Disclosure

R Neil Dalton
is a Director and minority shareholder in

Spoton Clinical Diagnostics
The application of electrospray tandem mass spectrometry to newborn haemoglobinopathy screening: the journey from theory to implementation

R Neil Dalton
The WellChild Laboratory
King’s College, London/
Evelina London Children’s Hospital

Guy’s and St Thomas’ NHS
NHS Foundation Trust

UK NEQAS for General Haematology – 18th Annual Participants’ Meeting
York Racecourse October 13th 2015
Newborn haemoglobinopathy screening

Ionisation
Electrospray (John Fenn)

Mass Spectrometry-Mass Spectrometry
Triple quadrupole - schematic

Sensitivity
Specificity
Newborn haemoglobinopathy screening

A range of scan modes/experiments possible using MSMS – MRM mode for quantitation
Newborn haemoglobinopathies screening

<table>
<thead>
<tr>
<th>Compound</th>
<th>Conc (µmol/l)</th>
<th>MRM (CV%)</th>
<th>Scan (CV%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenylalanine</td>
<td>242.5</td>
<td>1.8</td>
<td>20.5</td>
</tr>
<tr>
<td>Octanoylcarnitine</td>
<td>1.05</td>
<td>2.7</td>
<td>29.2</td>
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<tr>
<td>Tyrosine</td>
<td>479.2</td>
<td>4.1</td>
<td>23.2</td>
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<tr>
<td>Carnitine</td>
<td>6.5</td>
<td>4.5</td>
<td>44.8</td>
</tr>
</tbody>
</table>
Newborn haemoglobinopathy screening

The emergence of a new science

Newborn IEM metabolite screening

underivatised

The rest is history

Proteomics

Clinical proteomics?
Newborn haemoglobinopathy screening

Single analytical platform for metabolite and haemoglobinopathy screening
Efficient use of MSMS instrumentation
Potential for back-up instrumentation
Newborn haemoglobinopathy screening

Dilute whole blood with water and electrospray measuring m/z – charge series envelope

MS spectrum dominated by high abundance proteins α and β chains of haemoglobin
Newborn haemoglobinopathy screening

Charge series

Deconvolutional analysis
Newborn haemoglobinopathy screening
Newborn haemoglobinopathy screening

Target **MS** scanning

Hb β-chain 12 positive charged

Sickle - glutamic acid to valine, -30 daltons

Expected m/z shift 2.5 daltons

wild-type β-chain, m/z c.1322.5

sickle β-chain, m/z c.1320.0

scan range m/z 1315-1325

Quality check Hb β-chain 13 positive charged
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collaboration with Barbara Wild
King’s College Hospital NHS Trust

Significant sensitivity issues with **MS**
premature babies
Specificity
first line screening test?
Newborn haemoglobinopathy screening

At this stage forget everything I have said!

Analytical conclusion, newborn screening for sickle protein by MS insufficiently sensitive and not mutation specific

Re-evaluation of objectives
high sensitivity newborn sickle screening
heterozygote detection of other sickling haemoglobins
detection of other clinically significant haemoglobins, e.g. β-thalassaemia

Tryptic digestion, resultant peptides analysed as small molecules in MRM mode?
Newborn haemoglobinopathy screening

Detection of known mutations is different from determining complete protein sequence.

Endopeptidases, e.g. trypsin, act only at specific recognition sites and in a consistent and reliable manner.

A peptide consisting of 8 amino acids is a virtually unique entity.

Human β-globin, 15 peptides, T1-T15

Positions of mutations known, sickle mutation in T1

Digest proteins to informative peptides and analyse peptides as small molecules in MRM mode.
Newborn haemoglobinopathy screening

NHS Sickle Cell and Thalassaemia screening programme

<table>
<thead>
<tr>
<th>Beta chain point mutations</th>
<th></th>
<th></th>
<th>Δ Mass</th>
<th>Tryptic Peptide</th>
</tr>
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<tbody>
<tr>
<td>variant</td>
<td>Wild Type AA</td>
<td>Variant AA</td>
<td>Position</td>
<td></td>
</tr>
<tr>
<td>HbS</td>
<td>Glu</td>
<td>Val</td>
<td>6</td>
<td>-30</td>
</tr>
<tr>
<td>HbC</td>
<td>Glu</td>
<td>Lys</td>
<td>6</td>
<td>-1</td>
</tr>
<tr>
<td>HbD&lt;sub&gt;Punjab&lt;/sub&gt;</td>
<td>Glu</td>
<td>Gln</td>
<td>121</td>
<td>-1</td>
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<tr>
<td>HbO&lt;sub&gt;Arab&lt;/sub&gt;</td>
<td>Glu</td>
<td>Lys</td>
<td>121</td>
<td>-1</td>
</tr>
<tr>
<td>HbE</td>
<td>Glu</td>
<td>Lys</td>
<td>26</td>
<td>-1</td>
</tr>
</tbody>
</table>

