Standardization of hemoglobin A2: does HbA1c history repeat itself?

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Dip. Scienze e Tecnologie Biomediche
Outline

• Clinical relevance
• State-of-the-art of HbA₂ measurements
• IFCC HbA₂ standardization
• Conclusions
Clinical relevance

1.7 % of the world’s population is carrying thalassemic genes

- β-thal
  - Mediterranean regions: up to 8 %
  - Middle East: up to 10 %
  - India: 3 – 15 %
  - Southeast Asia: up to 9 %
Hb A₂ reference intervals
(2SD, Tietz)

normals: 1.5 - 3.5 %
β-thal trait: 3.7 - 7.0 %

Hb A₂ reference intervals
(Menarini HA8160)

normals: < 3.2 %
borderline: 3.3 - 3.8 %
β-thal trait: >3.8 %
Incidence of HbA₂ “borderline”
(between 3.3 and 3.7 %)
N = 194 over 8514 (2.3 %)

MCV <80 fL and Hb below the reference interval:
156 over 8514 (1.8 %)
## Genotype of 234 (over 1743) subjects with HbA₂ borderline

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Count</th>
</tr>
</thead>
<tbody>
<tr>
<td>NEG/-α3.7</td>
<td>2</td>
</tr>
<tr>
<td>NEG/IVS 1 nt 6</td>
<td>20</td>
</tr>
<tr>
<td>β*+δCd 27</td>
<td>7</td>
</tr>
<tr>
<td>NEG/ααα anti3,7</td>
<td>10</td>
</tr>
<tr>
<td>Hb Variants**</td>
<td>3</td>
</tr>
<tr>
<td>Cap +1570</td>
<td>1</td>
</tr>
<tr>
<td>β prom. (-101; -92)</td>
<td>10</td>
</tr>
</tbody>
</table>

* β-thal mutations: β 039, IVS I nt 1, IVS I nt 110
** Hb Variants: Hb Acharnes (cd 53 GCT>ACT); Hb Kokomo (cd 74 GGC>AGC); Hb Ernz (cd 123 ACC>AAC)
Genetic counselling

- HbA₂ borderline subjects should be always investigated in couples at risk
State-of-the-art of HbA$_2$ measurements

- EQAS data
EQAS – So.S.T.E.

N = 48 HPLC
April-June 2005

SOSTE, VEQ HbA₂
(distribuzione delle misure, maggio 2005)
Table 2 HbA$_2$ results reported in the study and grouped for method.

<table>
<thead>
<tr>
<th>HPLC systems</th>
<th>Sample</th>
<th>HbA$_2$, %</th>
<th>CV</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Mean</td>
<td>SD</td>
<td>Min</td>
</tr>
<tr>
<td><strong>D10</strong></td>
<td>A</td>
<td>2.6</td>
<td>0.2</td>
<td>2.3</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>5.1</td>
<td>0.3</td>
<td>4.6</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>3.9</td>
<td>0.2</td>
<td>3.6</td>
</tr>
<tr>
<td><strong>Variant</strong></td>
<td>A</td>
<td>2.6</td>
<td>0.2</td>
<td>2.0</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>5.0</td>
<td>0.2</td>
<td>4.5</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>3.9</td>
<td>0.2</td>
<td>3.4</td>
</tr>
<tr>
<td><strong>Variant II beta-thal</strong></td>
<td>A</td>
<td>2.6</td>
<td>0.2</td>
<td>2.2</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>4.9</td>
<td>0.4</td>
<td>4.2</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>3.8</td>
<td>0.3</td>
<td>3.2</td>
</tr>
<tr>
<td><strong>Variant II dual kit</strong></td>
<td>A</td>
<td>2.4</td>
<td>0.1</td>
<td>2.2</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>5.0</td>
<td>0.3</td>
<td>4.6</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>3.8</td>
<td>0.3</td>
<td>3.1</td>
</tr>
<tr>
<td><strong>All methods</strong></td>
<td>A</td>
<td>2.5</td>
<td>0.2</td>
<td>2.0</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>5.0</td>
<td>0.3</td>
<td>4.2</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>3.8</td>
<td>0.3</td>
<td>3.1</td>
</tr>
</tbody>
</table>
HbA₂, analytical goals

\[ TE = 1.65 \times CV_a + \frac{1}{4}(CV_i^2 + CV_G^2)^{1/2} \]

\[ CV_i = 2.8 - 3.4 \% \rightarrow 3.1 \% \]
\[ CV_G = 20 \% \]

\[ CV_a = 1.4 - 1.7 \% \rightarrow 1.6 \% \text{ (goal for imprecision)} \]

HbA₂ “true value”: 3.0 %
“acceptable” measured value: 2.8 – 3.2 %
campioni A 1 outlier
15/47 (31.9 %): bias > 7.8 %

