period will allow inclusion of these patients in clinical trials and appropriate therapy services to determine effectiveness of early treatment.

DMD and Becker muscular dystrophy (BMD) are dystrophinopathies presenting with a spectrum of phenotypes caused by mutations in the dystrophin gene, which result in either a deficiency, in BMD, or absence, in DMD, of the dystrophin protein. This deficiency leads to the loss of muscle integrity, weakening, and rupturing of the sarcolemma and, as a result, passive leakage of muscle enzymes including creatine kinase (CK) from muscle into the blood. Since the 1970s there have been several pilot studies to detect DMD in newborns[4-12] and most have used assays that measure total CK enzyme activity in blood, which is elevated in boys with DMD.[13,14] Determination of total CK level to detect DMD suffers from a lack of specificity because CK consists of three isoenzyme forms (CK-MM, CK-BB, and CK-MB). CK-MM is the isoform found predominantly in skeletal muscle and is increased in patients with DMD and other muscular dystrophies.[15-17] Although CK-MM is a more specific marker than total CK, it is not a diagnostic marker. In addition, serum CK changes with disease progression. It is highest in 3- to 5-year-old patients and decreases with age and disease progression, potentially reflecting the rate of muscle decay.[18,19]

The New York State (NYS) Newborn Screening (NBS) Program, in partnership with Parent Project Muscular Dystrophy and the DMD Steering Committee, initiated a consented pilot study to screen newborns in NYS for DMD in October 2019. A 2-tier approach using the CK-MM immunoassay as a first-tier screen followed by reflex genetic analysis of the DMD gene to minimize false positive results was implemented.[10] Because of the novelty of the first-tier assay, there was a paucity of published information regarding CK-MM measurement in dried blood spots (DBS). Two reports of newborn screening for DMD using the newly FDA-approved assay noted that CK-MM concentration decreased with age at sampling in unaffected newborns.[12,20] Here we expand on those studies by examining CK-MM concentration in DBS from over 33 000 specimens submitted to the NYS NBS Program and propose age-related cut-offs for the CK-MM assay.

METHODS

In the assay validation process, CK-MM concentration was measured in 8584 3 mm de-identified DBS specimens from 8265 newborns using the GSP Neonatal Creatine Kinase-MM kit and analyzer (PerkinElmer) as per the manufacturer's protocol. The assay is a solid phase, two-site immunofluorometric assay. CK-MM results are reported in nanograms per milliliter of blood. The majority of de-identified specimens were tested for CK-MM on day of receipt at the NBS Program. Specimens from random plates were duplicate punched each day and demographic information such as age at time of specimen collection, sex, birthweight, and neonatal intensive care unit (NICU) status were captured. In addition to testing deidentified specimens, consent was obtained to retrospectively screen newborn DBS from 15 boys who were known DMD patients to ensure that appropriate cut-offs were selected to differentiate confirmed DMD newborns from the general population. The affected boys were born between 2008 and 2015, and the 15 specimens were all collected when the boys were 1 to 3 days of age. DMD was confirmed by the treating physicians by molecular genetic testing and clinical features. CK-MM testing for the specimens from confirmed patients was performed in 2019, on specimens that had been in storage at 4°C with desiccant for between 4 and 11 years before this study.

Data from de-identified specimens screened during the assay validation process were analyzed. Factors influencing CK-MM levels were investigated. Mean, median, and 99.5th percentile were calculated and cut-offs for the assay were determined.

The consented pilot study was initiated on October 1, 2019 at select hospitals in NYS. The institutional review boards of the NYS Department of Health and participating hospitals approved the study. Informed consent was obtained from the parents or guardians of the newborns participating in the study. CK-MM screening was performed on the DBS specimens that were collected for routine NBS. In general, CK-MM testing was performed on the day of specimen receipt. However, occasionally, testing was done up to 4 weeks later mainly due to late consents. In these cases, specimens were stored at 4°C with desiccant before testing. After 18 months of the pilot study, CK-MM measurements were available for 28 495 specimens from 26 135 newborns. A repeat specimen was requested for any specimen that was collected when the newborn was less than 24 hours of age even though the specimen was tested, per routine newborn screening procedures in NYS.[21] Similarly, a repeat specimen was requested if a specimen was considered suboptimal for testing (eg, those with blood clots or serum rings) even though these specimens were also tested. Exclusion criteria for the study included newborns whose families did not understand English, Spanish, or Chinese (due to unavailability of translation services), families who did not consent to the study and newborns in the NICU who had congenital anomalies or complex medical concerns. Other newborns in the NICU had the opportunity to participate in the study.

