Disclosure

R Neil Dalton

is a Director and minority shareholder in

Sp⊙t⊙n Clinical Diagnostics

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

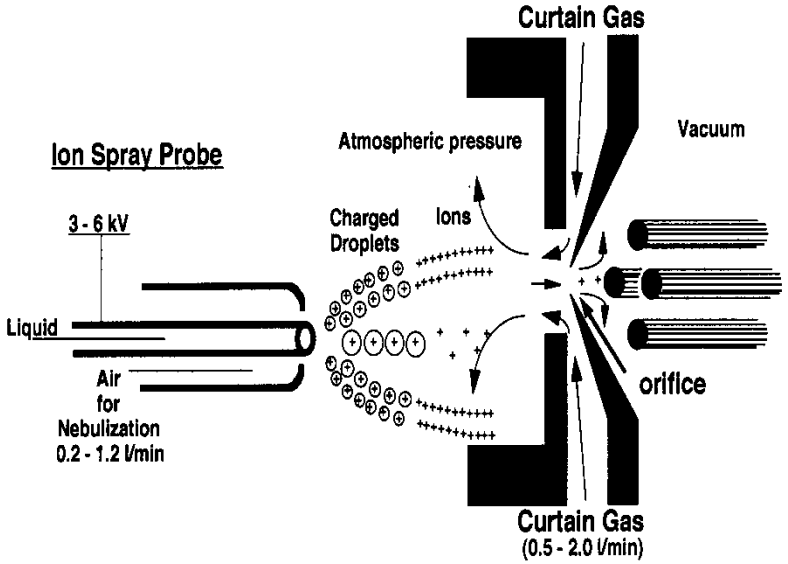
R Neil Dalton
The WellChild Laboratory
King's College, London/
Evelina London Children's Hospital

UK NEQAS for General Haematology – 18th Annual Participants' Meeting
York Racecourse October 13th 2015

Newborn haemoglobinopathy screening

Ionisation

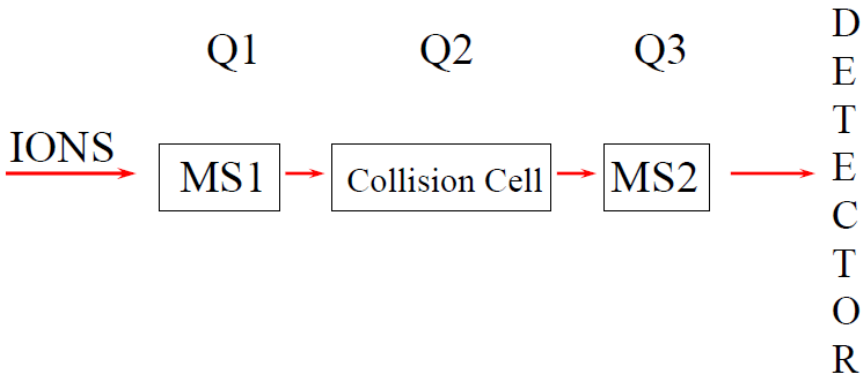
Electrospray (John Fenn)



Sensitivity

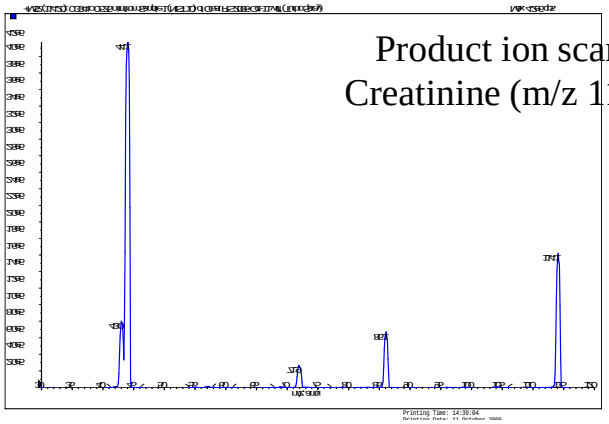
Mass Spectrometry-Mass Spectrometry

Triple quadrupole - schematic

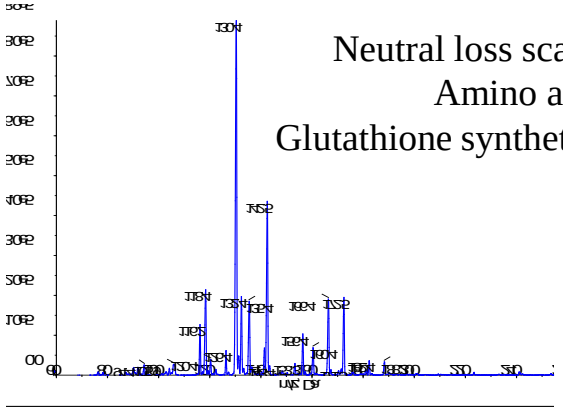


Specificity

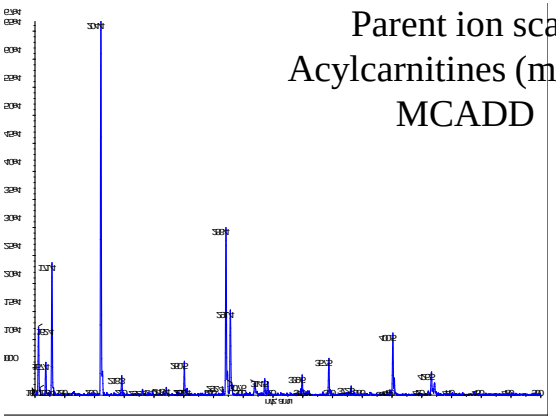
Newborn haemoglobinopathy screening



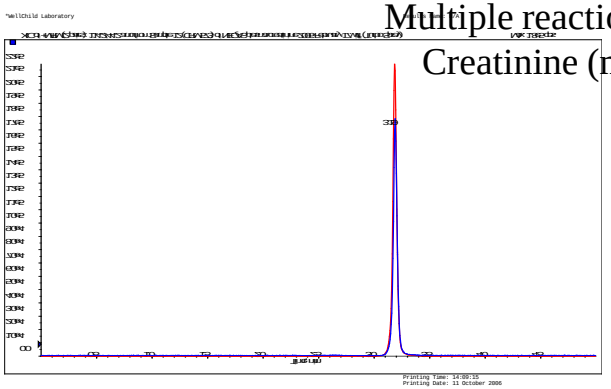
Product ion scan
Creatinine (m/z 114)



Neutral loss scan (m/z 46)
Amino acids
Glutathione synthetase deficiency



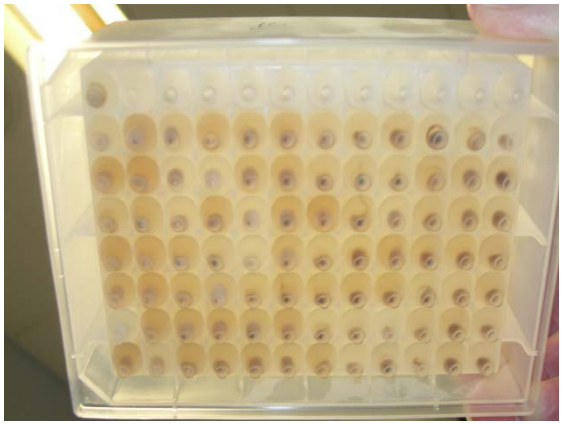
Parent ion scan
Acylcarnitines (m/z 85)
MCADD



