Neonatal Screening for Lysosomal Storage Disorders (LSD) by Tandem Mass Spectrometry (MSMS)

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1 Head, National Referral Laboratory (DG&MP)
### Summary Statistics 2008/2009

- **Budget**: 36.5 million
- **Emergency attendances**: 55,502
  - Women: 20,850
  - Children: 35,652
- **Admissions**: 41,595
  - Women: 19,480
  - Children: 22,115
- **Births**: 5,895
- **Beds**: 316
  - Women: 123
  - Children: 220
  - ICU/SC: 54
- **Average bed Occupancy**: 91.5%
SAPathology at the Women’s & Children’s Hospital

**Directorate of Genetics and Molecular Pathology**

168 Tenured and 86 Research funded Staff

Tertiary Paediatric & Obstetric/Gynaecological teaching Hospital affliations with the University of Adelaide

Departments of Paediatrics and Obstetric & Gynaecology.

**Departments**

Clinical Genetics  Molecular Genetics  Biochemical Genetics

Clinical Genetics  Cytogenetics & Cancer  Paediatric Biochemical Genetics

Metabolic Clinic  National Referral Laboratory  National Referral Laboratory

Antenatal Screening  Metabolic Laboratory  Antenatal Screening

Neonatal Screening  Metabolic Laboratory  Neonatal Screening
Screening & Diagnosis of LSD on Dried Blood-spots

- Undertaken a study to investigate the use of the PE-LSD 6-Plex MSMS substrates for NBS
  - GAUCHER-β-Glucocerebrosidase (ABG)
  - KRABBE-Galactocerebrosidase β-Galactosidase (GALC)
  - NIEMAN-PICK A/B - Sphingomyelinase (ASM)
  - FABRY-α-Galactosidase (GLA)
  - POMPE-α-Glucosidase (GAA)
  - MPS I- Mucopolysaccharidosis type I Iduronidase (IDUA)

- Perform analysis on a Sciex MSMS
  - API5000
    - Analysis method by Flow injection (FIA) & LC-MSMS

- Determine enzyme activity from dried blood-spots in:
  - Normal newborn population
  - Confirmed true positive (CTP) cases
Relative incidence of LSD in Australia 1 in 7,000

Based on Australian diagnoses (1981-96) Meikle et al. 1999 JAMA

Diagram showing the relative incidence of LSD in Australia, with the highest incidence being in MPS I and NCL (combined).*
Substrates Stored in Lysosomal Storage Disorders

- Glycosaminoglycan (sulphated saccharides)
- Sphingosine analogues
- Glycogen
- Amino acid
- Monosaccharide
- Others

Based on Australian diagnoses (1981-96)
Cultured skin fibroblasts from normal & an LSD affected.
**PE Six-Plex FIA-MSMS substrates & internal stable isotopes.**

**GAUCHER**

β-Glucocerebrosidase (ABG)

\[
\text{HO-CH}_{2-\text{CH}}(\text{CH}_2)_8\text{CH}_3\text{C}_2\text{C}_\text{CD}_3
\]

ABG-IS 390.38 g/mol

**NIEMANN-PICK A/B**

Sphingomyelinase (ASM)

\[
\text{HO-CH}_{2-\text{CH}}(\text{CH}_2)_6\text{CH}_3\text{C}_2\text{C}_\text{CD}_3
\]

ASM-IS 404.40 g/mol

**KRABBE**

Galactocerebrosidase β-Galactosidase (GALC)

\[
\text{HO-CH}_{2-\text{CH}}(\text{CH}_2)_{12}\text{CH}_3
\]

GALC-IS 416.70 g/mol

**FABRY**

α-Galactosidase (GLA)

\[
\text{HO-CH}_{2-\text{CH}}(\text{CH}_2)_6\text{CH}_3\text{C}_2\text{C}_\text{CD}_3
\]

GLA-IS 488.31 g/mol
PE Six-Plex FIA-MSMS substrates & internal stable isotopes.

