Measurand characteristics, Reference measurement procedures and Reference materials for HbA1c

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HbA1c/Glycohemoglobin

* independent parameter of metabolic control
* risk parameter for development of complications
* treatment goal in disease management
* independent cardiovascular risk parameter in non-diabetics

AN IMPORTANT ANALYTE >>>STANDARDISE!!!!
Fast haemoglobin's and HbA1c

1968: Rahbar detects ‘diabetic’ haemoglobin's

1971: Trivelli describes routine method

1978: HbA1c = glycosylated/glycated Hb

In HbA1c is glucose attached to the N-terminal valine of the ß-chain of HbA0.
Schematic ion exchange chromatogram HbA1c
Assay principles for HbA1c - 1

* ion-exchange chromatography based on very small differences in iso-electric point

* affinity chromatography based on covalent binding of cis-diols of glucose in GHb to a boronate matrix

* immune assays based on the antigenic properties of β-N-Val glycation
Definition of the analyte:

1) Cation exchange chromatography measures β-chain glycation of the N-terminal Valine.

2) Affinity chromatography measures ‘total’ glycation (α and β chain, Val and ε-Lys).

3) Immune assays measures β-chain glycation of the N-terminal Val.
**Ion-exchange vs affinity chromatography**

- Glycated fraction of affinity elutes in HbA1a+1b, HbA1c and HbA0
- Part of non-glycated fraction of affinity elutes with HbA1c
- HbA1c by IEC is NOT specific!
About HbA1c assays

* ion-exchange chromatography is not specific, contains up to 40 % non-A1c material. (It’s a chromatographic illusion!)

* affinity chromatography measures in principle all (↑ and β-val and θ-lys) glycated haemoglobin

* immune assays differ in specificity of the monoclonal and/or polyclonal antibodies to the different antigenic sites (4, 6 or 8 aa of β-N-Hb)
Standardisation of HbA1c results

Three approaches:

1 - harmonisation-by-calibration

2 - harmonisation by method comparison

3 - standardisation by a reference system
Harmonisation-by-calibration

[Diagram with labelled points and lines indicating comparison between 'zonder' and 'met']
Harmonisation by method comparison

Based on a careful but conscious chosen method to act as Designated Comparison Method when:

* the analyte is unstable and not available in purified form

* the analyte is heterogeneous

* the analyte is measured by a variety of methods based on different properties of the analyte
Harmonisation schemes based on DCM

- (NGSP) National Glycohemoglobin Standardisation Program, based on BioRex 70 method used in DCCT

- Swedish system, based on a very specific Mono-S ion exchange chromatography

- Japanese system, based on consensus between the two leading IEC companies, now the value obtained with KO500
NGSP vs Mono-S ion exchange

BioRex 70 Ion Exchange Chromatography

Goldstein et al in Clark WL, Larner J, Pohl SL eds
Methods of Diabetes Research Vol 2: 1986:475-504

Mono S Ion Exchange Chromatography

Clin Chem 1986;35:1867-72
The choice of a reference method

- several assay principles used today
- every method has its own drawback

None of the existing methods can be used as an universal reference method, a new reference method had to be developed
ANALYTE QUANTITY SI-UNIT

Value Transfer ↓ Primary Measurement Procedure

PRIMARY REFERENCE MATERIAL (Primary Calibrator) Pure or Purified Analyte

Value Transfer ↓ International Reference Measurement Procedure

SECONDARY REFERENCE MATERIAL (Biological, Matrixed Calibrator) INTERNATIONAL CERTIFIED MATERIAL

Value Transfer ↓ International Reference Measurement Procedure

INDUSTRIAL MASTER CALIBRATOR WORKING CALIBRATOR PRODUCT CALIBRATOR

Value Transfer ↓ Routine Measurement Procedure

PATIENT SAMPLE

International Protocol for Value Assignment

TRACEABLE to SI
Reference System for HbA1c

* Definition of the analyte
* Preparation of pure HbA0 and HbA1c
* Development of reference method
* Installation of a Reference Lab Network
* Preparation of secondary ref. material
Glycation sites in Glycohemoglobin

- In GHb, glycation occurs at
  - $\beta$-N Val 60%
  - $\alpha$-N-Val 6%
  - $\beta$-($\varepsilon$-Lys) 18%
  - $\alpha$-($\varepsilon$-Lys) 16%

- Glycation can occur at 44 different sites in the HbA0 molecule
  - Glycation ratio’s are always the same, despite the ultimate level.
**HbA1c reference system**

HbA1c is defined as β-N-Valine glycated Hb (β-N-(1-deoxy)-fructosyl-haemoglobin).

