Haemoglobin A_{1c} analysis: Understanding what is measured is fundamental to interpretation

Crossing Science and Education

Garry John
Norfolk and Norwich University Hospital
& The Norwich Medical School, UEA
UK

An abnormal haemoglobin in red cells of diabetics

Rahbar S

Clin Chim Acta 1968; 22: 296-298

International Federation of Clinical Chemistry and Laboratory Medicine

An abnormal hemoglobin in red cells of diabetics

In a survey carried out on 1200 patients from Tehran University Hospitals, in addition to three rare hemoglobins which are under investigation both in our department here and at the University of Cambridge, two patients also showed an abnormal fast moving hemoglobin fraction : both were suffering from diabetes mellitus. Studies were started to investigate the occurrence of this abnormal fraction in other diabetics, and in 47 cases examined in the last three months, including 11 children with severe diabetes mellitus, the additional fraction was detected. Routine hematological examination according to standard methods[2] gave normal results in the majority of cases.

Electrophoresis of hemoglobin was carried out on cellulose acetate according to Graham and Gruenbaum[3]; the abnormal fraction does not separate well by this method, but there is a broadening of the Hb A band. In starch gel electrophoresis with tris–EDTA–borate buffer pH 8.1 (ref. 1) the additional fraction moves a little faster than Hb A and slower than Hb J (Iran)[8] (Fig. 1).

Agar gel electrophoresis in citrate buffer pH 6.2 by the method of Robinson *et al.*[4] is the method of choice for the separation and demonstration of this fraction which moves in front of Hb A to the cathode in the same position as Hb F (Fig. 2).

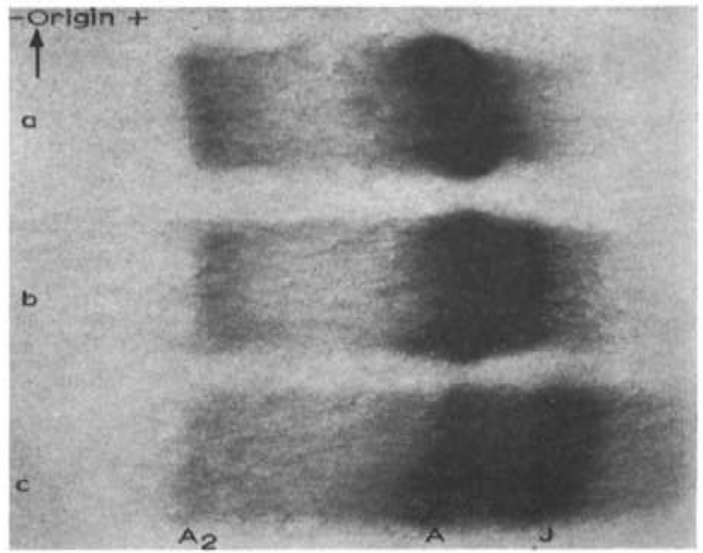


Fig. 1. Starch gel electrophoresis in tris–EDTA–borate buffer, pH 8.1. *o*-Dianisidine stain, ref. 7. a: normal; b: Hb A + Hb x; c: Hb A + Hb J (Iran).

Haemoglobin A_1 was first described in 1955

Kunkel and Wallenius using charge separaration identified a “new” fraction HbA_1. The amino acid sequence of HbA_1 was shown to be identical to HbA_0

The Evolution of the understanding of HbA_{1c} Biochemistry

1955: Kunkel and Wallenius: First description

1957: Rhinesmith *et al:* abnormality present in the N-terminal of the β-chain

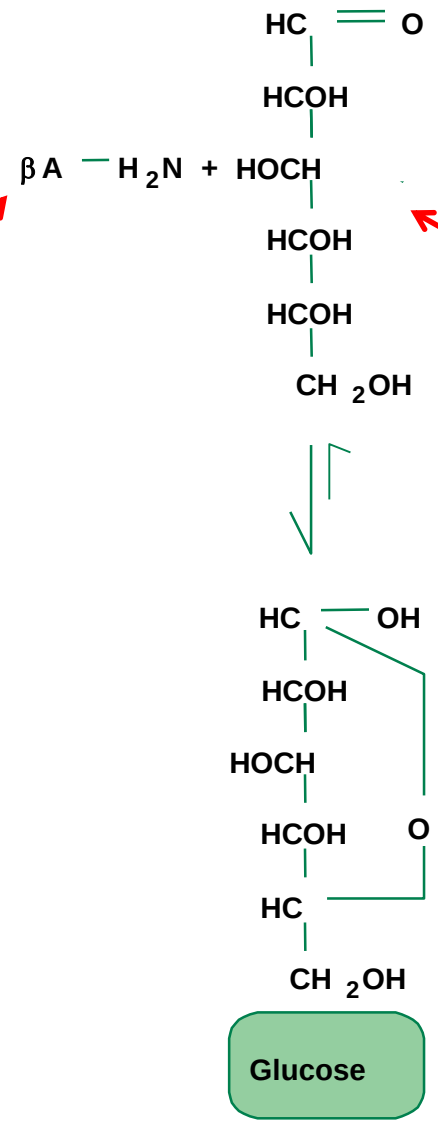
1958: Allen et al: resolved HbA_1 into three fractions HbA_{1a}, HbA_{1b} and HbA_{1c}

1966: Holmquist and Schroeder: concluded that HbA_{1c} is the condensation product (Schiff base) of HbA_0 and a ketone or an aldehyde

1968: Bookchin and Gallop: suggested that the N-terminal structure of the HbA_{1c} globin to be N-1-(deoxyhexitol)-valine

1975: Bunn et al: glucose reacted initially with the amino terminus to form an aldimine linkage; subsequently undergoing an Amadori rearrangement to form a stable ketoamine

1979: Bunn et al: indicated that the **extent of Amadori rearrangement of newly synthesised HbA_{1c} was 60% by 6 days and 91% by 22 days**

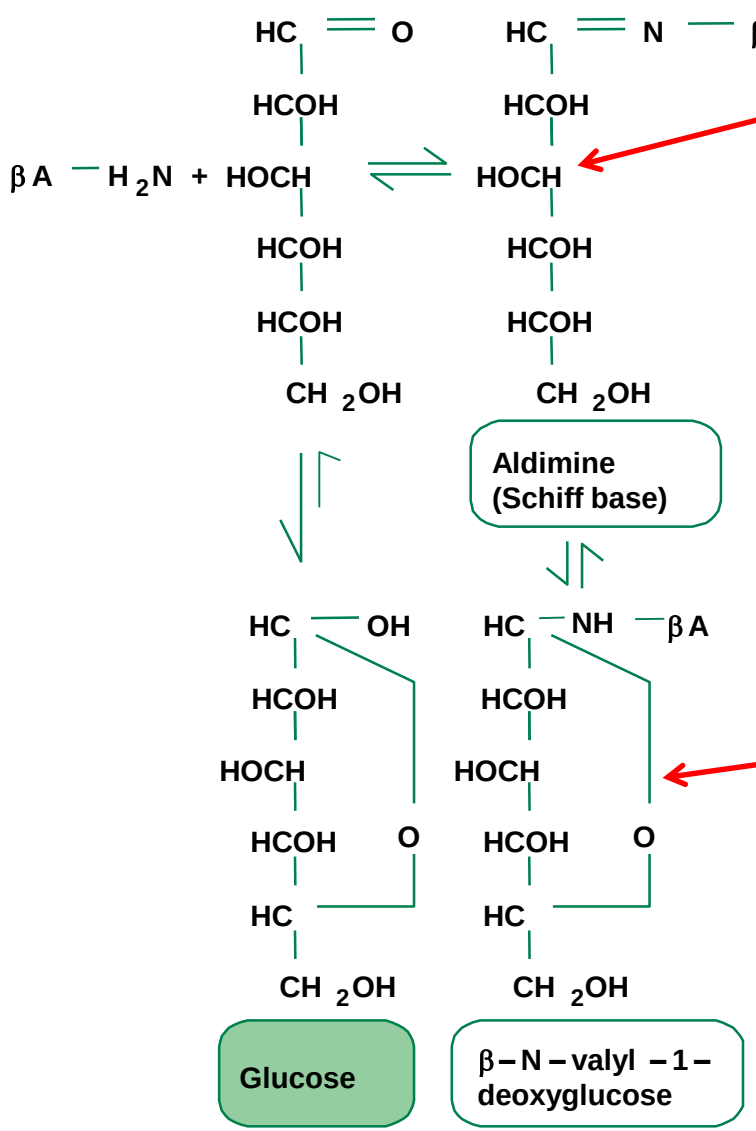


All proteins will react with glucose in the open chain format.

The amino acid Lys is the usual site of reaction: in Hb the N-terminal Val is favoured

Glucose is present in the body in two forms:
open chain format
a ring structure (majority of glucose)

Only glucose in the open chain format will react with protein.

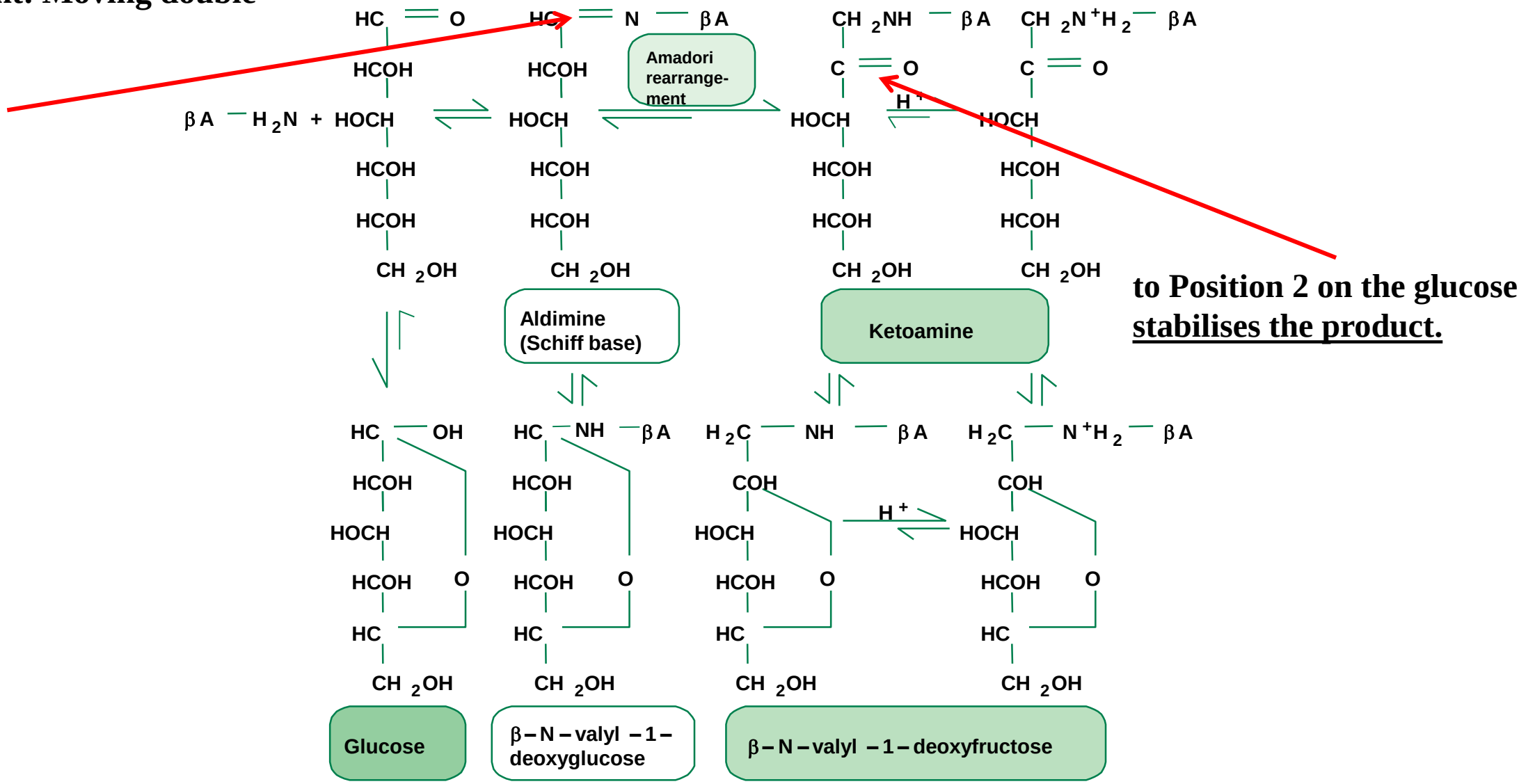


Protein reversibly reacts with the glucose forming a double bond at position 1 on the open glucose form.

