DIAGNOSTIC DILEMMAS RESULTING FROM THE IMMUNOREACTIVE
TRYPSINOGEN/DNA CYSTIC FIBROSIS NEWBORN
SCREENING ALGORITHM

RICHARD B. PARAD, MD, MPH, AND ANNE MARIE COMEAU, PhD

Objective To quantitate the proportion of infants identified through cystic fibrosis (CF) newborn screening (NBS) by an immunoreactive trypsinogen (IRT)/DNA screening algorithm who have an unclear diagnosis as defined by the findings of an elevated IRT level and either 1) 2 CF gene (CFTR) mutations detected and sweat chloride level <60 mEq/L; or 2) 0 or 1 CFTR mutations and a "borderline" sweat chloride level ≥30 and <60 mEq/L.

Study design Using the 4-year cohort of CF-affected infants recently described by the Massachusetts CF NBS program, we identified and described the number of infants with the diagnostic characteristics (diagnostic dilemmas) aforementioned.

Results Of infants with positive results on CF NBS who had 1 CFTR mutation detected and a borderline sweat chloride concentration, nearly 20% displayed a second CFTR mutation on further evaluation. Of all infants with positive CF NBS results considered affected with CF, 11% had a diagnosis that fell into 1 of the diagnostic dilemma categories aforementioned.

Conclusions Four problematic diagnostic categories generated by CF NBS are defined. In the absence of data on the natural history of such infants, careful follow-up is recommended for infants in whom a definitive diagnosis is elusive. (J Pediatr 2005;147:S78-S82)

The original algorithm for cystic fibrosis (CF) newborn screening (NBS) used 2 serial dried blood spot (DBS) immunoreactive trypsinogen (IRT) values (specimen collection separated by weeks) to confirm persistent neonatal IRT elevation before referral for definitive CF diagnosis with pilocarpine iontophoresis, or sweat testing.1,2 With the identification of the gene associated with CF, the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR), Gregg proposed using DNA testing in a new IRT/DNA 2-tier CF NBS algorithm that uses only a single DBS collection.3 This allowed for faster turnaround of screening results and a diminished risk of losing track of an infant who might not have the second DBS submitted for testing.

Although the IRT/DNA algorithm improves some aspects of CF NBS (eg, positive predictive value4), it draws attention to new issues. With over 1300 pathogenic mutations reported in the CFTR gene from a variety of racial and ethnic groups, any screening algorithm that is applied to a diverse population such as that in the United States may need to tailor the choice of the particular mutations included in the IRT/DNA screening assay. Use of only the most common CFTR mutation, ΔF508, in a subpopulation with a low ΔF508 allele frequency could miss a significant number of affected infants. The Massachusetts (MA) CF NBS algorithm,5,6 which includes a multiple CFTR mutation panel, uses a single, early DBS, and demonstrates a low false-negative results rate for identifying CF. The use of multiple mutations allows for DNA-based diagnosis from the screening test, leading to earlier initiation of therapy. In addition, use of a multiple rather than single CFTR mutation panel lowers the risk that the detection of only 1 CFTR mutation in the CF NBS will be associated with positive sweat test results, which allows for more reassuring pretest counseling.

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The 2 main considerations in applying DNA testing in the CF NBS are: 1) choosing an IRT cutoff value that prompts DNA testing and 2) choosing a mutation panel appropriate to a given population that will minimize the false-negative results rate and ideally only identify infants in whom classic CF will develop. When single mutation testing is chosen, a higher false-negative results rate occurs with the DNA component of the algorithm. There is an option to use a “failsafe” for identifying affected infants with rare mutations by referring infants with an extremely high IRT concentration for sweat testing5 regardless of DNA results.

Although the gold standard for CF diagnosis is still the sweat test, this test is also not perfect.7,8 For a small percentage of infants with positive CF NBS results, testing too early might result in inaccurate readings because sweat chloride concentration ([Cl\textsuperscript{-}]) falls within days of birth9 or because too little sweat is obtained to generate a reliable measurement. Repeating the test at a later time can solve these problems. In some frustrating instances, the sweat [Cl\textsuperscript{-}] value is in a “borderline” range. For infants, this has been defined in the MA CF NBS program as [Cl\textsuperscript{-}] values from 30 to 59 mEq/L, because 30 mEq/L is approximately 5 SDs higher than the mean of infants who are known CF carriers.10 The natural history of these infants with borderline results is not well defined, which suggests the need for a follow-up protocol to better understand the risks in this group.

