Glycated Hemoglobin
Challenges of assays & clinical use

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Agenda

1. Importance of diagnosis & monitoring of diabetes mellitus
2. Different terminology for glycated hemoglobin
3. HbA1C for monitoring of glycemic control
4. HbA1C as a diagnostic: advantages & considerations
5. Standardization of HbA1C assays & reports
6. Methods of HbA1C measurement & their limitations
7. Interfering conditions in HbA1C assays & interpretation
8. Blood glucose testing

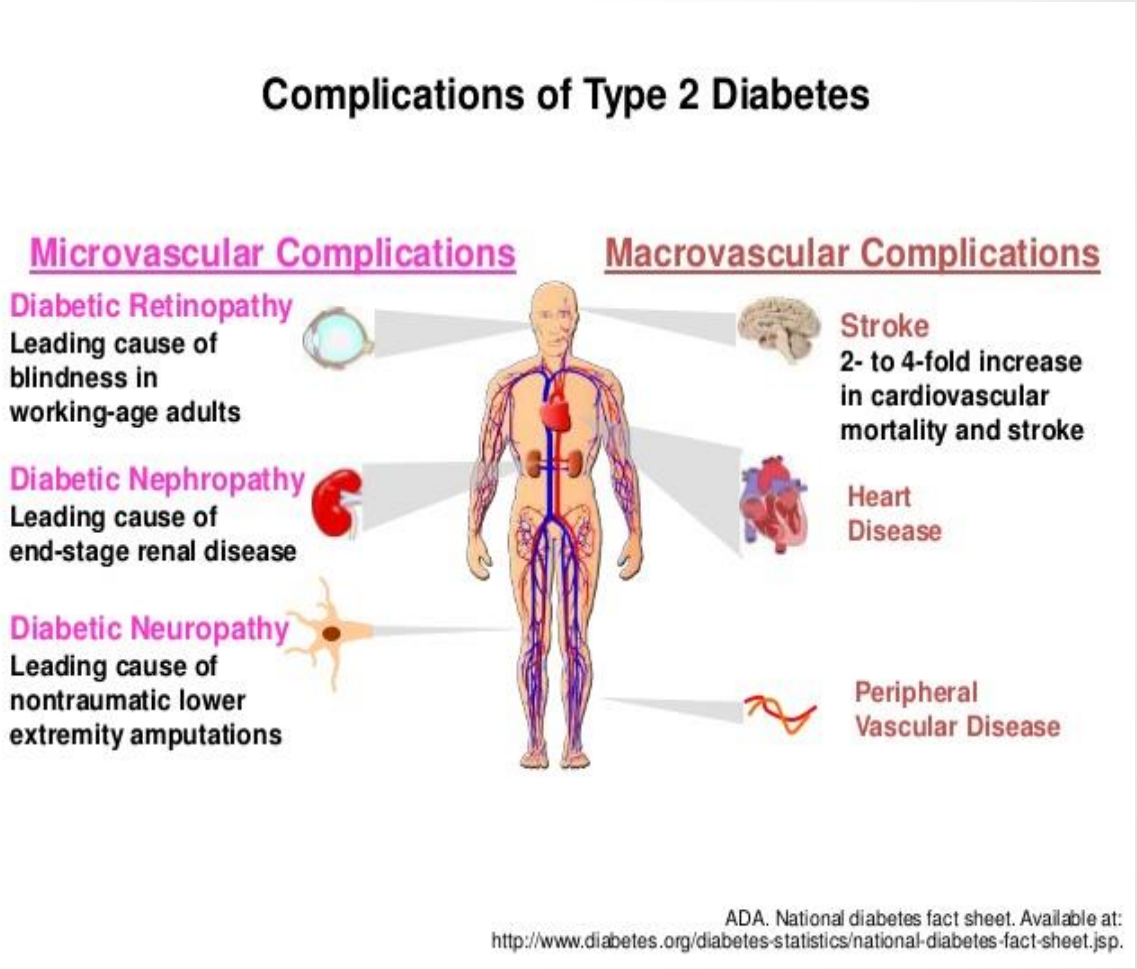
Importance of diagnosis & monitoring of diabetes mellitus

- ✓ A common & serious disease
- ✓ Increasing prevalence
- ✓ Significant morbidity
- ✓ Premature mortality
- ✓ Considerable economic impact

Importance of diagnosis & monitoring of diabetes mellitus

- ✓ Correct & well-timed diagnosis of diabetes is important & has important implication for individual patient.