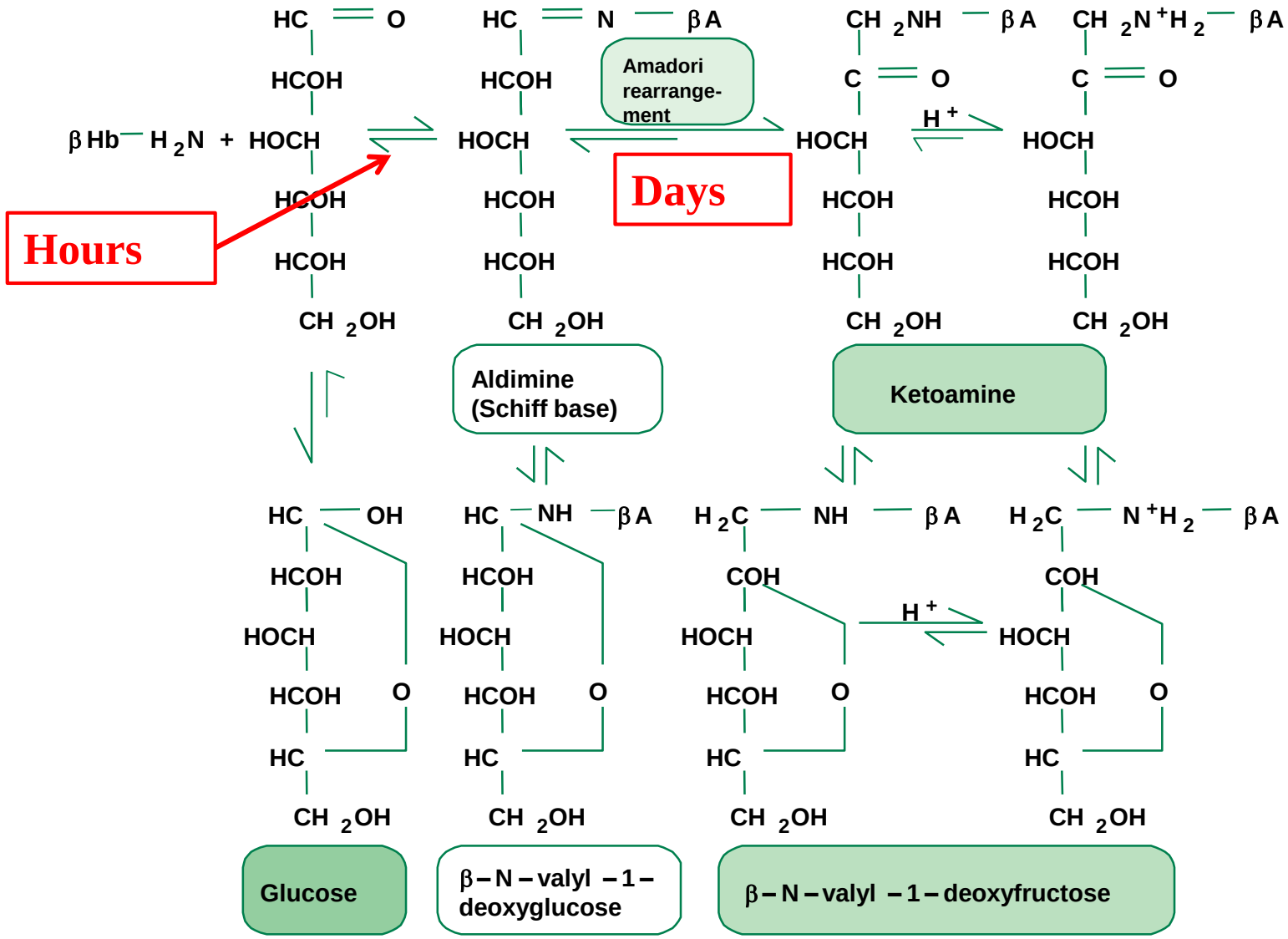
Termed a Shiff Base

The glucose may form a ring structure at this point. No further reaction will take place.

The almost irreversible Amadori rearrangement: Moving double bond from Position 1

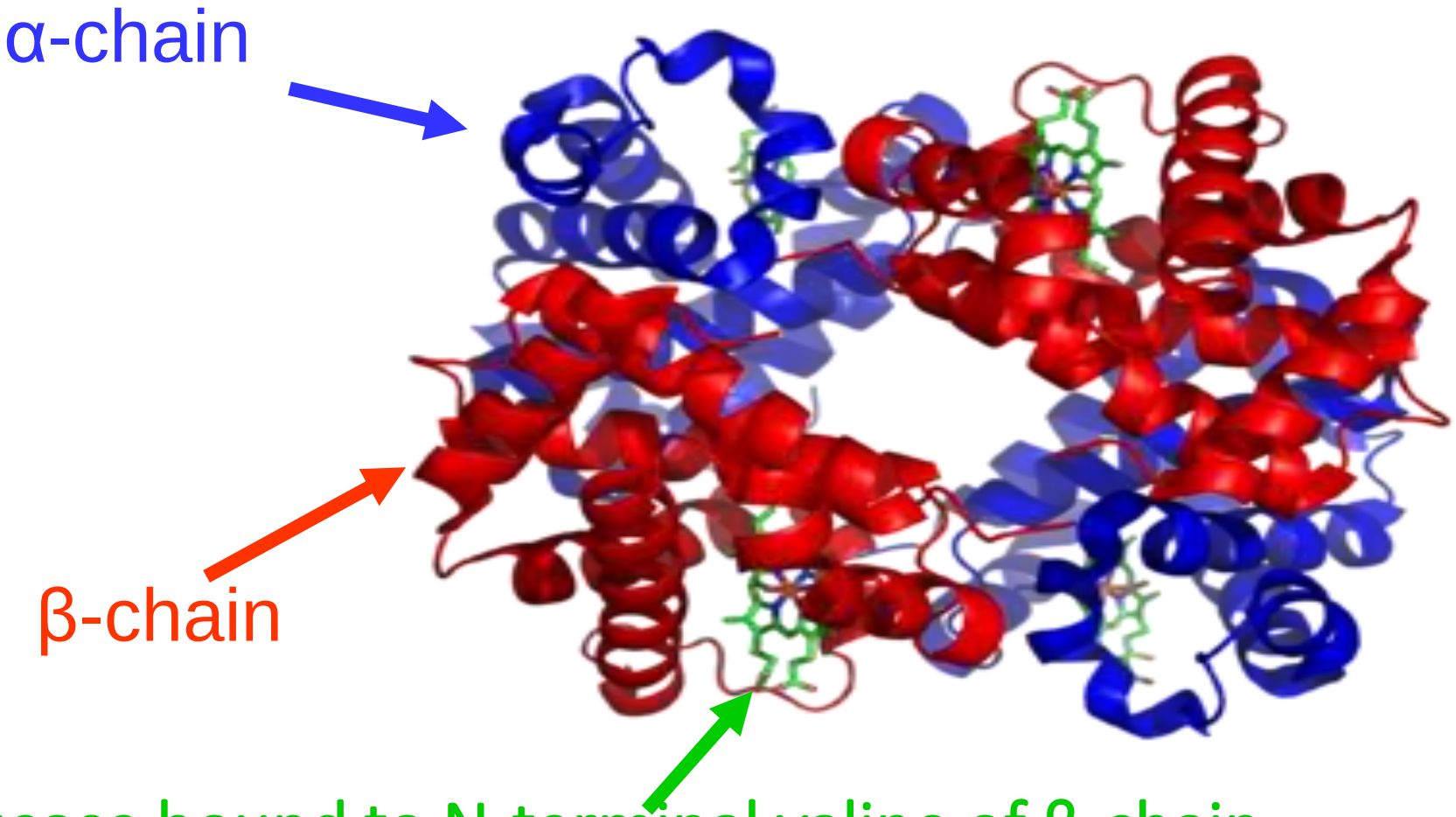


to Position 2 on the glucose <u>stabilises the product.</u>



A Non-enymatic reaction following the Law of Mass Action.

HbA_{1c} – Structure



Early Cation-Exchange Chromatography

1958: Allan *et al*

Described a large column ion-exchange chromatography method for measuring HbA_1

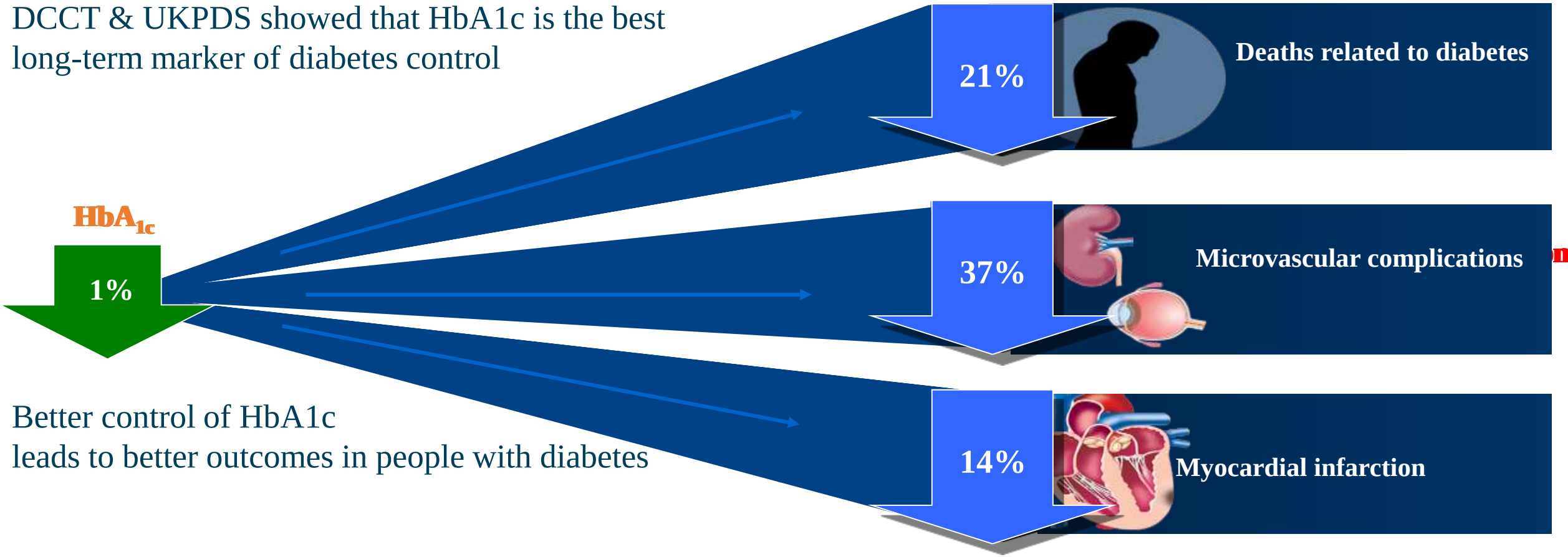
BUT

- Columns were over a meter long
- Over a litre of blood required for separation

Only another half litre of blood then we can measure your HbA_1

Why is HbA_{1c} so Important?

DCCT & UKPDS showed that HbA1c is the best long-term marker of diabetes control



Better control of HbA1c
leads to better outcomes in people with diabetes

Stratton IM, *et al*. *BMJ* 2000; 321:405–412.

The late 70s early 80s

All methods				
n	14	17	16	15
Mean	9.33	6.71	6.74	6.26
SD	1.68	1.35	1.30	0.80
CV%	18.0	20.1	19.3	12.7
Affinity Chromatography				
n	4	6	6	5
Mean	11.25	6.82	6.60	6.30
SD	1.20	2.03	1.96	1.14
CV%	10.6	29.7	29.7	18.1
Electroendosmosis				
n	7	9	8	7
Mean	8.70	6.79	7.01	6.50
SD	1.29	0.95	0.82	0.57
CV%	4.8	14.0	11.7	8.7
Ion-exchange Chromatography				
n	3	2	2	3
Mean	8.23	6.00	6.10	5.63
SD	0.81	0.28	0	0.27
CV%	9.8	4.7	0	5.1

The late 70s early 80s

All methods				
n	14	17	16	15
Mean	9.33	6.71	6.74	6.26
SD	1.68	1.35	1.30	0.80
CV%	18.0	20.1	19.3	12.7
Affinity Chromatography				
n	4	6	6	5
Mean	11.25	6.82	6.60	6.30
SD	1.20	2.03	1.96	1.14
CV%	10.6	29.7	29.7	18.1
Electroendosmosis				
n	7	9	8	7
Mean	8.70	6.79	7.01	6.50
SD	1.29	0.95	0.82	0.57
CV%	4.8	14.0	11.7	8.7
Ion-exchange Chromatography				
n	3	2	2	3
Mean	8.23	6.00	6.10	5.63
SD	0.81	0.28	0	0.27
CV%	9.8	4.7	0	5.1

Range of HbA_{1c} results: 4.4% - 9.7%

Difference between laboratories: 5.3% HbA_{1c}

Range of HbA_{1c} results: 4.3% - 9.4%

Difference between laboratories: 5.1% HbA_{1c}

Range of HbA_{1c} results: 5.9% - 8.8%

Difference between laboratories: 2.9% HbA_{1c}

Harmonisation of HbA_{1c} results

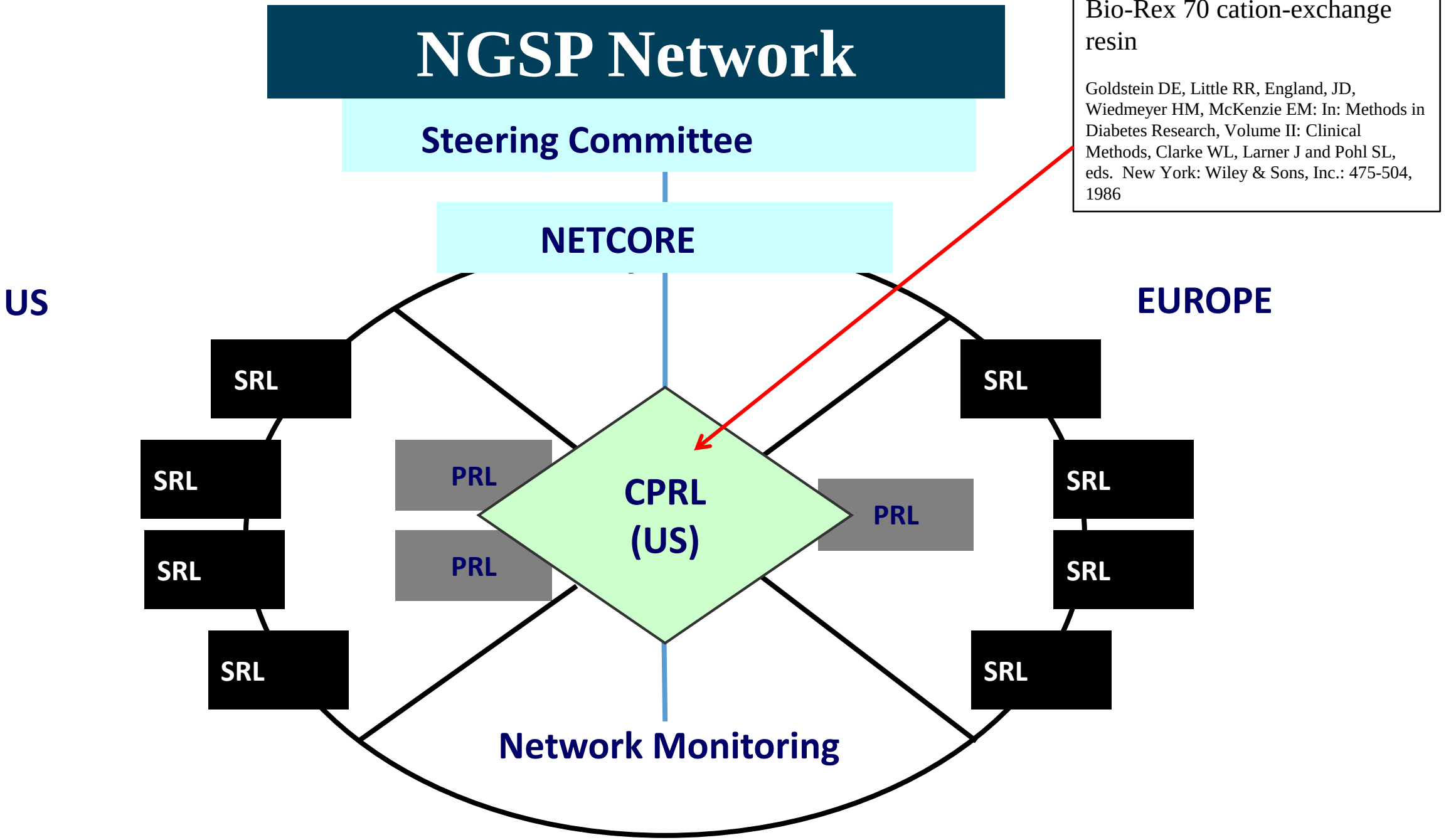
Lack of global standardisation resulted in National Schemes being developed. Notably:

