Markedly elevated neonatal immunoreactive trypsinogen levels in the absence of cystic fibrosis gene mutations is not an indication for further testing

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Abbreviations
CF cystic fibrosis
CFTR cystic fibrosis transmembrane conductance regulator
MI meconium ileus
NBS newborn screening
Abstract

Objective: to report the immunoreactive trypsinogen (IRT) values above the usual 99th percentile laboratory cut-off and determine the value of offering further testing to those infants with a markedly elevated IRT but no cystic fibrosis transmembrane regulator (CFTR) gene mutation identified by the screening program.

Design: population study

Setting and patients: all babies screened in Victoria, Australia between 1991-2003

Interventions: screening of newborns by IRT followed by CF gene mutation analysis

Main outcome measures: the diagnosis of CF

Results: There were 806520 babies born between 1991-2003, of whom 9268 with the highest IRT levels had CFTR mutation analysis. There were 123 ΔF508 homozygotes and 703 heterozygotes (86 with CF, 617 carriers). There were 8442 babies with no CF mutation of whom 18 (0.21%) had CF. The total CF babies with IRT greater than the laboratory cut-off was 227 (2.4%). The IRT results of the CF patients were distributed normally, with the majority above the laboratory cut-off of newborn IRT results. There was no evidence of an excess of babies with CF in the very highest levels of IRT above the 99th percentile.

Conclusions: Only a small proportion of babies with a neonatal IRT >99th percentile have CF. Additional CF testing for infants with an elevated IRT but no CF mutation has an extremely low yield, no matter how high the IRT result.
Introduction

Newborn screening (NBS) for cystic fibrosis (CF) was introduced in Victoria, Australia in 1989 to facilitate early diagnosis and provide genetic counseling for affected families. The primary screen is immunoreactive trypsinogen (IRT) from day 3 heel-prick blood collected on a filter paper card. Infants with an elevated IRT (>99th percentile of results) have cystic fibrosis transmembrane conductance regulator (CFTR) gene mutation analysis for the commonest mutation, ∆F508, from the same card. Other centres with NBS for CF screen with additional mutations, depending on the frequency of these mutations in their community. Infants with two CFTR mutations have CF while infants with one copy of the common CFTR mutation are referred for a sweat test. Infants without an identified mutation are not tested further. Some of these infants may have CF with two mutations not included in the screen and subsequently present with clinical features.

There is increasing interest in the introduction of NBS for CF by many centres around the world and paediatricians will be faced with dilemmas created by screening. One of these is the detection of infants with markedly elevated IRT values in the absence of one or two CFTR mutations. This frequently prompts the question as to whether further testing (usually a sweat test) should be performed to exclude CF.

The aim of this study is to report the IRT values above the usual 99th percentile cut-off and determine the value of offering further testing to those infants with a markedly elevated IRT but no CFTR mutation.

Methods

We examined the IRT values for CF NBS in Victoria, Australia between 1991 and 2003. This period was chosen because all elevated IRT values were followed up with ∆F508 mutation analysis (only) from 1991 and most children missed by NBS are likely to have presented at the time of the study (2005). The screening laboratory is centralized at Genetic Health Services Victoria and only two clinics provide specialized CF care in Victoria. We reviewed the patient lists of the two Victorian paediatric CF clinics to find infants missed by screening.

IRT was measured by enzyme-linked immunoassay with dual anti-IRT monoclonal antibodies to IRT (Biocline neonatal IRT ELISA: Cat No. 40502400) from day 2-4 heel-prick blood collected on a filter paper card. The absolute values of IRT vary between assays so a statistical approach is taken to determine elevated levels, and the 99th percentile taken as the cut-off. In Victoria, Australia only the common CFTR gene mutation, ∆F508 is tested as part of the NBS program. ∆F508 is analysed using polyacrylamide gel electrophoresis. If space was available on the gel after samples with the highest IRT had been loaded, babies with the IRT levels nearest the 99th centile were included. Therefore, the total number of babies who underwent ∆F508 analysis was slightly greater than the top 1%. The IRT data are presented as multiples of batch median (MOM) to eliminate between batch variation.
Results

The screening results for 1991-2003 are presented in figure 1. There were 806520 babies born between 1991-2003, of whom 9268 with the highest IRT levels had CFTR mutation analysis. There were 123 ΔF508 homozygotes and 703 heterozygotes (86 with CF, 617 carriers). There were 8442 with an elevated IRT but no ΔF508, of whom 18 (0.21%) had CF. The details of these patients are presented in Table 1.