Laboratorio (N. progressivo)

bass. %

-20
-10
0
10
20

A. Mosca

CIRM
Università degli Studi di Milano

A. Mosca

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IFCC HbA$_2$ standardization

• To prepare a reference material for hemoglobin A$_2$ in conjunction with IRMM.
# IFCC WG-HbA₂ Membership

<table>
<thead>
<tr>
<th>Name</th>
<th>Position</th>
<th>Country</th>
<th>Term</th>
<th>Time in Office</th>
</tr>
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<tbody>
<tr>
<td>A. Mosca</td>
<td>Chair</td>
<td>IT</td>
<td>1st</td>
<td>2004 01 – 2006 12</td>
</tr>
<tr>
<td>E. Bissé</td>
<td>Member</td>
<td>DE</td>
<td></td>
<td></td>
</tr>
<tr>
<td>D. Caruso</td>
<td>Member</td>
<td>IT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B. Green</td>
<td>Corp. Rep.</td>
<td>UK</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A. Van Dorsselaer</td>
<td>Member</td>
<td>FR</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B. Wild</td>
<td>Member</td>
<td>UK</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
IFCC Reference System for HbA$_2$

- **Primary reference materials** (pure HbA$_0$ and HbA$_2$)
- **Secondary reference materials** (lyophilized)
- **Manufacturer's internal reference measurement procedure**
- **Manufacturer's working calibrator**
- **Manufacturer's product calibrator**
- **Patient Sample**

**IFCC Reference System**

- **IFCC reference measurement procedure** (HPLC-MS)
- **Manufacturer's standing measurement procedure**
- **Routine measurement procedure**

**Manufacturer**

**Individual laboratory**
Primary Reference Materials

- Pure HbA_0 and HbA_2 (three batches produced so far)
- Liquid solutions (buffer with sucrose) at -80 °C
- Available c/o Dept. Science and Biomedical Technology, University of Milano
- Small aliquots will be transferred to IRMM
Preparation of pure HbA₀ and HbA₂

Whole blood

3x wash

RBC

hypotonic lysis

hemolysate

Sephadex G25

DE-52

HbA₂

HbA₀

3x wash

A. Mosca
Tests for assessing purity of the primary calibrators

- HPLC
- IEF
- Capillary EF
- ESI-MS
PolyCATA HPLC analysis (EB)

A. Mosca
ESI-MS of purified HbA₀ and HbA₂

Impurities < 1 %

Mw Measured  | Mw Predicted
---|---
Alpha | 15126,22  | 15126,38
Beta | 15867,15  | 15867,24

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Candidate Reference Measurement Procedure for HbA$_2$

**Principle**

HbA$_2$ ratio to whole hemoglobin is determined as ratio of a delta chain specific peptide to an alpha chain specific peptide.

Peptides are obtained by treating total red blood cell lysate with trypsin.

Peptide mixture is analyzed by HPLC-ESI/MS.

Calibration is performed against primary calibrators made of mixtures of HbA$_2$ and HbA$_0$ primary reference materials.
A. Mosca

blood

↓

erthrocytes

↓

hemolysate

↓

enzymatic cleavage with trypsin

↓

HPLC - Mass spectrometry

↓

quantification of specific peptides

\[ \delta T2 \text{ (TAVNALWGK) or } \delta T14 \text{ (EFTPQMQAAYQK)} \]

\[ \alpha T11 \text{ (VDPVNFK)} \]
Optimization

1) The method has been optimized with regard to several steps (digestion conditions, separation of tryptic peptides by RP-HPLC, determination of LOD and QOD, MS tuning, etc.)

2) A comparison between the results obtained in 2 MS labs has been performed in two steps during 2006

3) The SOP has been defined

4) Another comparison is planned in 2007

5) Further development to be discussed
HPLC conditions optimised to separate specific peptides for α and δ chains:

HPLC instrument: Agilent 1100 Chemstation, UV detector (MicroCell: 1µl)

**Column**: Tosoh TSKgel super ODS 2µm (2mmx10cm)

Gradient: 2% B à 40% B en 76 min Flow: 0.55ml/min (T=40°C)
Selected peptides

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence</th>
<th>RT</th>
<th>MH+</th>
<th>M2H+</th>
</tr>
</thead>
<tbody>
<tr>
<td>αT11</td>
<td>VDPVNFK</td>
<td>14.02 min</td>
<td>818.4</td>
<td>409.3</td>
</tr>
<tr>
<td>δT2</td>
<td>TAVNALWGK</td>
<td>20.19 min</td>
<td>959.5</td>
<td>480.6</td>
</tr>
<tr>
<td>δT3</td>
<td>VNVDAVGGEALGR</td>
<td>18.59 min</td>
<td>1256.7</td>
<td>629.1</td>
</tr>
<tr>
<td>δT14</td>
<td>EFTPQMQAAYQK</td>
<td>16.56 min</td>
<td>1441.4</td>
<td>721.5</td>
</tr>
</tbody>
</table>
2006 exercise
6 calibrators
29 samples
2 digestions, 2 analyses per digestion