- National Glycohemoglobin Standardization Programme (now NGSP)
- Swedish HbA_{1c} Standardisation Programme
- Japanese Standardisation Programme

The major problems of these National schemes were:

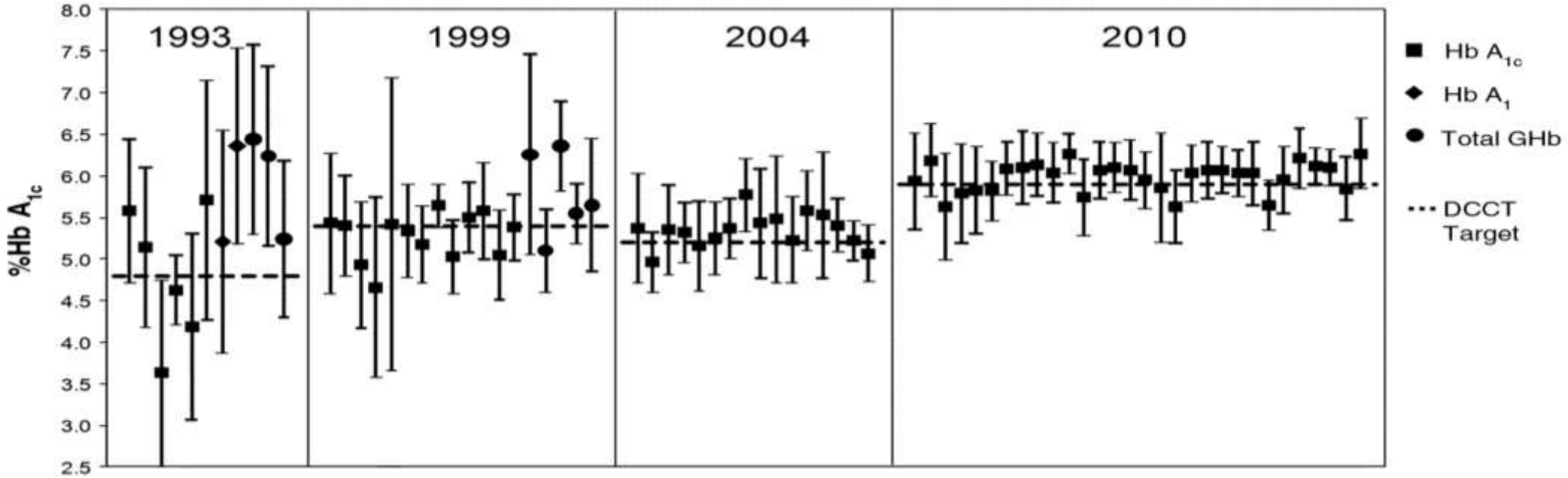
- **The lack of a “true” reference method.**
- **No primary reference material.**

NGSP Network



Bio-Rex 70 cation-exchange resin

Goldstein DE, Little RR, England, JD, Wiedmeyer HM, McKenzie EM: In: Methods in Diabetes Research, Volume II: Clinical Methods, Clarke WL, Larner J and Pohl SL, eds. New York: Wiley & Sons, Inc.: 475-504, 1986



Year	CAP Limit	Year	CAP Limit	Year	CAP Limit
1996	±15%	2009	±10%	2011	± 7%
2008	±12%	2010	± 8%	**2013**	**± 6%**

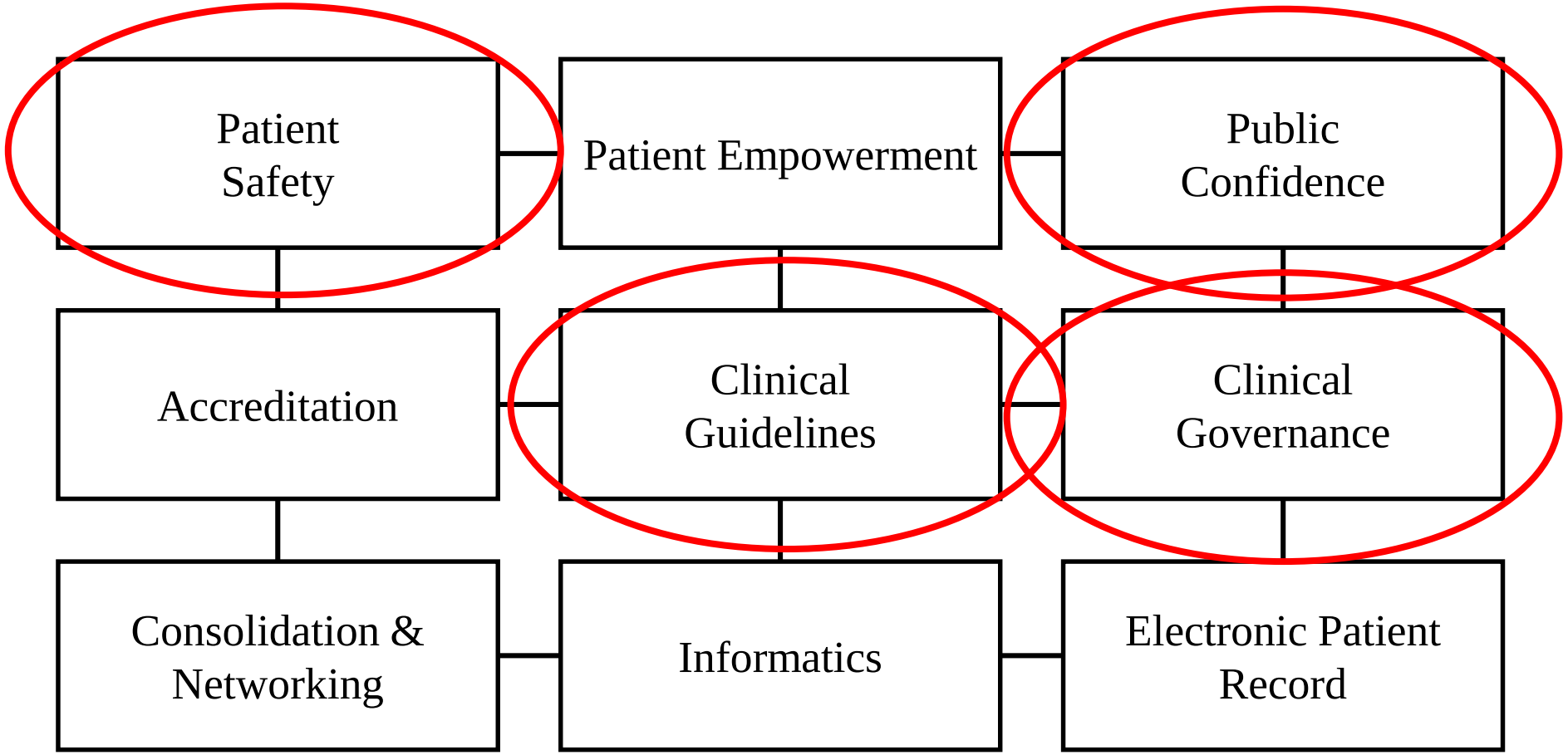
Little et al., Clin Chem 2011;57(2):205-214

Reference Measurement System

Metrological requirements:

- Identification of the “Measurand”
- Reference Measurement Procedure
- Reference materials / laboratories
- Traceability

Why Should We Standardise?



Dr. Graham Beastall, Past President IFCC and Chair JCTLM WG Traceability

Adapted from Plebani, *Clin Chem Lab Med* 2013; **51**: 741-51

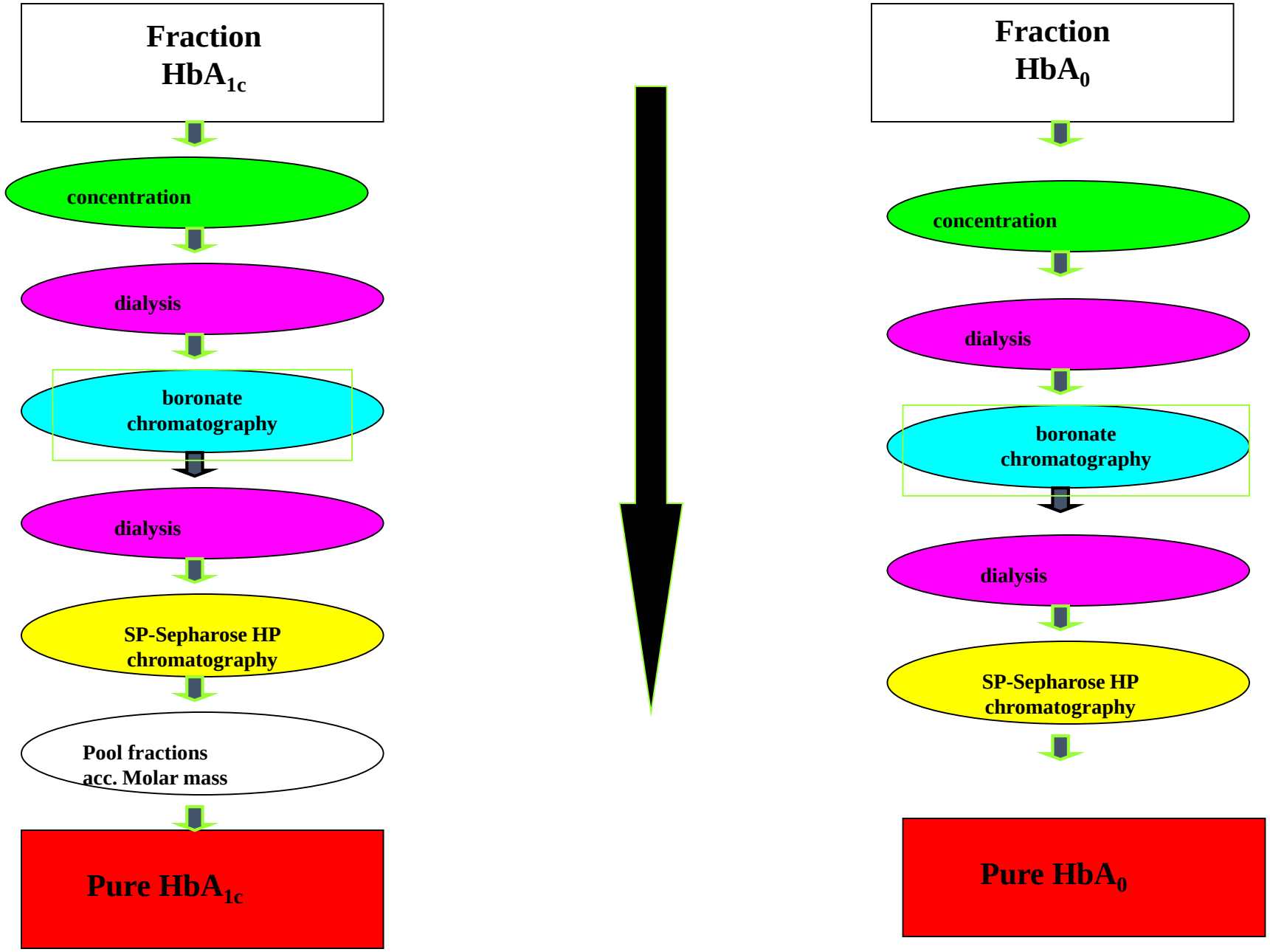
IFCC International Federation of Clinical Chemistry and Laboratory Medicine

Clinical Chemistry
CLCHAU 47 (1) 1–152 (2001)