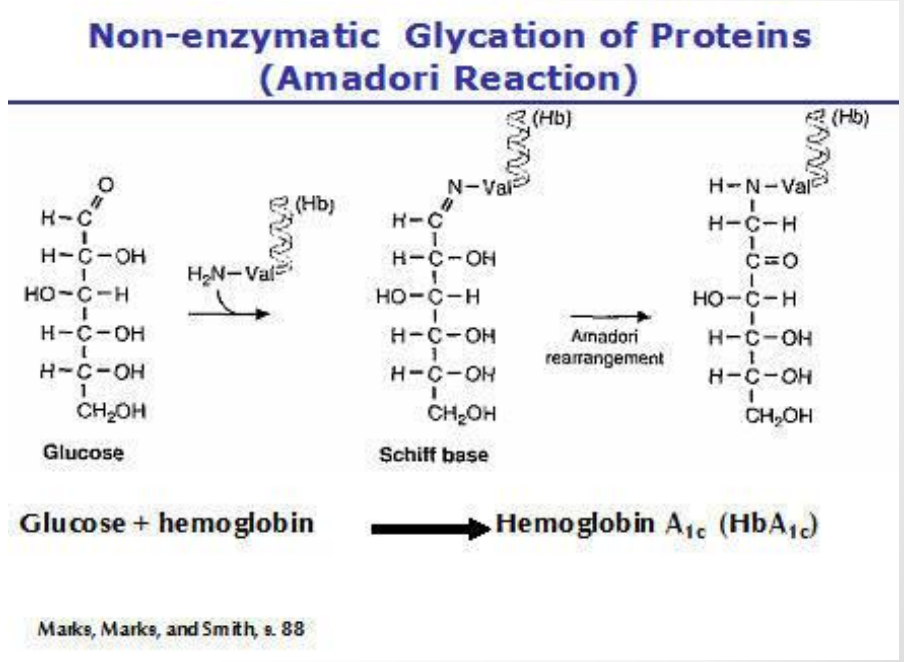
- ✓ Improved glycemic control prevents the progression of microvascular complications & probably macrovascular complications.



Different terminology for glycated hemoglobin

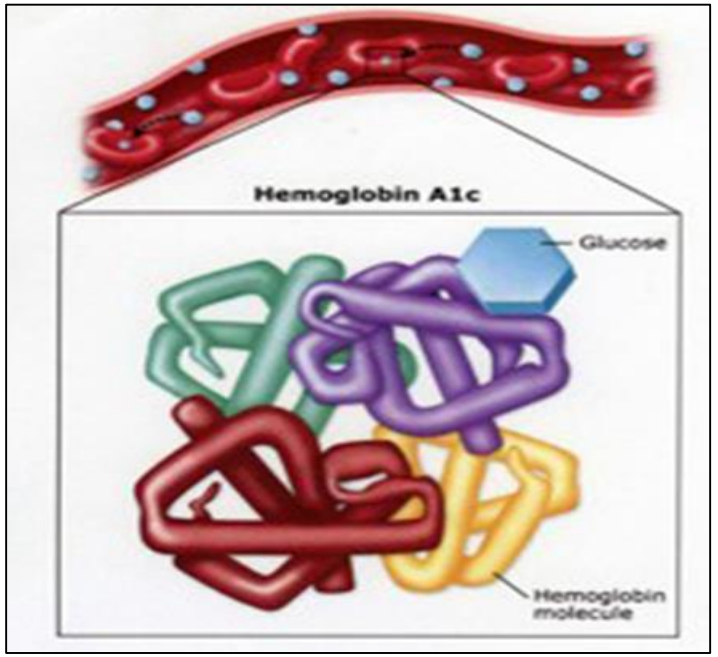
Glycated hemoglobin, Fast hemoglobins

- ✓ Fast hemoglobins: based on migration in electrophoresis
- ✓ HbA1a, HbA1b, HbA1c, in the order in which they were eluted.
- ✓ Glycated haemoglobin: result from the non-enzymatic attachment of
  - ✓ glucose (in HbA1c)
  - ✓ fructose 1, 6-diphosohate (in hbA1a)
  - ✓ glucose-6-phosphate in (in HbA1b)



Glycated hemoglobin, different terms

- ✓ glycated hemoglobin
- ✓ glycohemoglobin
- ✓ glycosylated hemoglobin
- ✓ HbA1
- ✓ HbA1C



All of these terms have been used to refer to hemoglobin that has been modified by the non-enzymatic addition of glucose.

✓ The IFCC group on HbA1C definition:

Hemoglobin A that is irreversibly glycosylated at one or both N-terminal valines of the β-chains of Hb molacule, including that may be also (but not solely) be glycosylated on lysine residues.

IFCC: International Federation of Clinical Chemistry

- ✓ The current acceptable term is glycated hemoglobin (GHb).
- ✓ HbA1C is the specific glycated species and is also interchangeably accepted term for reporting all GHb results.
- ✓ Major clinical diabetes organizations recommend use of the term A1C or “A1C test" to describe HbA1c in clinical practice.

HbA1C for monitoring of glycemic control

✓ Since 1976, as an index of mean blood glucose levels over the past 2–4 months.

✓ Also as a measure of risk for development of diabetes complications

✓ Youngest erythrocytes contributing to a greater extent than older ones so HbA1C levels represent a “weighted” average of glucose levels.