**POMPE**
α-Glucosidase (GAA))

GAA- IS 502.33 g/mol

**MPS I**
Mucopolysaccharidosis type I (IDUA)

MPS-I-IS 430.26 g/mol
### MRM pairs for each LSD substrate and product

<table>
<thead>
<tr>
<th>Analyte</th>
<th>MRM Pairs</th>
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<tbody>
<tr>
<td></td>
<td>Q1 Mass</td>
<td>Q2 Mass</td>
</tr>
<tr>
<td>ABG-S</td>
<td>546.440</td>
<td>264.200</td>
</tr>
<tr>
<td>ABG-IS</td>
<td>391.400</td>
<td>271.300</td>
</tr>
<tr>
<td>ABG-P</td>
<td>384.400</td>
<td>264.300</td>
</tr>
<tr>
<td>ASM-S</td>
<td>563.400</td>
<td>184.000</td>
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<tr>
<td>ASM-IS</td>
<td>405.400</td>
<td>264.300</td>
</tr>
<tr>
<td>ASM-P</td>
<td>398.400</td>
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<tr>
<td>GAA-S</td>
<td>660.400</td>
<td>560.300</td>
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<tr>
<td>GAA-IS</td>
<td>503.400</td>
<td>403.300</td>
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<tr>
<td>GAA-P</td>
<td>498.300</td>
<td>398.300</td>
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<tr>
<td>GALC-S</td>
<td>574.500</td>
<td>264.300</td>
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<tr>
<td>GALC-IS</td>
<td>417.400</td>
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<td>GALC-P</td>
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<td>264.300</td>
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<tr>
<td>GLA-S</td>
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<tr>
<td>GLA-IS</td>
<td>489.300</td>
<td>389.300</td>
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<tr>
<td>GLA-P</td>
<td>484.300</td>
<td>384.200</td>
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<tr>
<td>IDUA-S</td>
<td>602.260</td>
<td>317.200</td>
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<tr>
<td>IDUA-IS</td>
<td>431.300</td>
<td>322.200</td>
</tr>
<tr>
<td>IDUA-P</td>
<td>426.200</td>
<td>317.200</td>
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</table>
1. Addition 30µL of reagent to each well in a microtitre plate.
2. Incubate at 37°C for 18h and shake at 400rpm.
3. Add 100µL methanol:ethyl-acetate (MeOH:EA, 50:50) to quench followed by centrifugation.
4. Transfer to deep-well microtitre plates and add 400µL of EA & 200µL water.
5. Centrifuge for 5 minutes to separate layers.
6. Transfer 75µL of the top layer to a microtitre plate.
7. Evaporate at room temperature under a nitrogen stream.
8. Reconstitute in 150µL solvent flow buffer.
9. Mix at 400rpm and analyse on API5000 MSMS.
FIA of LS Substrates, Products & Stable Isotopes

XIC of +MRM (18 pairs (Turbo Spray))

Max. 1.8e6 cps.

Order of XIC in the TIC
1. ASM-S
2. GAA-S
3. GLA-S
4. ABG-S
5. GAA-S
6. GAA-IS
7. ABG-IS
8. GLA-IS
9. ASM-IS
10. GALC-IS
11. IDUA-S
12. IDUA-IS
13. ABG-P
14. ASM-P
15. GALC-P
16. GLA-P
## Analytical Performance

Normal dried blood spot- 10 repeated estimates (3mm)