HbS/HPFH, HbS/β-thalassaemia (β+, β0, δβ, Lepore)

Problem
Complexity of traditional tryptic digestion procedures

Solution
PhD student

Primary questions
Can we detect the mutant peptides in MRM mode?
How far can we minimise sample preparation and tryptic digestion?
Newborn haemoglobinopathy screening

Sickle mutation in T1 position 6 glutamic acid to valine

Wild-type  VHLTPEEK  MW 951.5
Sickle        VHLTPVEK  MW 921.5

Singly charged peptides
m/z 952.5 922.5

Doubly charged peptides
m/z 476.8 461.8

Patient samples, HbA, HbSS – not peptide standards
API4000, inject 2µl, usual solvent, flow rate 75µl/min
Full scan MS of tryptic digest
Newborn haemoglobinopathy screening

HbA MS full scan to detect wild type βT1
Newborn haemoglobinopathy screening

HbSS MS full scan to detect sickle βT1
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**MSMS** – product ion scan of doubly charged wild type βT1, m/z 476.8
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Isolate doubly charged peptide ion in Q1, fragment, Q3 product ion scan

Amino acids lost from N and C-terminal ends of peptide

\( y \) series, N-terminal AA sequence

- VHLTPE(V)EK, HLTPE(V)EK (y7),
- LTPE(V)EK (y6), TPE(V)EK (y5),
- PE(V)EK (y4), E(V)EK (y3), EK (y2), K

\( b \) series, C-terminal AA sequence

- VHLTPE(V)EK, VHLTPE(V)E (b7),
- VHLTPE(V) (b6), VHLTP (b5), VHLT(b4),
- VHL (b3), VH (b2), V

**MSMS** – product ion scan m/z 476.8

MRM for wild-type \( \beta T1 \), m/z 476.8/502.3 (y4)

MRM for sickle \( \beta T1 \), m/z 461.8/472.5 (y4)
Newborn haemoglobinopathy screening
Newborn haemoglobinopathy screening

The same afternoon, developed MRMs for HbC, HbD\textsuperscript{Punjab}, HbO\textsuperscript{Arab}, HbE
Newborn haemoglobinopathy screening

200 informative adult blood samples (not DBS) analysed:
AA 52   AS 57   AC 44   SS 14
SC 16   AE 10   AD\textsuperscript{Punjab} 2
CC 1    DD\textsuperscript{Punjab} 1   EE 1
AO\textsuperscript{Arab} 1   OO\textsuperscript{Arab} 1

Sensitivity 100%   Specificity 100%

Primary questions
Can we detect the mutant peptides in MRM mode? Yes
How far can we minimise sample preparation and tryptic digestion? Sufficient to make it practical in a routine clinical laboratory

New question
How will the assay perform in real-time newborn DBS screening?

Patented by King’s College London
Rapid and Specific Detection of Clinically Significant Haemoglobinopathies using Electrospray Mass Spectrometry-Mass Spectrometry,
British Journal of Haematology:130, 635-643
Newborn haemoglobinopathy screening

NHS Sickle Cell & Thalassaemia Screening Programme funded
Allison Streetly

1y technical evaluation in collaboration with a centre outside London
(Lisa Farrar, Leeds St James’s Hospital)

then current screening method IEF

Potential advantages

Efficient use of MSMS equipment

Integration of screening processes to improve efficiency of analysis/reporting

System targeted to only detect clinically significant conditions

High sensitivity sickle protein detection

Simple detection of transfusion

Screening pathway costs lower than for HPLC/IEF

Screen for other conditions simultaneously, e.g., biotinidase deficiency, type 1 tyrosinaemia
Newborn haemoglobinopathy screening

Collaboration with Leeds Neonatal Screening service (Lisa Farrar)

Blood spots punched in duplicate in Leeds, 1 replicate analysed by IEF
2nd anonymised replicate transported overnight dry in 96 well plates

Sample preparation
To each well: 125µl incubation reagent added and plates incubated for 30min at 37ºC
1ml of stop reagent added
MSMS analysis
Sample volume 2µl, flow injection, 1min MSMS acquisition (API 4000, API 4000 Q trap)