✓~ 50% of the HbA1C ⟶ plasma glucose levels over the last month

✓ ~ 75% of the HbA1C ➡ plasma glucose levels during the last 2 months

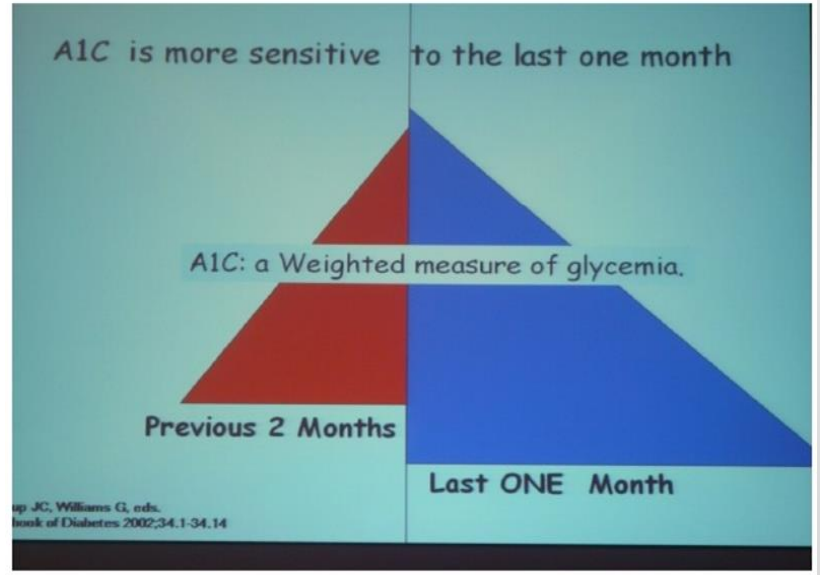


Table 5.1—Mean glucose levels for specified A1C levels (24,28)

A1C	Mean plasma glucose*		Mean fasting glucose		Mean premeal glucose		Mean postmeal glucose		Mean bedtime glucose	
% (mmol/mol)	mg/dL	mmol/L	mg/dL	mmol/L	mg/dL	mmol/L	mg/dL	mmol/L	mg/dL	mmol/L
6 (42)	126	7.0								
<6.5 (48)			122	6.8	118	6.5	144	8.0	136	7.5
6.5–6.99 (48–53)			142	7.9	139	7.7	164	9.1	153	8.5
7 (53)	154	8.6								
>7.0–7.49 (53–58)			152	8.4	152	8.4	176	9.8	177	9.8
7.5–7.99 (58–64)			167	9.3	155	8.6	189	10.5	175	9.7
8 (64)	183	10.2								
>8.0–8.5 (64–69)			178	9.9	179	9.9	206	11.4	222	12.3
9 (75)	212	11.8								
10 (86)	240	13.4								
11 (97)	269	14.9								
12 (108)	298	16.5								

A calculator for converting A1C results into eAG, in either mg/dL or mmol/L, is available at http://professional.diabetes.org/eAG.
*These estimates are based on ADAG data of ~2,700 glucose measurements over 3 months per A1C measurement in 507 adults with type 1, type 2, and no diabetes. The correlation between A1C and average glucose was 0.92 (28).

↑ 1 % HbA1C ~ 30 mg/dL

Estimated average glucose (eAG)

A1C (%)	mg/dL*	mmol/L
5	97 (76–120)	5.4 (4.2–6.7)
6	126 (100–152)	7.0 (5.5–8.5)
7	154 (123–185)	8.6 (6.8–10.3)
8	183 (147–217)	10.2 (8.1–12.1)
9	212 (170–249)	11.8 (9.4–13.9)
10	240 (193–282)	13.4 (10.7–15.7)
11	269 (217–314)	14.9 (12.0–17.5)
12	298 (240–347)	16.5 (13.3–19.3)

Data in parentheses are 95% CI. A calculator for converting A1C results into eAG, in either mg/dL or mmol/L, is available at professional.diabetes.org/eAG. *These estimates are based on ADAG data of ~2,700 glucose measurements over 3 months per A1C measurement in 507 adults with type 1, type 2, or no diabetes. The correlation between A1C and average glucose was 0.92 (12,13). Adapted from Nathan et al. (12).

Glycemic Goals and Hypoglycemia: Standards of Care in Diabetes— 2016 & 2025, Diabetes Care

Recommendations of American Diabetes Association (ADA)

- ✓Assess glycemic status (A1C or other glycemic measurement such as time in range or glucose management indicator) at least two times a year in patients who are meeting treatment goals (and who have stable glycemic control).

- ✓Assess glycemic status at least quarterly and as needed in patients whose therapy has recently changed and/or who are not meeting glycemic goals.

Glycemic Targets: Standards of Medical Care in Diabetes, *Diabetes Care 2025*

HbA1C as a diagnostic tool

advantages & considerations

HbA1C for diagnosis of diabetes mellitus

- ✓ 2009, International expert began to recommend HbA1C for diagnostic purpose.
- ✓ 2010, the ADA incorporated HbA1c into clinical practice guidelines.
- ✓ This recommendation is also supported by other major diabetes organizations:
  - ✓ International Diabetes Federation
  - ✓ European Association for the Study of Diabetes
  - ✓ World Health Organization

Diagnostic cutoff value
≥ 6.5% (≥ 48 mmol/mol)

Prediabetes (or high risk for diabetes)
HbA1c level of 5.7% to 6.4%
(39 to 46 mmol/mol)

Table 2.2—Criteria for the diagnosis of diabetes

FPG ≥126 mg/dL (7.0 mmol/L). Fasting is defined as no caloric intake for at least 8 h.*
OR
2-h PG ≥200 mg/dL (11.1 mmol/L) during OGTT. The test should be performed as described by WHO, using a glucose load containing the equivalent of 75 g anhydrous glucose dissolved in water.*
OR
A1C ≥6.5% (48 mmol/mol). The test should be performed in a laboratory using a method that is NGSP certified and standardized to the DCCT assay.*
OR
In a patient with classic symptoms of hyperglycemia or hyperglycemic crisis, a random plasma glucose ≥200 mg/dL (11.1 mmol/L).