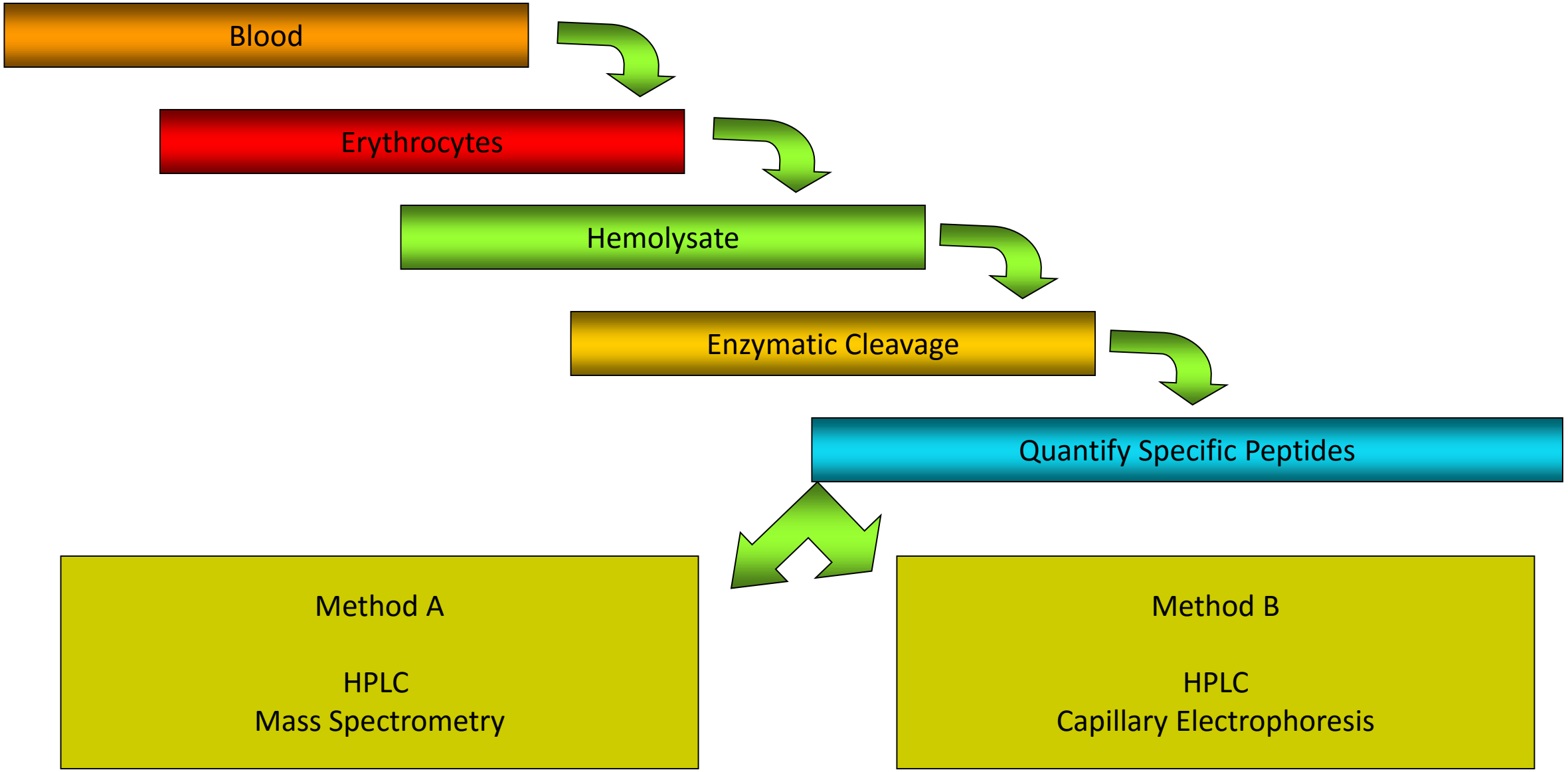
Clin Chem 2008

The IFCC Reference Measurement System for HbA1c: A 6-Year Progress Report

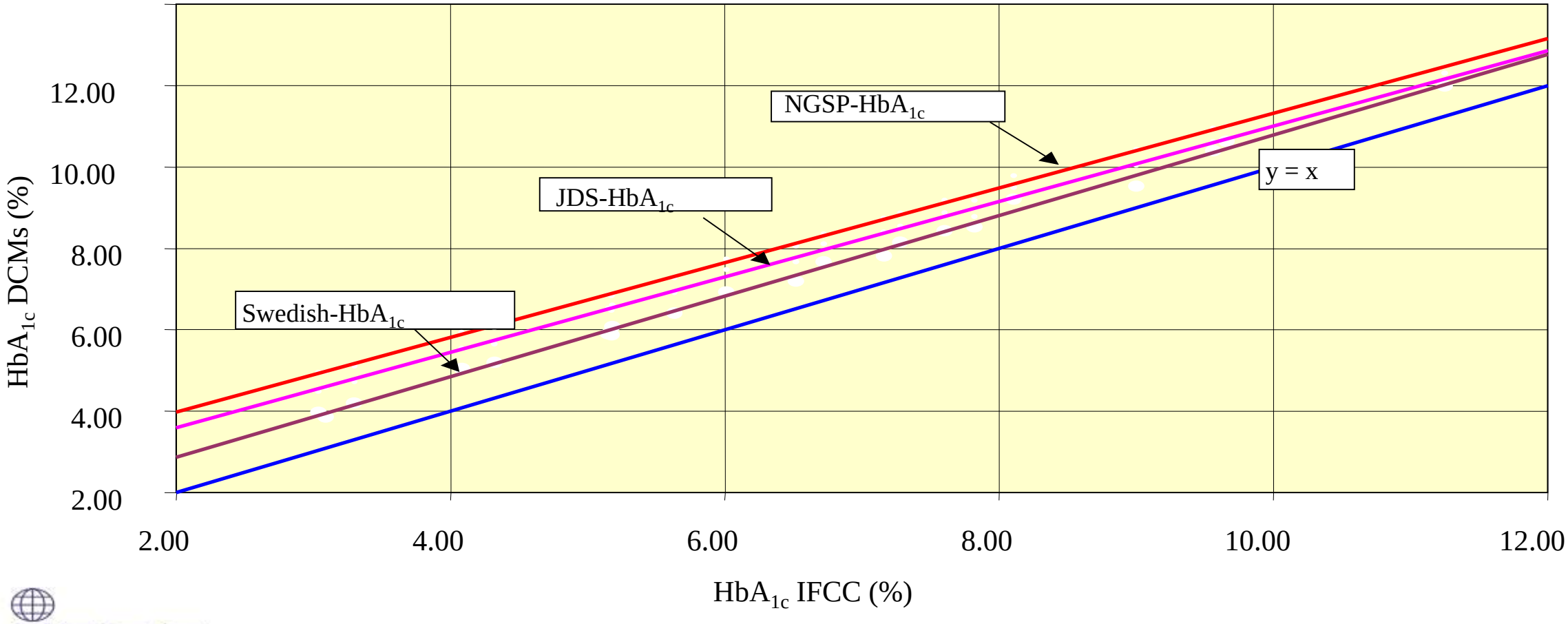
Cas Weykamp (1*), W. Garry John (2), Andrea Mosca (3)
Tadao Hoshino (4), Randie Little (5), Jan-Olof Jeppsson (6)
Kor Miedema (8), Gary Myers (9), Hans Reinauer (10)
David Sacks (11), Robbert Slingerland (8), Carla Siebelder (1)


Fraction HbA1c
concentration
dialysis
boronate chromatography
dialysis
SP-Sepharose HP chromatography
Pool fractions acc. Molar mass
Pure HbA1c
Fraction HbA0
concentration
dialysis
boronate chromatography
dialysis
SP-Sepharose HP chromatography
Pure HbA0

Reference Method



Outcome of increased Specificity of RMP



IFCC International Federation of Clinical Chemistry and Laboratory Medicine

Adapted from Hoelzel *et al Clin Chem* 2004; 50: 166-74

IFCC/IUPAC Committee on Nomenclature, Properties and Units (C-NPU)

Proposed the units for reporting HbA_{1c} should be:

Millimole per mole (mmol/mol)

mmol HbA_{1c} / mol (HbA_0 + HbA_{1c})

IFCC International Federation of Clinical Chemistry and Laboratory Medicine

Consensus Statement on the Worldwide Standardization of the Hemoglobin A1C Measurement

The American Diabetes Association, European Association for the Study of Diabetes, International Federation of Clinical Chemistry and Laboratory Medicine, and the International Diabetes Federation

1. The HbA_{1c} results should be standardized worldwide, including the reference system and results reporting.

2. The IFCC reference system for HbA_{1c} represents the only valid anchor to implement standardization of the measurement.

3. The HbA_{1c} assay results are to be reported worldwide in IFCC unit (mmol/mol) *and* derived NGSP unit (%), using the IFCC-NGSP master equation.

4. If the ongoing "average plasma glucose study" fulfils its *a priori* specified criteria, an HbA_{1c}-derived average glucose (ADAG) value will also be reported as an interpretation of the HbA1c result.

5. Glycaemic goals appearing in clinical guidelines should be expressed in IFCC units, derived NGSP units, and as ADAG.

Diabetes Care 2007;30:2399

Major method principles for Measuring HbA_{1c}

Charge difference

- Cation Exchange HPLC
- Capillary Electrophoresis

Structural difference

- Affinity Chromatography HPLC
- Immunoassay

Chemical difference

- Enzymatic

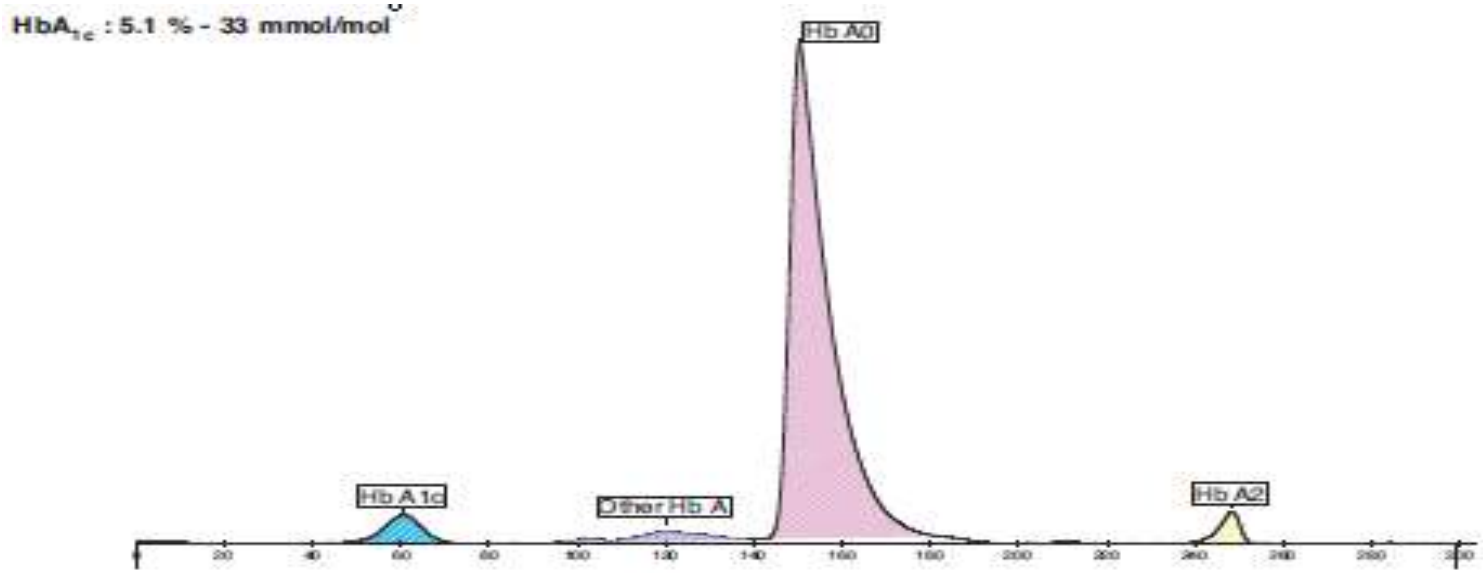
>100 methods/platforms available

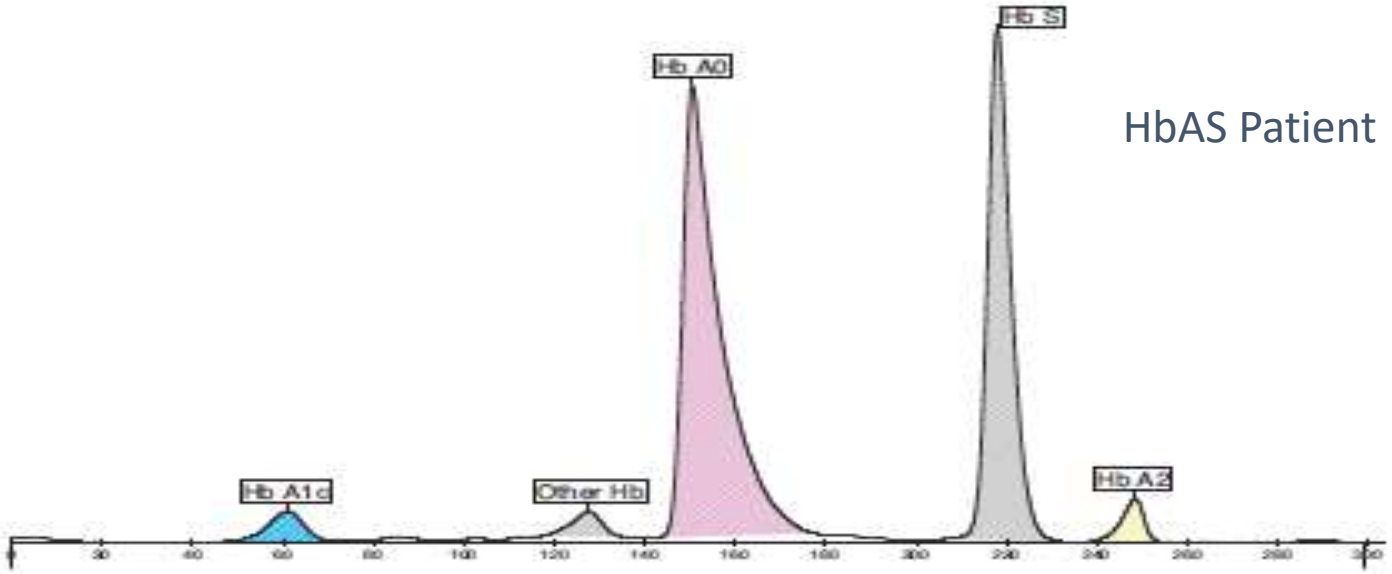
>20 international manufacturers (many more National)