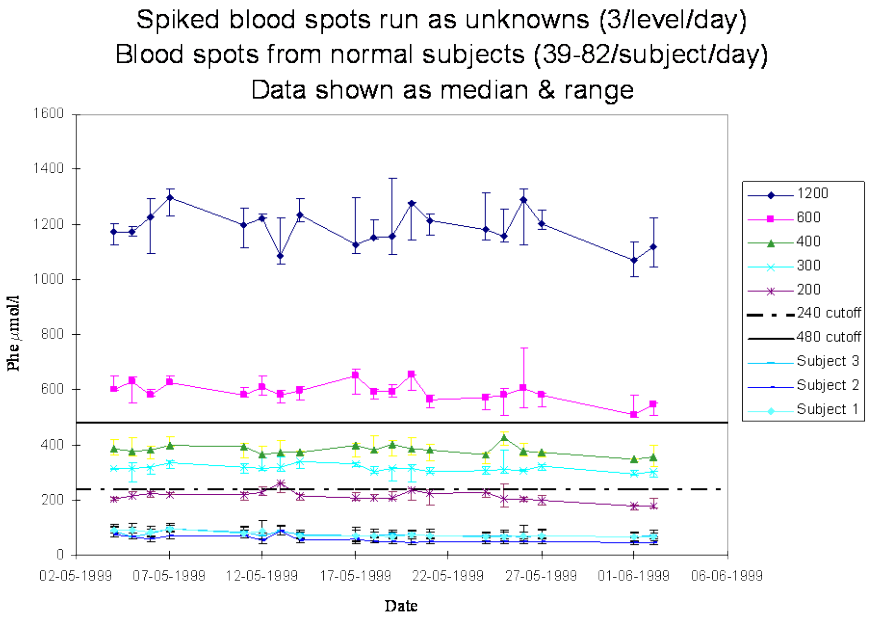
Multiple reaction monitoring
Creatinine (m/z 114/44)

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

Newborn haemoglobinopathy screening



	Conc (µmol/l)	MRM (CV%)	Scan (CV%)
Phenylalanine	242.5	1.8	20.5
Octanoylcarnitine	1.05	2.7	29.2
Tyrosine	479.2	4.1	23.2
Carnitine	6.5	4.5	44.8



Newborn haemoglobinopathy screening

The emergence of a new science

Newborn IEM metabolite screening

underivatised

The rest is history

Proteomics

Clinical proteomics?

Newborn haemoglobinopathy screening

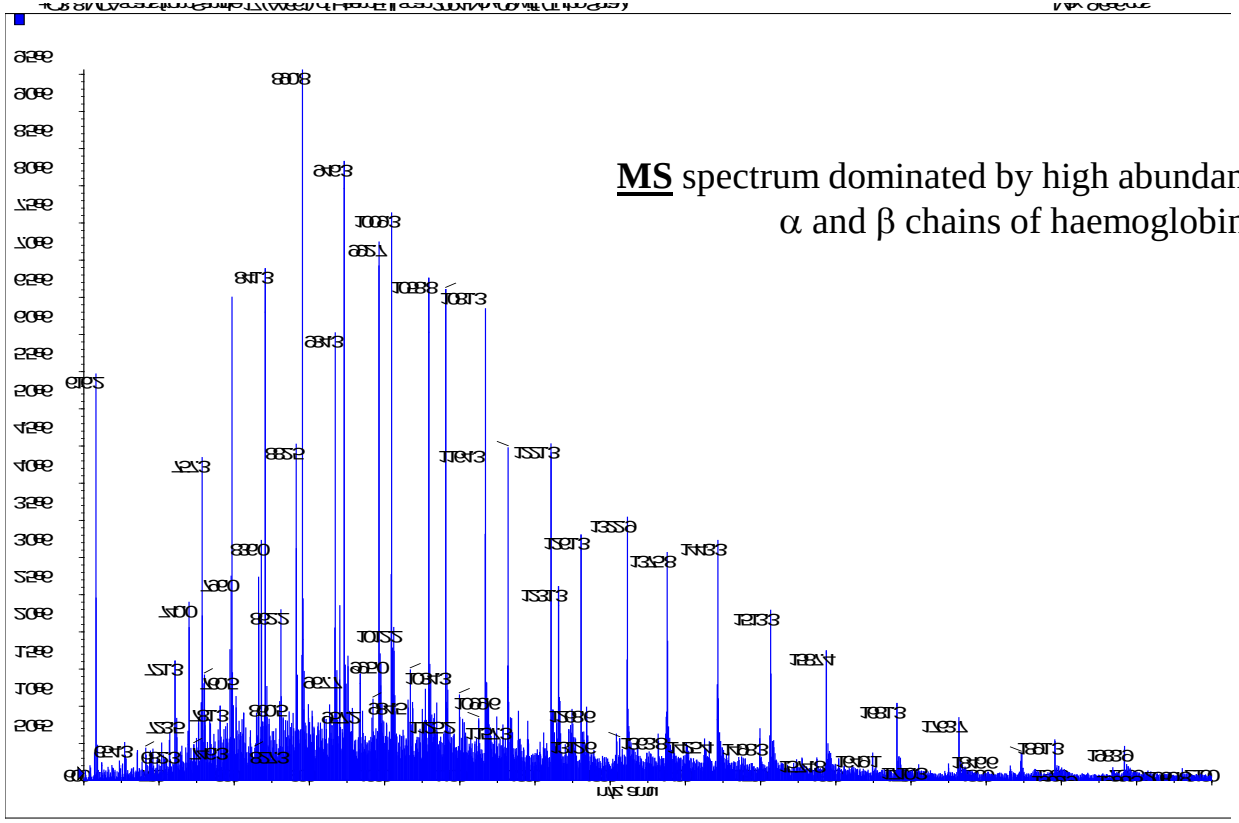
Single analytical platform for metabolite and haemoglobinopathy screening

Efficient use of MSMS instrumentation

Potential for back-up instrumentation

Newborn haemoglobinopathy screening

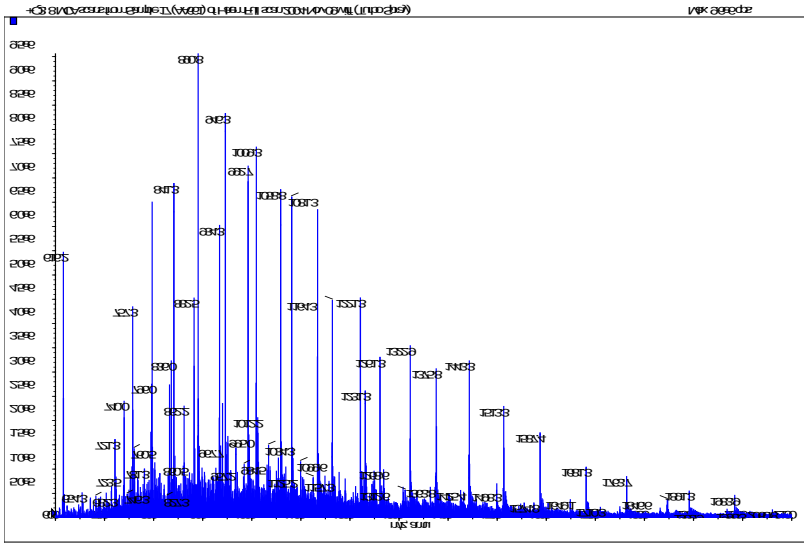
Dilute whole blood with water and electrospray measuring m/z – charge series envelope