DCCT, Diabetes Control and Complications Trial; FPG, fasting plasma glucose; OGTT, oral glucose tolerance test; WHO, World Health Organization; 2-h PG, 2-h plasma glucose. *In the absence of unequivocal hyperglycemia, diagnosis requires two abnormal test results from the same sample or in two separate test samples.

Classification and Diagnosis of Diabetes: Standards of Medical Care in Diabetes. *Diabetes Care 2025*
Guidelines and Recommendations for Laboratory Analysis in the Diagnosis and Management of Diabetes Mellitus. Clinical Chemistry, Special Report 1–61 (2023).

Advantages of HbA1C as a diagnostic tool

- ✓ Non-fasting state
- ✓ Less variable

Within-individual day to day variability < 2 % for HbA1C.
12-15% for FPG.
16.6% for OGTT.

- ✓ Fairly stable after collection
- ✓ Unaffected by short lifestyle changes
- ✓ Being a marker related to the onset of diabetic complications

Standardization of HbA1C reports

- ✓ The NGSP express HbA1C values as a proportion of the total Hgb (% HbA1C).
- ✓ The IFCC recommends presenting HbA1C values as mmol of HbA1C per mole of total Hgb.
- ✓ From 2007, based on a globally accepted consensus, HbA1C should be presented in both IFCC (mmol/mol) & NGSP (%) units.
- ✓ Conversion between IFCC & NGSP units can be performed using a mathematical equation due to a linear relationship.
- ✓IFCC units can be converted to NGSP units as follows: NGSP = (0.915 × IFCC) + 2.152.

IFCC: International Federation of Clinical Chemistry; NGSP: National Glycohemoglobin Standardization Program

HENRY'S, Clinical Diagnosis and Management by Laboratory Methods.
Guidelines and Recommendations for Laboratory Analysis in the Diagnosis and Management of Diabetes Mellitus. Clinical Chemistry, Special Report 1–61 (2023).

Certificate of Traceability to the DCCT Reference Method

To obtain and retain a "Certificate of Traceability to the DCCT Reference Method" in the NGSP, the laboratory must annually satisfy:

- ✓ Precision criteria:
  - ✓ CV ≤ 5%; ≤ 3% for Level 1 laboratories
- ✓ Bias criteria (95% CI of differences between test method and Secondary Reference Laboratories (SRL) must fall within ±1% GHb of the SRL [±0.70% GHb for Level 1 laboratories])
- ✓ Outlier criteria (greater than mean +3 standard deviation of absolute differences between pairs)

Quality control

To get these goals, at least two QC materials with different average concentrations should be used at the beginning and at the end of the day's run to monitor performance and quality control of assay.

HENRY'S, Clinical Diagnosis and Management by Laboratory Methods.
Guidelines and Recommendations for Laboratory Analysis in the Diagnosis and Management of Diabetes Mellitus. Clinical Chemistry, Special Report 1–61 (2023).

Limitations of HbA1C

- ✓ Underestimation of average plasma glucose concentration by HbA1C in patients with poor glycemic control due to a decrease in RBC life span.

- ✓ HbA1C cannot provide information about glycemic variability or hypoglycemia.

- ✓ For patients who are prone to glycemic variability, especially type 1 diabetic patients or type 2 diabetic patients with severe insulin deficiency, it is preferable to evaluate glycemic control with a combination of self-monitoring and A1C results.

Methods of HbA1C measurement & their limitations

Methods of HbA1C measurement

- ✓ Based on charge difference

- ✓ Based on structural difference

The major difference among these methods lies the way Hb is separated from its glycated form.

Methods based on charge difference

- ✓ Ion-exchange chromatography / HPLC
- ✓Electrophoresis
- ✓Isoelectric focusing

Among methods based on charge difference

✓ Ion- exchange HPLC is highly efficient, reliable & widely standardized

Limitation:

- Occasional problem with some less common Hb variants
- HPLC is a stand-alone & not multipurpose instrument for general laboratories

Methods based on structural difference

- ✓ Immunoassays
- ✓Affinity chromatography
- ✓Enzymatic assays

Immunoassay & affinity chromatography

- ✓ easily performed
- ✓ less expensive than other methods

❖ Limitation of immunoassys:

- ✓ HbF interference (20% underestimation of HbA1C)
- ✓ Immunoassay needs frequent multilevel calibration
- ✓ Limited stability of reagents

❖ Limitation of affinity chromatography:

- ✓ All glycohemoglobin binds to the resin

Interfering conditions in

HbA1C assays
&
interpretation

Impact of age on HbA1C

✓ Age-related increases in mean Hb A1c in people without diabetes:

approximately 0.1% per decade after age 30 years

✓The increase in Hb A1c levels with age generally parallel other measures of glycemia.

