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Standard of Care in Diagnosis of Diabetes Mellitus: HbA1c

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Abstract

Objective: The HbA1c concentration is a crucial component of routine diabetes therapy since it serves both as a risk predictor and an indicator of long-term glycemia and represents the average glycemic history of the previous two to three months. The HbA1c assay was approved as the preferred approach for diagnosing diabetes by the American Diabetes Association, the European Association for the Study of Diabetes, and the International Diabetes Federation. This article provides an overview of HbA1c to comprehend its importance in the routine monitoring of diabetic patients. Topics discussed include measurement techniques, advantages and disadvantages of different measurement techniques, clinical significance of HbA1c, rapid diagnosis of HbA1c, and alternative means available to assess glycaemic control.

Methods: This review highlights in detail the structure and formation of HbA1c, clinical importance factors influencing HbA1c levels, methods of measurement, the challenges associated with measuring the HbA1c concentration and describes the current state of the art of analytical and clinical aspects of the process.

Results: Recent developments have made HbA1c assays more sensitive, simple to use, and less expensive. In comparison to previous technologies, the point-of-care HbA1c testing devices deliver quantifiable findings while limiting inference of other Hb variations, making diagnosis far more reliable.

Conclusion: A person with diabetes can live a long and high -quality life if regular monitoring and treatment of diabetes should be done properly. This review emphasizes the shortcomings faced by recent diagnostics techniques and the correlation between falsely elevated and lowered HbA1c results with different physical conditions.

Keywords: Glycated Haemoglobin; Blood Glucose; Glycemic Control; Diabetes Mellitus; Reference Standards; Reference Values

Abbreviations

Abbreviations	*Full Forms*
HbA1c	Glycated haemoglobin
Hb/ Hgb	Haemoglobin
T1DM	Type 1 diabetes mellitus
T2DM	Type 2 diabetes mellitus
GDM	Gestational Diabetes Mellitus
SOC	Standard of Care
RBCs	Red Blood Cells
HbA	Hemoglobin A
HbA2	Haemoglobin A2
HbF	Haemoglobin F
KDA	Korean Diabetes Association
DM	Diabetes Mellitus
UKPDS	United Kingdom Prospective Diabetes
DCCT	Diabetes Control and Complications Trial
HPLC	High-Performance Liquid Chromatography
IFCC	International Federation of Clinical Chemistry
IFCC-WG	International Federation of Clinical Chemistry Working Group
CE	Capillary Electrophoresis
NGSP	National Glycohemoglobin Standardization Program
PBA	Phenylboronic acid
pI	Isoelectric Point
IA	Immunoassay
CNT	Carbon Nanotubes
RVC	Reticulated Vitreous Carbon
CS	Chitosan
TEOS	Tetraenthoxyl Silica
FAO	Fructosyl Amino-Acid Oxidase
MNPs	Magnetic Binananoparticles
EA	Enzymatic Assay
LFIA	Lateral Flow Immunoassay
FIA	Fluorescence Immunoassay
ELISA	Enzyme Linked Immunosorbent Assay
LRET	Luminescence Resonance Energy Transfer
UCNPs	Upconversion Nanoparticles
Hb E	Haemoglobin E
Hb S	Haemoglobin S
Hb D	Haemoglobin D
Hb C	Haemoglobin C

AACC	American Association for Clinical Chemistry
IFCC-RMP	International Federation of Clinical Chemistry Reference Measurement Process
Eag	Estimated Average Glucose
AG	Average Glucose
IDF	International Diabetes Federation
WHO	World Health Organization
CVD	Cardiovascular Disease
GLP-1	Glucagon-like peptide 1
DPP4	Dipeptidyl peptidase 4
eGFR	Estimated Glomerular Function
ADA	American Diabetes Association
FPG	Fasting Plasma Glucose
PG	Plasma Glucose
PDM	Persatuan Diabetes Malaysia
OGTT	Oral Glucose Tolerance Test
GHb	Glycated Hb