IFCC International Federation of Clinical Chemistry and Laboratory Medicine

Cation-Exchange HPLC

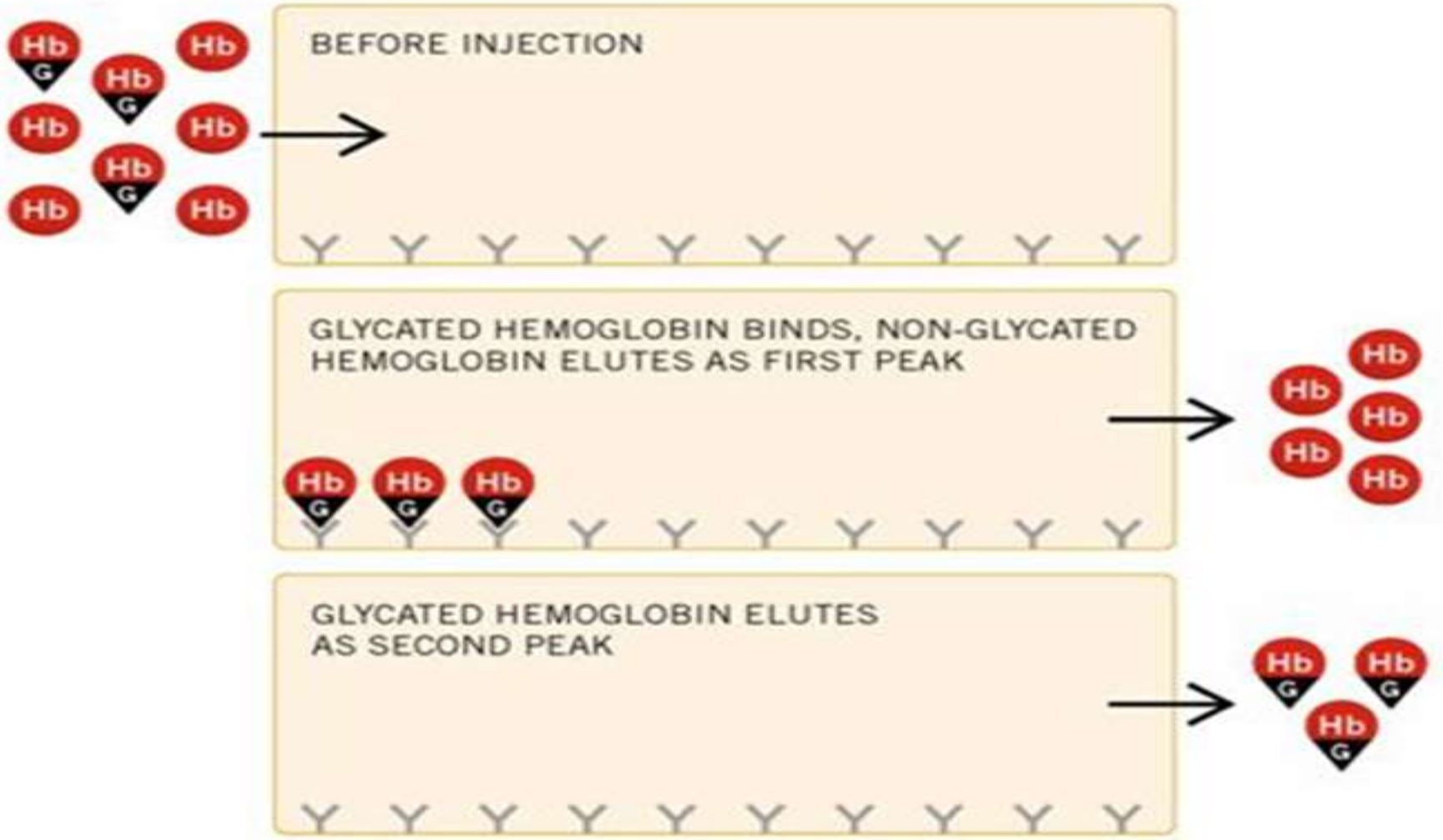
Capillary Electrophoresis



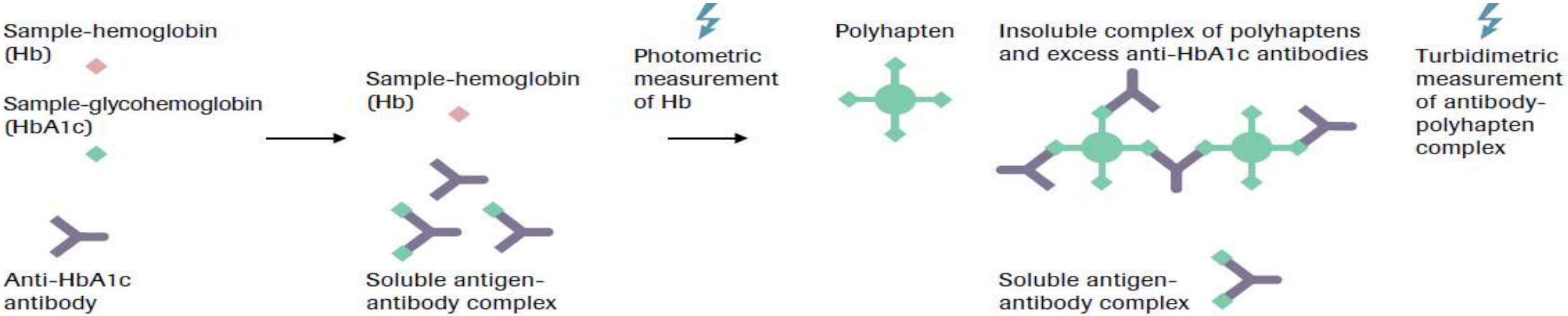


HbAS Patient

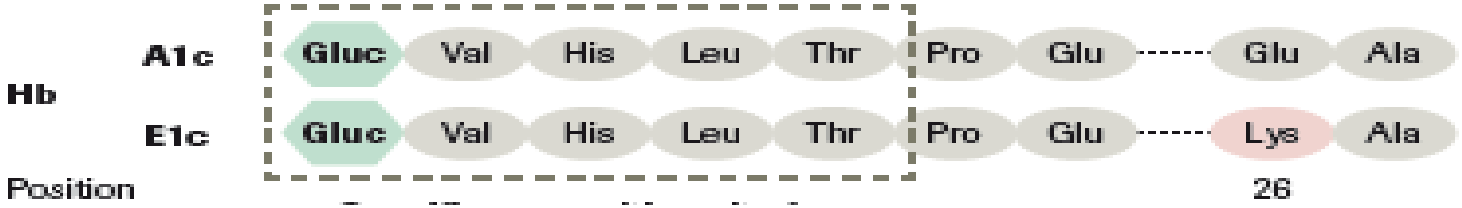
Affinity Chromatography



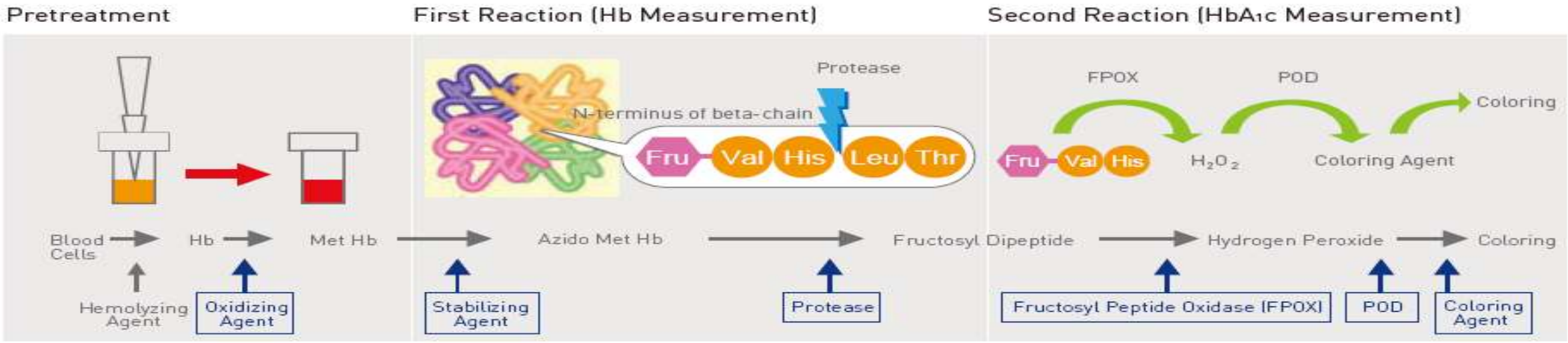
Immunoassay



HbA_{1c} Antibody – 4 amino acids



Enzyme Immunoassay HbA_{1c}



a. Pretreatment (Pretreatment Solution): Whole blood is hemolyzed. Hemoglobin is oxidized with sodium nitrite to produce 'Met Hb'.

b. First Reaction (Reagent 1): Protease is added to cleavage and produce fructosyl dipeptide from the N-terminal of the beta-chain of HbA1c. Sodium azide is added to 'Met Hb' to produce 'Azido Met Hb'. Absorbance at 476 nm of 'Azido Met Hb' is measured to calculate Hemoglobin concentration.

c. Second Reaction (Reagent 2): FPOX is added to Fructosyl Dipeptide to produce Hydrogen Peroxide. The Hydrogen Peroxide reacts with the coloring agent DA-67 in the presence of POD to develop color. The change in absorbance at 660 nm is measured to calculate HbA1c concentration.

Whole blood specimens are lysed automatically on the ARCHITECT c8000 and c4000 instruments with the Whole Blood application OR may be lysed manually using the Hemoglobin A1c Diluent with the Hemolysate application

HbA_{1c} – Point of Care Systems

- The DCA Vantage™ (Siemens Medical Solutions Diagnostics), which is based on **latex agglutination inhibition immunoassay** methodology and provides results in 6 min.
-
- The B-analyst (Menarini Diagnostics), which is based on **latex agglutination immunology turbidimetric** methodology, with results available in 8 min.

- The Afinion™ (Alere Technologies), which is based on **boronate affinity separation**, with results available in 5 min.

- The Quo-Test (Quotient Diagnostics an EKF Diagnostics Holding Company), which is based on **boronate affinity separation** and the use of fluorescence quenching with results available in 3 min.

- The Quo-Lab (Quotient Diagnostics an EKF Diagnostics Holding Company), which is based on **boronate affinity separation** and the use of fluorescence quenching with results available in 3 min. This method is the same as the Quo-Test but needs some manual handling.

- The InnovaStar (DiaSys Diagnostics), which is based on **agglutination immunoassay** and provides results in 11 min.

- The Cobas B101 (Roche Diagnostics), which is based on **latex agglutination inhibition** immunoassay methodology and provides results in 5 min.

Science

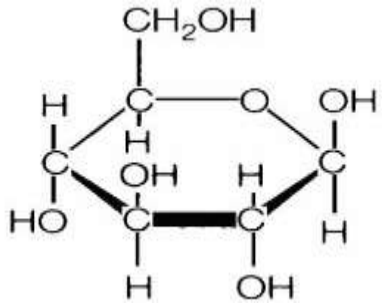
Education

IFCC International Federation of Clinical Chemistry and Laboratory Medicine

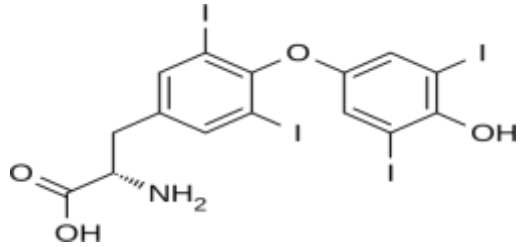
The use of Haemoglobin A_{1c} in Clinical Practice

HbA_{1c} is not like:

Glucose



Thyroxine



These are <u>single</u> molecular structures of known composition; their concentration in the body is tightly regulated and <u>concentrations controlled</u>.

The use of Haemoglobin A_{1c} in Clinical Practice

HbA_{1c} is:

A product of a non-enzymatic Glycation reaction; the reaction follows the Law of Mass action. The reaction is not controlled and the Glycated Haemoglobin formed is not a single molecular structure.

IFCC International Federation of Clinical Chemistry and Laboratory Medicine

The use of Haemoglobin A_{1c} in Clinical Practice

Interpretation of a Biochemical result (any result) requires:

- Understanding of Biochemistry of the analyte measured. How is it formed / controlled
- Understanding of the limitations of the analyte measured
- Understanding of the analytical ability of the method used to measure the analyte. The Quality Procedures.

IFCC International Federation of Clinical Chemistry and Laboratory Medicine

The use of Haemoglobin A_{1c} in Clinical Practice

ifcc
International Federation of Clinical Chemistry and Laboratory Medicine

The use of Haemoglobin A_{1c} in Clinical Practice

The Good: Full understanding of the analyte measured. Good analytical quality and standardisation.

The Bad: Poor understanding of the analyte measured. Good analytical quality and standardisation.

The Ugly: Poor understanding of the analyte measured. Poor analytical quality and standardisation.