✓The clinical implications of this small, but statistically significant, progressive increase of “normal” Hb A1c levels with aging : not determined

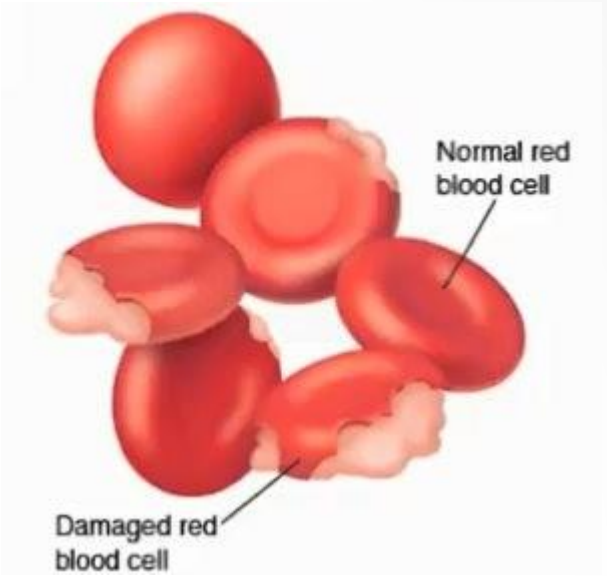
✓ There is controversy regarding racial differences in A1C.

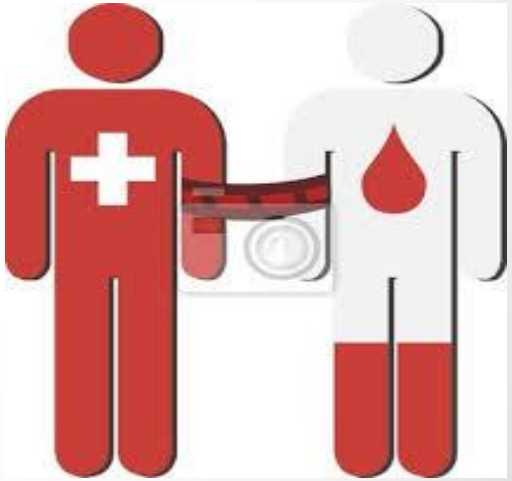
African American individuals have slightly higher A1C levels.

Guidelines and Recommendations for Laboratory Analysis in the Diagnosis and Management of Diabetes Mellitus. Clinical Chemistry, Special Report 1–61 (2023).

✓ *Altered erythrocyte turnover*

- ✓ anemia
- ✓ iron status
- ✓ Splenectomy
- ✓ blood loss
- ✓ transfusion
- ✓ hemolysis
- ✓ glucose-6phosphate dehydrogenasedeficiency
- ✓erythropoietin



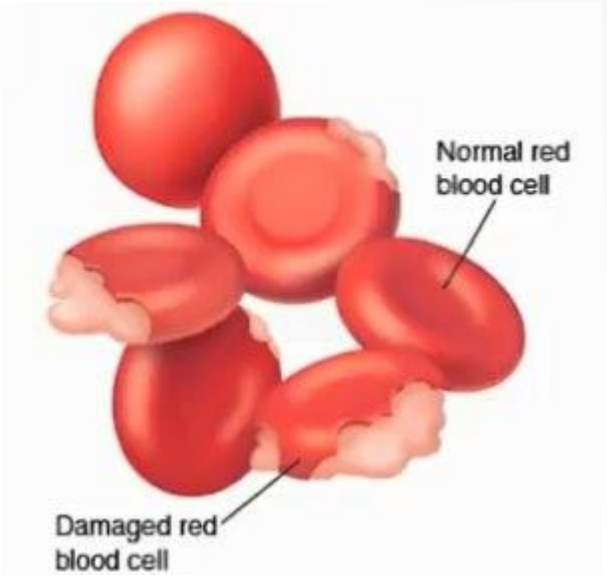


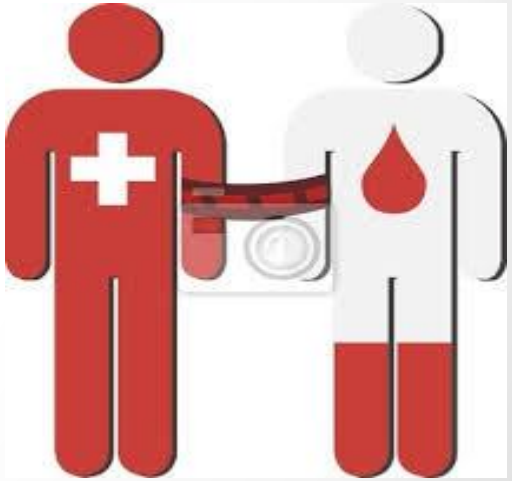
✓Any condition that shorten RBC survival

