The long and winding road to the standardization of HbA$_2$

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Contents

• Why HbA₂ is important
• State of the art
• Activities of the IFCC WG-HbA2
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• Why HbA$_2$ is important
  • State of the art
  • Activities of the IFCC WG-HbA2
World Distribution, Population Genetics, and Health Burden of the Hemoglobinopathies

Thomas N. Williams\textsuperscript{1} and David J. Weatherall\textsuperscript{2}

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Table 1. A breakdown of the annual number of births with the different hemoglobin disorders

<table>
<thead>
<tr>
<th>Annual births with major hemoglobin disorders</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>β-thalassemia major</td>
<td>22,989</td>
</tr>
<tr>
<td>HbE β thalassemia</td>
<td>19,128</td>
</tr>
<tr>
<td>HbH disease</td>
<td>9,568</td>
</tr>
<tr>
<td>Hb Bart’s hydrops (α\textsuperscript{0}/α\textsuperscript{0})</td>
<td>5,183</td>
</tr>
<tr>
<td>SS disease</td>
<td>217,331</td>
</tr>
<tr>
<td>S β thalassemia</td>
<td>11,074</td>
</tr>
<tr>
<td>SC disease</td>
<td>54,736</td>
</tr>
</tbody>
</table>

From available data (Modell and Darlison 2008; Weatherall 2010).

Figure 2. The world distribution of the origins of the α\textsuperscript{0} and β-thalassemias. (From Weatherall and Clegg 2001; reprinted, with permission, from the author.)
The importance of Hb A$_2$ measurement

- **Hb A$_2$ measurement is used as a marker for beta thalassemia trait**

- **Carrier detection is important because:**
  - β-thalassemia carriers are asymptomatic but homozygous β-thalassemia is a life-threatening disorder
  - Women should be screened for β-thal trait (high risk areas)
  - Carriers: recommend partner testing prediction of genetic risk

- **Failure to detect condition may result in newborn with a medically significant condition**
HbA₂ in various β-thalassemia genotypes

0 1 2 3 4 5 6 7

HbA₂ (%)

- non β
- 101 C→T
- IVS2- 844 C→G
- IVS1-6 T→C
- β° 39 C→T

0     1     2     3     4     5     6     7

silent

mild

severe

(R. Galanello, 2002)
NSC&TSP: High Prevalence Screening

FBC and HPLC

Hb Variant

- HbS, HbC, HbD, HbE, Hb OArab Hb Lepore
- Other variant

Test partner

refer to Consultant Haematologist*

No variant

MCH < 27

- HbA2 >= 3.5 beta thal trait
  - Test partner
- HbA2 < 3.5
  - MCH < 25
    - Consider ethnic group
  - MCH >= 25
    - Iron deficiency alpha thal

MCH >= 27

- HbA2 > 4.0 or HbF > 5%
  - Refer to Consultant Haematologist*
- HbA2 <= 4.0 HbF <= 5%
  - Test partner
- HbF > 5%
  - MCH < 27
  - HbA2 < 3.5
  - MCH >= 27

MCH < 25

- MCH < 25
- MCH >= 25

Iron deficiency alpha thal

Test partner

No further action

High risk of alpha zero thalassaemia**

No further action***

Low risk of alpha zero thalassaemia**

No further action***

Test partner

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Contents

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- State of the art
- Activities of the IFCC WG-HbA2
Interlaboratory comparison of current high-performance methods for HbA₂

R. Paleari*, B. Gulbis†, F. Cotton†, A. Mosca*
UKNEQAS
UK National External Quality Assessment Scheme

Borderline sample: Hb A₂ 3.7%
Analisi: HbA2
Risultato atteso: 3.0

<table>
<thead>
<tr>
<th></th>
<th>N.</th>
<th>Out</th>
<th>Media</th>
<th>C.V.</th>
<th>S.D.</th>
<th>Med. na</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tutti</td>
<td>101</td>
<td>3</td>
<td>3.33</td>
<td>13.26</td>
<td>0.44</td>
<td>3.30</td>
</tr>
<tr>
<td>Tuo Metodo</td>
<td>25</td>
<td>1</td>
<td>3.03</td>
<td>4.41</td>
<td>0.13</td>
<td>3.00</td>
</tr>
</tbody>
</table>

Campioni
Tuo risultato: 2.9

<table>
<thead>
<tr>
<th></th>
<th>Diff S</th>
<th>Diff %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tutti</td>
<td>-0.97</td>
<td>-12.91</td>
</tr>
<tr>
<td>Tuo Metodo</td>
<td>-1.00</td>
<td>-4.29</td>
</tr>
</tbody>
</table>

Riepilogo x Metodo risultati numerici (> 7 Centri)