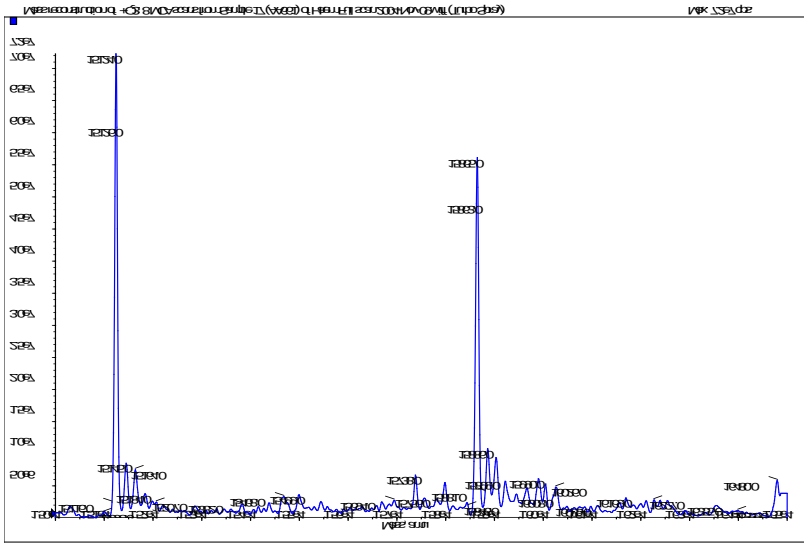
MS spectrum dominated by high abundance proteins
α and β chains of haemoglobin