Reference methods are developed based on peptide mapping of Hb after proteolytic cleavage of haemoglobin by endoprotease Glu-C.

There is no alternative!!
The premisse is:

ratio of glycated to non-glycated hexapeptide

equals

the ratio of β-glycated HbA0 to total HbA0
The Analytical Challenge

Proteolytic cleavage of β-chain (146 amino acids)

HbAo-peptide

Val His Leu Thr Pro Glu Glu Lys Ser

Glu-C

HbA1c-peptide

Glc Val His Leu Thr Pro Glu Glu Lys Ser
blood → erythrocytes → hemolysate → enzymatic cleavage → quantify specific peptides

Method A: HPLC - Mass Spectrometry
Method B: HPLC - Capillary Electrophoresis
Photometric detection of peptides

- HbA0 Hexapeptide
- HbA1c Hexapeptide

Graph showing peaks with labels for HbA0 and HbA1c hexapeptides.
ESI-MS spectra of hexapeptides

Uwe Kobold, Boehringer Mannheim GmbH, Tutzing, Germany.
Two-dimensional separation of N-terminal hexapeptides of hemoglobin

C 18 Chromatography

Capillary Electrophoresis
Comparison HPLC-MS versus HPLC-CE Reference Method
(HPLC-MS 4 reference labs, HPLC-CE 6 reference labs)

$y = 0.997x - 0.005$

$R^2 = 1.000$
The reference methods are calibrated with sets of calibrators, each year a new lot, from the Primary Reference Materials:

> 99.5% pure HbA1c

> 97% pure HbA0

Integrity and purity checked by several and different procedures
The HbA1c Reference System

With the analyte defined, and the method and materials developed, the next step is in the network:

- Method validation
- International method comparison studies
- Value assignment to calibrators
- Implementation
IFCC Network of HbA1c Reference Labs

The main task of the IFCC Network is the reliable assignment of HbA1c target values to reference materials, reference panels of blood samples and control materials which are necessary for the implementation and maintenance of the system.
IFCC Network of HbA1c Reference Labs

State of affairs:

- 13 intercomparison studies performed
- results of HPLC/CE and ESI-MS identical
- stability of the system proven
- value assignment with very low uncertainty
- controls included in every study
Analytical performance of network labs

**Precision Profile Reference Method -**
Intra-Lab Standard Deviation (Florence I and II, 10 Reference Laboratories)

**Precision Profile Reference Methods**
Intra-Lab CV (Results Florence I and II studies, 10 reference laboratories)
Method Comparison Studies

a: 8 method comparison studies were performed with the existing DCM schemes in USA (NGSP), Sweden and Japan.

B: 3 method comparison studies were performed with all the major manufacturers.
About anchoring DCM’s

Needed:

- multiple method comparison
- participation of the whole network on both sides
- statistical validation of the master equation

Ends with very precise value assignment to IFCC SRM and DCM materials.
Master equation (Marrakech)
Correlation between IFCC and DCM’s

\[
\begin{align*}
y \text{ HbA1c/NGSP} &= 0.915 \text{ HbA1c/IFCC} + 2.15 \\
y \text{ HbA1c/JDS} &= 0.927 \text{ HbA1c/IFCC} + 1.27 \\
y \text{ HbA1c/MonoS} &= 0.989 \text{ HbA1c/IFCC} + 0.88
\end{align*}
\]

Converting % HbA1c to mmol/l glucose:

\[
\begin{align*}
\text{NGSP:} & \quad \text{Glu} = 1.98 \times \text{HbA1c/NGSP} - 4.29 \\
\text{IFCC:} & \quad \text{Glu} = 1.84 \times \text{HbA1c/IFCC} - 0.01
\end{align*}
\]
X-mean SRL's DCCT vs individual SRL DCCT calibrated

- Roche $Y=0.99X + 0.00$
- Primus $Y=1.02X - 0.17$
- Tosoh $Y=0.99X + 0.17$
- Linear ($X=Y$)
Anchoring Manufacturers Methods

- blood panels with assigned values
- different certified reference material according to assay design and principle
- manufacturers should use their own in-house calibration principle
- correlation with DCM’s will be provided
- manufacturers asks certification of traceability
Nature of the SRMs

6-8 pools of at least 10 single donations each

no interferences

no Hb-variants, no Hb-derivatives

normal Hb levels

intended range 3-12 (IFCC)%
Measurement System of HbA1c in Japan Society of Clinical Chemistry (July, 199

Human Hemolysate

Primary Reference Material (Primary Calibrator; P.Cal)

Reference Method by IFCC (SOP)

Human Whole Blood

Secondary Reference Material

Standard Method by JSCC (KO500 Procedure)

Manufacturer's Standard Method

Human Whole Blood

Working Reference Material

Manufacturer's Product Calibrator

Field Method
(IFCC Traceable Method)
“diagnostic manufacturers must guarantee the traceability of their routine tests to reference methods and materials of higher metrological order”.

