Tandem Mass Spectrometry in Newborn Screening

David S. Millington, PhD
Director, Biochemical Genetics Lab
Pediatrics, Medical Genetics Division
Duke University Medical Center
The World of Newborn Screening prior to Tandem Mass Spectrometry (MS/MS)
• 1930’s: dietary treatment was proposed
• 1950’s: dietary treatment became available
  – greatest cognitive improvement seen in youngest patients

*Pediatrics, 105:89, 2000.*
Newborn Screening for PKU

• Introduced in United States in 1963
• Uses heel stick method to collect blood specimen → dried blood spots (c 50 µL) on filter paper
• Phenylalanine measured in a 1/8” blood dot by using a simple and inexpensive bacterial inhibition assay (Guthrie test)
• Specimens with Phe > 4 mg/dL prompt diagnostic follow-up testing for PKU
The “PKU Paradigm”

• Set the basis and standard for introducing new screening tests:

  – The disorder has severe consequences if not treated soon after birth – esp. MR
  – A simple and inexpensive biochemical test is applicable for mass screening
  – The disorder is treatable and treatment is available and effective (prevents MR)
Summary of the first 30 years (1964-1994)

- PKU detected in almost all ethnic groups – screening performed worldwide
- Frequency of PKU in US determined to be c. 1:15,000 (approx 200 new cases/yr)
- Successful diagnosis and treatment of thousands of PKU has dramatically altered natural course of disease
- Single tests added for other disorders associated with MR:
  - Congenital Adrenal Hyperplasia
  - Congenital Hypothyroidism
  - Galactosemia
- States view newborn screening and long-term treatment as a public health issue
The Paradigm Shift:

“Technology Driven” Analyte Screening by MS/MS – a Multiplex Assay
How MS/MS Became Involved in Newborn Screening

• Historically, MS/MS was developed as a clinical diagnostic test for disorders of fatty acid oxidation, by detection of abnormal acylcarnitines (Duke Laboratory 1984-89)
• The method was found to be applicable to dried blood spots (Millington, et al. JIMD 1990 13:321-324)
• MS/MS targeted to detect amino acids at the same time as acylcarnitines, and can recognize over 30 metabolic diseases at once (including PKU)
• Commercial Lab (Neogen) founded in 1994 by Dr. Edwin Naylor – motivated interest in expansion of NBS using MS/MS
• Dr M Rashed (Riyadh) applied microplate technology and an automated analytical protocol (1994)
• Collaboration between NC state lab, Duke and Neogen led to first state-wide expanded screening program in 1997
Challenges of MS/MS – Based Expanded Newborn Screening

- Significant “paradigm shift” – MS/MS is expensive, technologically complex and can detect disorders for which no effective treatment is available
- Multiple analytes and possible disorders detected with one test – sudden expansion of presumptive positive cases
- Many detectable disorders are very rare – natural course poorly understood.
- Confusion as to how many disorders/conditions are detectable by MS/MS – the “numbers game”
- Detection of “mild/variant” forms of diseases (e.g. SCAD, MCAD)
- Staff re-training for unfamiliar and difficult roles
- Significant stress on financial and personnel resources that are already limited
Organizational Chart for MS/MS Expansion

Advisory Committee

MS/MS Consultant

Lab Director

Coordinator (Reporting)

Section Manager: MS/MS Lab

Section Manager Hb Lab

Section Manager Gal Lab

MS/MS consultant

Technical staff MS/MS (2)
The MS/MS is set up to specifically target the analytes of interest using “scan functions” that detect compounds within the same chemical class.

