Haemoglobinopathies – case studies
11th Annual Sickle Cell and Thalassaemia Conference 11 – 13 October 2017

Chris Lambert
Haematology Service Delivery Manager
Viapath Laboratories
Kings College Hospital
HUMAN ALPHA AND BETA GLOBIN GENES
GLOBIN GENE EXPRESSION

Embryonic
Gower 1 $\zeta_2\epsilon_2$
Gower2 $\alpha_2\epsilon_2$
Portland $\zeta_2\gamma_2$

Fetal
HbF $\alpha_2\gamma_2$

Adult
HbA $\alpha_2\beta_2 \sim 96\%$
HbA2 $\alpha_2\delta_2 2.4 - 3.3\%$
HbF $\alpha_2\gamma_2 < 1.0\%$
HAEMOGLOBIN

• Tetramer made up of 2 alpha and 2 non alpha globins
• Iron containing oxygen transport protein
• Oxygen binds to heme on the haemoglobin molecule
• Transports oxygen from the lungs to the tissues – reversible binding
• Oxygen binds in a co-operative manner – as one subunit binds it makes it easier for the others via conformational changes – classical sigmoid oxygen dissociation curve
Haemoglobin Variants
- The result of an amino acid substitution in a globin chain
- Over 1000 different haemoglobin variants described to date
- Majority of variants are harmless
- Functionality may be affected:
  - Solubility
  - Oxygen dissociation
  - Stability

Thalassaemias
- Arise due to the impaired production of globin
- $\alpha$, $\beta$, $\gamma$ or $\delta$ chains may be affected
- Over 200 mutations described for $\beta$ thalassaemia
- Over 200 mutations described for $\alpha$ thalassaemia
Detection of Hb Variants at KCH

First line method
• High Performance Liquid Chromatography
  Automated HPLC

Second line methods
• Sickle solubility testing
• Haemoglobin electrophoresis:
  – Acid pH, agarose gel (AAG)
  – Isoelectric focusing (IEF)

Third Line
• DNA analysis

Other methodologies
• Mass spectrometry
• Capillary electrophoresis
PHENOTYPIC METHODS

HPLC

Cellulose Acetate Membrane
- HbAA
- HbSS
- HbSC
- Control

Sickle Solubility test
- Tube 1 negative
- Tube 2 positive

Acid Agarose Gel
- HbS+S
- HbS+C
- HbS+S
- HbA+F
- Control

Iso-electric focusing
- AF D C
- AF S E
- HbFAS
- HbFS
- HbFAS

controls
ANTENATAL SCREENING - PARENTAL CARRIER STATE COMBINATIONS

Is there a risk to the fetus or not?

From Sickle Cell and Thalassaemia Screening Programmes - Handbook for Laboratories – October 2012
General

- Retention times and even appearances of the chromatogram can vary between resin batches, individual columns and even between two machines running the same column batch.

- Use of the relative elution time (\(\text{RET} = \text{RT of X - RT of A2 in the same sample}\)) can compensate for this to a certain extent, but even this relationship does not always survive a resin batch change, so historical data may need reviewing.

- Many different haemoglobins have the same HPLC characteristics; with more than 900 variants described it is unrealistic to expect otherwise.

- However, careful study of all the features can suggest the direction of further tests.
Relative Variant Percentages in Carriers

- Depends on which chain, and how many chains are involved
  - Beta chain variants (1 chain of 2 affected) 35-50%
  - Alpha chain variants (1 chain of 4 affected) 18-24%
  - Delta chain variants (1 chain of 2 affected) 1-2%
  - Fusion chain variants Variable

- HOWEVER
  - Instability reduces the amount of the variant
  - Iron deficiency reduces the amount of the variant
  - Some variants have reduced production
  - Co-existing Thalassaemia usually reduces the amount of a variant of the opposite chain, and increases the amount of a variant of the same chain
Hb SC Disease