Results generated “blind”, available within 24h. Comparison carried out in Leeds

<table>
<thead>
<tr>
<th>Tryptic peptide</th>
<th>Target Peptide Ion (m/z)</th>
<th>Target fragment ion (m/z)</th>
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<tbody>
<tr>
<td>Wild Type (Beta) T1</td>
<td>476.9</td>
<td>y4 502.3</td>
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<tr>
<td>HbS T1</td>
<td>461.9</td>
<td>y4 472.4</td>
</tr>
<tr>
<td>HbC T1</td>
<td>694.5</td>
<td>b4 451.3</td>
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<tr>
<td>Wild Type T13</td>
<td>689.9</td>
<td>b3 378.1</td>
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<tr>
<td>HbDPunjab T13</td>
<td>689.4</td>
<td>b3 377.1</td>
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<tr>
<td>Wild Type T13</td>
<td>689.9</td>
<td>y9 1001.4</td>
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<tr>
<td>HbOArab T13</td>
<td>625.3</td>
<td>y9 1001.4</td>
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<td>Wild Type T3</td>
<td>657.9</td>
<td>y9 887.5</td>
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<tr>
<td>HbE T3</td>
<td>458.7</td>
<td>y5 489.3</td>
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<tr>
<td>Delta chain T2</td>
<td>480.3</td>
<td>y6 688.4</td>
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<tr>
<td>Delta chain T14</td>
<td>721.4</td>
<td>y9 1064.3</td>
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<tr>
<td>Wild Type T2</td>
<td>466.8</td>
<td>y6 675.4</td>
</tr>
<tr>
<td>Gamma Chain T2</td>
<td>488.6</td>
<td>y6 691.6</td>
</tr>
<tr>
<td>Alpha Chain T1</td>
<td>365.2</td>
<td>y4 430.4</td>
</tr>
<tr>
<td>Alpha Chain T13</td>
<td>626.9</td>
<td>y10 992.5</td>
</tr>
</tbody>
</table>
Newborn haemoglobinopathy screening
Newborn haemoglobinopathy screening

Mutations detected by IEF & MSMS
confirmed by HPLC

T1 S peptide 196
   HbS/HbF 8
   HBSC 3
   HbS trait 185

T1 C peptide 39 (including the 3 HbSC)
T13 D\textsuperscript{Punjab} peptide 51
T13 O\textsuperscript{Arab} peptide 0
T3 E peptide 47

β-thalassaemia major (no T1, T3, T5, & T13 β-chain peptides) 4
confirmed by IEF/ante-natal history/6 month follow up

Total 334

Incidence of sickle peptide 1:204
Incidence of SCD 1:3,641
Incidence of HbC 1:1,082
Incidence of HbD\textsuperscript{Punjab} 1:817
Incidence of HbE 1:834
Incidence of all mutations 1:120
Incidence of β-thalassaemia major 1:10,012

Patients identified with clinical disease causing mutations 15, 1:2,670

No false negatives by MSMS
Newborn haemoglobinopathy screening

Abstract Poster Presentation at ASH 2008
A comparison of IEF and MSMS for clinical haemoglobinopathy screening in 40,000 newborns

Technical case for newborn DBS haemoglobinopathy screening by electrospray tandem mass spectrometry rigorously established

Implementation?

Kit development

2 reagents:
- internal standard (50µl)
  (releases stable isotope sickle peptide on tryptic digestion)
- trypsin reagent (50µl)

- system suitability check: MRM and sensitivity confirms tryptic digestion for every DBS
- monitors stability of instrumentation throughout run

Incubate with mixing at 37°C for 30-45min

Add 1ml running solvent MeCN:water (1:1) with 0.025% formic acid

Peptide standards provided for instrument set-up
Method set-up by SpOtOn within a day
Fully electronic data analysis using Chemoview and NeoLynx
Kit CE marked
Newborn haemoglobinopathy screening

Implementation

Wales Newborn Screening Laboratory
Dr Stuart Moat and colleagues

NHS Sickle Cell & Thalassaemia Screening Programme pilot studies

Daniel YA, Henthorn JS

Newborn Screening for Haemoglobin Disorders using Tandem Mass Spectrometry – pilot study to evaluate multicentre implementation and integration with existing platforms.

Submitted for publication

Current sites
Cardiff
Leeds
Great Ormond Street pilot
Acknowledgements

Charles Turner
Yvonne Daniel
Sue Bird
Barbara Wild

NHS Sickle Cell & Thalassaemia Screening Programme
Allison Streetly
Lisa Farrar

Stuart Moat and the Wales Newborn Screening Laboratory
The pilot sites
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The Evelina London Children’s Hospital Appeal

Guy’s and St Thomas’ NHS Foundation Trust