- Up to 12 acylcarnitines and 8 amino acids are simultaneously targeted as primary analytes.
- Stable isotope-labeled internal standards added to the extract to enable quantification of the targeted analytes.
- The quantitative values are compared with control ranges ("cut-offs") for each analyte.
- Presumptive positives are referred for further testing to determine whether or not a disease is present.
Disorders Accessible to Newborn Screening by MS/MS

<table>
<thead>
<tr>
<th>Type of disorder</th>
<th>Clinical Manifestations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fatty acid oxidation disorders (e.g. MCAD, VLCAD, LCHAD deficiencies)</td>
<td>Hypoglycemia, coma, sudden death, seizure, cardiomyopathies</td>
</tr>
<tr>
<td>Branched-chain amino acid disorders (e.g. propionic, methylmalonic, isovaleric acidemias)</td>
<td>Metabolic acidosis, lethargy, coma, respiratory distress, recurrent metabolic crises</td>
</tr>
<tr>
<td>Aminoacidemias (e.g. PKU, MSUD, Urea Cycle disorders)</td>
<td>Mental Retardation, Hyperammononemia, failure to thrive</td>
</tr>
</tbody>
</table>
The Technology and How it Works
Analytical Process for Newborn Screening by MS/MS

• Specimens are 3mm diameter blood dots punched from each newborn’s blood spot into a 96-well tray
• Analytes (amino acids, acylcarnitines etc.) extracted into organic solvent with added internal standards
• Extracts are dried, esterified, analyzed by MS/MS at the rate of about 40 specimens per hr per instrument
• Signals from acylcarnitines (~12 primary analytes) and amino acids (~8 primary analytes) are ratio-ed to their respective internal standards, then converted to concentration values and compared with normal ranges (“cut-offs”)
• Out-of-range values reported as presumptive positives
Components of a Tandem Mass Spectrometer

Samples injected into solvent stream – analytes ionized intact by electrospray

Region where new ions formed

Second mass analyzer

Additional “ion source” and mass analyzer enables analysis of complex mixtures without prior separation
Fragmentation of acylcarnitines in MS-MS derivatized as n-butyl esters

\[
\begin{align*}
\text{CID (MS/MS)} & \quad \rightarrow \quad - \text{N(CH}_3\text{)}_3 \\
& \quad - \text{RCO}_2\text{H} \\
& \quad - \text{C}_4\text{H}_8 \\
\end{align*}
\]

\[\text{m/z 85}\]
Precursor ion scan - detects all precursors of a common fragment -
Used for the analysis of acylcarnitines

- Ion Source
- Mass Analyzer I
- Collision Cell
- Mass Analyzer II
- Detector

Precursor ion mixture

Full scan of MS-I

Fragmentation of M1-M7, etc

MS-II transmits only selected F*

Masses of precursor ions recorded
MS/MS targeted to acylcarnitines using precursor ion scan of m/z 85 – analysis time ~30 seconds!

Internal standards marked by *

Precursors: long-chain fatty acids (C16 = palmitoyl, etc.)

End-products: from catabolism of amino and fatty acids
A block in the metabolic pathway of fatty acid oxidation produces abnormally elevated intermediates.

**MCAD Deficiency**

- C2
- C6
- C8
- C10
- C10:1
- C16
- C18
- C18:1
MS/MS Targeted to Amino Acids – Using “Neutral Loss of 102” Scan Function (30s)

Normal Blood
Marked Elevation of Phenylalanine is Seen in PKU
Cut-offs and Result Reporting
“Cut-offs”

- A laboratory term meaning the upper limit of the normal (control) range of age-matched specimens (sometimes a lower limit is defined).

- Cut-offs are determined initially by statistical analysis of large data sets generated in a pilot study.

- A cut-off of mean + 4σ approximates to 99.95th percentile: 5 results per ten thousand per analyte and 20 primary analytes implies up to 1% of results could be flagged as abnormal. Most programs use a two-tier cut-off system to assign risk (e.g. “abnormal” and “alert”).

- Cut-offs are laboratory-specific variables, not universal constants, and may need to be adjusted periodically.
Impacts of The Cut-off Decisions

- Affects presumptive positive detection rates (compounded by detection of multiple analytes)
- Affects work load (repeating initial tests, repeat NBS specimens and/or diagnostic tests)
- Most initial positives turn out to be false - too many of these tend to affect attitude of health providers, credibility of NBS program
- Affects costs of implementing the program
### Population Distribution