<table>
<thead>
<tr>
<th>Peak Name</th>
<th>Calibrated Area</th>
<th>Area %</th>
<th>Retention Time (min)</th>
<th>Peak Area</th>
</tr>
</thead>
<tbody>
<tr>
<td>F</td>
<td>2.2*</td>
<td>---</td>
<td>1.07</td>
<td>45365</td>
</tr>
<tr>
<td>P1</td>
<td>---</td>
<td>0.2</td>
<td>1.28</td>
<td>3513</td>
</tr>
<tr>
<td>P3</td>
<td>---</td>
<td>0.1</td>
<td>1.80</td>
<td>2943</td>
</tr>
<tr>
<td>Unknown</td>
<td>---</td>
<td>0.6</td>
<td>2.20</td>
<td>12435</td>
</tr>
<tr>
<td>Ag</td>
<td>---</td>
<td>1.8</td>
<td>2.36</td>
<td>38534</td>
</tr>
<tr>
<td>A2</td>
<td>5.0*</td>
<td>---</td>
<td>3.64</td>
<td>113073</td>
</tr>
<tr>
<td>S-window</td>
<td>---</td>
<td>45.1</td>
<td>4.42</td>
<td>974872</td>
</tr>
<tr>
<td>Unknown</td>
<td>---</td>
<td>0.6</td>
<td>4.91</td>
<td>13929</td>
</tr>
<tr>
<td>C-window</td>
<td>---</td>
<td>44.3</td>
<td>5.13</td>
<td>959211</td>
</tr>
</tbody>
</table>

F Concentration = 2.2*%
A2 Concentration = 5.0*%

*Values outside of expected ranges

Analysis comments:

Sex: Male
Age: 43
Rbc: 6.20
Hb: 13.7
MCV: 62.8
MCH: 22.1
Sol: POS
HbS: 45.1%
HbC: 44.4%
Haemoglobin E trait

Bio-Rad Variant II HPLC

<table>
<thead>
<tr>
<th>Peak Name</th>
<th>Calibrated Area %</th>
<th>Area %</th>
<th>Retention Time (min)</th>
<th>Peak Area</th>
</tr>
</thead>
<tbody>
<tr>
<td>F</td>
<td>3.2</td>
<td></td>
<td>1.07</td>
<td>14285</td>
</tr>
<tr>
<td>P2</td>
<td></td>
<td>0.5</td>
<td>1.26</td>
<td>6963</td>
</tr>
<tr>
<td>Unknown</td>
<td></td>
<td>2.8</td>
<td>1.35</td>
<td>33310</td>
</tr>
<tr>
<td>Unknown</td>
<td></td>
<td>0.5</td>
<td>1.52</td>
<td>5869</td>
</tr>
<tr>
<td>Unknown</td>
<td></td>
<td>2.0</td>
<td>1.70</td>
<td>23358</td>
</tr>
<tr>
<td>P3</td>
<td></td>
<td>2.0</td>
<td>1.83</td>
<td>23540</td>
</tr>
<tr>
<td>A2</td>
<td>45.2</td>
<td></td>
<td>2.42</td>
<td>786240</td>
</tr>
<tr>
<td>HbS+C</td>
<td></td>
<td>24.0+</td>
<td>3.65</td>
<td>313637</td>
</tr>
</tbody>
</table>

Total Area: 1,206,421

F Concentration = 1.2 %
A2 Concentration =24.0%*

*Values outside of expected ranges

Analysis comments:

RBC 4.79
Hb 11.8
MCV 74.9
MCH 24.6

Cellulose Acetate Electrophoresis

[Image of electrophoresis gel with bands labeled F, A2, SC, HbS+C]
Haemoglobin E Trait - Electrophoresis

Iso-electric focussing

Normal control
HbA + HbA2

Acid Agarose gel

Normal control
HbA
VARIANTS IN THE HbA2 WINDOW

RBC  4.53 10^12/L  3.8 - 5.8
Hb   11.8 g/dL    11.5 - 15.5
MCV  87.0 fL      77.0 - 95.0
MCH  26.0 pg      25 - 34

