Standardization of HbA₂: a long way to succeed

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Università degli Studi di Milano

* Why HbA₂ is important
* State of the art
* Activities of the IFCC WG-HbA₂
* Reducing inter-laboratory variability
* Future perspectives
* Why HbA₂ is important
* State of the art
* Activities of the IFCC WG-HbA₂
* Reducing inter-laboratory variability
* Future perspectives

**contents**

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**World Distribution, Population Genetics, and Health Burden of the Hemoglobinopathies**

Thomas N. Williams¹ and David J. Weatherall²

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

<table>
<thead>
<tr>
<th>Disorder</th>
<th>Annual Number of Births</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-thalasemia major</td>
<td>22,989</td>
</tr>
<tr>
<td>HbE/β thalasemia</td>
<td>19,128</td>
</tr>
<tr>
<td>HbH disease</td>
<td>9,568</td>
</tr>
<tr>
<td>Hb Bart's hydrops (α²/α²)</td>
<td>5,183</td>
</tr>
<tr>
<td>SS disease</td>
<td>217,331</td>
</tr>
<tr>
<td>S/β thalasemia</td>
<td>11,074</td>
</tr>
<tr>
<td>SC disease</td>
<td>54,276</td>
</tr>
</tbody>
</table>

¹From available data (Skodell and Harbison 2008; Weatherall 2010).
The role of haemoglobin A\textsubscript{2} testing in the diagnosis of thalassaemias and related haemoglobinopathies

A Mosca,\textsuperscript{1} R Paleari,\textsuperscript{1} G Ivaldi,\textsuperscript{1} R Galienello,\textsuperscript{2} P C Giordano\textsuperscript{*}

**Review**

- Hemoglobin A\textsubscript{2} (HbA\textsubscript{2})
  - Reduced (< 2.3%)
  - Normal (2.3% - 4.6%)
  - Increased (> 4.6%)
-MCV, MCH
  - Normal
  - Reduced
  - Normal or increased
- HbF
  - < 1%
  - 1 - 3%
  - > 3%
- Iron markers
  - Normal
  - Increased
  - Decreased
- Possible diagnosis
  - Iron deficiency
  - 2 or more gene defects
  - Thalassemia
  - Family history
  - That can trigger Thalassemia
  - alpha Thalassemia
  - beta Thalassemia

Molecular confirmation
- Q-PCR
  - a or b sequence
  - a and b sequence
- C-PCR
  - a or b sequence
  - a and b sequence
- Normal HbA\textsubscript{2} / no major gene implications
- Hb-E variant
- Hb-D variant
- Hb-M variant

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Andrea Mosca, Renata Paleari, Barbara Ivaldi, on behalf of the IFCC Working Group on Standardization of HbA\textsubscript{2}

**Analytical goals for the determination of HbA\textsubscript{2}**

<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 HbA\textsubscript{2} measurement derived from data on biologic variation.
* Why HbA₂ is important
* State of the art
* Activities of the IFCC WG-HbA₂
* Reducing inter-laboratory variability
* Future perspectives

Interlaboratory comparison of current high-performance methods for HbA₂
R. PALEARI*, B. GULBIS†, F. COTTON†, A. MOSCA*

International Journal of Laboratory Hematology
The Official Journal of the International Society for Laboratory Hematology
* Why HbA$_2$ is important
* State of the art
* Activities of the IFCC WG-HbA$_2$
* Reducing inter-laboratory variability
* Future perspectives

IFCC
Scientific Division
Standardisation of Haemoglobin A2 (WG-HbA$_2$)

A. Mosca - UniMI
1. Definition of a reference measurement procedure using mass spectrometry associated with proteolytic degradation

Approved IFCC Reference Method for the Measurement of HbA1c in Human Blood

International Federation of Clinical Chemistry and Laboratory Medicine (IFCC)

Scientific Division
Working Group on HbA1c Standardisation and Network of Reference Laboratories for HbA1c

Prepared for publication by
Jan-Olof Jöpppe1, Uwe Kobal2, John Bar1, Andreas Pfaller1, Wladimir Hidalgo3, Fred Hochheinzel1, Ker skyde3, Andreas Mosca2, Michael Mau1, Rita Parent1, Linda Thompson1, Massi Umemoto5 and Cas Weyhampen6

\[ \text{Figure 1: An overview of the reference method procedure} \]

\[ \text{Figure 2: Principle of the proteolytic digestion of HbA1c chains} \]
Development of the methods

- Choice of the proteolytic enzyme (endoproteinase Lys C, Trypsin)
- Definition of digestion protocol (denaturation step with acetonitrile, trifluoroethanol, rapigest, digestion time, temperature, time course)
- Choice of marker peptides (δT2, δT3, δT14, αT4, αT5, αT11)
- Choice of column (Tosoh TSK gel, Zorbax)
- Analytical condition
- ESI-MS detection (double-charge, mono-charge)

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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

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recombinantly expressed, intact
HbA$_2$ and $^{15}$N-labeled HbA$_2$
HbA$_0$ and $^{15}$N-labeled HbA$_0$

The metrological traceability of measurement using the HbA$_2$ and HbA$_0$ 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

Paleari et al, Clin Chima Acta 2017;467:21-6
Arsene et al, Clinica Chimica Acta 2018

Repeatability and within-lab precision of HbA2 determination using IDMS (EP15-A3)