Introduction

Diabetes is a heterogeneous group of metabolic disorders that has been characterized by rise in blood glucose level refer as hyperglycemia due to defects in insulin production or action or both. If diabetes is becoming more severe, increasing the likelihood of developing additional illnesses including obesity, cataracts, erectile dysfunction, non-alcoholic fatty liver disease, peripheral artery, and cerebrovascular disease [3, 56, 133, 139]. Type 1 diabetes mellitus (T1DM), type 2 diabetes mellitus (T2DM), and gestational diabetes mellitus (GDM) are the most common forms of diabetes [41]. T1DM diabetes is commonly known as insulin-dependent diabetes or juvenile-onset diabetes or autoimmune diabetes categorized by insulin deficiency, only 5-10% of people with diabetes have type 1 diabetes. It is caused by an autoimmune reaction that damages the pancreatic beta cells on a cellular level [5]. T1DM is classified into three stages stage1 and stage 2 (Presymptomatic) and stage 3 (Symptomatic), in all stage's autoantibodies are present and clear β-cell loss [57].

T2DM diabetes is also referred to as adult-onset diabetes or non-insulin-dependent diabetes, individuals with insulin resistance and relative shortage of insulin affected with this type of diabetes. Type 2 diabetes accounts for 90-95% of people where autoimmune destruction of β-cells does not occur [5]. Gestational Diabetes Mellitus (GDM) is defined as glucose intolerance that causes hyperglycemia during pregnancy and has negative effects on both mothers and new-borns [32]. The occurrence of GDM was made more common by the obesity [85] and diabetes epidemics [84], lack of physical activity, and older age marriages in developing countries like US and China [143]. In India, women who inhabit urban rather than rural areas are more likely to have GDM [146].

For diagnosing and monitoring diabetes, specifically type 2 diabetes, the HbA1c is now suggested as a Standard of Care (SOC) [140]. Glycated haemoglobin (HbA1c) in blood analysis reveals information about a person's typical blood glucose level over the last two to three months which is expected as half-life of red blood cells (RBCs), hence giving a proper reading of HbA1c [80]. When the physiological conditions are favourable, proteins are regularly glycated during numerous enzymatic processes. The nonenzymatic glycation of haemoglobin i.e., glycosylation occurs when glucose interacts with the N-terminal end of the beta-chain, producing a Schiff base [93, 144]. The Schiff base undergoes rearrangement to produce Amadori products i.e., HbA1c. In the first step of the reversible synthesis of glycated haemoglobin, blood glucose and haemoglobin combine to form aldimine. Aldimine eventually converts to the stable ketoam-

ine form in the irreversible secondary step (see Fig.1) [1].

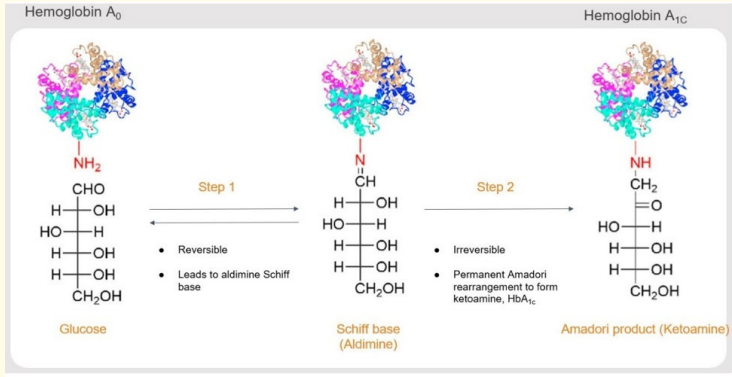

Figure 1: The interaction of Glucose and Hb beta chain N-terminal valine to form HbA1c [140].

The normal range for non-diabetic patients is 4.0% to 5.6% HbA1c, readings between 5.7% and 6.4% are typically indicative of pre-diabetes, while levels of 6.4% or more indicate diabetes [7]. The risk of having problems correlated with diabetes is strongly associated with HbA1c level as level rises. According to earlier research, women are more at risk than men are for developing cardiovascular problems caused by T2DM [27, 49, 50, 82, 98, 99]. That indicates HbA1c levels and diabetes complications are directly correlated; as levels rise, the chances of developing diabetes-related complications increase.