Assumptions for use of HbA_{1c} as a monitor of glycaemia:

Haemoglobin is present at a constant concentration

- Within an individual this is probably true (? Anaemia)

Life span of Red Blood Cell is a constant

- Within an individual this is probably true; This may not be true between individuals

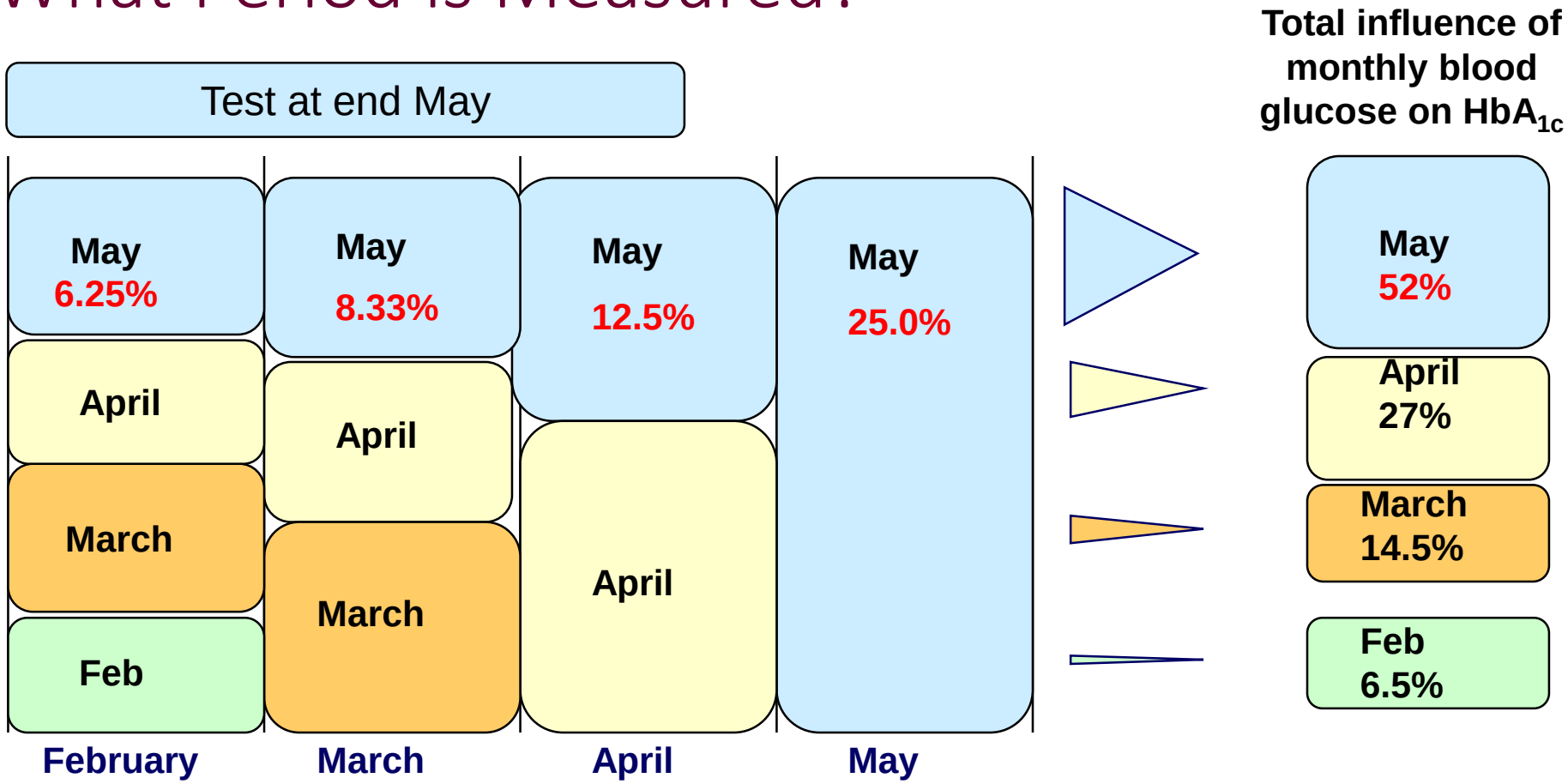
Micro-environment is constant

- Within an individual this is probably true.

Hence: Glucose is the ONLY variable.

IFCC International Federation of Clinical Chemistry and Laboratory Medicine

What Period is Measured?



Month red blood cell produced

International Federation of Clinical Chemistry and Laboratory Medicine

What affect does anaemia have on HbA_{1c}?

Diabetologia (2015) 58:1409–1421
DOI 10.1007/s00125-015-3599-3

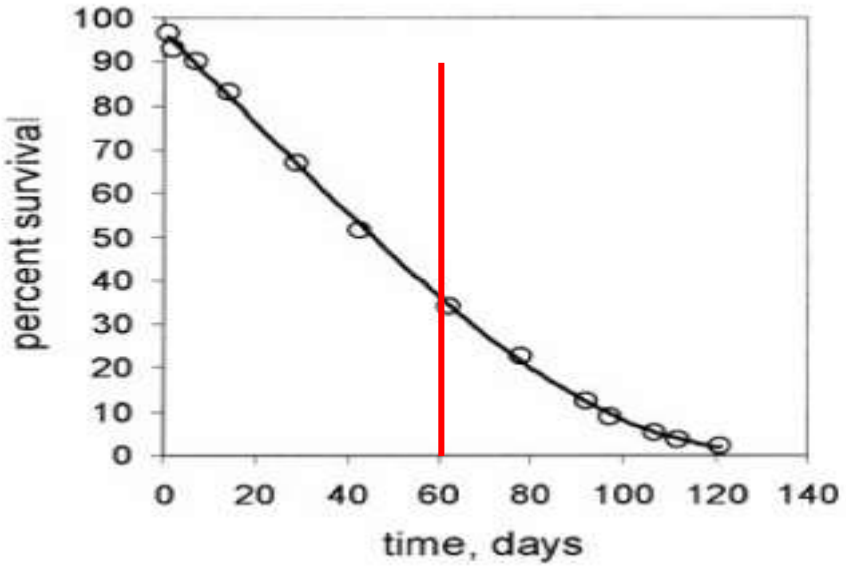
SYSTEMATIC REVIEW

The effect of anaemia and abnormalities of erythrocyte indices on HbA_{1c} analysis: a systematic review

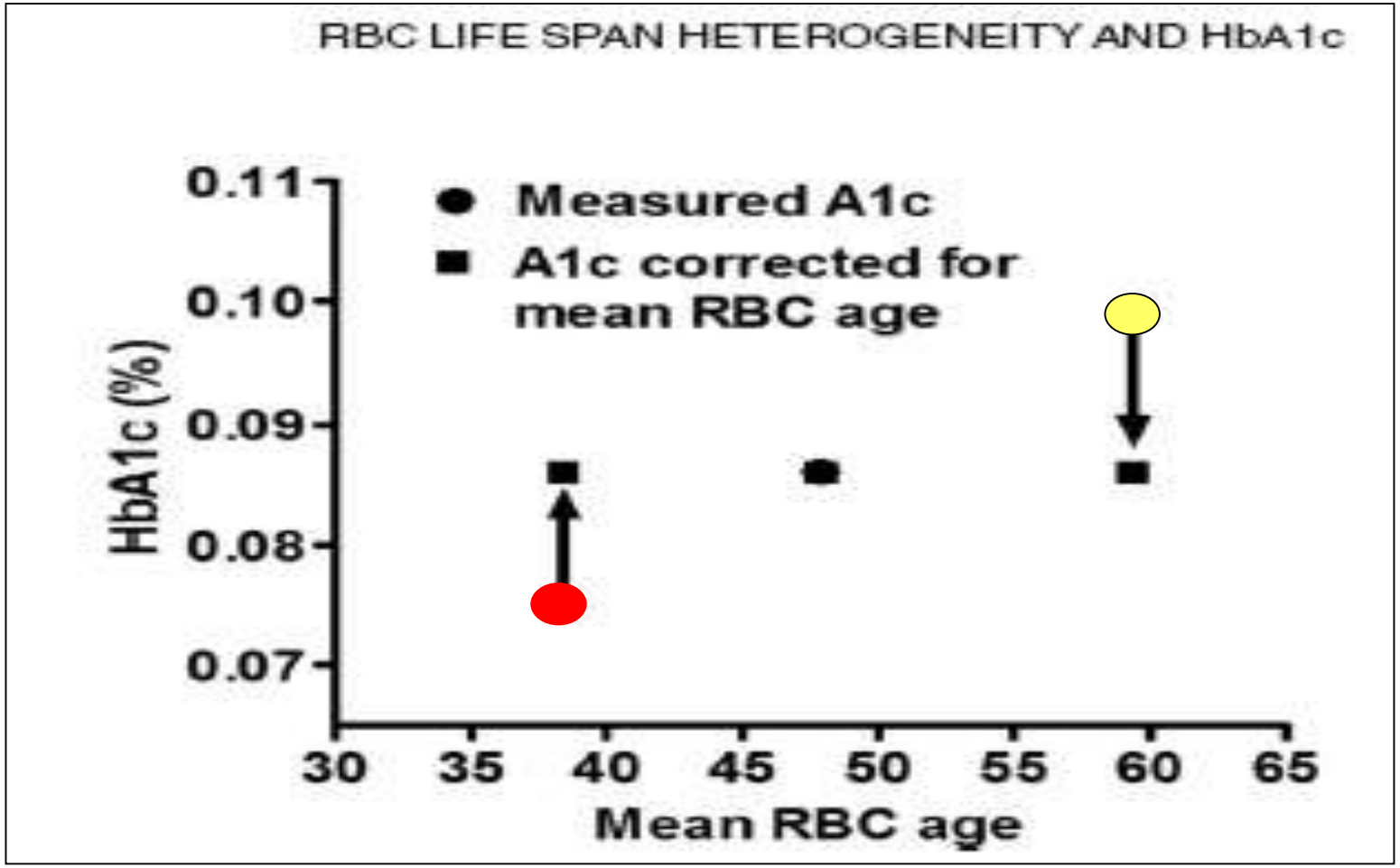
Emma English[1] · Iskandar Idris[1] · Georgina Smith[1] · Ketan Dhatariya[2] · Eric S. Kilpatrick[3] · W. Garry John[4]

Red cell life span heterogeneity in haematologically normal people is sufficient to alter HbA_{1c}

Red cell survival



Franco RS. Am J Hematol 2009;84:109-114



Cohen et al. Blood 2008;112:4284-4291

International Federation of Clinical Chemistry and Laboratory Medicine

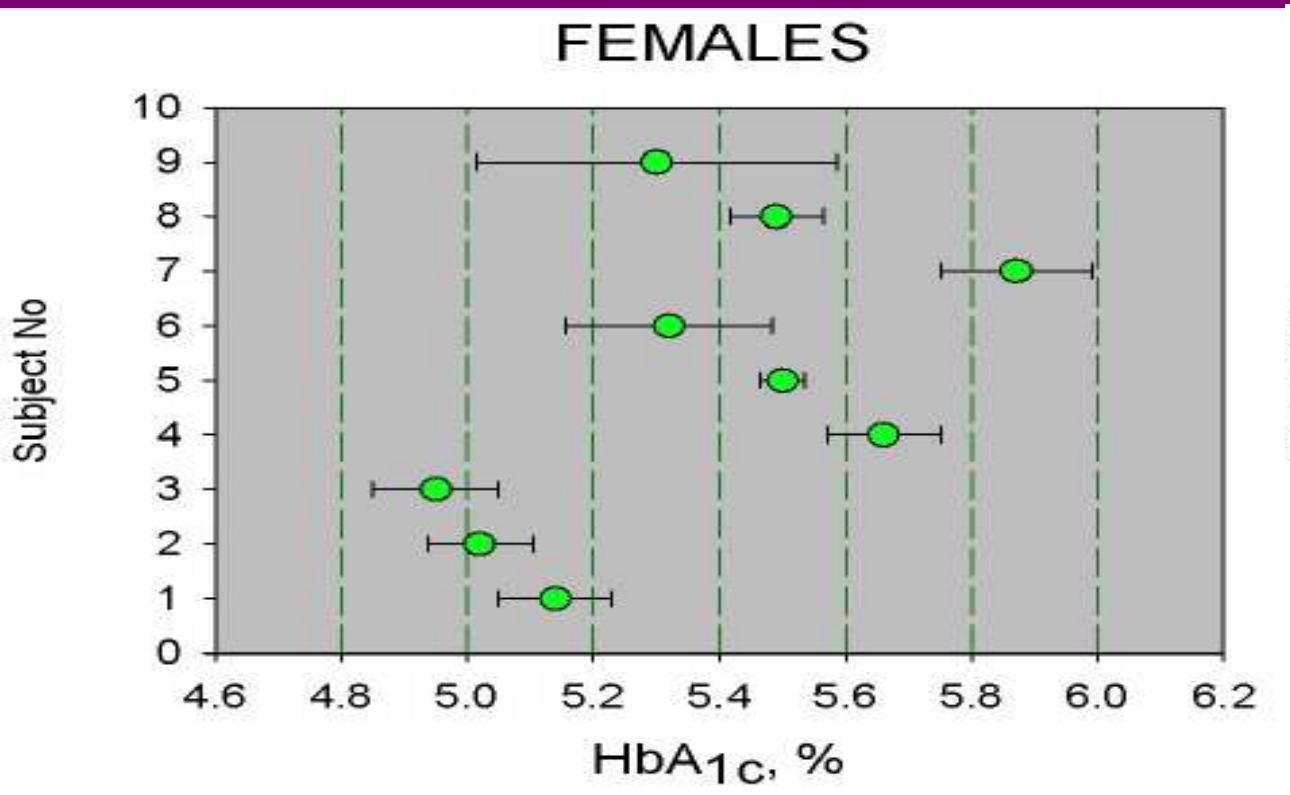
Predicting average glucose (eAG) using HbA_{1c}