<table>
<thead>
<tr>
<th>Blood-Spot</th>
<th>Stats</th>
<th>ABG</th>
<th>ASM</th>
<th>GAA</th>
<th>GALC</th>
<th>GLA</th>
<th>IDUA</th>
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<tbody>
<tr>
<td>Normal A</td>
<td>Mean</td>
<td>17.2</td>
<td>6.1</td>
<td>15.1</td>
<td>5.2</td>
<td>34.2</td>
<td>4.5</td>
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<tr>
<td></td>
<td>CV%</td>
<td>8.7</td>
<td>6.2</td>
<td>6.4</td>
<td>9.4</td>
<td>11.7</td>
<td>6.4</td>
</tr>
<tr>
<td>Normal B</td>
<td>Mean</td>
<td>10.3</td>
<td>3.2</td>
<td>6.5</td>
<td>2.2</td>
<td>23.5</td>
<td>2.9</td>
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<tr>
<td></td>
<td>CV%</td>
<td>16.0</td>
<td>9.9</td>
<td>8.3</td>
<td>8.3</td>
<td>15.6</td>
<td>5.2</td>
</tr>
<tr>
<td>Normal C</td>
<td>Mean</td>
<td>8.4</td>
<td>3.6</td>
<td>10.5</td>
<td>5.5</td>
<td>32.7</td>
<td>4.7</td>
</tr>
<tr>
<td></td>
<td>CV%</td>
<td>8.4</td>
<td>3.6</td>
<td>10.5</td>
<td>5.5</td>
<td>13.7</td>
<td>4.7</td>
</tr>
<tr>
<td>Normal D</td>
<td>Mean</td>
<td>11.9</td>
<td>6.4</td>
<td>10.7</td>
<td>6.1</td>
<td>30.3</td>
<td>3.9</td>
</tr>
<tr>
<td></td>
<td>CV%</td>
<td>11.1</td>
<td>9.0</td>
<td>8.8</td>
<td>7.5</td>
<td>15.3</td>
<td>6.7</td>
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</table>
Blood-spot Normal vs Affected

Gaucher (ABG)

<table>
<thead>
<tr>
<th>Normal (592)</th>
<th>Gaucher (20)</th>
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</thead>
<tbody>
<tr>
<td>40.0</td>
<td>20</td>
</tr>
<tr>
<td>30.0</td>
<td></td>
</tr>
<tr>
<td>20.0</td>
<td></td>
</tr>
<tr>
<td>10.0</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td></td>
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</tbody>
</table>

Pompe (GAA)

<table>
<thead>
<tr>
<th>Normal (592)</th>
<th>Pompe (20)</th>
</tr>
</thead>
<tbody>
<tr>
<td>30.0</td>
<td>20</td>
</tr>
<tr>
<td>25.0</td>
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<tr>
<td>20.0</td>
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<tr>
<td>15.0</td>
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<td>10.0</td>
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<tr>
<td>5.0</td>
<td></td>
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<tr>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>
Blood-spot Normal vs Affected

Niemann-Pick A/B (ASM)

MPS-I (IDUA)

IDUA Enzyme Activity

Normal (592)  MPS-I (6)

ASM Enzyme Activity

Normal (592)  NP-A/B (1)

Blood-spot Normal vs Affected
Blood-spot Normal vs Affected

Krabbe (GALC)

- Normal: 592
- Krabbe: 5

GLC Enzyme Activity

- Normal: 80.0
- Fabry: 14
- Fabry-Hets: 28

Fabry (GLA)

- Normal: 592
- Fabry: 14
- Fabry-Hets: 28
Blood-spot Normal vs Affected

Krabbe (GALC)

GALC Enzyme Activity

Normal     Krabbe
(592)          (5)

GLA Enzyme Activity

Normal       Fabry     Fabry-Hets
(592)          (14)             (28)

Fabry (GLA)

GLA Enzyme Activity

Normal (592)     Fabry (14)     Fabry-Hets (28)
Developed an On-line LC-MSMS method
- C18 column (100mm) on a Sciex API5000 MSMS instrument
- Gradient of: buffer A; Water + 0.1% Formic acid & buffer B; 50:50 Acetonitrile/Methanol + 0.1% Formic acid

Perform analysis on a Sciex MSMS
- API5000
  - Analysis method by Flow injection (FIA) & LC-MSMS

Determine enzyme activity from dried blood-spots in:
- Normal newborn population
- Confirmed true positive (CTP) cases
LC-MSMS for IDUA MPS I- α Iduronidase

XIC of +MRM (19 pairs): 431.300/322.200 Da ID: IDUA IS from Sample 2 (mix) of 150107e.wiff (Turbo Spray)

Max. 1.4e6 cps.