Data analysis was performed using 37 079 CK-MM values from two data sets: (1) 8584 de-identified specimens screened during the assay validation process in 2019; and (2) 28 495 specimens from consented newborns received from October 1, 2019 to March 31, 2021 and screened for DMD.

RESULTS

Demographic information of babies whose specimens were included in the data analysis are tabulated in Table 1. During the assay

validation stage of the pilot, the influence of several variables, including age at collection, sex, and weight, on CK-MM concentration were investigated based on testing over 8000 specimens. It was determined the time of specimen collection had the greatest influence on CK-MM concentration. Therefore, normal ranges were established based on age of collection (Table 2). The cut-off for the normal range was based on the 99.5th percentile, a conservative cut-off that would lead to a recall rate of approximately 1 in 200 newborns. A higher cut-off was set for referrals to reduce the number of families requiring genetic counseling and the need for second-tier molecular testing. The cut-off for referrals was based on the CK-MM results of newborns with confirmed DMD, the CK-MM frequency distribution of the de-identified specimens included in the validation (with 7 of 8584 specimens at referral level), and the expected frequency of DMD in the general population (approximately 1 in 5000 males[10]). Of the 15 confirmed newborns tested during the validation phase, 2 had a CK-MM value of approximately 2000 ng/mL, 3 were in the range of 3600 to

6000 ng/mL, and 10 had a CK-MM value of greater than 6800 ng/mL. The highest CK-MM concentration was approximately 19,000 ng/mL.

Once the pilot was initiated, specimens in the normal range were reported as screen negative. Specimens within the borderline range (above the normal range and below the referral cut-off) were reported as borderline and an additional specimen was requested for repeat CK-MM testing. The presumption was that if the newborn was unaffected the CK-MM concentration would normalize, and, if the baby was affected, the CK-MM would remain elevated. Last, newborns with elevated CK-MM above the referral cut-off were referred for genetic counseling and second-tier genetic testing.

After 18 months of the pilot study, the CK-MM data from the pilot screening and validation data were analyzed together (total, 37 079 specimens). The CK-MM concentration distribution for the specimens was determined (Figure 1).

The 2.5th, 50th, 97.5th, and 99.5th percentiles were calculated based on age at specimen collection for the entire data set (Figure 2A). When the 99.5th percentile was calculated for each age category for the entire data set, the 99.5th percentile value for the 0- to 47-hour category had increased from 2050 to 2390 ng/mL (Table 2). This category includes most newborn specimens submitted to the program (33 774 of 37 079 = 91.1%). The 99.5th percentile CK-MM value had decreased for the other three age categories (Table 2).

The mean and median CK-MM were determined based on the age of newborn at specimen collection (Figure 2B). CK-MM was marginally elevated at 1 hour after birth (median, 109 ng/mL), increased considerably within the first 25 hours of life to a high of 499 ng/mL, and decreased precipitously to 47 hour of life (median, 200 ng/mL). CK-MM continued to decrease more gradually and then stabilize at approximately 1 week of life (median of approximately 40 ng/mL) (Figure 2A).