- recovery from acute blood loss
- hemolytic anemia
- poorly controlled diabetes

✓ Blood transfusion

✓ ***An method-independent interference***





Falsely low HbA1C

regardless of assay method

Iron deficiency anemia

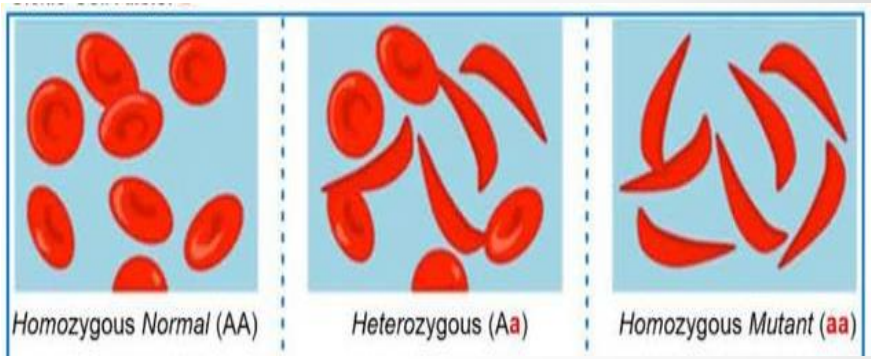
Malondialdehyde, which is increased in patients with iron deficiency anemia enhances the glycation of hemoglobin.

✓Elevated HbA1C but not glycated albumin in late pregnancy in non-diabetic women is related to iron deficiency.

Higher HbA1C

Hashimoto K, et al.: A1C but not serum glycated albumin is elevated in late pregnancy owing to iron deficiency. *Diabetes Care 2008;31:1945-8.*

Hemoglobin variants



- ✓ Hemoglobin variants may affect Hb A1c test results
- ✓ An method-dependent interference
- ✓ Homozygous hemoglobin variants (such as Hb SS or Hb EE) & absence of HbA
- ✓ Presence of 2 Hb variant, like Hb SC
- ✓ Boronate affinity chromatographic assay methods are generally considered to be less affected by Hb variants.

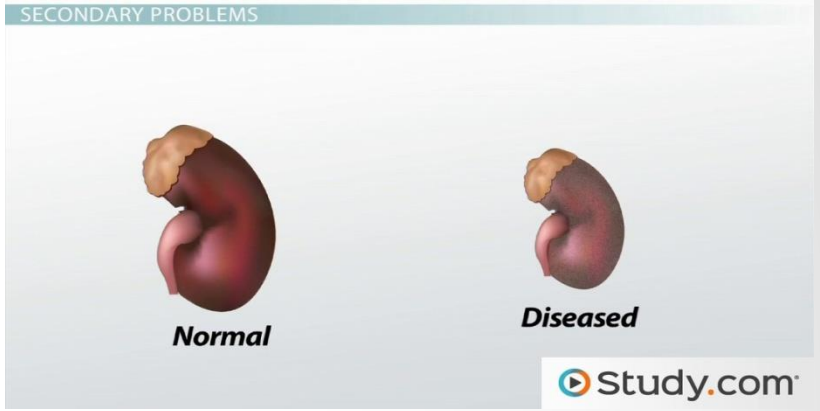
✓ *Chronic renal failure*

- Interference from carbamylated Hb, a chemically modified derivative of Hb

- Renal anemia

- Erythropoetin intake

- Hemodialysis



These factors make it difficult to interpret the HbA1C results in diabetic patients with chronic renal failure.

Other interferences in HbA1C

❖ **Falsely high HbA1C**

- ✓ Hypertriglyceridemia
- ✓ Hyperbilirubinemia
- ✓ Salicylates
- ✓ Chronic alcohol or opiate use
- ✓ Lead poisoning
- ✓ Elevated fetal hemoglobin (HbF)
  - ✓ Hereditary persistence of fetal hemoglobin
  - ✓ Refractory anemia
  - ✓ Chronic bone marrow failure syndrome
  - ✓ Some hemoglobinopathies
  - ✓ …

❖ **Falsely low HbA1C**

- ✓ Vitamin C & E

Other interferences in HbA1C

- ✓ Treatment with for HIV by different potential interference such as:
  - ✓ anemia
  - ✓ altered RBC life span
  - ✓ altered lipid metabolism
  - ✓ drug-induced hyperglycemia

- ✓ Cirrhosis:

(both under- & overestimation)

- ✓ decreased erythropoetin production
- ✓ GI bleeding
- ✓ Hypersplenism
- ✓ Reduced RBC life
- ✓ Bone marrow suppression
- ✓ Hyperbilirubinemia

Conclusions

- ✓ Using HbA1c as a diagnostic tool for diabetes as a substitute for FPG & OGTT will depend on local considerations including:

  - ✓ Population characteristics
  - ✓ Availability of a national quality assurance program
  - ✓ Accessibility
  - ✓ Cost of the test

- ✓ To achieve accurate and precise HbA1C values, laboratories must:
  - ✓ Select a NGSP certified method
  - ✓ Set optimal quality control protocols
  - ✓ Being aware of the factors that affect HbA1C measurement including:
    - ✓ Sample storage
    - ✓ RBC survival
    - ✓ Interfering conditions or substances

- ✓ Although efforts of the NGSP and IFCC work group on standardization of HbA1C have been provided improvements in the consistency of HbA1C assays but do not guarantee the best performance in all laboratories.

Blood Glucose Testing

In the absence of unequivocal hyperglycemia, diagnosis requires two abnormal results from different tests which may be obtained at the same time (e.g., A1C and FPG), or the same test at two different time points.