Table 1: Details of cystic fibrosis patients with IRT > 99th percentile but no ΔF508 mutation. Victoria, Australia 1991-2003.

<table>
<thead>
<tr>
<th>Patient</th>
<th>IRT (MoM)</th>
<th>Genotype*</th>
<th>Presentation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patient 1</td>
<td>2.77</td>
<td>R117H/-</td>
<td>Sibling with CF</td>
</tr>
<tr>
<td>Patient 2</td>
<td>4.57</td>
<td>N1303K/-</td>
<td>Meconium ileus/sibling with CF</td>
</tr>
<tr>
<td>Patient 3</td>
<td>3.28</td>
<td>-/-</td>
<td>Failure to thrive</td>
</tr>
<tr>
<td>Patient 4</td>
<td>18.16</td>
<td>N1303K/N1303K</td>
<td>Failure to thrive/ recurrent cough</td>
</tr>
<tr>
<td>Patient 5</td>
<td>2.98</td>
<td>V520F/-</td>
<td>Meconium Ileus</td>
</tr>
<tr>
<td>Patient 6</td>
<td>3.79</td>
<td>-/-</td>
<td>Meconium Ileus</td>
</tr>
<tr>
<td>Patient 7</td>
<td>6.65</td>
<td>G551D/3849</td>
<td>Failure to thrive/ recurrent cough</td>
</tr>
<tr>
<td>Patient 8</td>
<td>8.32</td>
<td>-/-</td>
<td>Failure to thrive</td>
</tr>
<tr>
<td>Patient 9</td>
<td>6.45</td>
<td>-/-</td>
<td>Failure to thrive</td>
</tr>
<tr>
<td>Patient 10</td>
<td>3.69</td>
<td>-/-</td>
<td>Clinical details not available</td>
</tr>
<tr>
<td>Patient 11</td>
<td>13.81</td>
<td>-/-</td>
<td>Failure to thrive</td>
</tr>
<tr>
<td>Patient 12</td>
<td>6.64</td>
<td>G542X/-</td>
<td>Recurrent chest infection</td>
</tr>
<tr>
<td>Patient 13</td>
<td>5.51</td>
<td>-/-</td>
<td>Affected sibling</td>
</tr>
<tr>
<td>Patient 14</td>
<td>3.95</td>
<td>G542X/-</td>
<td>Meconium Ileus</td>
</tr>
<tr>
<td>Patient 15</td>
<td>6.92</td>
<td>-/-</td>
<td>Recurrent chest infection</td>
</tr>
<tr>
<td>Patient 16</td>
<td>6.82</td>
<td>-/-</td>
<td>Failure to thrive</td>
</tr>
<tr>
<td>Patient 17</td>
<td>7.31</td>
<td>-/-</td>
<td>Failure to thrive</td>
</tr>
<tr>
<td>Patient 18</td>
<td>7.66</td>
<td>-/-</td>
<td>Sibling with CF</td>
</tr>
</tbody>
</table>

IRT = immunoreactive trypsinogen, MoM = multiple of median
All patients had sweat chloride values >60mmol/L.


The total number of babies with CF and an IRT greater than the laboratory cut-off was 227 (2.4% of babies with an elevated IRT).