Because the specimens used in validation were de-identified, the babies with elevated CK-MM were not followed up and there is no information as to the outcome for these babies. Of the babies in the pilot study, 237 had borderline results and 29 were referred. Of the babies with borderline results, CK-MM values had normalized in repeat specimens from 220 babies. Repeats were not received for 15 babies. Two babies were referred after elevated CK-MM was observed on testing the repeat specimens (CK-MM of 1958 and

TABLE 1 Demographics of babies whose specimens were included in the data analysis

	Number (%[a])
Total specimens	37 079
Gender	
Male	18 903 (51.0%)
Female	18 023 (48.6%)
Weight	
<2500 g	3850 (10.4%)
≥2500 g	33 198 (89.5%)
Gestation age	
<37 weeks	4426 (11.9%)
≥37 weeks	32 615 (88.0%)
NICU status	
NICU	2963 (8.0%)
Non-NICU	34 012 (91.7%)

Abbreviation: NICU, neonatal intensive care unit.
[a]The numbers in each category do not add to 100% due to submission of incomplete demographic information to the NBS Program.

TABLE 2 CK-MM mean, median, 99.5th percentile, and reference ranges based on age at specimen collection

Age of collection (hours)	Specimens used in validation				Reference ranges based on validation specimens		Total specimens used in validation and routine screening			
	No. specimens analyzed	Mean CK-MM (ng/mL)	Median CK-MM (ng/mL)	99.5% (ng/mL)	Normal range (ng/mL)	Referral cut-off (ng/mL)	No. specimens analyzed	Mean CK-MM (ng/mL)	Median CK-MM (ng/mL)	99.5% (ng/mL)
0-47	7304	466.1	382	2050	<1990	≥4000	33 774	520.3	431.0	2390
48-71	497	209.2	151	1430	<1430	≥4000	1083	190.5	130.5	1330
72-167	342	101.4	60.1	860	<571	≥860	551	92.7	56.9	842.7
≥168	441	61.1	41.8	571	<571	≥571	1671	54.1	43.4	294.5

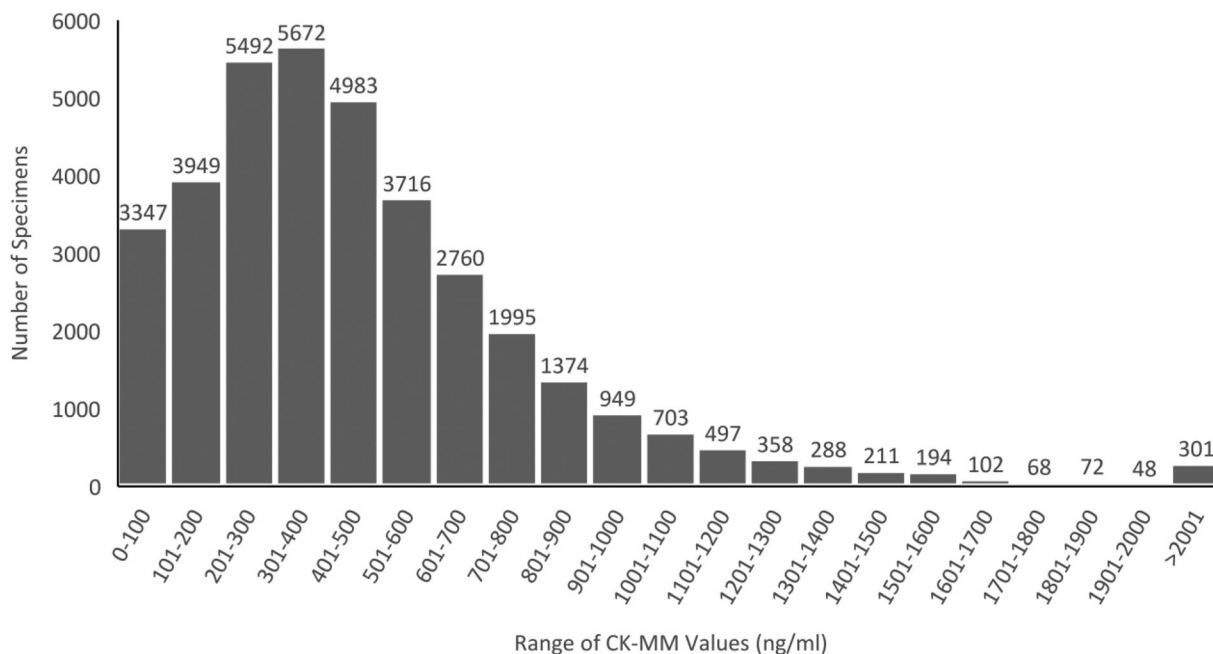Abbreviation: CK-MM, creatine kinase.