Newborn haemoglobinopathy screening

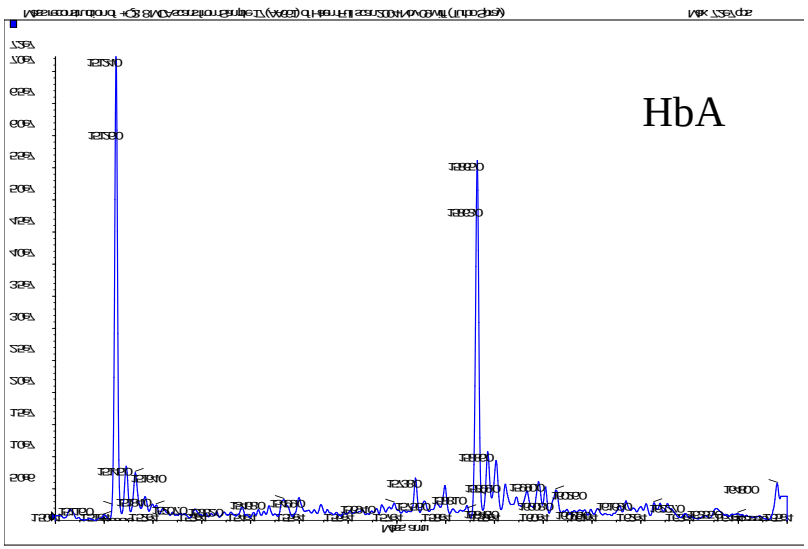
Charge series



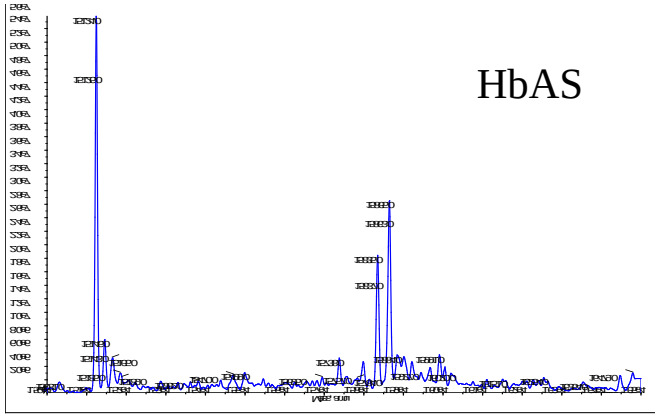
Deconvolutional analysis



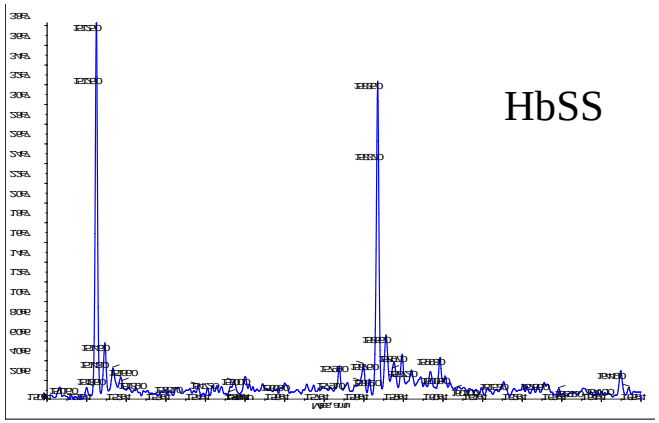
Newborn haemoglobinopathy screening



HbA

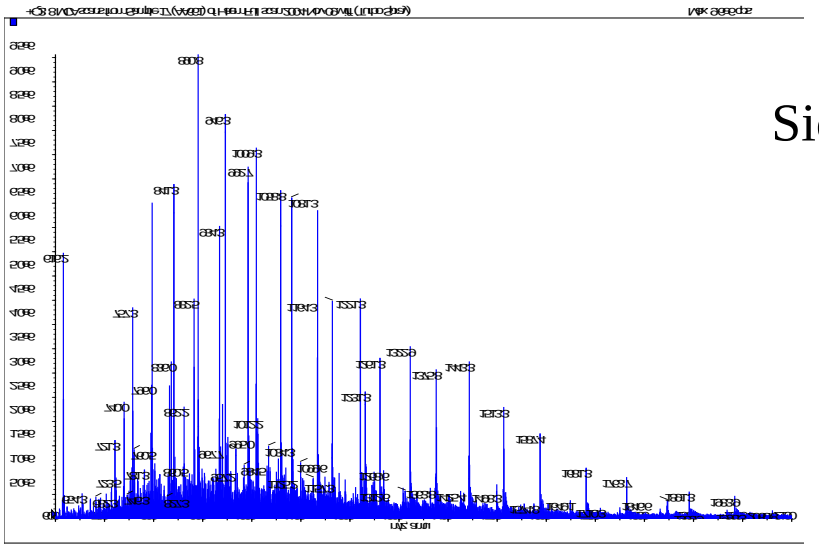


HbAS



HbSS

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

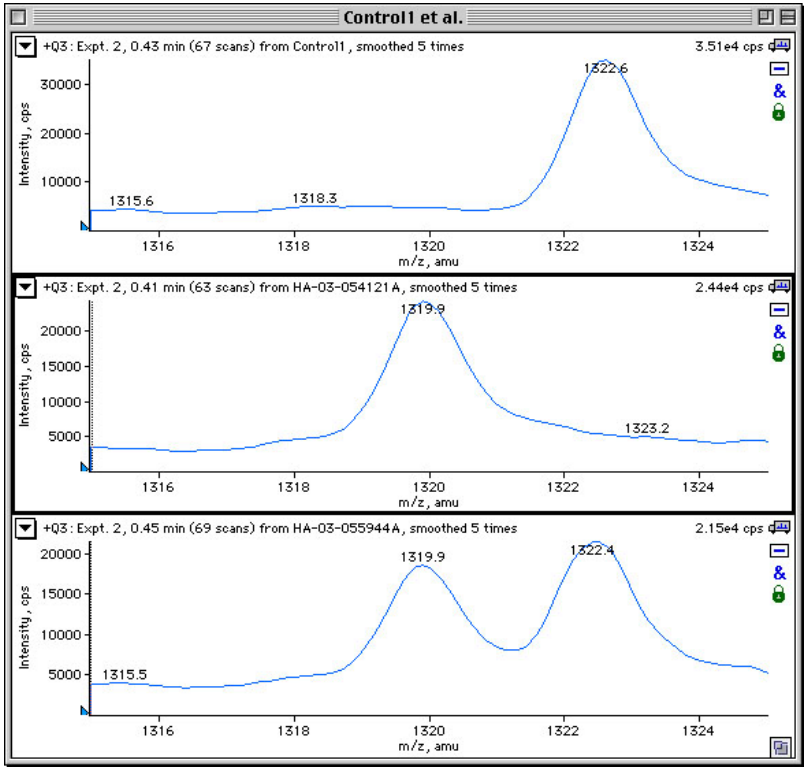
wild-type β-chain, m/z c.1322.5

sickle β-chain, m/z c.1320.0

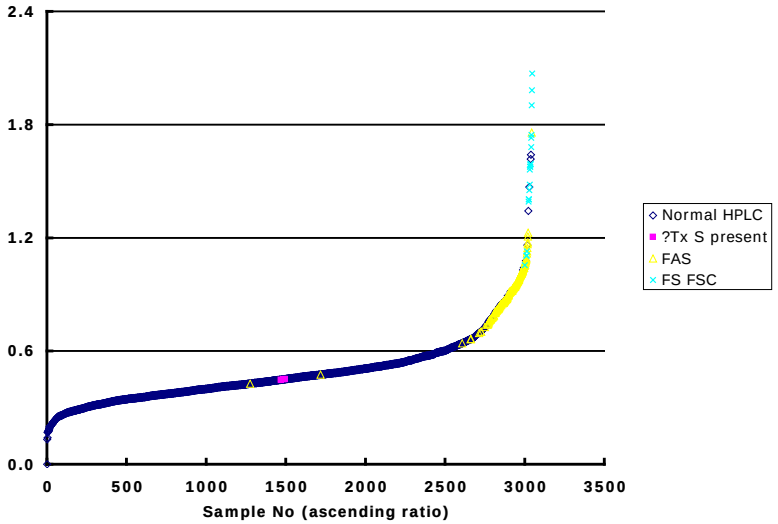
scan range m/z 1315-1325

Quality check Hb β-chain 13 positive charged

Newborn haemoglobinopathy screening



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

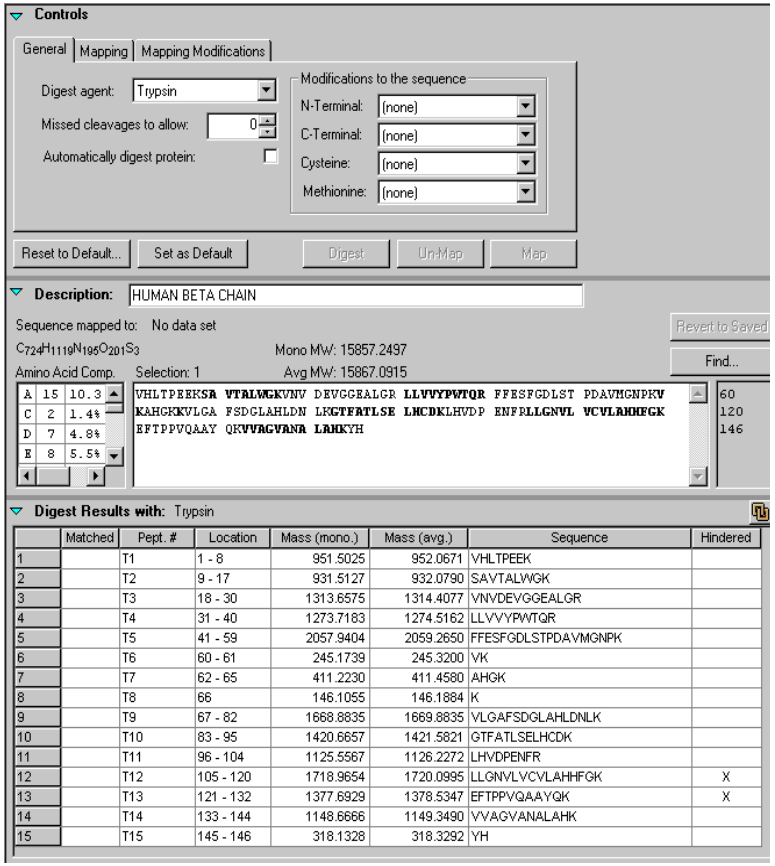
Endopeptidases, e.g. trypsin, act only at specific recognition sites and in a consistent and reliable manner

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



	Matched	Pept. #	Location	Mass (mono.)	Mass (avg.)	Sequence	Hindered
1		T1	1 - 8	951.5025	952.0671	VHLTPEEK	
2		T2	9 - 17	931.5127	932.0790	SAVTALWGK	
3		T3	18 - 30	1313.6575	1314.4077	VNVDEVGGEALGR	
4		T4	31 - 40	1273.7183	1274.5162	LLVVYPWTQR	
5		T5	41 - 59	2057.9404	2059.2650	FFESFGDLSTPDAVMGNPK	
6		T6	60 - 61	245.1739	245.3200	VK	
7		T7	62 - 65	411.2230	411.4580	AHGK	
8		T8	66	146.1055	146.1884	K	
9		T9	67 - 82	1668.8835	1669.8835	VLGAFSDGLAHLDNLK	
10		T10	83 - 95	1420.6657	1421.5821	GTFATLSELHCDK	
11		T11	96 - 104	1125.5567	1126.2272	LHVDPENFR	
12		T12	105 - 120	1718.9654	1720.0995	LLGNVLVCVLAHHFGK	X
13		T13	121 - 132	1377.6929	1378.5347	EFTPPVQAAYQK	X
14		T14	133 - 144	1148.6666	1149.3490	VVAGVANALAHK	
15		T15	145 - 146	318.1328	318.3292	YH	

Newborn haemoglobinopathy screening

NHS Sickle Cell and Thalassaemia screening programme

Beta chain point mutations					
variant	**Wild Type AA**	**Variant AA**	**Position**	**Δ Mass**	**Tryptic Peptide**
HbS	Glu	Val	6	-30	T1
HbC	Glu	Lys	6	-1	T1 (new peptide)
HbD^{Punjab}	Glu	Gln	121	-1	T13
HbO^{Arab}	Glu	Lys	121	-1	T13 (new peptide)
HbE	Glu	Lys	26	-1	T3 (new peptide)

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

Problem

Complexity of traditional tryptic digestion procedures

Solution

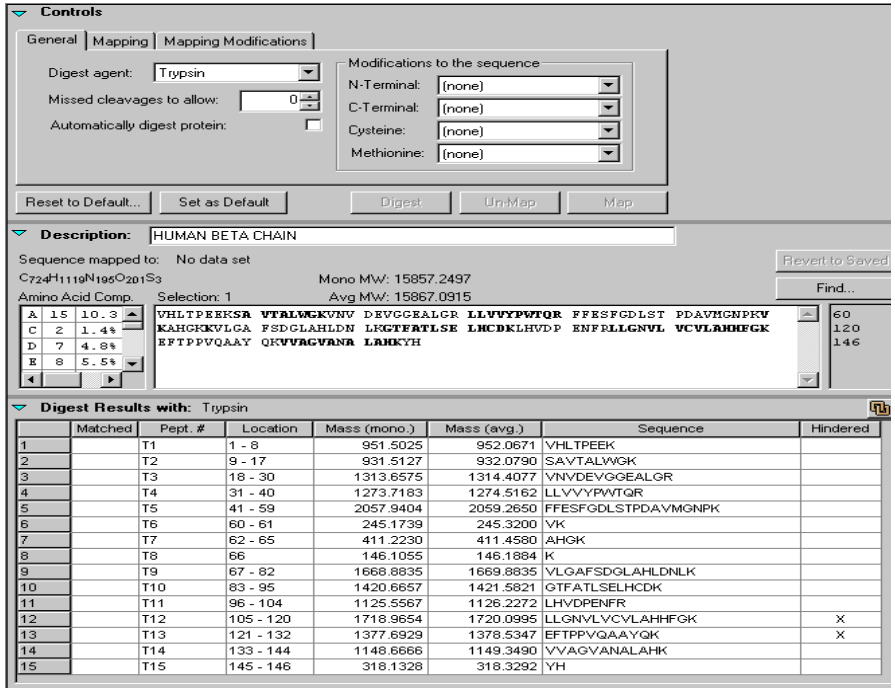
PhD student

Primary questions

Can we detect the mutant peptides in MRM mode?