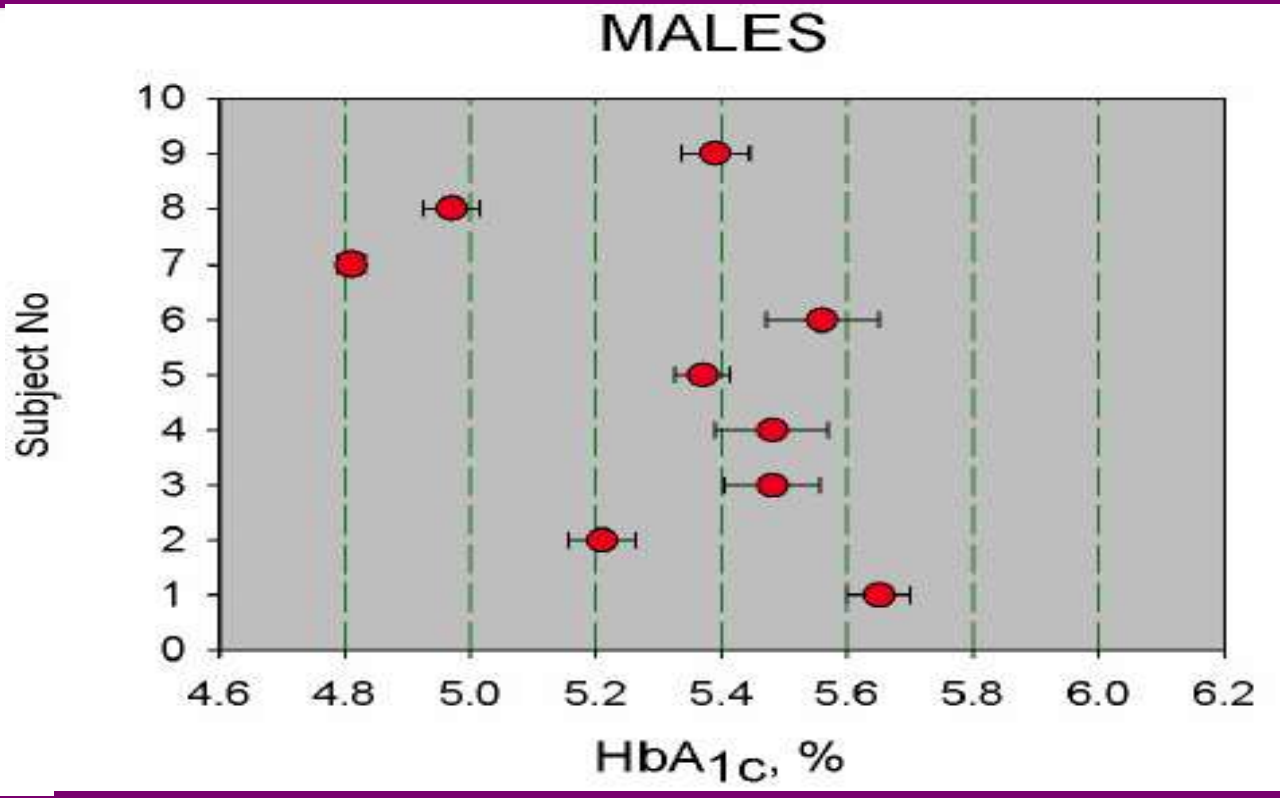
(Results from the ADAG Study)

HbA_{1c} (%)	eAG (mmol/L)	95% Predictive Limits for individual glucose (mmol/L)
5.0	5.4	(4.2 to 6.7)
6.0	7.0	(5.5 to 8.5)
7.0	8.6	(6.8 to 10.3)
8.0	10.1	(8.1 to 12.1)
9.0	11.7	(9.4 to 13.9)
10.0	13.3	(10.7 to 15.7)
11.0	14.9	(12.0 to 17.5)
12.0	16.5	(13.3 to 19.3)

Diabetes Care 2008;31:1–6

Biological Variability HbA_{1c}



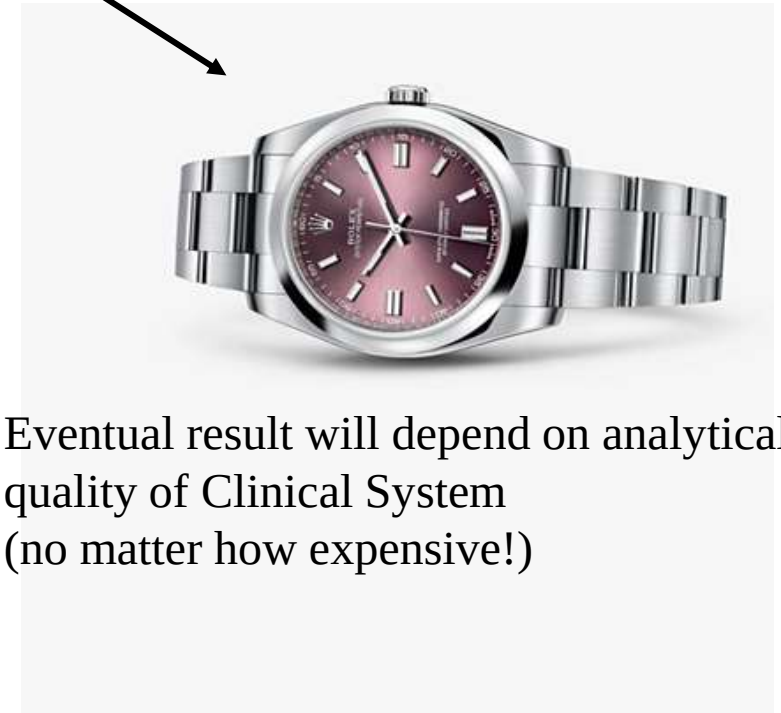


(Mosca et al.)

HbA_{1c} Are we Accurate enough

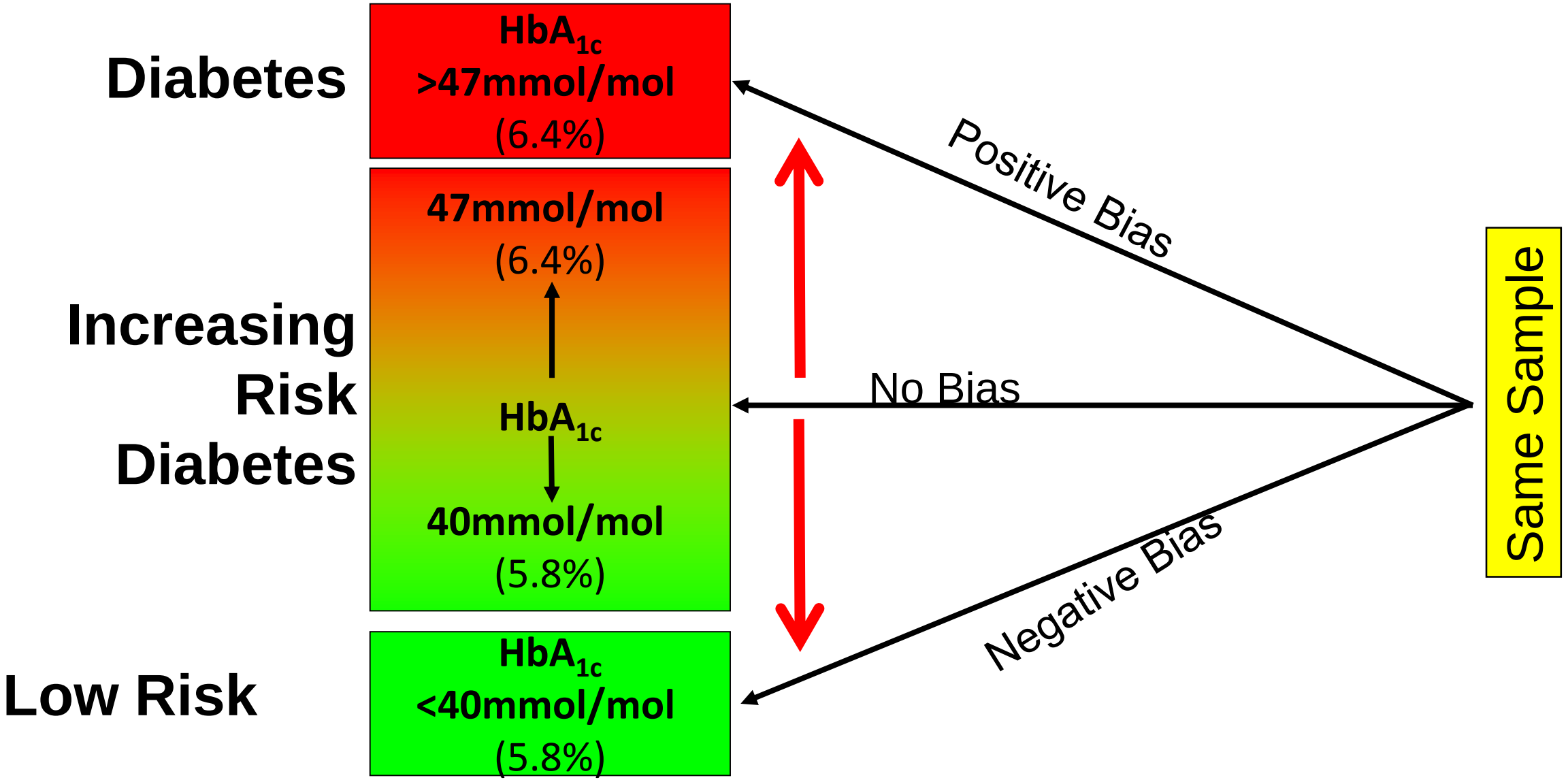
A continuous cold caesium fountain atomic clock in Switzerland, started operating in 2004 at an uncertainty of one second in 30 million years

Set accuracy



Eventual result will depend on analytical quality of Clinical System
(no matter how expensive!)

Impact Bias on Interpretation



College of American Pathologists (CAP) Survey

45 mmol/mol

48mmol/mol: Cut point for diagnosis

52 mmol/mol

7 mmol/mol difference around diagnosis cut point:

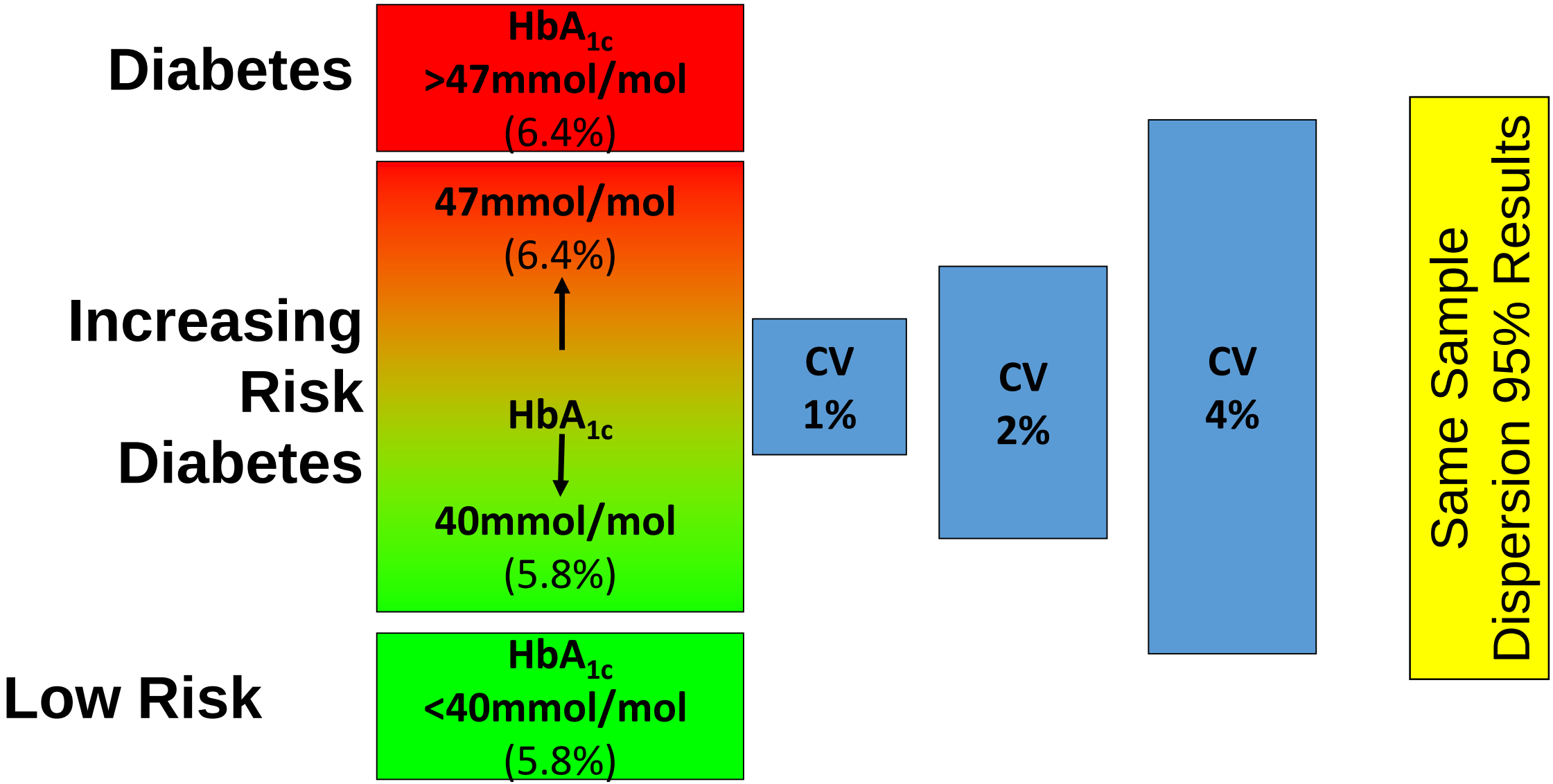
Hemoglobin A_{1c} – %

GH2-01

Method	No. Labs	Mean	S.D.	C.V.	Median	Low Value	High Value
	58	6.69	0.31	4.7	6.7	5.9	7.6
	8	-	-	-	6.5	6.5	6.7
	53	6.44	0.30	4.7	6.4	5.7	7.4
	58	6.47	0.24	3.7	6.5	6.1	7.1
	6	-	-	-	6.2	6.0	6.3
	[illegible]	6.28	0.24	3.8	6.3	5.9	7.0
	191	6.50	0.20	3.0	6.5	6.0	7.1
	146	6.51	0.18	2.8	6.5	6.1	7.1
	209	6.58	0.15	2.3	6.6	6.2	7.0
	5	-	-	-	6.3	6.2	6.8
	84	6.44	0.15	2.3	6.4	6.0	6.8
	151	6.57	0.16	2.5	6.6	6.2	7.0
	103	6.45	0.13	2.0	6.5	6.1	6.7
	7	-	-	-	6.4	6.1	6.8
	9	-	-	-	6.9	5.9	7.1
	29	6.60	0.21	3.1	6.6	6.1	7.0
	316	6.52	0.16	2.5	6.5	6.0	7.0
	50	6.46	0.19	2.9	6.4	6.1	6.9
	136	6.57	0.14	2.2	6.6	6.2	6.9
	12	6.38	0.09	1.4	6.4	6.2	6.5
	40	6.78	0.26	3.8	6.8	6.2	7.3
	30	6.60	0.20	3.0	6.6	6.1	7.0
	305	6.46	0.17	2.6	6.5	6.0	6.9
	188	6.85	0.22	3.3	6.9	6.2	7.5
	[illegible]	6.88	0.25	3.7	6.9	6.2	7.5
	270	6.84	0.23	3.3	6.8	6.2	7.4
	65	6.82	0.21	3.1	6.8	6.3	7.4
	102	6.81	0.13	1.9	6.8	6.6	7.2
	327	6.77	0.12	1.8	6.8	6.4	7.0
	20	6.39	0.12	1.9	6.4	6.1	6.5
	41	6.42	0.16	2.5	6.4	6.1	6.7
	[illegible]	6.37	0.17	2.7	6.4	5.9	6.8
Reference Method*		6.49					

* Samples were analyzed by the National Glycohemoglobin Standardization Program (NGSP) network laboratories.