Solubility – Negative

Hb elect. pH 8.6
Bands in HbA + HbS positions

Hb elect.pH 6.0
Single band in position of HbA

Isoelectric Focusing
HbA + cathodal band -5.5mm + HbA2

Haemoglobin Lepore
delta-beta hybrid (delta through 22; beta from 50)
Can give rise to significant disorders
Hb S/O-Arab

SOL - POS
Fraction in the HbD window

<table>
<thead>
<tr>
<th>Peak Name</th>
<th>Area %</th>
<th>Area $%$</th>
<th>Time (min)</th>
<th>Area</th>
</tr>
</thead>
<tbody>
<tr>
<td>F</td>
<td>0.3</td>
<td>---</td>
<td>1.09</td>
<td>3932</td>
</tr>
<tr>
<td>Unknown</td>
<td>---</td>
<td>0.7</td>
<td>1.25</td>
<td>8516</td>
</tr>
<tr>
<td>P2</td>
<td>---</td>
<td>2.3</td>
<td>1.36</td>
<td>28457</td>
</tr>
<tr>
<td>Unknown</td>
<td>---</td>
<td>0.5</td>
<td>1.52</td>
<td>6537</td>
</tr>
<tr>
<td>P3</td>
<td>---</td>
<td>3.8</td>
<td>1.73</td>
<td>46205</td>
</tr>
<tr>
<td>Unknown</td>
<td>---</td>
<td>2.0</td>
<td>2.13</td>
<td>24696</td>
</tr>
<tr>
<td>Ao</td>
<td>---</td>
<td>56.8</td>
<td>2.50</td>
<td>698875</td>
</tr>
<tr>
<td>A2</td>
<td>1.6*</td>
<td>---</td>
<td>3.64</td>
<td>21205</td>
</tr>
<tr>
<td>D-window</td>
<td>---</td>
<td>31.9</td>
<td>4.12</td>
<td>392396</td>
</tr>
</tbody>
</table>

Total Area: 1,230,820

F Concentration = 0.3 %
A2 Concentration =1.6%

*Values outside of expected ranges

Analysis comments:

Retention time 4.12

31.9%
Probable Hb D Punjab carrier

DNA analysis: Codon 121 (GAA;Glu > CAA;Gln) mutation in exon 3 of the beta globin gene
# Multiple Fractions

<table>
<thead>
<tr>
<th>Peak Name</th>
<th>Calibrated Area %</th>
<th>Area %</th>
<th>Retention Time (min)</th>
<th>Peak Area</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unknown</td>
<td>0.0</td>
<td>0.0</td>
<td>0.99</td>
<td>662</td>
</tr>
<tr>
<td>F</td>
<td>0.1</td>
<td>---</td>
<td>1.08</td>
<td>2037</td>
</tr>
<tr>
<td>Unknown</td>
<td>---</td>
<td>0.5</td>
<td>1.24</td>
<td>535</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>RBC</th>
<th>Hb</th>
<th>MCV</th>
<th>MCH</th>
<th>SOL</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.07</td>
<td>13.5</td>
<td>83.0</td>
<td>26.6</td>
<td>POS</td>
</tr>
</tbody>
</table>

---

**Notes:**
- The table above shows the results for different fractions of blood, including HbA, HbD, HbS, and an unknown fraction.
- The graph illustrates the peaks corresponding to each fraction, with retention times and peak areas indicated.
- The peaks are labeled with their corresponding hematological values.
Multiple Fractions
HbA + S with G-Philadelphia

<table>
<thead>
<tr>
<th>Alpha globin</th>
<th>Alpha G-Phil</th>
</tr>
</thead>
<tbody>
<tr>
<td>Beta globin</td>
<td>HbA</td>
</tr>
<tr>
<td>Beta S globin</td>
<td>HbS</td>
</tr>
<tr>
<td>Delta globin</td>
<td>HbA2</td>
</tr>
</tbody>
</table>
Previously tested as a pre-operative screen
HbS 14% assumed transfused HbAS