$\delta T2 \ / \ \alpha T11$

All samples - July 2006

Fig. 01 all

$y = 0.9436x + 0.11$

$n = 28$

$r = 0.9907$
2007 exercise
6 calibrators
3 controls
20 samples
2 digestions, 2 analyses per
digestion

\[ y_1 = 1.194x - 0.7 \]
\[ n = 20 \]
\[ r = 0.972 \]

\[ y_2 = 1.178x - 0.7 \]
\[ n = 20 \]
\[ r = 0.974 \]
Reproducibility

<table>
<thead>
<tr>
<th></th>
<th>$\delta T2/\alpha T11$</th>
<th>$\delta T14/\alpha T11$</th>
</tr>
</thead>
<tbody>
<tr>
<td>HbA$_2$, % (CV, %)</td>
<td>2.7 (4.9)</td>
<td>5.5 (6.3)</td>
</tr>
<tr>
<td></td>
<td>2.7 (6.3)</td>
<td>5.6 (8.1)</td>
</tr>
</tbody>
</table>

y = 0.979x + 0.03
n = 20
r = 0.995
[Secondary Reference Materials]

- **Lyophilized hemolysates**
- Process started on May 15, 2007
- Previously tested for stability and commutability against 3 HPLC methods
- Probably available in 2008
### HUMAN SERUM

<table>
<thead>
<tr>
<th>Concentration</th>
<th>Certified value</th>
<th>Uncertainty 2)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>[µg/L]</td>
<td>[nmol/L]</td>
</tr>
<tr>
<td>Progesterone</td>
<td>8.5</td>
<td>26.9</td>
</tr>
</tbody>
</table>

1) The certified value is the concentration of progesterone determined by Isotope Dilution Gas Chromatography coupled to Mass Spectrometry (ID-GC-MS). This value is the unweighted mean of 4 sets of results, independently obtained from 4 laboratories. The material must be reconstituted according to the specified procedure (see below). The certified value is traceable to the International System of Units (SI).

2) The certified uncertainty is the expanded uncertainty estimated in accordance with the Guide to the Expression of Uncertainty in Measurement (GUM). It is expressed with a coverage factor \( k = 2 \), corresponding to a level of confidence of about 95%.
Secondary reference materials for HbA₂ (IRMM)

- **Expression of interest (2006)**
  - Analis
  - Bio-Rad Laboratories
  - Drew
  - Helena
  - Menarini
  - Sebia
  - Tosoh

- **Pilot lyophilization (100 vials, 1 level): November 2007**
- **Working Lyophilization (1500 vials, 2 levels): 2008**
- **IRMM/reference labs activities**
  - Homogeneity, stability, commutability
  - Certification
Project Planning Form

Project Title
IRMM/IFCC-xxx (HbA2)

Project Information
Action Number: 15012
Action Leader: Stefanie Trapmann
Project Responsible: Amalia Muñoz-Piñeiro
Further developments

• **Reference method**
  - Isotopic dilution
  - More reference labs (4?)
  - Establishing a network (?)

• **Implementation**
  - Change of units (mmol/mol Hb) (?)
### Standardization of hemoglobin A2: does HbA1c history repeat itself?

<table>
<thead>
<tr>
<th></th>
<th>HbA$_{1c}$</th>
<th>HbA$_2$</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Primary reference materials</strong></td>
<td>IRMM</td>
<td>Milano</td>
</tr>
<tr>
<td><strong>Reference method</strong></td>
<td>IFCC official</td>
<td>under development</td>
</tr>
<tr>
<td><strong>Secondary reference materials</strong></td>
<td>under dev.</td>
<td>IRMM (PPF)</td>
</tr>
<tr>
<td><strong>Network</strong></td>
<td>implemented</td>
<td>--</td>
</tr>
<tr>
<td><strong>Implementation</strong></td>
<td>under dev.</td>
<td>probably not dramatic</td>
</tr>
</tbody>
</table>
References

• Mosca A. Development of a reference system for HbA2. EARCR, 15th meeting, Muerten (CH), April 2005.


Aknowledgments

Renata Paleari (CIRME, Dip.Sc. Tecnol. Biom., Università degli Studi di Milano)

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  Christine Schaeffer

IFCC Scientific Division

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Cristina Passarello, Antonio Giambona, Aurelio Maggio,
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