#### Table:

<table>
<thead>
<tr>
<th>Statistic</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>0.204</td>
</tr>
<tr>
<td>SD</td>
<td>0.134</td>
</tr>
<tr>
<td>Mean + 1 SD</td>
<td>0.338</td>
</tr>
<tr>
<td>Mean + 2 SD</td>
<td>0.472</td>
</tr>
<tr>
<td>Mean + 3 SD</td>
<td>0.606</td>
</tr>
<tr>
<td>Mean + 4 SD</td>
<td>0.74</td>
</tr>
<tr>
<td>Sample V</td>
<td>0.018</td>
</tr>
<tr>
<td>Kurtosis</td>
<td>68.048</td>
</tr>
<tr>
<td>Skewness</td>
<td>5.213</td>
</tr>
<tr>
<td>Range</td>
<td>3.119</td>
</tr>
<tr>
<td>Minimum</td>
<td>0.0109</td>
</tr>
<tr>
<td>Maximum</td>
<td>3.13</td>
</tr>
</tbody>
</table>

**Cut-off**

**Alert**
Controlling the Factors Affecting Presumptive Positive Rate

• Report the Primary Analytes only - Up to 12 acylcarnitines and 8 amino acids
• Use secondary metabolites or biochemically rational analyte ratios in conjunction with primary analyte results to assign risk
• Isolated elevations of secondary markers are considered unimportant
### Interpretation: Primary Markers in the Acylcarnitine Profile

<table>
<thead>
<tr>
<th>Acylcarnitine species</th>
<th>Disorder to be considered</th>
</tr>
</thead>
<tbody>
<tr>
<td>C0↓</td>
<td>CUD (CTD)</td>
</tr>
<tr>
<td>C3↑</td>
<td>PA, MMA, MCD</td>
</tr>
<tr>
<td>C4 ↑</td>
<td>IBCD, SCAD, MAD</td>
</tr>
<tr>
<td>C5 ↑</td>
<td>IVA, 2MBCD, MAD</td>
</tr>
<tr>
<td>C5:1 ↑</td>
<td>SKAT</td>
</tr>
<tr>
<td>C5-OH ↑</td>
<td>3-MCC, 3-HMGL, SKAT, MCD, 3-HMG</td>
</tr>
<tr>
<td>C3-DC ↑</td>
<td>MA</td>
</tr>
<tr>
<td>C5-DC ↑</td>
<td>GA-I</td>
</tr>
<tr>
<td>Acylcarnitine species</td>
<td>Disorder to be considered</td>
</tr>
<tr>
<td>-----------------------</td>
<td>---------------------------</td>
</tr>
<tr>
<td>C8 ↑</td>
<td>MCAD</td>
</tr>
<tr>
<td>C14:1 ↑</td>
<td>VLCAD</td>
</tr>
<tr>
<td>C16 ↑ (usually with C18:1)</td>
<td>CPT-II, CAT</td>
</tr>
<tr>
<td>C16-OH ↑</td>
<td>LCHAD, TFP</td>
</tr>
</tbody>
</table>
Secondary Metabolites and Ratios Used When Primary Marker Elevated

Some examples:

• For PKU: PHE/TYR ratio > 3 (or as defined by lab)
• For PA and MMA: C3/C2 ratio (as defined by lab)
• For MCAD: (C8/C10 ratio); +/- C6, C10:1
• For VLCAD: (C14:1/C12:1) ratio; +/- C16, C18:1
• No suitable secondary markers for C3-DC, C4, C5, C5-OH, C5-DC

Caveat: some programs are introducing more ratios based on “data mining” after review of missed cases – generally without scientific basis or rationale
The Tiered Approach to Result Reporting

• Define high-risk based on elevations above the alert value (based on known positives, if available) or on elevated primary marker plus at least one secondary marker or indicator
• Define low risk as mild elevation of primary marker only (exceptions: C5-DC and C16-OH plus some others - always high risk)
Action Taken by Coordinator

- **Low risk:** contact physician of record, check clinical status of pt., request second blood spot specimen for repeat newborn screen. Mention possible disorder(s) & recommend follow-up testing only if child symptomatic.