All infants with positive IRT/DNA CF NBS results who have undergone sweat testing will fall into 1 of 9 outcome categories (Table I). Most of them are predicted to have clear-cut outcomes. In this paper, we report the frequency of outcomes that fall into 1 of 4 categories that constitute diagnostic dilemmas (shown by the shaded cells in Table I).

### METHODS

Massachusetts began a CF NBS as a supplementary, optional program with approval for research from Human Subjects Committees on February 01, 1999, which required informed parental consent for CF NBS testing. The IRT/DNA algorithm using a multiple mutation panel is described elsewhere in detail.5 IRT and DNA data were maintained in an Access (Microsoft) database at the New England Newborn Screening Program (NENSP), where testing was performed. More than 99% of sweat tests required on infants with positive CF NBS results were performed at 1 of the 5 MA CF Foundation centers in a National Committee for Clinical Laboratory Standards-certified sweat laboratory. A sweat [Cl\textsuperscript{-}] of >60 mEq/L on \(\geq 75\) mg or \(\geq 15\) \(\mu\)L of collected sweat resulted in recommendation for treatment at a CF care center. The presence of 2 CF–causing mutations or a sweat [Cl\textsuperscript{-}] of 30

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**Table I. A cohort of newborns with elevated serum IRT concentrations divided into diagnostic categories by results of sweat Cl\textsuperscript{-} concentration and CFTR mutation analysis (total number of infants with positive CF NBS = 1338, but sweat data only available on 1214). Total number considered CF true positives = 110 (data not included on 2 false negative and 1 not screened affected infants who would bring the total known affecteds to 113). Four shaded areas contain infants considered diagnostic dilemmas.**

<table>
<thead>
<tr>
<th>Elevated serum IRT (&gt;95%)</th>
<th>Number of mutations detected on CFNBS</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Sweat Chloride (mEq/L)</strong></td>
<td><strong>2</strong></td>
</tr>
<tr>
<td>(\geq 60) (Abnormal)</td>
<td>CF (n = 75)</td>
</tr>
<tr>
<td>30 - 59 (Borderline)</td>
<td>CF Spectrum(^1)</td>
</tr>
<tr>
<td>Group I (n = 4)</td>
<td>Group III (n = 4)</td>
</tr>
<tr>
<td>CF Spectrum(^1)</td>
<td>Carrier (n = 904)(^2)</td>
</tr>
<tr>
<td>Group II (n = 3)</td>
<td></td>
</tr>
</tbody>
</table>

\(^1\)10 infants in this group with 2 Pancreatic Insufficient CF mutations (e.g., ΔF508 homozygote) have not yet completed sweat testing but are included in this group.

\(^2\)CF Spectrum refers to a CFTR dysfunction related phenotype which might range from severe multi-organ to mild single organ involvement. “Classic CF” and “Atypical or Variant CF” are included.

\(^3\)114 infants included in these 2 categories have missing sweat data, but are presumed not to have CF for this analysis because they have not come to clinical attention at a CF center.

The Figure. Diagnostic dilemma follow-up protocol.
to 59 mEq/L was considered abnormal (borderline or indeterminate) and prompted entry into a follow-up protocol for which agreement from all 5 CF center directors was obtained (Figure). All follow-up data (clinical diagnoses, sweat test data, genetics evaluation) were reported by the CF centers for entry into the central database at NENSP, where outcomes of infants with positive screening results were prospectively tracked.

The MA CF NBS program follow-up on infants with positive CF NBS results assigns the infants to one of the 9 categories in Table I, 4 of which are considered to be “diagnostic dilemma” categories. These 4 categories are: group I = IRT >95%, 2 CFTR mutations, and a borderline sweat test result; group II = IRT >95%, 2 CFTR mutations, and a negative sweat test result; group III = IRT >95%, 1 CFTR mutation, and a borderline sweat test result; and group IV = IRT >99.8%, 0 CFTR mutations, and a borderline sweat test result.

A protocol for follow-up of both “quantity not sufficient” (QNS) and borderline sweat values (Figure) was agreed on by the MA CF NBS Workgroup (a group including newborn screeners and CF center directors) before the initiation of the MA CF NBS program.

### RESULTS

Between February 1, 1999, and January 31, 2003, 323,506 newborns were screened for CF in Massachusetts over 98% of infants born in that period. Of the infants screened in 4 years, 1338 infants had a positive CF newborn screen result,5 and in 110 of these infants CF was diagnosed. Two infants with CF (1 newborn with meconium ileus, and a 4-month-old infant with respiratory and gastrointestinal symptoms) were identified as having false-negative results through the follow-up system. CF was diagnosed in 1 infant after the parents had refused CF NBS. Results of 1214 sweat tests were available. Missing data were caused either by death before sweat testing, parental refusal to perform sweat test, sweat testing performed out-of-state, loss to follow-up (testing not requested), or multiple QNS sweat tests without resolution.