<table>
<thead>
<tr>
<th>Metodo</th>
<th>N.</th>
<th>Out</th>
<th>Media</th>
<th>C.V.</th>
</tr>
</thead>
<tbody>
<tr>
<td>TPLC TOSOH 08</td>
<td>34</td>
<td>0</td>
<td>3.47</td>
<td>6.40</td>
</tr>
<tr>
<td>BIORAD VARIANT II DUAL KIT</td>
<td>25</td>
<td>1</td>
<td>3.03</td>
<td>4.41</td>
</tr>
<tr>
<td>SEBIA CAPILLARY5 HEMOGLOBIN</td>
<td>13</td>
<td>0</td>
<td>2.88</td>
<td>6.68</td>
</tr>
<tr>
<td>TPLC</td>
<td>12</td>
<td>0</td>
<td>4.23</td>
<td>16.46</td>
</tr>
</tbody>
</table>

Nota: I valori di Diff S > 4 o < -4 non rientrano nel grafico.
Andrea Mosca*, Renata Paleari, Barbara Wild, on behalf of the IFCW Working Group on Standardization of HbA₂

**Analytical goals for the determination of HbA₂**

<table>
<thead>
<tr>
<th>Quality level</th>
<th>Imprecision, %</th>
<th>Bias, %</th>
<th>Total error, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Optimal</td>
<td>0.2</td>
<td>1.0</td>
<td>1.5</td>
</tr>
<tr>
<td>Desirable</td>
<td>0.3</td>
<td>1.9</td>
<td>3.0</td>
</tr>
<tr>
<td>Minimal</td>
<td>0.5</td>
<td>2.9</td>
<td>4.5</td>
</tr>
</tbody>
</table>

**Table 1** Analytical goals for HbA₂ measurement derived from data on biologic variation.
Contents

• Why HbA₂ is important
• State of the art
• Activities of the IFCC WG-HbA2
IFCC Reference System for HbA$_2$

Metrological traceability chain
1. Definition of a reference measurement procedure using mass spectrometry associated with proteolytic degradation
Based on the quantification of target peptides of $\delta$ and $\alpha$ chains

1. Whole blood
   - Hemolysate
   - Enzymatic cleavage with trypsin
   - Mass spectrometry
   - Quantification of specific target peptides $\delta T2$ and $\alpha T11$

\[
[HbA_2]\% = \frac{[\delta T2]}{[\alpha T11]} \times 100
\]

Calibration

- External calibration
- Calibrators consisting of mixtures of highly purified $HbA_2$ and $HbA_0$
- Target values assigned volumetrically on the base of their purity

First approach (2005-2009)
Development of the protocol for HbA$_2$ and HbA$_0$ purification

- Blood (from healthy donor)
  - Red cell lysate
    - DE cellulose
      - Raw HbA$_2$ (purity 94%)
      - Raw HbA$_0$ (purity 86%)
  - CM cellulose
    - Pure HbA$_2$ (purity >99%)
    - Pure HbA$_0$ (purity > 98.5%)

- Calibrators
Interlaboratory exercises

2006: 6 calibrators, 29 samples

2007: 6 calibrators, 20 samples
  (2 digestions, 2 replicates/digested)

2008: 4 calibrators, 3 samples
  (3 digestions, 3 replicates/digested)

2009: 1 calibrators, 1 samples
  (centralized digestion, measurements over 5 days)

Inter-laboratory variability
Problems:
- Digestion not completed
- Not defined and reproducible yield for tryptic digest
  (different kinetic for $\alpha$T11 and $\delta$T2 peptides)
The new approach is based on the use of

- Isotope dilution-mass spectrometry
- Recombinantly expressed, intact $\text{HbA}_2$ and $^{15}\text{N}$-labeled $\text{HbA}_2$
  $\text{HbA}_0$ and $^{15}\text{N}$-labeled $\text{HbA}_0$
- Target peptides specific for $\delta$ and $\alpha$ chains: $\delta T2$, $\alpha T5$
- **HbA$_2$** and **HbA$_0$** intact proteins
Metrological traceability

The metrological traceability of measurement using the HbA₂ and HbA₀ protein standards is ensured by:

1. determination of content of peptide by LC-ID-MS (amino acid analysis)
2. determination of purity by LC-TOF -MS
ID-MS-based amino acid analysis of Hb-calibrator material

<table>
<thead>
<tr>
<th>amino acid</th>
<th>Leu</th>
<th>Phe</th>
<th>Val</th>
</tr>
</thead>
<tbody>
<tr>
<td>concentration of Hb-reference solution [nmol/g]</td>
<td>14.96</td>
<td>15.23</td>
<td>15.19</td>
</tr>
<tr>
<td>mean: 15.13 nmol/g</td>
<td>U= 2.6%</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Purification of HbA₀ reference material