The IRT values of the entire population (which includes carriers and CF affected infants) are slightly skewed to the right (ie towards the higher IRT values) (figure 1) On closer examination of the IRT results in the higher values, the CF patients were distributed normally, with the majority above the 99th percentile (figure 2). The mean IRT value for CF group was around a MoM of 7 (figure 3). Table 2 presents data on the Positive Predictive Value (PPV) of IRT at increasing percentile cut-offs.

**Table 2: IRT level and risk of CF in absence of ΔF508**

<table>
<thead>
<tr>
<th>Cutoff</th>
<th>No. of confirmatory tests</th>
<th>No. CF without ΔF508 (unexpected cases)*</th>
<th>Positive Predictive value (unexpected cases)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>IRT (percentile)</td>
<td>MoM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>98.8</td>
<td>2.77</td>
<td>7345</td>
<td>18 (11)</td>
</tr>
<tr>
<td>99</td>
<td>2.93</td>
<td>6750</td>
<td>17 (10)</td>
</tr>
<tr>
<td>99.2</td>
<td>3.14</td>
<td>5817</td>
<td>16 (10)</td>
</tr>
<tr>
<td>99.4</td>
<td>3.39</td>
<td>4768</td>
<td>15 (9)</td>
</tr>
<tr>
<td>99.6</td>
<td>3.83</td>
<td>3289</td>
<td>13 (8)</td>
</tr>
<tr>
<td>99.8</td>
<td>4.77</td>
<td>1504</td>
<td>11 (8)</td>
</tr>
<tr>
<td>99.9</td>
<td>6.49</td>
<td>532</td>
<td>9 (8)</td>
</tr>
</tbody>
</table>

*unexpected cases are those that were diagnosed only by NBS, and did not have MI or a family history of CF.

This is compared to the PPV of infants with an IRT above the cut-off and who have one ΔF508 mutation (table 3)
Table 3: IRT level and risk of CF in ΔF508 heterozygotes

<table>
<thead>
<tr>
<th>Cutoff (IRT percentile)</th>
<th>MoM</th>
<th>No. of confirmatory tests</th>
<th>No. CF detected</th>
<th>Positive Predictive value</th>
</tr>
</thead>
<tbody>
<tr>
<td>98.8</td>
<td>2.77</td>
<td>703</td>
<td>88</td>
<td>1 in 8</td>
</tr>
</tbody>
</table>

Discussion

Only a small proportion of infants with a neonatal IRT >99th percentile have CF. The ones with CF are normally distributed across the range of IRT results >99th percentile. Seven of the 18 patients with CF missed by screening in the >99th percentile IRT were detected in the newborn period due to meconium ileus or having a sibling with CF. Of the remaining 11, 3 infants could have been detected if additional CFTR mutations were analysed. Table 2 demonstrates the relatively poor PPV of elevated IRT in the absence of a CFTR mutation compared to a PPV of 1 in 8 based on an elevated IRT and one CFTR mutation (Table 3). On this basis, there is no indication to recall the infants with an elevated IRT, but no CFTR mutation, for further testing, no matter how high the IRT.

There are a number of explanations for an elevated neonatal IRT apart from CF. Contamination of the heel prick site with meconium is the likely explanation in most cases. We have NBS filter paper cards with elevated IRT results taken from sections of the card well away from the blood spots from non-CF infants (unpublished data). Other poorly understood physiological factors resulting in an elevated IRT include neonatal stress (low Apgars), respiratory distress, hypoglycaemia or serious congenital abnormalities.(5;6) Persisting hypertrypsinogeneamia has been associated with congenital infections, bowel atresias and trisomies 13 and 18.(7) In most cases, there is no recognized cause of a falsely elevated IRT, they merely represent the high end of the normal distribution.