FIGURE 1 Distribution of CK-MM concentration in newborns. CK-MM, creatine kinase-MM
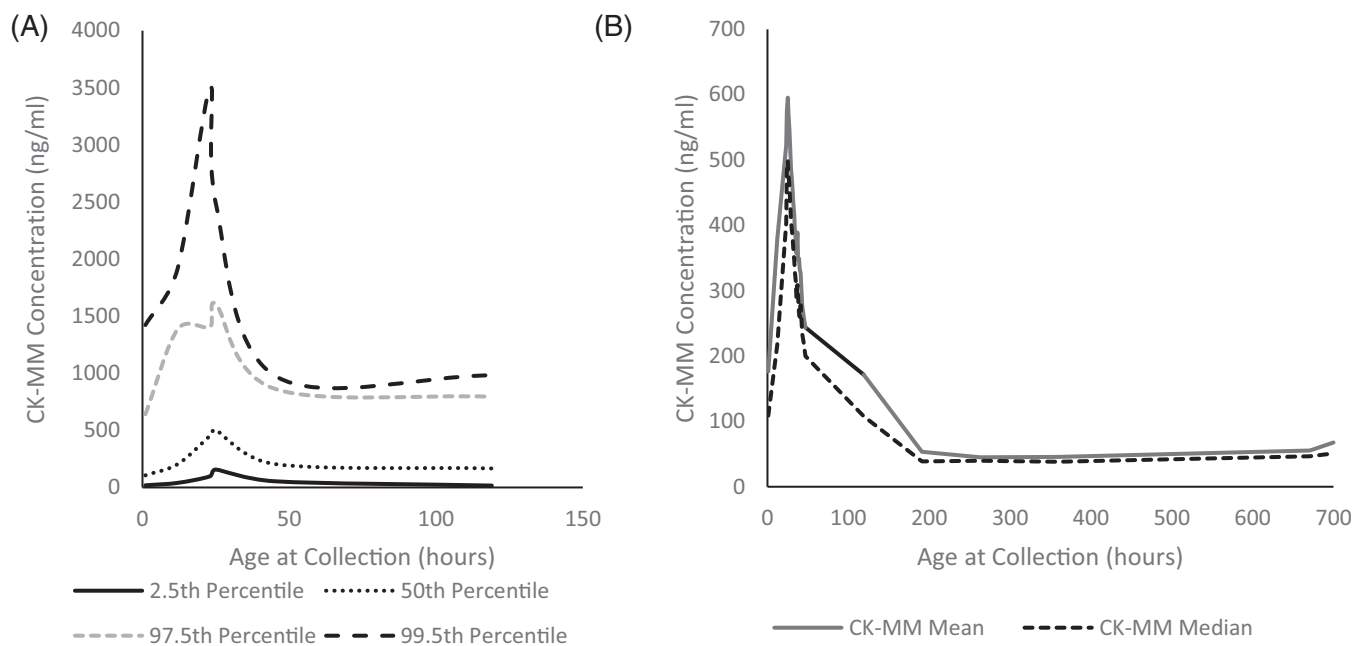

FIGURE 2 A, Trendline of 2.5th, 50th, 97.5th, and 99.5th percentiles of CK-MM based on age at specimen collection. Data from 1 to 120 hours of age are presented. B, CK-MM mean, and median based on age at specimen collection. CK-MM, creatine kinase-MM

993 ng/mL; normal range, <571 ng/mL). After molecular testing, one baby was confirmed with a deletion in the *DMD* gene consistent with DMD/BMD, and the second baby was found to be a carrier. Of the remaining 27 referred babies, 3 had deletions or duplications consistent with DMD/BMD (CK-MM between 6384 and 18,574 ng/mL; normal range, <1990 ng/mL), some families refused follow-up testing, and other babies are being followed.