Impact Imprecision on Interpretation



College of American Pathologists (CAP) Survey

Between Laboratory Agreement within a single method group:

Poorest CV: 4.7%

Best CV: 1.4%

Mean CV: 3.8%

Hemoglobin A_{1c} – %

GH2-01

Method	No. Labs	Mean	S.D.	C.V.	Median	Low Value	High Value
	58	6.69	0.31	4.7	6.7	5.9	7.6
	8	-	-	-	6.5	6.5	6.7
	53	6.44	0.30	4.7	6.4	5.7	7.4
	58	6.47	0.24	3.7	6.5	6.1	7.1
	6	-	-	-	6.2	6.0	6.3
	55	6.28	0.24	3.8	6.3	5.9	7.0
	191	6.50	0.20	3.0	6.5	6.0	7.1
	146	6.51	0.18	2.8	6.5	6.1	7.1
	209	6.58	0.15	2.3	6.6	6.2	7.0
	5	-	-	-	6.3	6.2	6.8
	84	6.44	0.15	2.3	6.4	6.0	6.8
	151	6.57	0.16	2.5	6.6	6.2	7.0
	103	6.45	0.13	2.0	6.5	6.1	6.7
	7	-	-	-	6.4	6.1	6.8
	9	-	-	-	6.9	5.9	7.1
	29	6.60	0.21	3.1	6.6	6.1	7.0
	316	6.52	0.16	2.5	6.5	6.0	7.0
	50	6.46	0.19	2.9	6.4	6.1	6.9
	136	6.57	0.14	2.2	6.6	6.2	6.9
	12	6.38	0.09	1.4	6.4	6.2	6.5
	40	6.78	0.26	3.8	6.8	6.2	7.3
	30	6.60	0.20	3.0	6.6	6.1	7.0
	266	6.46	0.17	2.6	6.5	6.0	6.9
	188	6.85	0.22	3.3	6.9	6.2	7.5
	86	6.88	0.25	3.7	6.9	6.2	7.5
	270	6.84	0.23	3.3	6.8	6.2	7.4
	65	6.82	0.21	3.1	6.8	6.3	7.4
	102	6.81	0.13	1.9	6.8	6.6	7.2
	327	6.77	0.12	1.8	6.8	6.4	7.0
	20	6.39	0.12	1.9	6.4	6.1	6.5
	41	6.42	0.16	2.5	6.4	6.1	6.7
	208	6.37	0.17	2.7	6.4	5.9	6.8
Reference Method*		6.49					

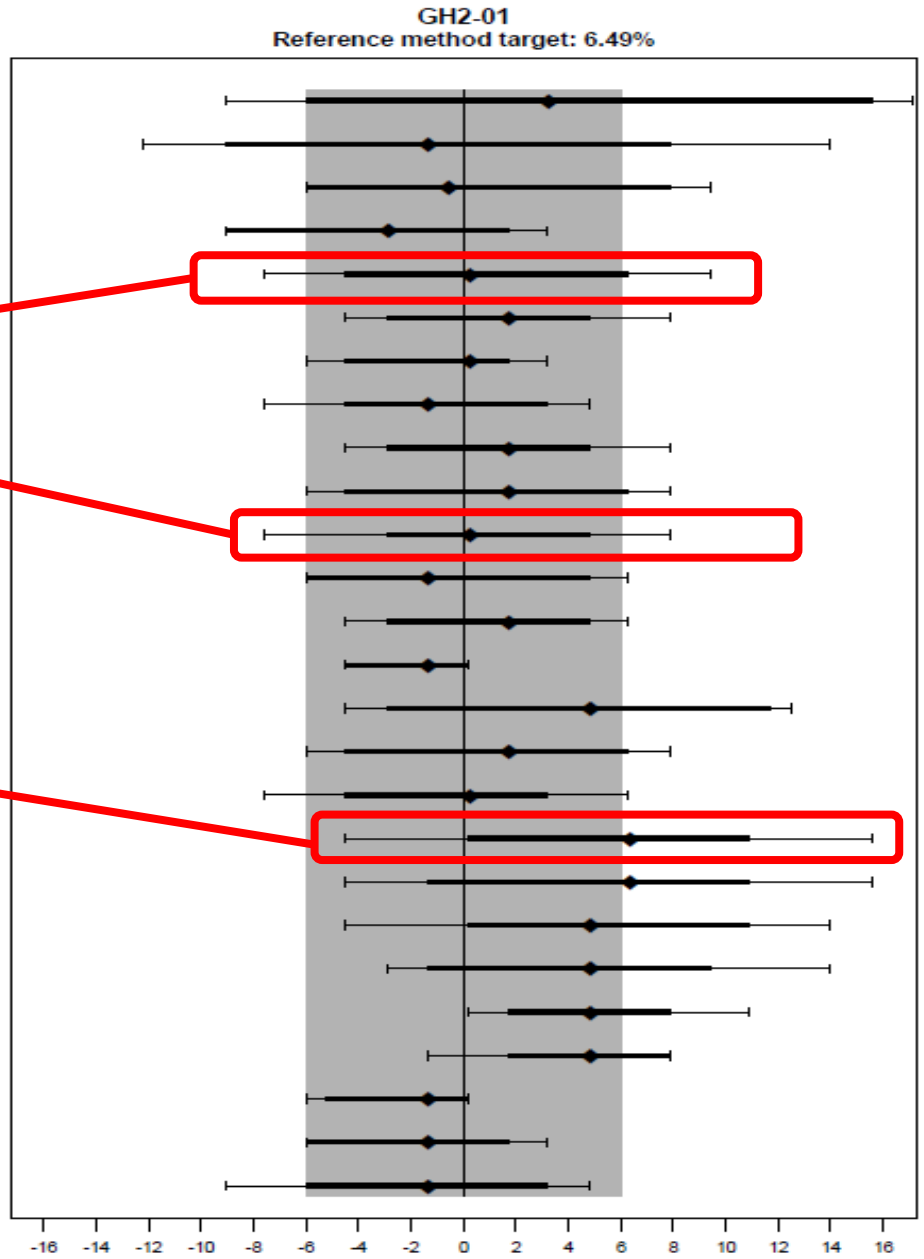
* Samples were analyzed by the National Glycohemoglobin Standardization Program (NGSP) network laboratories.

College of American Pathologists (CAP) Survey

"Method mean" displays no Bias; But outliers will result in wrong diagnosis

Poor accuracy and poor between laboratory agreement (imprecision).

Figure 1A



Lines extend from the min to max difference with the middle 90% range represented by the thicker line. The median is the solid diamond. Outliers were excluded from this summary.

International Federation of Clinical Chemistry and Laboratory Medicine

Clinical Chemistry 61:5
752–759 (2015)

Investigation of 2 Models to Set and Evaluate Quality Targets for Hb A_{1c}: Biological Variation and Sigma-Metrics

Cas Weykamp,[1,2*] Garry John,[3] Philippe Gillery,[4] Emma English,[5] Linong Ji,[6] Erna Lenters-Westra,[7,8] Randie R. Little,[9] Gojka Roglic,[10] David B. Sacks,[11] and Izumi Takei,[12] on behalf of the IFCC Task Force on Implementation of HbA1c Standardization

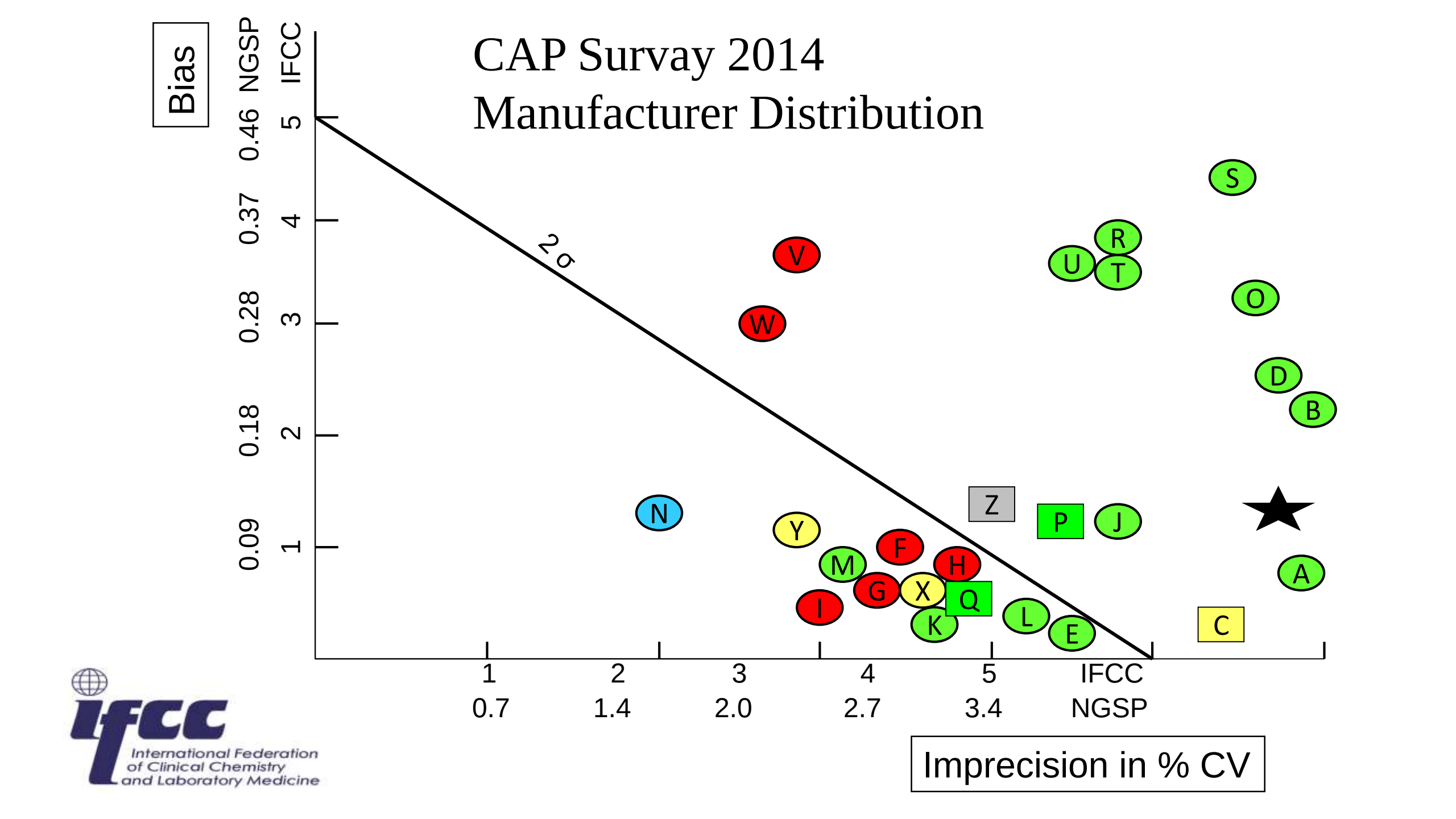
BACKGROUND: A major objective of the IFCC Task Force on Implementation of HbA_{1c} Standardization is to develop a model to define quality targets for glycated hemoglobin (Hb A_{1c}).

METHODS: Two generic models, biological variation and sigma-metrics, are investigated. We selected variables in the models for Hb A_{1c} and used data of external quality assurance/proficiency testing programs to evaluate the suitability of the models to set and evaluate quality targets within and between laboratories.

RESULTS: In the biological variation model, 48% of individual laboratories and none of the 26 instrument groups met the minimum performance criterion. In the sigma-metrics model, with a total allowable error (TAE) set at 5 mmol/mol (0.46% NGSP), 77% of the individual laboratories and 12 of 26 instrument groups met the 2σ TAE and risk levels of 2σ and 4σ for routine laboratories and laboratories performing clinical trials, respectively. These goals should serve as a starting point for discussion with international stakeholders in the field of diabetes.

A major objective of the IFCC Task Force on Implementation of HbA1c Standardization (TF-HbA1c)[13] is the following:

> Develop quality targets for the measurement of Hb A_{1c} [glycated hemoglobin], and, on the basis of these targets, and in conjunction with professional bodies, advise on the use of Hb A_{1c} for monitoring, diagnosis and screening of diabetes and glucose intolerance ["Hb A_{1c}" substituted for other spellings used in the original]. *(1)*


Bias
CAP Survay 2014
Manufacturer Distribution
NGSP
IFCC
0.46
0.37
0.28
0.18
0.09
5
4
3
2
1
2σ
V
W
R
U
T
S
O
D
B
N
Z
P
J
Y
F
M
H
G
X
Q
I
K
L
E
A
C
1
2
3
4
5
IFCC
0.7
1.4
2.0
2.7
3.4
NGSP
Imprecision in % CV
International Federation
of Clinical Chemistry
and Laboratory Medicine

HbA_{1c} – Not so straightforward!