(ISO TCT 212 demands documentation about how to trace back assigned values)
<table>
<thead>
<tr>
<th>Material</th>
<th>Calibration value assignment</th>
<th>Procedure</th>
<th>Implementation</th>
<th>Uncertainty of measurement</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>a) Definition of Si unit by CGPM</td>
<td>b) Primary reference measurement procedure</td>
<td>BIPM, NMI(^a), ARML(^b)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>c) Primary calibrator</td>
<td>d) Secondary reference measurement procedure</td>
<td>BIPM, NMI(^b)</td>
<td>NMI(^a), ARML(^b)</td>
</tr>
<tr>
<td>e) Secondary calibrator</td>
<td>f) Manufacturer’s selected measurement procedure</td>
<td>NMI(^a), ARML(^b), ML</td>
<td>ML</td>
<td>ML</td>
</tr>
<tr>
<td>g) Manufacturer’s working calibrator</td>
<td>h) Manufacturer’s standing measurement procedure</td>
<td>ML</td>
<td>ML</td>
<td>ML</td>
</tr>
<tr>
<td>i) Manufacturer’s product calibrator</td>
<td>j) End-user’s routine measurement procedure</td>
<td>Manufacturer and/or end-user</td>
<td>End-user</td>
<td>End-user</td>
</tr>
</tbody>
</table>

b. Accredited measurement reference laboratory. Such a laboratory may be an independent or a manufacturer’s laboratory.
Figure 6. Results of the Reference Range Study HbA1c
(n = 120; 7 reference laboratories, x = mean value, s = standard deviation)
Translation of good clinical diabetes practice in IFCC HbA1c terms:

non-diabetics \( 3 - 4 \% \text{ HbA1c}^1 \)

target for therapy \( 5 \% \text{ HbA1c}^2 \)

change of therapy \( 6 \% \text{ HbA1c}^2 \)

\(^1\) reference range determined by network
\(^2\) diabetic targets recalculated from DCCT
About the name:

- Glycated Hemoglobin  HbA1c
- Glycated Hemoglobin  GHb
- β Glycated Hemoglobin  β-GHb
- Glycation Index  GI
- Diabetes Risk Number  DRN
- β-1-N-Deoxy Fructosyl Hb  β-DFH

The IFCC WG prefers to keep on the name HbA1c.
We are measuring

Specific β-N terminal glycation of hemoglobin.

Chemical name 1-N-β-deoxyfructosyl hemoglobin

Name it: HbA1c(IFCC)

Unit: mmolHbA1c/mol tot Hb

IS:

30 – 100 in practical life.
Standardisation of Manufacturers

Methods - 1

a) Method Comparison Biorad-HPLC versus IFCC Reference Method

\[ y = 0.979x + 1.650 \]

\[ R^2 = 0.994 \]

b) Comparison Biorad-HPLC (transformed)

\[ y = 0.94x \]

\[ y = 1.06x \]
Method Comparison IFCC Reference Method Versus Standardized Designated Comparison Methods

- Diamat
- Immunoassays
- Primus
- Mono S
- CE
- Menarini
- Tosoh
- Kyoto-Daiichi
- K0500

Equations:
- \( y = 1.03x \)
- \( y = 0.97x \)
- \( y = x \)
Implementation of the Reference System

1. Blood panels (certified reference materials) with assigned values are available for manufacturers for internal calibration.

2. IFCC Reference Method will be introduced as anchor for DCM-systems.

3. Introducing the IFCC system = IFCC numbers.
The IFCC Reference System for HbA1c

- clear definition of the analyte
- primary reference material prepared
- a new reference method developed
- an international network established
- suitability of calibrating routine procedures investigated
- relationship to the old trueness established in order to maintain clinical experience
Figure 1—MPG versus HbA$_{1c}$: $n = 1,439$; $r = 0.82$; \( \text{PG (mmol/l)} = (1.98 \times \text{HbA}_{1c}) - 4.29 \). The dashed line indicates the regression line.
HbA1c & Glucose

HbA1c vs Fasting Glucose

n=36,264

AVG = 1.57HbA1c - 3.54
STD = 0.42HbA1c - 1.05
Risk of Sustained Retinopathy and Mean of HbA1c / IFCC in Type 1 Diabetes (DCCT study)