Table 2: repeatability and within-lab precision of HbA2 determination using IDMS

<table>
<thead>
<tr>
<th>day</th>
<th>aliquot 1</th>
<th>aliquot 2</th>
<th>aliquot 3</th>
<th>mean</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HbA2 (%)</td>
<td>HbA2 (%)</td>
<td>HbA2 (%)</td>
<td>HbA2 (%)</td>
<td>HbA2 (%)</td>
</tr>
<tr>
<td>1</td>
<td>2.95</td>
<td>3.00</td>
<td>2.91</td>
<td>2.95</td>
<td>1.53</td>
</tr>
<tr>
<td>2</td>
<td>2.96</td>
<td>2.95</td>
<td>3.01</td>
<td>2.97</td>
<td>1.08</td>
</tr>
<tr>
<td>3</td>
<td>2.94</td>
<td>2.91</td>
<td>3.02</td>
<td>2.96</td>
<td>1.92</td>
</tr>
<tr>
<td>4</td>
<td>2.99</td>
<td>2.99</td>
<td>3.07</td>
<td>3.02</td>
<td>1.53</td>
</tr>
<tr>
<td>5</td>
<td>3.03</td>
<td>2.97</td>
<td>3.06</td>
<td>3.02</td>
<td>1.52</td>
</tr>
<tr>
<td>sample 1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>repeatability (%)</td>
<td>1.50</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>within-lab precision (%)</td>
<td>1.68</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Correlation between IDMS-results obtained in two laboratories

\[ y = 0.98x + 0.09 \]
\[ r = 0.998 \]

Correlation between IDMS and routine methods

\[ y = 0.93x - 0.14 \]
\[ r = 0.987 \]

\[ y = 0.85x + 0.026 \]
\[ r = 0.989 \]

\[ y = 1.03x - 0.59 \]
\[ r = 0.987 \]

\[ y = 0.60x + 1.05 \]
\[ r = 0.975 \]
2. Preparation of a secondary reference material for hemoglobin A\textsubscript{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 O\textsubscript{2} to limit oxidation
- accelerated degradation experiments
- Long term stability
No unexpected peaks due to preparation/lyophilization process

Good commutability

Stability of the lyophilized material

Storage
Temp = -20 °C

Storage
Temp = +4 °C
* Why HbA₂ is important
* State of the art
* Activities of the IFCC WG-HbA₂
* Reducing inter-laboratory variability
* Conclusions and future perspectives

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**contents**

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Calibration by commutable control materials is able to reduce inter-method differences of current high-performance methods for HbA².*

<table>
<thead>
<tr>
<th>WHO</th>
<th>Lyphochek 1</th>
<th>Lyphochek 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td><img src="image1.png" alt="Image" /></td>
<td><img src="image2.png" alt="Image" /></td>
</tr>
<tr>
<td>2</td>
<td><img src="image3.png" alt="Image" /></td>
<td><img src="image4.png" alt="Image" /></td>
</tr>
<tr>
<td>3</td>
<td><img src="image5.png" alt="Image" /></td>
<td><img src="image6.png" alt="Image" /></td>
</tr>
<tr>
<td>4</td>
<td><img src="image7.png" alt="Image" /></td>
<td><img src="image8.png" alt="Image" /></td>
</tr>
<tr>
<td>5</td>
<td><img src="image9.png" alt="Image" /></td>
<td><img src="image10.png" alt="Image" /></td>
</tr>
<tr>
<td>6</td>
<td><img src="image11.png" alt="Image" /></td>
<td><img src="image12.png" alt="Image" /></td>
</tr>
<tr>
<td>7</td>
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<tr>
<td>8</td>
<td><img src="image15.png" alt="Image" /></td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>RP 1</th>
<th>RP 2</th>
<th>RP 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td><img src="image17.png" alt="Image" /></td>
<td><img src="image18.png" alt="Image" /></td>
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<tr>
<td>2</td>
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</tr>
<tr>
<td>3</td>
<td><img src="image21.png" alt="Image" /></td>
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<tr>
<td>4</td>
<td><img src="image23.png" alt="Image" /></td>
<td><img src="image24.png" alt="Image" /></td>
</tr>
<tr>
<td>5</td>
<td><img src="image25.png" alt="Image" /></td>
<td><img src="image26.png" alt="Image" /></td>
</tr>
<tr>
<td>6</td>
<td><img src="image27.png" alt="Image" /></td>
<td><img src="image28.png" alt="Image" /></td>
</tr>
<tr>
<td>7</td>
<td><img src="image29.png" alt="Image" /></td>
<td><img src="image30.png" alt="Image" /></td>
</tr>
<tr>
<td>8</td>
<td><img src="image31.png" alt="Image" /></td>
<td><img src="image32.png" alt="Image" /></td>
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</tbody>
</table>

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A. Mosca - UniMI
Why HbA₂ is important

State of the art

Activities of the IFCC WG-HbA₂

Reducing inter-laboratory variability

Future perspectives

Table 3
Overall variability of HbA₂ results before (raw) and after (calib) common calibration of the different methods by RP1 and RP2 materials.

<table>
<thead>
<tr>
<th>Group 1</th>
<th>Group 2</th>
<th>Group 3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HbA₂</td>
<td>Raw</td>
<td>Calib</td>
</tr>
<tr>
<td>2.46</td>
<td>2.46</td>
<td>2.46</td>
</tr>
<tr>
<td>0.17</td>
<td>0.08</td>
<td>0.17</td>
</tr>
<tr>
<td>6.6</td>
<td>3.4</td>
<td>6.6</td>
</tr>
</tbody>
</table>
* 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)

* Definition of new reference intervals for HbA$_2$ (?)

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

* Outcome: screening procedures to be optimized, more careful requirements for molecular analysis

**Next steps**

* IFCC WG members (R. Paleari, C, Arsene, P, Kaiser)
* C. Hartefeld (Leiden University, NL)
* I. Zegers, H. Schimmel (JRC, BE)

**Acknowledgements**