Purification of Hb A by centrifugal filtration using filter units with molecular weight cut-off (MWCO): 50 kDa

size-exclusion-chromatography of Hb A₀
Workflow
Peptides selected for LC-MSMS measurements

**deltaT2**


15N Internal standard

**alphaT5**


M* – F* – L* – S* – F* – P* – T* – T* – K2*

15N Internal standard
HbA$_2$ measurement results on blood samples
2. Preparation of a certified reference material for hemoglobin $A_2$ (in cooperation of IRMM)
Development of a candidate certified reference material (CRM)

- Lyophilized material

First pilot batch (April 2008)
- homogeneity
- total Hb content
- MetHb
- stability at +4°/-20 °C
- Commutability

Second batch (November 2010)
- Storage without O₂ to limit oxidation
- accelerated degradation experiments
- Long term stability
No unexpected peaks due to preparation/lyophilizing process

Good commutability
- Stability of the lyophilized material

- Storage Temp = -20 °C

- Storage Temp = +4 °C
Based on the quantification of intact globin chains by LC-ESI/MS (without protein digestion)

Modified from:
Fetal hemoglobin: assessment of glycation and acetylation status by electrospray ionization mass spectrometry
Andrew S. Davison¹*, Brian N. Green² and Norman B. Roberts¹
Clin Chem Lab Med 2008;46(9):1230–1238

Alternative approach (harmonization)
Protocol for sample preparation

Fresh blood in EDTA

- Filtration through cellulose column
- Removal of WBC, platelets, plasma

RBC (overnight at -80°C)

- Lysis with H₂O
- Removal of cellular stroma

Hemolysate

- Dilution with acetonitrile/formic acid

Desalting on cation exchange resin bead

Sample for MS
Hb 1 mg/mL
Acetonitrile 50 %
Formic acid 0.18 %
Results from experiment June 2014

Close correlation with HPLC (except for 1 sample)
Improvement in the reproducibility

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>HbA₂, %</th>
<th>CV, %</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HPLC</td>
<td>MS</td>
</tr>
<tr>
<td>P2</td>
<td>3.05</td>
<td>3.23</td>
</tr>
<tr>
<td>P3</td>
<td>2.58</td>
<td>2.56</td>
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<td>P7</td>
<td>3.25</td>
<td>3.04</td>
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<tr>
<td>P10</td>
<td>5.95</td>
<td>5.86</td>
</tr>
<tr>
<td>P12</td>
<td>2.35</td>
<td>2.45</td>
</tr>
<tr>
<td>P15</td>
<td>5.10</td>
<td>4.33</td>
</tr>
<tr>
<td>P16</td>
<td>5.55</td>
<td>5.54</td>
</tr>
<tr>
<td>P20</td>
<td>3.93</td>
<td>3.89</td>
</tr>
<tr>
<td>P21</td>
<td>4.48</td>
<td>4.15</td>
</tr>
<tr>
<td>P23</td>
<td>2.93</td>
<td>2.71</td>
</tr>
</tbody>
</table>
Harmonization of Measurement Results of the Alcohol Biomarker Carbohydrate-Deficient Transferrin by Use of the Toolbox of Technical Procedures of the International Consortium for Harmonization of Clinical Laboratory Results

Fig. 2. Success of harmonization: bias for routine MPs in individual patient samples after calibration. The bias of routine MPs of individual patients after calibration with the frozen CRM in IFCC CDT units is on the y axis. The CDT concentration in IFCC CDT units is on the x axis. Samples with an increased triosaccharotransferrin concentration are indicated with an arrow and the percentage of triosaccharotransferrin. Solid, dotted, and broken lines are the limits for optimum, desirable, and minimum TEa, respectively.
Conclusions

• Reference measurement procedure: under way to be finalized and validated

• Alternative reference method: to be validated

• Certified reference material
  – Defined the optimal condition for sample preparation and lyophilization
  – Composition in Hb similar to that of blood (Hbtot, MetHb)
  – Good commutability (for the methods tested)
  – HbA$_2$ stable at least for 4 years at +4°C or -20°C (lyophilized form)
Next steps

• **Reference measurement procedure:** to be approved by IFCC (ballot)

• **Certified reference material**
  – to be prepared in at least one large batch
  – to be distributed and used (manufacturers)

• **State-of-the-art:** to be monitored on a regular base by adequate EQAS studies and/or surveys
Acknowledgments

IFCC WG on Standardization of HbA$_2$

Andrea Mosca IT
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Maria Ospina US
Victor De Jesus US