Red = substrate
Blue = internal std

<table>
<thead>
<tr>
<th>Step</th>
<th>Time (min)</th>
<th>μl/min</th>
<th>A (%)</th>
<th>B (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.01</td>
<td>300</td>
<td>100.0</td>
<td>0.0</td>
</tr>
<tr>
<td>1</td>
<td>0.99</td>
<td>300</td>
<td>0.0</td>
<td>100.0</td>
</tr>
<tr>
<td>2</td>
<td>5.99</td>
<td>300</td>
<td>0.0</td>
<td>100.0</td>
</tr>
<tr>
<td>3</td>
<td>6.00</td>
<td>300</td>
<td>100.0</td>
<td>0.0</td>
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<tr>
<td>4</td>
<td>11.00</td>
<td>300</td>
<td>100.0</td>
<td>0.0</td>
</tr>
</tbody>
</table>

Buffer A: Water, 0.1% formic
Buffer B: 50% ACN, 50% MeOH, 0.1% formic
IDUA MPS I- α Iduronidase Normal vs Affected

- **Affected (n=5)**
- **Normal (n=28)**

**IDUA activity (μmol/L/h)**
LC-MSMS for KRABBE- Galactocerebrosidase β-Galactosidase (GALC)

XIC of +MRM (19 pairs): 417.400/264.300 Da ID: GALC IS from Sample 2 (mix) of 150107e.wiff (Turbo Spray)

Max. 1.9e6 cps.

<table>
<thead>
<tr>
<th>Step</th>
<th>Time(min)</th>
<th>μl/min</th>
<th>A (%)</th>
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<td>11.00</td>
<td>300</td>
<td>100.0</td>
<td>0.0</td>
</tr>
</tbody>
</table>

Buffer A: Water, 0.1% formic
Buffer B: 50% ACN, 50% MeOH, 0.1% formic

Red= substrate
Blue= internal std
KRABBE- GALC activity Normal/Affected/Hets/Psuedo
LC-MSMS for FABRY α-Galactosidase (GLA)

XIC of +MRM (19 pairs): 489.300/389.300 Da ID: GLA IS from Sample 2 (mix) of 150107e.wiff (Turbo Spray)

Max. 3.1e6 cps.

<table>
<thead>
<tr>
<th>Step</th>
<th>Time(min)</th>
<th>µl/min</th>
<th>A (%)</th>
<th>B (%)</th>
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<td>0.0</td>
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Buffer A: Water, 0.1% formic
Buffer B: 50% ACN, 50% MeOH, 0.1% formic

Red= substrate
Blue= internal std

Intensity, cps

Time, min
GLA FABRY $\alpha$-Galactosidase Normal/Affected & Heterozygote

![Box plot showing GLA activity forAffected (n=5), Normal (n=23), and Heterozygote (n=5).]
Summary from the Adelaide Study

- The **PE-6-Plex LSD reagents** on dried blood-spot assays
  - Performs well with good assay analytical CV% on a Sciex API5000 instrument (also on an API3200)
  - Capable of detecting true confirmed LSD from an unaffected population
  - In a limited cohort potential to distinguish carriers and pseudo deficiencies.
    - Larger studies are required to confirm

- LC-MSMS method provides a confirmatory and diagnostic assay from dried blood-spots
  - A solid phase solid-phase-extraction (SPE-silica) is also being investigated

- Proposal for a larger study to be conducted in the future

- *Towards replacing existing 4-MU assays for diagnostic assays*