How far can we minimise sample preparation and tryptic digestion?

Newborn haemoglobinopathy screening



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11		T11	96 - 104	1125.5567	1126.2272	LHVDPENFR	
12		T12	105 - 120	1718.9654	1720.0995	LLGNVLVCVLAHHFGK	X
13		T13	121 - 132	1377.6929	1378.5347	EFTPPVQAAYQK	X
14		T14	133 - 144	1148.6666	1149.3490	VVAGVANALAHK	
15		T15	145 - 146	318.1328	318.3292	YH	

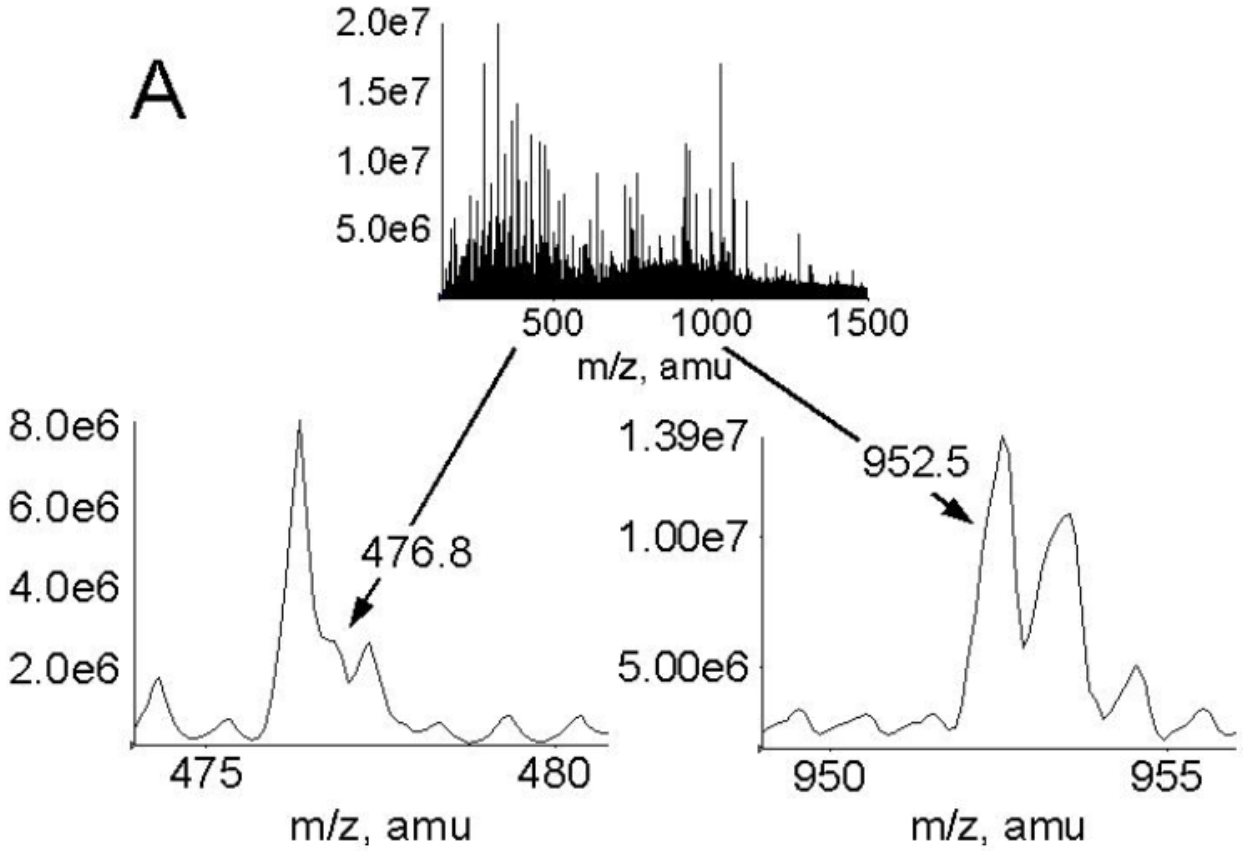
Sickle mutation in T1 position 6
glutamic acid to valine

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

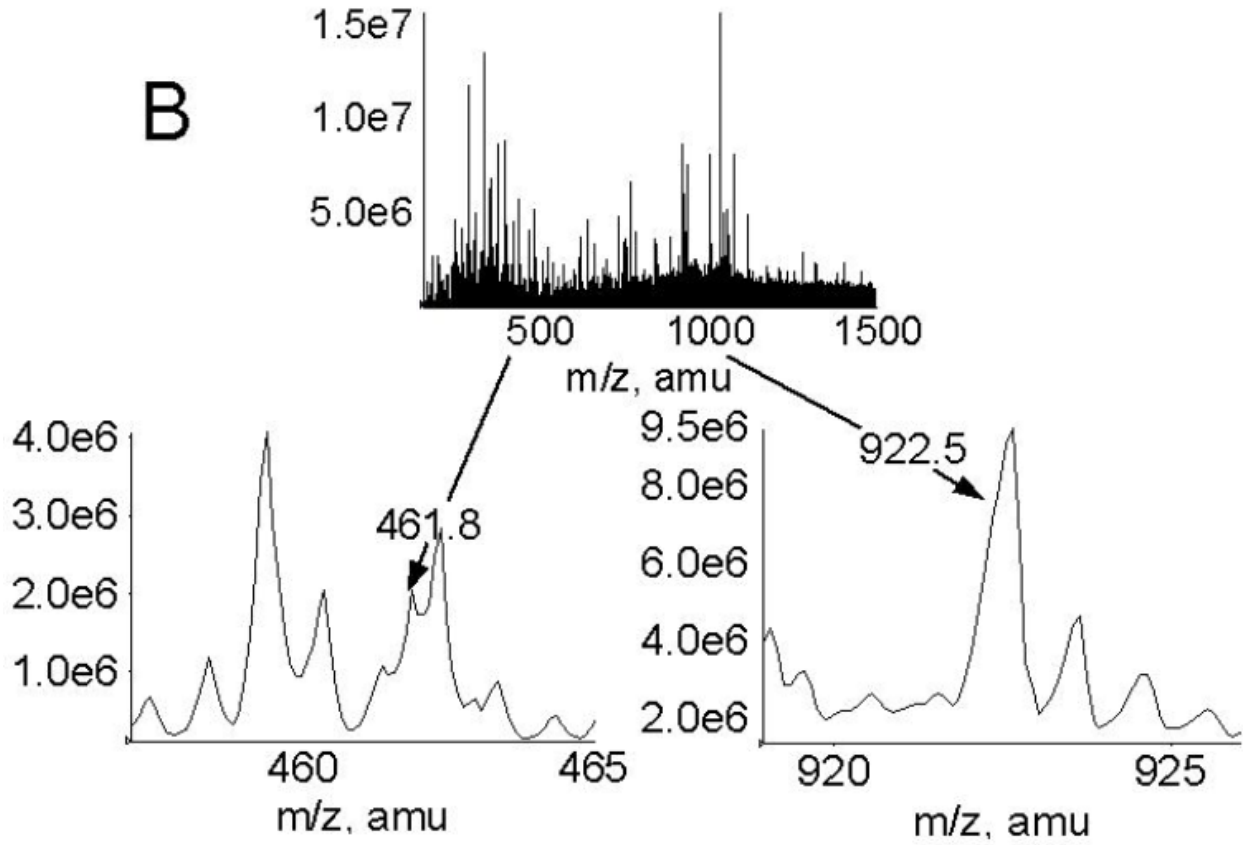
Patient samples, HbA, HbSS – not peptide standards
API4000, inject 2µl, usual solvent, flow rate 75µl/min
Full scan **MS** of tryptic digest