The three forms of haemoglobin that make up 97%, 2.5%, and 0.5% of normal adult haemoglobin are HbA, HbA2 and HbF respectively. The HbA1 fraction accounts for around 6% of all HbA, which is composed of HbA1a1, HbA1a2, HbA1b, and HbA1c based on chromatographic and electrophoretic characteristics [58]. The most prevalent of these fractions, HbA1c makes up about 5% of the overall HbA fraction in healthy individuals [115]. The physiological function cycle naturally includes the synthesis of glycated haemoglobin. However, the quantity of glycated haemoglobin in the plasma also rises as the average plasma glucose level climbs. An epidemic global survey estimated that the prevalence could rise to 10.2% (578 million) by 2030 and to 10.9% (700 million) by 2045, up from 4.7% in 1980 and 9.3% (463 million) in 2019, respectively [112]. Due to the increased prevalence of diabetes and the expenditures per diabetic patient rises the economic cost by 26% in US between 2012 and 2017 [6, 19]. According to a study performed by Korean Diabetes Association (KDA) in 2007, the number of people with diabetes increased by 10% annually [96]. Another study in 2019 showed that the overall incidence of diabetes rapidly increased to 10.9% by 2013 in China [47], similarly in last 15 years in India prevalence of diabetes is risen from 5.9 % to 7.3% as more diabetic cases are reported [11, 37, 42, 111, 116]. Corresponding with increasing diabetic patient numbers the annum economic cost simultaneously rises in India 31.9 billion in 2010 [127]. China (102.9 million) and India (64.5 million) had the largest prevalence of diabetes mellitus, followed by the United States (22.4 million) in 2014, as per research published in 2020 [91]. To overcome this epidemic problem there is a need for proper management. HbA1c is a biochemical marker that is frequently used in the treatment of people with Diabetes Mellitus (DM) to track long-term glycemia and determine the chance of developing further complications. According to the United Kingdom Prospective Diabetes Study (UKPDS), the Diabetes Control and Complications Trial (DCCT) risks of developing health complications are directly correlated with glycemic control, as evaluated by HbA1c concentration [5, 71, 87]. Currently, the HbA1c concentration serves as a helpful diagnostic tool for establishing specific treatment goals and decision thresholds as well as for keeping track of long-term glycemic management. There are more than 700 Hb variants, of which half are silent and the rest affect HbA1c [51]. Interference of these variants provide inaccurate results in High-Performance Liquid Chromatography (HPLC) method although it is considered as the gold standard for HbA1c determination. So, it is recommended to standardize the procedure to ensure correct results [122]. International Federation of Clinical Chemistry (IFCC)

developed a worldwide reference system for an International Reference method as well as the purified HbA1c standards or calibrators in 1994 [85]. According to IFCC, HbA1c is the haemoglobin that is a glycated molecule that binds to the beta chains of one or both N-terminal valines [2]. Different methods are also used to detect HbA1C levels depending on the chemical, structural features, and charge as, electrophoresis, ion-exchange chromatography, affinity chromatography and isoelectric focusing other than HPLC [138]. The International Federation of Clinical Chemistry Working Group (IFCC-WG) has established two reference methods for HbA1c detection and analysis, i.e., mass spectroscopy and Capillary Electrophoresis (CE). The National Glycohemoglobin Standardization Program (NGSP) standardization program began in 1996. The NGSP approached HbA1c standardization and evaluated the results in %HbA1c whereas the IFCC network evaluated the HbA1c results in mmol/mol [90].

Methods for Measurement of HbA1c in Samples (Diabetic Patients)

More than 300 techniques have been developed for the analysis of HbA1c due to the high importance of HbA1c monitoring [21]. Methods of HbA1c measurement classified into two groups techniques based on structural differences and based on chemical differences; a) Separation based on charge difference (ion-exchange chromatography [61, 128], electrophoresis [61, 78] and isoelectric focusing), b) Separation based on structural characteristics of glycogroups on haemoglobin (immunoassay [10, 118] and affinity chromatography [66, 101, 132]), and, c) Separation based on chemical reactivity (photometry, electrospray mass spectrometry)(See Fig.2).

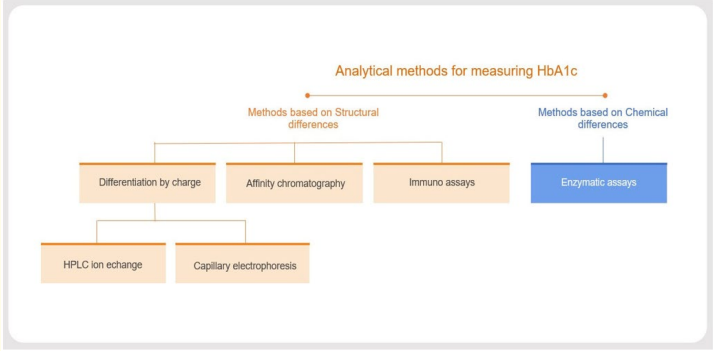

Figure 2: Analytical methods for measuring HbA1c.