DISCUSSION

Our data indicate that, in normal newborns, CK-MM is elevated soon after birth, declines, and then stabilizes at 1 week of life. The fraction of specimens (8.9%) submitted for older newborns (≥48 hours) was considerably smaller than for those submitted from newborns within the first 2 days of life and, therefore, mean, median, and 99.5th

percentile may not be as accurate. Additional data from a greater number of specimens collected at later time-points will assist in confirming our results. The NYS NBS Program requests specimen collection between 24 and 36 hours after birth and, even though a high percentage of specimens in this study were collected during this period (82.3%), a substantial number (17.7%) were also collected outside this period. Most NBS programs in the United States recommend specimen collection at 24 to 48 hours after birth, but worldwide specimen collection times vary widely,[22] and invariably NBS programs receive specimens collected between minutes to several days or even several weeks after birth. Our data indicate that one set CK-MM referral cut-off for all newborns is not appropriate. Such a strategy would lead to false negative results or an inordinate number of recalls. In most specimens in our study (73.2%) the CK-MM concentration was less than 600 ng/ml. For specimens with higher CK-MM concentrations (eg, ≥571 ng/mL which is the most conservative CK-MM cut-off in our program [Table 2]), it is important to determine whether the elevated CK-MM is due to early specimen collection or due to a neuromuscular abnormality. If 571 ng/mL had been selected as a cut-off for all specimens, then 10 912 of 37 079 (29.4%) results would have led to a referral or a request for a repeat specimen, whereas using the age-adjusted cut-offs would lead to 320 of 37 079 (0.86%) results requiring any follow-up. Timonen et al used a cut-off of the 99.5th percentile (675 ng/mL) for screening a Danish population whose specimens were collected at 2 to 3 days of age or later.[20] Ke et al reported a pilot study from the Zhejiang Province of China using the CK-MM assay and used a set cut-off of 700 ng/mL of blood.[12] They reported that NBS specimens were collected at 3 to 7 days of age.[12] Unlike those two studies, we used age-adjusted cut-offs due to the variability of age of collection of the specimens submitted to our program. It should be noted that, in a pilot study performed in Ohio from 2007 to 2011 using CK activity as a first-tier screen for DMD, age of collection had little effect on enzyme activity out to 5 days.[10] This is in stark contrast to the variability we observed in CK-MM concentration in DBS within the first days of life, possibly underscoring differences between measuring total CK activity and measuring CK-MM concentration in DBS.

Determination of outcomes of borderline and referred infants through additional CK-MM testing of borderline infants and second-tier genetic testing of referred infants will assist in evaluating the assay cut-offs and aid in reassigning cut-offs, if necessary. Each NBS program will need to establish its own range of normal concentration for the birth population for which screening is performed. The cut-offs selected by our program were based on the NYS population. The NYS NBS Program automatically requests a repeat for any baby who had a specimen collected at less than 24 hours of age and therefore we chose not to have a lower cut-off for this group of newborns. We lowered the initial borderline cut-off of 1990 ng/mL in three stages to mirror the decrease in mean and median CK-MM based on age at specimen collection. In addition, having retrospectively screened DBS specimens from confirmed DMD patients, we selected cut-offs that would flag the confirmed newborn with the lowest CK-MM. There were two confirmed newborns with CK-MM values of approximately 2000 ng/mL. One specimen was 6 years old when tested for CK-MM and was collected when the newborn was 71 hours old. The other specimen was 10 years old when tested and the accurate age of collection is unknown, but it is in the range 25 to 71 hours. Both specimens had been stored at 4°C before testing, but it is possible that CK-MM may have degraded over time and the measured CK-MM values may be underestimated. Sample stability studies show that CK-MM degrades at high temperature and high humidity.[23] Furthermore, CK-MM degradation has also been observed in samples that were frozen for over 7 years.[20] Data from additional screening will show whether freshly collected specimens from confirmed DMD newborns, as opposed to 6- to 10-year-old specimens, will have CK-MM concentrations in the 2000-ng/mL range for specimens collected at up to 47 hours of age. Thus far, none have been detected in this pilot study. The remaining 13 specimens from confirmed DMD patients with CK-MM values over 3600 ng/mL were clearly elevated compared with most specimens screened and well above the 99.5th percentile.