Newborn haemoglobinopathy screening



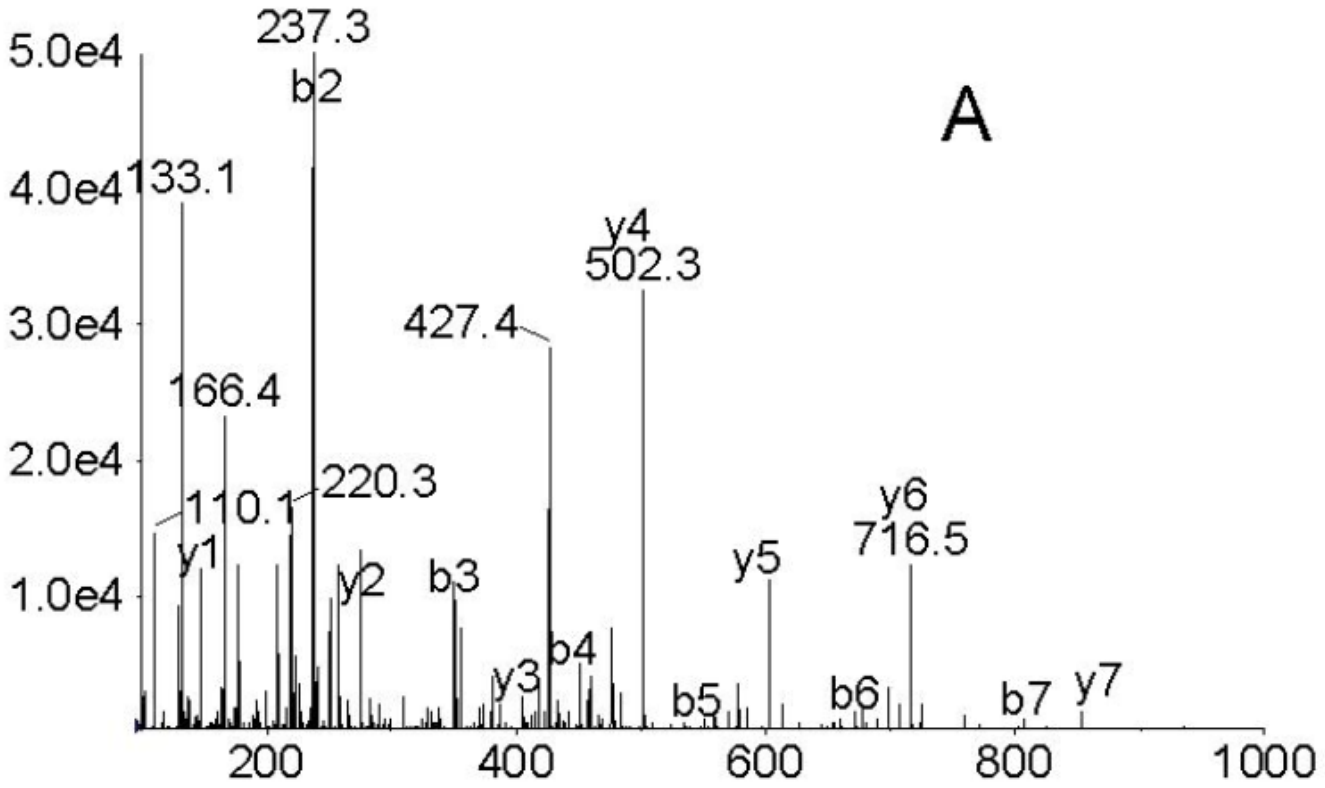
HbA **MS** full scan to detect wild type βT1

Newborn haemoglobinopathy screening



HbSS **MS** full scan to detect sickle βT1

Newborn haemoglobinopathy screening



MSMS – product ion scan of doubly charged wild type βT1, m/z 476.8

Newborn haemoglobinopathy screening

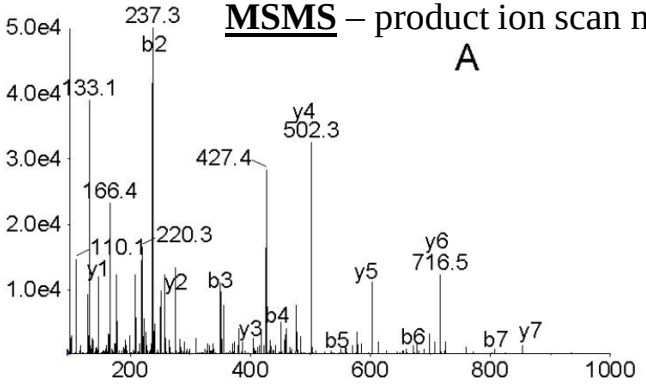
Isolate doubly charged peptide ion in Q1, fragment, Q3 product ion scan

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

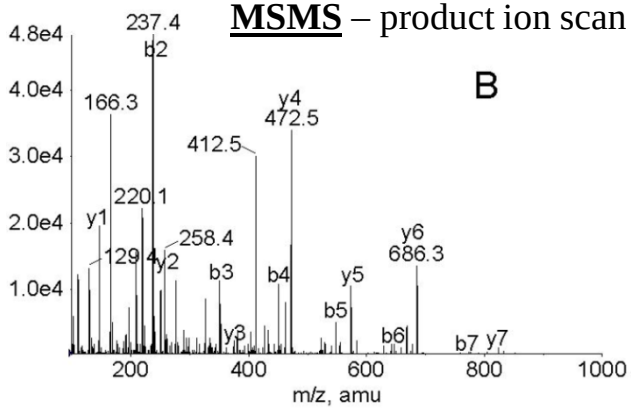
y series, N-terminal AA sequence
VHLTPE(V)EK, HLTPE(V)EK (y7), LTPE(V)EK (y6), TPE(V)EK (y5), PE(V)EK (y4), E(V)EK (y3), EK (y2), K