Methods based on Structural differences

Ion-exchange chromatography and high-performance liquid chromatography

Based on charge differences, Hb species can be separated using ion-exchange chromatography. Hb species elute from the cation-exchange column at different times depending on their charge when exposed to various buffers of increasing ionic strength. In such columns, the glycosylated forms elute earlier than the non-glycosylated ones because they are more negatively charged. After Hb has been eluted from the column, its concentration is calculated, and the area under each peak is utilised to calculate the amount of HbA1c [17]. Therefore, to detect HbA1c levels in many patients, high performance liquid chromatography (HPLC) has been used widely along with other techniques. Ion exchange or affinity chromatography coupled to an HPLC instrument was required for the separation of the HbA1c molecules from other haemoglobins in the chromatography based HPLC assay. Assay give the result based on the ration of HbA1c peak area to the total haemoglobin peak areas. It is used to detect HbA1c % in blood which provides high reproducibility. It has been designated as the comparative method in the national glycohemoglobin standardisation programme. In a pregnant woman, Capillary Electrophoresis (CE)-HPLC was used to evaluate HbA1c levels and along with some Hb variants (HbA2 and HbA0), 5.4% HbA1c could be detected in blood [149]. Along with this HPLC has some disadvantages required specific instrument, time consum-

ing, required high maintenance, limitation of processing capabilities [128]. Sometimes it may lead to produce false results due to the presence of Hb interferences mostly from Hb carbamylation (reported in uremic patients) or acetylation (due to the presence of large dosages of salicylic acid), that must be considered [77]. Many HbA1c methods apart from HPLC have been developed recently to address these problems.

Boronate affinity chromatography

Based on the biological interactions present in the sample, analytes are separated and analysed using boronate affinity chromatography. This method is used to detect small saccharide molecules such as glucose and fructose which form boronate ester bond with phenylboronic acid (PBA) [60, 123, 125, 129]. Glucose binds to the heamoglobin and forms a unique and stable cis-diol configuration to which borate binds and is detected easily. The obtained result is known as real or total HbA1c, and the patient's value is given as a percentage of this total HbA1c. The measuring range for the HbA1c detection is 5.3-17% [38]. Boronate affinity chromatography is used to differentiate between Hb and HbA1c by specific binding with PBA [132]. These chromatographic methods are time- and money-consuming, even though they produce results that are quite reliable as well as precise. They require skilled workers and sophisticated instruments and might provide incorrect results due to the coexistence of genetic variations and other chemically modified derivatives of haemoglobin [9, 74, 102, 117]. It is preferable to create low-cost, simple-to-use, robust, and fast HbA1c detection technologies.

Capillary electrophoresis (CE)

In general, there are two ways to separate HbA1c in CE based on charge-to-mass ratio. Firstly, analysis as cations in acidic buffers with pH level below the haemoglobin isoelectric point (pI), which is around 7.0. HbA1c and A0 (mixture of non-glycated and glycated haemoglobin fraction) are separated from one another due to a difference in charge caused by the removal of one positively charged amino group from the HbA1c molecule by the attachment of the glucose moiety. Second, Hb analysis as anions in an alkaline environment with selectivity to HbA1c generated by a cis diol contact of its glucose unit with an electrolyte background borate anion [38]. Electrophoretic mobility governs the resolution of charged molecules. Positively charged Hb molecules elute first in decreasing order of their charge to mass ratio. If multiple molecules of the same charge exist, then the resolution based on size is done by the system [119].