Table 2.1—Criteria for the diagnosis of diabetes in nonpregnant individuals

A1C ≥6.5% (≥48 mmol/mol). The test should be performed in a laboratory using a method that is NGSP certified and standardized to the DCCT assay.*
OR
FPG ≥126 mg/dL (≥7.0 mmol/L). Fasting is defined as no caloric intake for at least 8 h.*
OR
2-h PG ≥200 mg/dL (≥11.1 mmol/L) during OGTT. The test should be performed as described by the WHO, using a glucose load containing the equivalent of 75 g anhydrous glucose dissolved in water.*
OR
In an individual with classic symptoms of hyperglycemia or hyperglycemic crisis, a random plasma glucose ≥200 mg/dL (≥11.1 mmol/L). Random is any time of the day without regard to time since previous meal.

DCCT, Diabetes Control and Complications Trial; FPG, fasting plasma glucose; OGTT, oral glucose tolerance test; NGSP, National Glycohemoglobin Standardization Program; WHO, World Health Organization; 2-h PG, 2-h plasma glucose. *In the absence of unequivocal hyperglycemia, diagnosis requires two abnormal results from different tests which may be obtained at the same time (e.g., A1C and FPG), or the same test at two different time points.

Advantage of glucose testing

✓inexpensive and widely available

Disadvantages of glucose testing

✓ fasting requirement

✓ high diurnal variation in glucose

✓ affected by recent physical activity, illness, or acute stress

✓ glycolysis in samples not handled properly and promptly prior to analysis

	Glucose	A1C
Cost	Inexpensive and available in most laboratories across the world	More expensive than glucose and not as widely available globally
Time frame of hyperglycemia	Acute measure	Chronic measure of glucose exposure over the past 2–3 months
Preanalytical stability	Poor; plasma must be separated immediately or samples must be kept on ice to prevent glycolysis	Good
Sample	Measurement can vary depending on sample type (plasma, serum, whole blood) and source (capillary, venous, arterial)	Requires whole-blood sample
Assay standardization	Not standardized	Well standardized
Fasting	Fasting or timed samples required	Non-fasting test; no participant preparation is needed
Within-person variability	High	Low
Acute factors that can affect levels	Food intake, stress, recent illness, activity	Unaffected by recent food intake, stress, illness, activity

Diagnosis and Classification of Diabetes: Standards of Care in Diabetes—2025

✓ *Intra-individual biologic variability*

Definition: a difference in the glucose concentration of a person under the same conditions on different days (5-7%).

✓It is necessary to repeat the sampling and testing of the patient on another day in cases where plasma glucose is within the range of decision cut offs.

✓People with FBS around the maximum normal range should be followed up at shorter intervals.

✓ ***Intra- & inter-assay coefficient of variation for glucose***

The reproducibility index

Target: 2/2%

Acceptable ≤ 3/3 %

✓ *Measurement of glucose*

- ✓ Application: in screening, diagnosis and follow-up of disorders of glucose metabolism

- ✓ Glucose can be measured in whole blood, serum and plasma.

- ✓ Standard sample: Venous blood plasma

- ✓ Common samples tested in the laboratories: Venous blood sample serum

- ✓ Decrease glucose level in whole blood: 5-7 mg/dL/h at room temperature
- ✓ Decrease glucose level in whole blood: 2 mg/dL/h at 4 °C
- ✓ Higher rate of metabolism in the presence of more cells in the sample (bacteremia and leukocytosis)
- ✓ Separation of serum or plasma as soon as possible (30 minutes) after sampling is ideal.
- ✓ In conditions without leukocytosis and bacterial contamination, the results are clinically acceptable up to 90 minutes between sampling and isolation.

- ✓ Glucose stability, in sterile and non-hemolyzed isolated serum, without preservatives:
  - ✓ 25 °C: up to 8 hours
  - ✓ 4 °C: 48 to 72 hours
- ✓ For long-term storage, a temperature of - 20°C is recommended, although it will drop in the long term.
- ✓ If it is not possible to separate the serum quickly, sodium fluoride (as a glycolysis inhibitor) should be added to the sample. (2-2.5 mg per ml of whole blood, effective for 48 hours in refrigerator)

Patient preparation

❖ ***Fasting blood sugar***

✓ 8 hours of overnight fasting (no calorie intake)

✓ Diurnal variation: Average FPG in the morning > evening

❖ ***OGTT test***

✓ 8-14 hours of night fasting including food, tea, coffee, alcohol and cigarettes

✓ taking a mixed diet with at least 150 gr of carbohydrates per day in the previous 3 days

Antecedent carbohydrate restriction in the days prior to OGTT can falsely elevate post-load glucose levels, potentially resulting in a false-positive OGTT .