<table>
<thead>
<tr>
<th>Test</th>
<th>Value</th>
<th>Normal range</th>
</tr>
</thead>
<tbody>
<tr>
<td>RBC</td>
<td>5.61 \times 10^{12}/L</td>
<td>3.8 - 5.8</td>
</tr>
<tr>
<td>Hb</td>
<td>14.5 g/dL</td>
<td>11.5 - 15.5</td>
</tr>
<tr>
<td>PCV</td>
<td>0.440 L/L</td>
<td>0.370 - 0.470</td>
</tr>
<tr>
<td>MCV</td>
<td>78.4 fL</td>
<td>77.0 - 95.0</td>
</tr>
<tr>
<td>MCH</td>
<td>25.9 pg</td>
<td>25 - 34</td>
</tr>
<tr>
<td>MCHC</td>
<td>33.0 g/dL</td>
<td>32.0 - 37.0</td>
</tr>
<tr>
<td>RDW</td>
<td>14.3 %</td>
<td>11.0 - 15.0</td>
</tr>
</tbody>
</table>
11B0187321 HPLC

Over a year later another sample was received

Antenatal request

No history of transfusion

- Sickle Solubility Test Positive
  - Hb elect. pH 8.6
    Bands in HbA + HbS positions
  - Hb elect.pH 6.0
    Bands in HbA + HbS positions
  - Isoelectric Focusing
    HbA, HbS + HbA2

**Analysis comments:**

<table>
<thead>
<tr>
<th>Peak Name</th>
<th>Calibrated Area %</th>
<th>Area %</th>
<th>Retention Time (min)</th>
<th>Peak Area</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unknown</td>
<td>---</td>
<td>0.1</td>
<td>0.98</td>
<td>951</td>
</tr>
<tr>
<td>F</td>
<td>0.2</td>
<td>---</td>
<td>1.07</td>
<td>3671</td>
</tr>
<tr>
<td>Unknown</td>
<td>---</td>
<td>1.0</td>
<td>1.21</td>
<td>18538</td>
</tr>
<tr>
<td>P2</td>
<td>---</td>
<td>3.3</td>
<td>1.34</td>
<td>61477</td>
</tr>
<tr>
<td>Unknown</td>
<td>---</td>
<td>0.4</td>
<td>1.51</td>
<td>7994</td>
</tr>
<tr>
<td>P3</td>
<td>---</td>
<td>3.5</td>
<td>1.68</td>
<td>66011</td>
</tr>
<tr>
<td>A0</td>
<td>---</td>
<td>74.2</td>
<td>2.39</td>
<td>1388548</td>
</tr>
<tr>
<td>A2</td>
<td>3.4*</td>
<td>---</td>
<td>3.62</td>
<td>67500</td>
</tr>
<tr>
<td>S-window</td>
<td>---</td>
<td>13.7</td>
<td>4.39</td>
<td>256808</td>
</tr>
</tbody>
</table>

F Concentration = 0.2 %
A2 Concentration = 3.4*%

*Values outside of expected ranges
Indices indicate possible co-existing alpha thalassaemia but unlikely to reduce variant by this amount

Novel unstable sickling mutation

Beta plus thalassaemia mutation inherited on the same chromosome as HbS mutation ie promoter mutation

Genetic mosaic
DNA analysis

- Beta gene sequencing
  - Heterozygous for HbS, codon 6 (GAG;Glu>GTG;Val) mutation in exon 1 of the beta globin gene

- Alpha thalassaemia multiplex analysis
  - Heterozygous for alpha + thal due to the 3.7Kb deletion. One of the 4 alpha globin genes has been deleted from one chromosome

- Multiplex Ligation-dependent Probe Amplification
  - MLPA Beta globin gene analysis indicates the entire beta globin cluster is duplicated on one chromosome from the upstream HS3 element in the LCR through the end of the beta globin gene. This duplication spans approx 60.5Kb on chromosome 11p15.5

The extra beta gene cluster is fully functional and hence the additional production of normal beta globin is “diluting” the beta S globin.

The alpha + thalassaemia mutation is further reducing the amount of HbS present via affinity competition.
QUESTIONS