- **High risk:** (includes positive test on repeat specimen from above): recommend referral to metabolic specialist/center, order follow-up testing and initiate appropriate therapy regardless of clinical status.
Follow-up Diagnostic Testing From Abnormal MS/MS Results

**Abnormal amino acid results:**

amino acid analysis of appropriate fluids and urine organic acids

**Abnormal acylcarnitine results:**

analysis of urine organic acids with plasma carnitine levels and acylcarnitine profiles

**Important:** These tests should be entrusted to a single, competent reference lab

Further testing is often required to establish a diagnosis – includes enzyme assays, DNA testing and various *in vitro* tests – these are performed only in a few specialist labs
Results: Impact of MS/MS
Incidence of Metabolic Disorders Detected by Newborn Screening Using MS/MS in North Carolina

Total screened = 904,207 (7/28/97 to 4/31/05)
Initial presumptive positive rate: ~0.4%

- Overall: 207 cases with confirmed diagnoses
  Overall Incidence 1:4,400

  PPV ~ 4% (>60% after repeat NBS!)

- Organic acidemias (55): 1:19,000
- Fatty acid oxidation disorders (92): 1:9,000
- Amino acid disorders (60): 1:13,000

CLINICAL AND LABORATORY STANDARDS INSTITUTE
Impact of The 2-tier NBS System in NC

- Approximately 120,000 live births recorded in NC annually.

- About 500 borderline NBS are reported by MS/MS per year of which 14-16 (3%) are typically +ve on second NBS test: of those, about half lead to a confirmed diagnosis.

- About 45-50 “alert” NBS reported by MS/MS per year: of which about 70% lead to a confirmed diagnosis.

- Based on conservative cost estimate of $1200 per infant for follow-up testing, at least $500,000 is saved annually.

- There are no known false negatives among the borderline cases that normalized on the second newborn screen.
Standard Protocols

• **Blood Collection on Filter Paper for Newborn Screening Programs; Approved Standard (LA04-A5)**
• **Newborn Screening Follow-up; Approved Guideline (I/LA27-A)**
• In between – not much! (actually nothing on MS/MS)
What to screen for?

- Advisory Committee decides for which disorders the program should screen
- Some selectivity is possible by limiting the analytes detected by MS/MS
- When all possible analytes are included, the list of possible disorders is a long one and varies from program to program
Some NBS programs are under pressure to list the
diseases they are screening for by MS/MS, even to
*maximize the number of disorders on the list regardless of whether or not there is evidence to support the claim.*

This tends to give the false impression that all the
disorders on the list can actually be identified by
newborn screening.

It also led to a bizarre game of political one-upmanship among programs, resulting in misunderstanding and confusion among the public and health professionals.
At a working group of experts, jointly convened by HRSA and ACMG, generated a ranking system for disorders and issued a “recommended” panel as a guideline for State labs: “Newborn Screening: toward a uniform screening panel”. (Watson, et al. Pediatrics 2006;117:S296)