The IRT distribution for the entire population in this study is similar to that reported by Larsen et al (1994).(8) In this paper the IRT values of CF infants were reported, but there were only 11 of them. The median IRT for their entire screened population was 250 ng/ml and the mean IRT of the CF group was 1570 ng/ml. Different IRT assays give different results, but it is possible to compare studies by using MOM. In this paper, the mean IRT of CF infants equates to an IRT MOM of around 7, similar to our population of CF infants. The highest IRT in this series was 1997 ng/ml (MOM 7.9).(8)

From the Wisconsin newborn screening program, the number of infants with CF increased in the highest IRT cohorts, up to 20 CF infants from 83 (CF risk 24.1%, 95%CI 14.9, 33.3) with an IRT >300ng/ml.(9) However the median IRT for the entire screened population was not reported to determine a MOM. There was no DNA
information to know whether these infants were all detected by mutation analysis but it is unlikely all 20 would have been missed by CFTR mutation analysis. In this case, the actual number of infants in the highest IRT cohort missed by screening is likely to be low and therefore does not justify recalling all of the infants for a sweat test.

We could have improved the diagnostic yield (identifying 7 extra patients) from NBS, by the addition of 4 or 5 extra CFTR mutations. However, of these 7 patients, 4 would have been detected on clinical grounds, regardless of the screening result (with meconium ileus or an affected sibling). This means that we would have had to test 8442 samples with an extended screen to find an additional 3 positive results. It is difficult to justify the cost of this in our screening program.

Another approach to identifying the missing infants is repeating the IRT from all those infants with an IRT >99\textsuperscript{th} centile who did not have a CFTR mutation. Again this amounts to the recall of 8442 infants (649 per year) for a second IRT to identify 11 unexpected CF patients (the other 7 missed patients were identified clinically in the newborn period). This recall is associated with significant parent anxiety and use of resources. It was to avoid the recall for a second IRT that most screening programs moved to an IRT/CFTR protocol from the same screening card. (7, 9). The initial IRT values of the missed CF infants was spread across the range of values above the 99\textsuperscript{th} centile so that we could not identify an IRT level above which there is justification for a second IRT.

Our data do not support additional CF testing for infants with a markedly elevated IRT but no CFTR mutation. Even at the highest levels of IRT we did not find an excess of infants with CF who had been missed by our screening program. If we contacted the family of each baby with an IRT >99\textsuperscript{th} centile without ΔF508, this would have required an extra 8442 patient contacts for testing and associated counselling. We recognize that a small number of infants will be missed by this approach. However the IRT/CFTR mutation analysis approach for neonatal CF detection is a screening program and the number of missed infants is small compared with the number of infants with a falsely elevated IRT, no matter how high the IRT result.
Competing Interests
There are no conflicts of interest by any of the authors.

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What is already known about this topic
- Newborn screening for cystic fibrosis with an IRT/DNA approach will miss some patients with CF who have an elevated IRT but no CFTR gene mutation
- There are a number of non-CF causes of an elevated IRT which include contaminated filter paper cards, neonatal stress and congenital infections. Some high values will simply be the upper end of the normal distribution.

What this study adds
- In the population studied, the addition of further mutations to ∆F508 as part of newborn screening would not have made a significant impact on detection of babies with CF who had an elevated IRT
- Recall of babies with an elevated IRT but no CFTR mutation is unnecessary

Figure Legends

Figure 1
Distribution of immunoreactive trypsinogen (IRT) values presented as multiples of median (MoM). Cystic fibrosis (CF) patients presented as ∆F508 heterozygotes (×) or ∆F508 homozygotes (*)

Figure 2
Detailed figure of immunoreactive trypsinogen (IRT) distribution above the 99th percentile of values. IRT presented as multiples of median (MoM).

Figure 3
Distribution of immunoreactive trypsinogen (IRT) values for patients with cystic fibrosis (CF). Cystic fibrosis (CF) patients presented as ∆F508 homozygotes (*), ∆F508 heterozygotes (×), or no ∆F508 mutation (○).
References


IRT distribution in CF cases

- ○ NO DF508
- × DF508 HOM
- × DF508 HET

- - - 99 percentile
- - - ALL CF
- - - MEDIAN

IRT MOM

Frequency

0 2 4 6 8 10 12 14 16 18 20
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