Before development of the CK-MM assay, other programs used CK activity as a marker for DMD.[4-11] Several groups have reported on normal CK activity in both adults and newborns.[4,24-26] In a study measuring normal serum CK activities in 663 normal Brazilian individuals in three age categories (newborns, children <15 years old, adults), activity was significantly higher in newborns than in the young children and adults.[24] Bodensteiner and Zellweger reported CK activity was highest during the first day of life and gradually decreased, but may remain slightly elevated for the first year of life.[25] In a study of 70 newborns, blood was collected from 10 minutes to 10 weeks of life and serum CK activity was measured and found to be highest during the first 24 hours after delivery.[26] There was a gradual decline of CK activity during the next 3 days.[26] A study from New Zealand also showed that CK was elevated on the first day of life and, by day 4, decreased to within three times the upper limit of normal range for adults.[4] In addition, in 10-day-old full-term newborns, serum CK activity levels similar to adults were observed.[27] Our data on CK-MM concentration in DBS from newborns support the elevated CK activity data reported previously in the first days of life.[4,25,26,28] We also observed an increase in CK-MM concentration from 1 to 25 hours of age. Notably, babies whose specimens are collected before 24 hours of age are generally cared for in the NICU. The lower CK-MM in the newborns of less than 24 hours compared with 25-hour-old newborns may also be attributed to low birthweight, prematurity, or illness, not only age of specimen collection. To prevent false negative results in low birthweight, premature, and ill infants, repeat DBS collection and CK-MM screening at a later time-point is warranted. A serial screening protocol is recommended for this special population because the newborn screen results for several disorders are unreliable using a single DBS specimen.[29]

Limitations of this study include the variability introduced by analyzing data from newborns with different weight, gestational age, ethnicity, sex, mode of delivery, and tested at various time-points after specimen collection. The majority of specimens were screened on day of receipt at the NBS program. However, specimens from confirmed DMD patients were tested for CK-MM after 4 to 11 years of storage

at 4°C. CK-MM values for these specimens may be an underestimation. Other limitations of the study include the need for additional CK-MM data at later ages of collection (ie, sequentially after 48 hours of age), as well as from non-NICU, healthy babies at less than 24 hours of age. In addition, not all specimens included in our data analysis underwent genetic testing and follow-up (in particular, the de-identified cohort), and therefore the DMD status of all the newborns is unknown. We are not aware of any newborns with false negative results.

The elevation of CK-MM in non-DMD newborns indicates a need for refining confirmatory testing for newborns with elevated CK-MM, and management of newborns who are identified with non-DMD muscular dystrophies.[30] Because CK is an indirect marker of DMD, the measurement of CK levels inevitably results in the identification of other muscular dystrophies, which may not have treatment options available.[31] In contrast, the increase in CK activity in normal newborns is transient and a consequence of the trauma experienced by the newborn during the birth process.[4,26,32] This increase could lead to false positive DMD screen results if serum CK activity is used for the screen. One option for DMD screening would be to postpone CK screening beyond the neonatal period as previously proposed.[26] As an example, a group in Italy plan to screen male infants aged between 6 and 42 months using a two-step CK/DNA screening process.[33] This option would not be practical for NBS programs, especially in the United States where specimens are collected within the first 2 days of life. In addition, it would lead to diagnostic delays. Careful consideration of cut-off values is warranted based on the elevation and variability of CK-MM values within the first week of life. We propose using multiple age-adjusted cut-offs based on age at specimen collection to prevent unreasonably high recall rates in newborns whose specimens are collected within the first 2 days of life, as well as false negative results in older newborns.

CONFLICT OF INTEREST
The authors declare no potential conflict of interest.

ETHICAL PUBLICATION STATEMENT
We confirm that we have read the Journal's position on issues involved in ethical publication and affirm that this report is consistent with those guidelines.

DATA AVAILABILITY STATEMENT
The data that support the findings of this study are available from the corresponding author upon reasonable request.

ORCID
Norma P. Tavakoli https://orcid.org/0000-0001-7758-820X

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