### CF-Affected Infants

The CF NBS resolved 1338 infants who were screen-positive in one of 9 categories defined in Table I (1 normal and 8 others). The available sweat test results showed most infants with a positive CF NBS result (1117/1214 or 92%) had normal sweat tests ([Cl$$^-$$] <30 mEq/L). For the purposes of subsequent considerations, in this Table, the 114 infants detected with 0 or 1 mutations who did not have sweat test data available were assigned as having a negative sweat test result. A diagnosis of CF was made in 8% of the infants with a positive CF NBS result. Within this group, presentations included: 2 mutations detected (82/929), 1 mutation detected and a [Cl$$^-$$] >60 mEq/L (n = 21), or no mutations detected, but an IRT >99.8% (2/327).

### Borderline Sweat Test Results

Forty-two infants (3.4%) had borderline sweat [Cl$$^-$$] on their initial visit to a CF center. Of these, 23 underwent the repeat sweat testing suggested by our guidelines. This low level of compliance with guidelines was often caused by the pediatricians’ choice not to pursue an ambiguous result in a healthy-appearing infant, but in some cases appeared also to be caused by suboptimal communication of the follow-up protocol between the sweat laboratories and the pediatricians. Of the 23 infants who underwent repeat sweat testing, 8 (approximately 1/3) dropped into the normal sweat [Cl$$^-$$] range, 1 had a
positive sweat test result (≥60 mEq/L), and 14 remained in the borderline range.

**Diagnostic Dilemma Group I and Group II**

These groups include infants in whom 2 CFTR mutations are defined, but the sweat [Cl\(^-\)] is not definitively abnormal. Some of these infants may have atypical forms of CF that will be associated with milder presentation\(^{11-13}\) and present at a later than average age.

**Diagnostic Dilemma Group III and Group IV**

In addition to the recommendation for repeat sweat testing, the follow-up protocol (Figure) also recommends using an expanded mutation panel for infants with persistently elevated sweat [Cl\(^-\)]. Twenty-three of the 42 infants with borderline sweat test results (not all in the group that had a repeat sweat test performed) had expanded genetic testing performed. Five of 24 (20%) unidentified chromosomes in these 23 infants (4 infants) had a second mutation detected on the Genzyme 86 or 87 CFTR mutation panel (Genzyme Genetics, Framingham, Mass). One of these infants later had [Cl\(^-\)] >60 mEq/L. The other 3 infants could be recategorized from group III to group I. An additional 2 infants had the intron 8 5T allele detected, presumably trans to the AF508 mutation. These 2 infants were not definitively considered as having a second CFTR mutation in this study, but were deemed appropriate to maintain under the follow-up protocol. Such infants might possibly be categorized in group I or III if 5T was shown to be acting as a true mutation.

With the detection of a second mutation in the setting of an elevated IRT, it appears that at least 17% (4/23) of infants in groups III and IV (Table II) with borderline or normal sweat [Cl\(^-\)] may be diagnosed as having CF or belonging to the CF spectrum of disease (a CFTR dysfunction-related phenotype that might range from severe multiorgan to mild single-organ involvement; “classic CF,” and “atypical, variant, or non-classical CF” are included).

Seven of the 110 infants (6.4%) reported as having positive screen results by the MA CF NBS program in this 4-year pilot interval and who were given a CF diagnosis had 2 CFTR mutations on the CF NBS, but sweat [Cl\(^-\)] that did not allow a classic CF diagnosis (groups I and II). An additional 5 (groups III and IV) of the 110 infants reported with positive screen results and given a CF diagnosis did not have 2 mutations identified by the initial MA CF NBS and had sweat [Cl\(^-\)] in the 30 to 59 mEq/L range, and 4 of those had expanded mutation analysis that detected a second mutation.

**DISCUSSION**

NBS, by definition, is designed to detect newborns who are affected but have no symptoms. Although the CF Foundation Consensus Guidelines\(^ {14}\) made provisions for diagnosis in infants with positive NBS results, not all group I to IV infants are covered by that scheme. The guidelines state that when the NBS results are abnormal and 2 CFTR mutations are detected, a diagnosis of CF can be made. The consensus panel stated, “…the diagnosis of CF should be based on the presence of one or more characteristic phenotypic features, a history of CF in a sibling, or a positive newborn screening test result plus laboratory evidence of a CFTR abnormality as documented by elevated sweat chloride concentration, or identification of mutations in each CFTR gene known to cause CF or in vivo demonstration of characteristic abnormalities in ion transport across the nasal epithelium.”\(^ {13}\)