Immunoassay (IA)

It is conventional method uses a specific latex-coated antibody (monoclonal) to the glucose and the antibodies that identify the N-terminal glycated amino acids in the first 4 to 10 amino acids of the beta-globin chain of the haemoglobin are used in immunoassay-based HbA1c assays [68]. In Immunoassay, to haemolyze the sample excessive antibodies are added, and these excess antibodies are agglutinated after binding to HbA1c. By using turbidimeter or nephelometer the turbidity of the associated immunocomplexes is determined photometrically which is further used to calculate HbA1c [55]. Other than this when the agglutinator reacts with the antibody and produces scattered light it increases the absorbance. From this absorbance the HbA1c can be calculated by the Soret absorption band or by Drabkins method. The disadvantage of immunoassays is that they are sensitive to haemoglobin F interference [8]. For example, it has been demonstrated that a high haemoglobin F concentration might result in an underestimation of the HbA1c levels of more than 20%. Furthermore, immunoassays require relatively frequent calibration [108].

Biosensors

Bioelectroanalytical sensors make the HbA1c determination much more robust, sensitive, specific, and cheaper. Electrochemical biosensors consist of a biorecognition element, transducer, and detection system. There are various nanomaterials like gold nanoparticles, carbon nanotubes (CNT), core-shell magnetic binanoparticles, nitrogen doped graphene etc., which are used as detection probes for the detection of glycated haemoglobin in different biosensors [22, 86, 94, 120, 131]. Microfluidic, optical, and electrochemical biosensors [33, 65, 81, 104, 109] are the most common biosensors for checking daily glucose level and help to control food consumption

and insulin usage. In comparison with chromatography based HPLC assay, antibody-based immunoassay, and other enzymatic assay these biosensors show better linear response. Molazemhosseini et al. developed a differential pulse voltammetry, a coated-screen printing electrode based amperometric biosensor where they used poly (terthiophene benzoic acid) (Pttba/AuNPs) which showed a linear range of 0.1-0.25 mg/mL of HbA1c [86]. Another study by Liu et al. investigated a reticulated vitreous carbon (RVC) electrode modified with 3-aminophenylboronic acid, chitosan (CS), and tetraenthoxyl silica (TEOS) worked as an electrochemical biosensor for detection of HbA1c. The flow injection and screen-printed electrode gave a linear relationship between HbA1c concentration of 002-2.2 mg/mL and electric charge at RVC electrode [73]. Chawla et al. also formed a high performance amperometric fructosyl valine (FV) biosensor where fructosyl amino-acid oxidase (FAO) immobilized on core-shell magnetic binananoparticles (MNPs) achieving a lower limit of 0.1 mM and linear range of 0-2 mM [23]. In 2015, Chang et al. developed an aptamer-based microfluidics tool for HbA1c detection along with Hb in 25 min [22].

Methods based on Chemical differences

Enzymatic Assay (EA)

A recently discovered direct enzymatic HbA1c assay gives report in %HbA1c directly, and it uses a single channel test, which means no use of total haemoglobin test. Most HbA1C measurements are carried out using HPLC and immunoassay. HPLC provides accurate and precise results, but it requires a large instrument and long running time. In comparison with HPLC and Immunoassay, Enzymatic assay can be measured large number of samples within short period of time, but the repeatability is poor, and the calibration curve is unstable. The enzymatic test technique for measuring HbA1C essentially entails two steps: (a) the proteolysis of HbA1C to release glycated amino acid (or glycated peptide), and (b) a reaction by an enzyme (such as oxidase or dehydrogenase) that has selectivity towards the glycated amino acid (or glycated peptide) [43]. In EA, lysis buffer is used for oxidizing the blood sample to remove low and high molecular weight signal interfering substances. Then the lysed blood is used for the proteolytic digestion process which leads to release of amino acids along with the glycated valine of β-chain. A specific recombinant enzyme fructosyl valine oxidase is a specific oxidizing agent for glycated valine. Fructosyl valine oxidase specifically cleaves N-terminal valines and produces hydrogen peroxide which will be measured using horseradish peroxidase catalysed reaction. It is then measured in %HbA1c using signal produced in the reaction by linear calibration curve [75]. Most HbA1C measurements are carried out using HPLC and immunoassay. HPLC provides accurate and precise results, but it requires a large instrument and long running time. In comparison with HPLC and Immunoassay, Enzymatic assay can be measured large number of samples within short period of time. Table 1 represents the advantages and disadvantages of the above-mentioned techniques.