A second panel of experts issued a more pragmatic guideline: “Counting disorders (conditions) for newborn screening panels” (Sweetman et al. Pediatrics 2006;117:S308)
<table>
<thead>
<tr>
<th>Condition/Disorder</th>
<th>ACMG Code</th>
<th>Primary Analyte/Biomarker</th>
<th>Preferred Screening Strategy</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Endocrine disorders</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thyroid disorders</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Primary congenital hypothyroidism</td>
<td>CH</td>
<td>T&lt;sub&gt;4&lt;/sub&gt; and TSH</td>
<td>T&lt;sub&gt;4&lt;/sub&gt; and TSH immunoassay</td>
</tr>
<tr>
<td><strong>Disorders of adrenal steroidogenesis</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Congenital adrenal hyperplasia (MIM 201910) (steroid 21-hydroxylase deficiency [EC 1.14.99.10]), salt-wasting, simple virilizing, or nonclassic</td>
<td>CAH</td>
<td>17-OHP</td>
<td>17-OHP immunoassay</td>
</tr>
<tr>
<td><strong>Metabolic disorders</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Organic acid disorders</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Propionic acidemia (MIM 606054) (propionyl-CoA carboxylase deficiency [EC 6.4.1.3])</td>
<td>PROP&lt;sup&gt;a&lt;/sup&gt;</td>
<td>C&lt;sub&gt;3&lt;/sub&gt;</td>
<td></td>
</tr>
<tr>
<td>Methylmalonic acidemia (MIM 251000) (methylmalonyl-CoA mutase deficiency [EC 5.4.99.2])</td>
<td>MUT&lt;sup&gt;a&lt;/sup&gt;</td>
<td>C&lt;sub&gt;3&lt;/sub&gt;</td>
<td></td>
</tr>
<tr>
<td>Methylmalonic acidemia (Cbl A, MIM 251100; Cbl B, MIM 251110; EC 5.4.99.2)</td>
<td>Cbl A,B&lt;sup&gt;a&lt;/sup&gt;</td>
<td>C&lt;sub&gt;3&lt;/sub&gt;</td>
<td></td>
</tr>
<tr>
<td>Methylmalonic acidemia (Cbl C, MIM 277400; Cbl D, MIM 277410; EC 5.4.99.2)</td>
<td>Cbl C,D&lt;sup&gt;a&lt;/sup&gt;</td>
<td>C&lt;sub&gt;3&lt;/sub&gt;</td>
<td></td>
</tr>
<tr>
<td>Malonic acidemia (MIM 248360) (malonyl-CoA decarboxylase deficiency [EC 4.1.1.9])</td>
<td>MAL</td>
<td>C&lt;sub&gt;3&lt;/sub&gt;DC</td>
<td></td>
</tr>
<tr>
<td>Isobutyrylglycinuria (MIM 604773) (isobutyryl-CoA dehydrogenase deficiency [EC 1.1.1.157])</td>
<td>IBG&lt;sup&gt;a&lt;/sup&gt;</td>
<td>C&lt;sub&gt;4&lt;/sub&gt;</td>
<td></td>
</tr>
<tr>
<td>Isovaleric acidemia (MIM 243500) (isovaleryl-CoA dehydrogenase deficiency [EC 1.3.9.107])</td>
<td>IVA&lt;sup&gt;a&lt;/sup&gt;</td>
<td>C&lt;sub&gt;5&lt;/sub&gt;</td>
<td></td>
</tr>
</tbody>
</table>
Lack of Procedural Guidelines

- Source of internal standards varies – kits are available but not universally employed – many programs use a “home brew”
- Derivatization of the analytes by butylation is not universally employed – some programs do not derivatize
- Laboratory equipment, including mass spectrometers and software, is not standardized
- Laboratory procedures are not standardized
- Reporting and follow-up procedures vary
MS/MS Can be Used for Second Tier Testing in the NBS Lab

(Eg Matern, et al. J Inherit Metab Dis 2007;30:585-592)

- Tyrosinemia type 1 (succinylacetone)
- CAH (17-OH progesterone/cortisol)
- Galactosemia (Gal-1-P)
- Hypothyroidism (T4)
- Hemoglobinopathies
Multiplex MS/MS assay of lysosomal enzymes (5) in dried blood spots for newborn screening.


- Take 5 punches (3 mm) from same specimen
- Incubate each with reagent cocktail specific for one targeted enzyme activity – includes a synthetic substrate and a product internal standard
- Quench after 24 h, recombine cocktails into a single well
- Clean-up reaction products by solid-phase cartridge
- Quantify all reaction products simultaneously by MS/MS
- 96-well technology, robot-friendly sample preparation
Resources

- Training programs (Duke & Baylor) sponsored by APHL, HRSA, NNSGRC, CDC
- CDC Quality Assurance and Proficiency Testing Program
- NNSGRC http://genes-r-us.uthscsa.edu
- Link to ACT Sheets (ACMG) – By Analyte & Disorder(s)
- Links to US NBS programs (clickable map), the regional collaboratives (7), information for parents & providers, Link to NNSIS (Database)
Summary

• MS/MS is fast becoming integrated routinely into NBS
• MS/MS has markedly enhanced the scope of NBS to include many disorders that were previously inaccessible
• Further enhancements, especially for treatable lysosomal storage diseases, are on the horizon
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