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

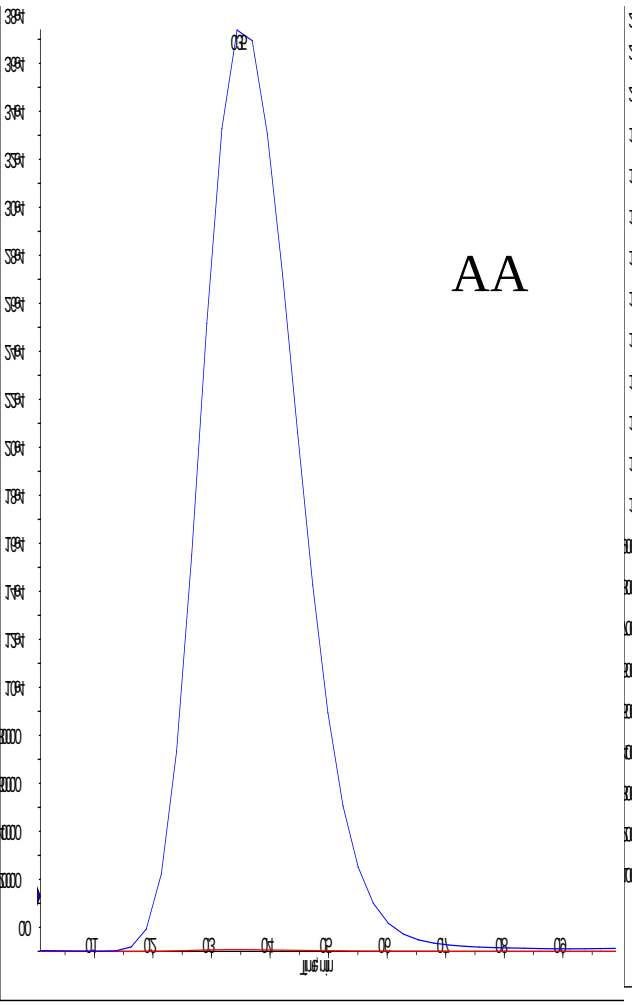


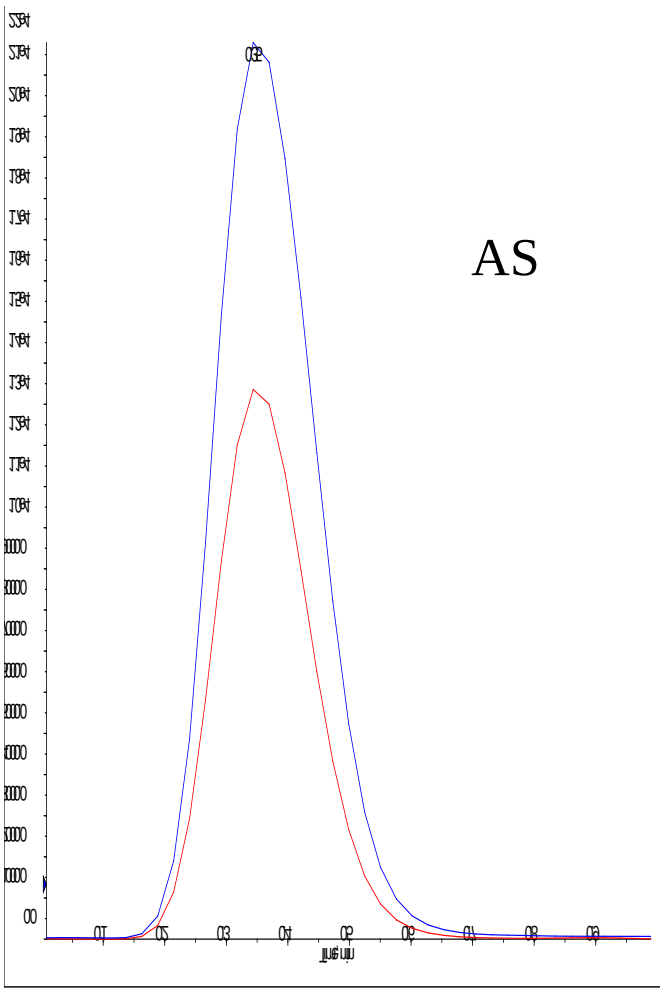
MSMS – product ion scan m/z 461.8

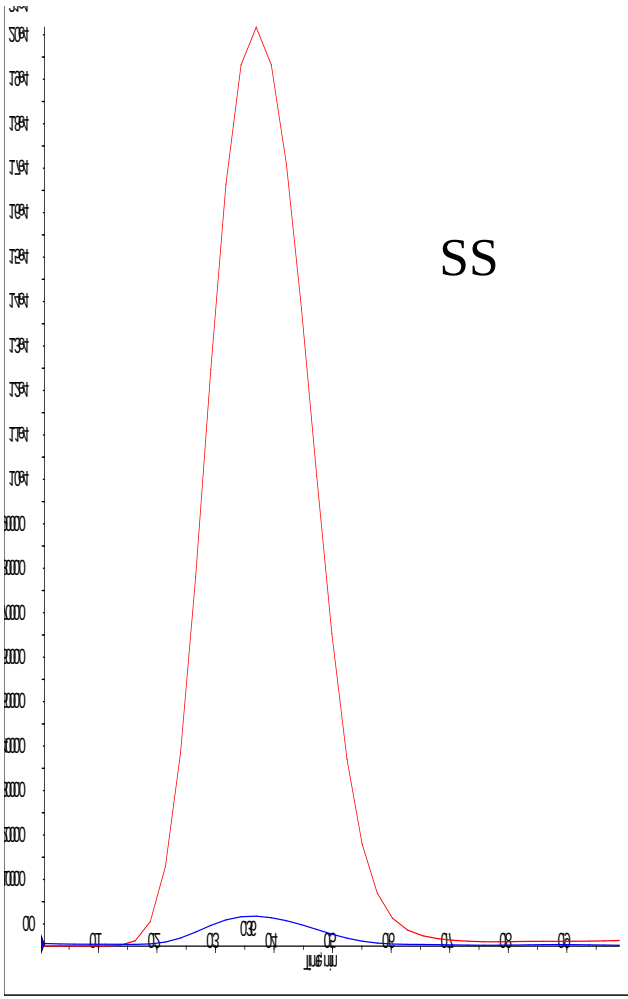


MRM for wild-type βT1, m/z 476.8/502.3 (y4)
MRM for sickle βT1, m/z 461.8/472.5 (y4)