Method for HbA1c Detection	***Advantages***	***Disadvantages***
HPLC Ion Exchange Chromatography (IEC) [18, 39, 124, 136]	• Able to search for Hb variations in chromograms. • Measurements with great precision • Gold standard method for HbA1c testing • HbA1c overestimation leads to aggressive glucose management, resulting in more frequent hypoglycaemic episodes.	• Haemoglobinopathies, HbF, and carbamylated Hb can all cause variable interference. • Changing the normal process of HbA glycation to A1C. • Causing an unexpected peak on chromatography, rendering A1C measurement incorrect. • Making the red blood cell more susceptible to hemolysis, resulting in a shorter time for glycosylation and a falsely low A1C result.

Capillary Electrophoresis (CE) [29, 59, 124, 136]	• CE is a very precise and accurate method to estimate Hb fractions. • Haemoglobin variants HbF, HbS, HbD, HbE and HbC do not interfere. • The CE separation technique is rapid, uses low amounts of reagents, and is easily automated.	• It has a longer runtime. • High cost as compared to HPLC
Affinity Chromatography [38, 106]	• HbF, carbamylated Hb, and hemoglobinopathies rarely interfere	• It measures the glycation of N-terminal valine on β-chain but also β-chains glycated at other places and glycated α-chains
Immunoassay (IA) [38, 95]	• Not impacted by haemoglobin E, haemoglobin D, or carbamylated haemoglobin; relatively simple to use in a variety of formats. • Reduces the scattering of lightand the absorbance	• Comparatively more time is required to complete the analysis. • Technical skills required for handling. • High price of reagents • May be impacted by hemoglobinopathies with changed amino acids at binding sites
Enzymatic assay [53, 75, 95]	• Enzymatic assay proved to be a robust and reliable method for HbA1c measurement suitable for routine practice in clinical chemistry laboratories. • It gives HbA1c values in percentage, and it is not adversely affected by interferences from common haemoglobin variants in samples. • Unaffected analytically by the presence of Hb variants	• A disadvantage of the enzymatic method is its relatively high cost

Table 1: Advantages and Disadvantages of Methods for HbA1c Detection.

HbA1c Lateral Flow Fast Test: Rapid Diagnosis of HbA1c

There are two available methods for the detection of HbA1c by rapid test, i.e., Gold-based Lateral Flow Immunoassay (LFIA) and Fluorescence Immunoassay (FIA). Gold-based LFIA and FIA are simple flow devices which are based on the principle of Enzyme Linked Immunosorbent Assay (ELISA) [137]. Gold-based LFIA and FIA are point of care testing devices. These are available in quantitative and qualitative formats which are rapid, simple, and low-cost system devices. Gold-based LFIA uses coloured nanoparticles which are excellent labels and are most used, whereas the FIA uses fluorescent dye. There are different other fluorophores like lanthanides, up-converting nanoparticles, fluorescent proteins also can be utilised as label for HbA1c detection. Other than gold nanoparticles magnetic nanoparticles, carbon nanotubes, graphene oxide are also be used as a probe. The coloured nanoparticles which are used in gold based LFIA can be detected visually while fluorescence dye requires ultraviolet light to observe the result. Jain et al. constructed a biosensor where they immobilized ructosyl amino-acid oxidase (FAO) on the nitrogen-doped graphene/gold nanoparticles (AuNPs)/fluorine doped tin oxide (FTO) glass electrode [52]. For the detection of glycated haemoglobin Jo et al. reported a luminescence resonance energy transfer (LRET) based immunosensor. They utilised near-infrared to visible rare earth upconversion nanoparticles (UCNPs) as the

donor and acceptor is HbA1c for the LRET [54]. A comparison between these two techniques has been mentioned below (see Table 2).

Colloidal gold-based lateral flow immunoassay	*Fluorescence immunoassay*
Gold nanoparticles are used	Fluorescent dyes are used
Can be seen by the naked eye	Requires a reader/analyzer to observe results
Qualitative and quantitative detection	Qualitative and quantitative detection
Low sensitivity	High sensitivity
Device-free	Device dependent
The result interpretation can be biased	Result interpretation is AI (Artificial Intelligence) based which reduces chances of biasness

Table 2: Comparison between Gold -based lateral flow immunoassay and Fluorescence immunoassay.