✓ No physical activity restrictions

✓ Sampling in a sitting position

✓ Smoking is not allowed during the test

Oral glucose tolerance test (OGTT)

- ✓ 75 gr anhydrous glucose load for adults
  - ✓ in 250-300 cc water
  - ✓ in 5-10 min

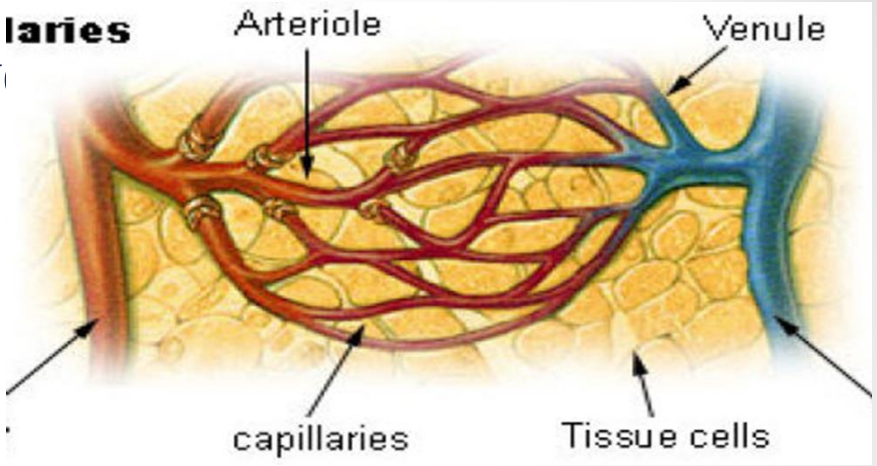
- ✓ Equivalent to 82.5 gr glucose monohydrate

  MW: glucose 180 , H2O 18

- ✓ 1.75 gr glucose/ Kg up to 75 gr for children

Comparison of glucose levels in different blood samples

✓ Glucose concentration in whole blood is 10-15% low(than plasma.



✓ This difference depends on the hematocrit value.

✓ Glucose concentration in capillary blood is similar to arterial blood.

Somogyi et al.

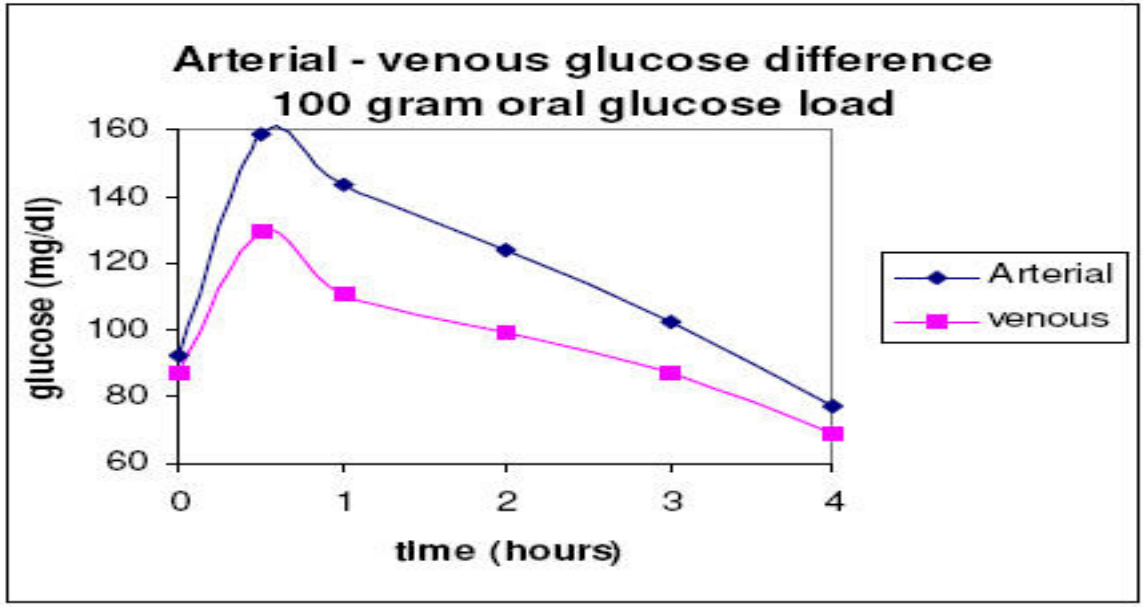
compared the glucose content of blood samples simultaneously drawn from the femoral artery and the fingertip of non-diabetics one-hour after ingestion of 50 grams of glucose.

Difference : less than 1 mg/dL

Comparison of glucose levels in different blood samples

- ✓ Comparing the glucose concentration in capillary blood with venous blood, depending on the time interval between sampling and eating, there can be a significant difference.

- ✓ The difference in glucose concentration in capillary blood and venous blood is 2 mg/dL after 8 hours of fasting and about 30 mg/dL after eating.



Comparison of glucose levels in different blood samples

Somogyi et al. study

- Aim: difference between fingerstick capillary and venous glucose

- 100 healthy individuals

- Fasting state (fasting for 10-14 hours)

- - Fingerstick capillary blood mean value: 89 mg/dL (78-97 mg/dL)

- Venous blood glucose mean value: 84 mg/dL (5 mg/dL lower)

1 mg/dL to 7 mg/dL in 93% of the patients studied

Methods for glucose measurement methods

- ✓ Enzymatic assays using :
  - ✓ Hexokinase
  - ✓ Glucose oxidase

- ✓ Reference method for measuring glucose: hexokinase enzymatic method

- ✓ The difference between hexokinase and glucose oxidase methods is small and has no effect on clinical decisions.


Thank you
for your
attention