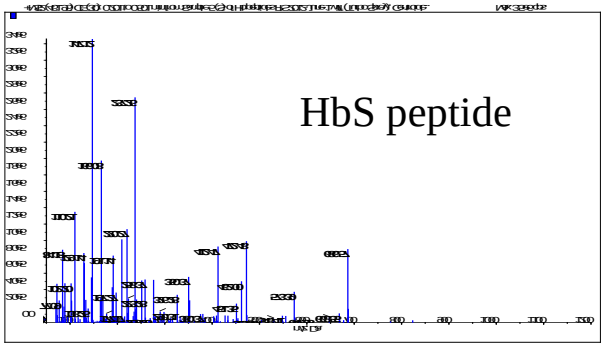
Newborn haemoglobinopathy screening

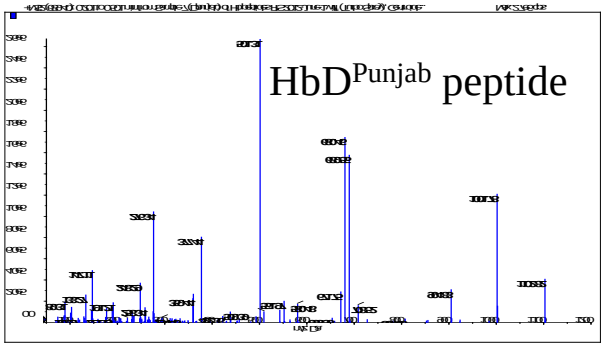


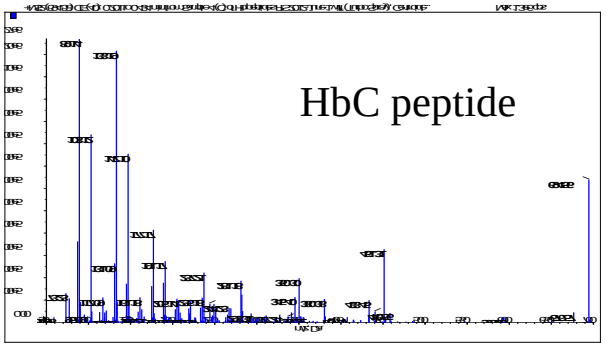


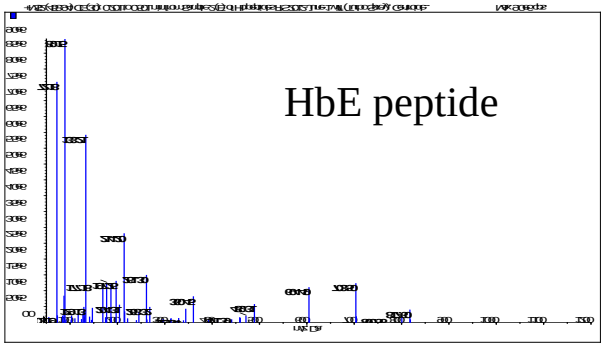


Newborn haemoglobinopathy screening









The same afternoon, developed MRMs for HbC, HbD^{Punjab}, HbO^{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^{Punjab} 2	
CC 1	DD^{Punjab} 1		EE 1
AO^{Arab} 1	OO^{Arab} 1		

Sensitivity 100% Specificity 100%

Patented by King's College London

Yvonne A Daniel, Charles Turner, Roberta M Haynes, Beverley J Hunt, R Neil Dalton (2005)
Rapid and Specific Detection of Clinically Significant Haemoglobinopathies using Electrospray Mass Spectrometry-Mass Spectrometry,
British Journal of Haematology:130, 635-643

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?

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 37°C

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.

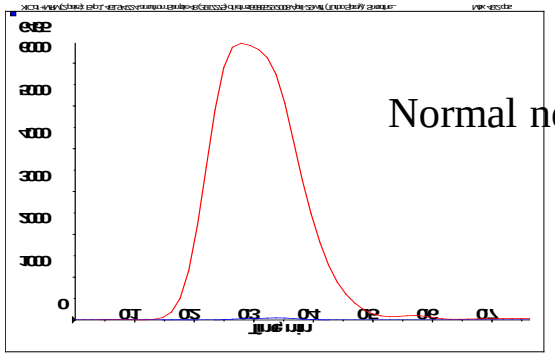
Comparison carried out in Leeds

MRM acquisitions

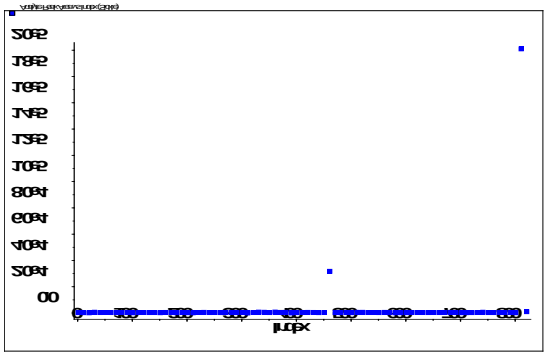
Tryptic peptide	Target Peptide Ion (m/z)	Target fragment ion (m/z)
Wild Type (Beta) T1	476.9	y4 502.3
HbS T1	461.9	y4 472.4
HbC T1	694.5	b4 451.3
Wild Type T13	689.9	b3 378.1
HbD^{Punjab} T13	689.4	b3 377.1
Wild Type T13	689.9	y9 1001.4
HbO^{Arab} T13	625.3	y9 1001.4
Wild Type T3	657.9	y9 887.5
HbE T3	458.7	y5 489.3
Delta chain T2	480.3	y6 688.4
Delta chain T14	721.4	y9 1064.3
Wild Type T2	466.8	y6 675.4
Gamma Chain T2	488.6	y6 691.6
Alpha Chain T1	365.2	y4 430.4
Alpha Chain T13	626.9	y10 992.5

Newborn haemoglobinopathy screening

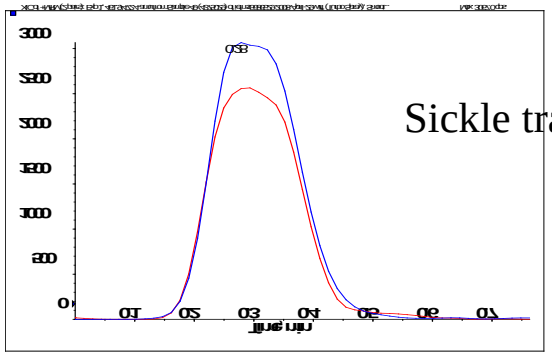
Outputs



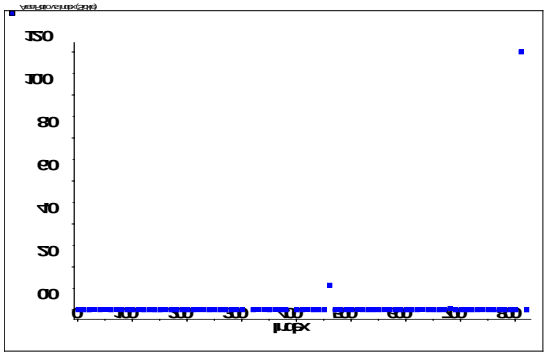
Normal neonate



Area



Sickle trait neonate



Area ratio S/WT T1

Newborn haemoglobinopathy screening

40,046 newborn DBS from Leeds screened (Aug 2007-Aug 2008)

Mutations detected by IEF & MSMS

confirmed by HPLC

T1 S peptide	196	Incidence of sickle peptide 1:204
HbS/HbF 8		Incidence of SCD 1:3,641
HBSC 3		
HbS trait 185		
T1 C peptide	39 (including the 3 HbSC)	Incidence of HbC 1:1,082
T13 D^{Punjab} peptide	51	Incidence of HbD^{Punjab} 1:817
T13 O^{Arab} peptide	0	
T3 E peptide	47	Incidence of HbE 1:834
β-thalassaemia major (no T1, T3, T5, & T13 β-chain peptides)	4	Incidence of all mutations 1:120
confirmed by IEF/ante-natal history/6 month follow up		Incidence of β-thalassaemia major 1:10,012
Total	334	

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

YA Daniel et al Blood (2008) 112, 2387

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

trypsin reagent (50µl)

Incubate with mixing at 37^{o}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

Stuart J. Moat[1], Derek Rees[1], Lawrence King[2], Adeboye Ifederu[3], Katie Harvey[3], Kate Hall[4], Geoff Lloyd[1], Christine Morrell[5] and Sharon Hillier[6]

(2014) Newborn Blood Spot Screening for Sickle Cell Disease by Using Tandem Mass Spectrometry: Implementation of a Protocol to Identify Only the Disease States of Sickle Cell Disease Clin Chem 60, 373-380

[1] *Wales Newborn Screening Laboratory, Department of Medical Biochemistry, Immunology & Toxicology, and*

[2] *Department of Haematology, University Hospital Wales, Cardiff, UK;*

[3] *Newborn Screening Laboratory, Department of Chemical Pathology, Great Ormond Street Hospital, London, UK;*

[4] *Newborn Screening & Biochemical Genetics, Birmingham Children's Hospital, Birmingham, UK;*

[5] *Directorate for Public Health and Health Professions, Welsh Government, Cardiff, UK;*

[6] *Screening Division, Public Health Wales, Cathedral Road, Cardiff, UK.*

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

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The pilot sites

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