Twelve (sum of infants in groups I-IV) of 110 of the infants (11%) detected by the CF NBS with elevated IRT concentrations had DNA or sweat [Cl\(^-\)] results that suggest the presence of atypical CF. The infants were designated as having true-positive results through a combination of the 3 tiers of CF NBS testing and the evaluation of a CF center. These infants might go on to have severe, life-threatening, morbidity-causing ramifications of their CFTR abnormalities. However, they do not fit neatly into the classic gold standard diagnostic guidelines of having a sweat [Cl\(^-\)] >60 mEq/L. It is also possible that some of these infants will go on to have such minimal phenotypes that they would never cross the threshold to come to clinical attention as part of the CF spectrum. Because the borderline group we have monitored ([Cl\(^-\)] = 30-59 mEq/L) does appear to contain a significant number of infants ultimately with 2 CFTR mutations, we have adopted this lower sweat chloride threshold for infants (30 mEq/L) for follow-up within the MA CF NBS algorithm. It is also possible that, within the cohort of babies with positive CF NBS results from the 4-year study period, an infant resolved to the carrier or normal status (sweat [Cl\(^-\)] <30 mEq/L) may actually harbor 1 or 2 mutations that were missed by the NBS mutation panel. That infant might have symptoms later in life that suggest CF spectrum and an atypical form of CF associated with a low or borderline sweat [Cl\(^-\)].

Because our evaluation of the infants in group III with borderline sweat [Cl\(^-\)] was not complete, an additional 4 infants who could be moved to group I (assuming that 20% of unidentified chromosomes in the 19 who did not receive expanded genotyping would have a second mutation) might be detected if further genotyping were performed. There may also be individuals present in the population with IRT less than the 95% cutoff value who have 2 CFTR mutations and in whom symptoms will develop. This additional group will include both individuals with false-negative results who have classic CF and individuals with mild atypical forms of CF. Late identification of any of these individuals could change the size of the affected cohort.

There may be an increase in the number of patients with CF identified in the population because of those infants detected who will be at the mild or atypical end of the CF spectrum. Critics of the identification of such individuals through CF NBS programs claim that this is a risk or harm, because it strays from identifying only the desired patients with classic CF, for whom the CF NBS was intended, in whom early severe disease will develop. To put the atypical CF problem into perspective, nearly 90% of the infants identified with the MA CF NBS have genotypes and sweat [Cl\(^-\)] that are consistent with becoming patients with classic CF, and those infants...
who might be at the atypical end of the spectrum are a relatively small number. Although the ultimate outcomes of these atypical patients is currently unclear, there is some evidence to support concerns that significant problems may develop in them, and thus early intervention could affect outcome.

It will be important to evaluate the impact of this information on both the psychosocial and the medical outcome of infants with non-classical CF who are detected early, because only very limited data are currently available. The concern that harm is being done, because there is not a clear answer to present to parents about whether the infant actually has CF, needs to be put into the perspective that the relative number in this group is small. Although one could modify cutoff values (IRT or sweat [Cl]) in a way that would diminish the detection of such infants, those changes could lead to a higher false-negative result rate in infants who will turn out to have classic CF. Modification of the mutation panel to exclude pancreatic sufficient mutations not associated with classic CF (eg, R117H) so that elevated IRT level and at least 1 severe mutation became the screening gate that would allow infants to proceed to sweat testing is another potential solution. The latter modification assumes either the risk/benefit ratio does not warrant identifying this group or that limited resources are better focused on infants with classic CF.

Our workgroup of newborn screeners and CF center directors developed recommendations for the follow-up of infants with uncertain diagnosis (groups I-IV) until more data are available on their outcome (Figure):

- Maintain a consistent follow-up approach between CF centers via a standardized protocol;
- Follow sweat [Cl] with time, and consider resolution criteria (eg, sweat [Cl] falling into the reference range leads to discharge of the infant as a carrier);
- Perform expanded genotyping on infants with persistently elevated sweat [Cl] to search for a second mutation using total gene screen approaches;
- Withhold a definitive diagnosis of classic CF, but explain to parents that a CF diagnosis may surface with time;
- Perform regular (every 6-12 months) clinical follow-up with a CF specialist who will work with the primary care provider to monitor the child for early CF symptoms and guide appropriate treatment.

Accumulation of long-term outcome data on infants followed with this protocol could allow the identification of factors that will predict who will ultimately have classic CF or atypical CF. Such information may also allow for fine-tuning of treatment protocols used to treat infants detected with CF through CF NBS programs.

REFERENCES