Interfering Hb Variants

The three most common analytical factors that interfere with HbA1c testing are elevated fetal haemoglobin (HbF), Hb adducts, and Hb variations. Hb variants have a small structural difference as well as a point mutation in the protein chain. Nine hundred variants have been discovered, although the majority (99%) belong to the four haemoglobin groups S, C, D, and E. Haemoglobin S shows high prevalence in black Africans, Americans, people of Mediterranean descent, and Indians. Haemoglobin C shows high prevalence in black Africans and Americans, Haemoglobin E shows high prevalence in South-East Asian, and Haemoglobin D is more evenly distributed throughout the world [138]. These interferences are method specific and variant specific, and it is very difficult to recognize the impact of each variant on method type and substitution. This is why the NGSP has made an effort to evaluate all methods for interference from these four haemoglobin variants [90]. HbF is made up of two α-chains and two γ-chains. The γ-chain contains terminal glycine instead of valine and HbF can only be glycated at lysine residues causing a glycation rate that is around one-third that of HbA. For every 1% HbF, IA reduces HbA1c concentration by 1%, while AC reduces HbA1c concentration by 0.7%. This interference becomes significant at levels greater than 10-15%. In general, HbF and HbA1c can be separated using IEC and CE [124]. Most of the cation exchange HPLC can separate Hb F from HbA1c [17]. Most recently found, Haemoglobin Wayne is a rare variant of haemoglobin (Hgb) that can also interfere with HbA1c result. Due to a frameshift mutation in the HBA2 gene produces alpha chain Hgb variant [4]. According to Ao et al. haemoglobin alteres haemoglobin charge and conquers HbA1c by interfering [12]. To overcome this problem boronate affinity employed m-aminophenylboronic acid which reacts with the cis-diol groups of glucose coupled to Hgb and detects total glycated Hgb lowering the interference. Therefore, before diagnosing and treating DM, clinicians should be aware of any abnormal cases, to avoid wrong interpretations [12]. Haemoglobin E (Hb E) is another type of variant inferring HbA1c, occurs when lysine for glutamic acid at position 26 of the beta globin chain is deleted. It is the second most occurring hemoglobinopathy prevalence in East and South-east Asia. A recent study by Little et al. showed people with high trait with Hb E give low HbA1c results, indicating that amino acid substitution at position 26 was far from the N terminus of the beta-globin chain where glycation and antibody binding occurred [72]. Haemoglobin S (Hb S) is another interferer caused by amino acid deletion from glutamic acid to valine at position 6 at beta globin chain showed prevalence in West and North Africa, Middle east, and Indian subcontinent [17]. Another subtype Haemoglobin D (Hb D) generated by substitution of glutamine for glutamic acid at position 121 of the beta globin chain usually found in Sikhs of the Punjab state of the Indian subcontinent. Little et al. also reported 75 5 of Hb D trait cases are silent and 25% are recognizable acquired by affinity chromatography [72]. Haemoglobin C (Hb C) also affects the HbA1c level, caused by the substitution of glutamic acid for lysine at position 6 of the beta globin chain commonly occurring in West Africa and Caribbean regions. HPLC (VARIANT II, Bio-Rad, Hercules), DNA Sequencing, alkaline and acid electrophoresis (HYDRASYS 2, Lisses, France) and capillary zone electrophoresis (CAPILLARYS 2, Sebia) are the tools used to detect hemoglobinopathy [107]. Table 3 covers the 20 most frequently used techniques to measure HbA1c along with information on how each technique is affected by elevated HbF levels, HbC, HbS, HbE, HbD traits. The manufacturer is listed after each method in alphabetical order. When a technique exhibits interference that is clinically significant (marked by "Yes"), the

criteria are >6% at 6 and/or 9% HbA1c.

Method	*Based on*	*Interference from HbC*	*Interference from HbS*	*Interference from HbE*	*Interference from HbD*	*Interference from elevated HbF*
Abbott Architect c Enzymatic	Enzymatic Assay	No	No	No	No	-
Alere Afinion	Boronate affinity assay	No	No	No	No	$
Arkray ADAMS A1c HA-8180V (Menarini)	HPLC	No	No	HbA1c not quantified.	HbA1c not quantified.	No<30%
Beckman HbA1c Advanced B00389 Manual Application on DxC 700 AU AU system	IA	No	No	No	No	$
Beckman HbA1c Advanced B93009 Online Application on DxC 700 AU	Clinical Biochemistry based	No	No	No	No	$
Beckman Synchron System Unicel DxC	Chemistry Analyzer	No	No	No	No	$
Bio-Rad D-100 (A1c program)	HPLC	No	No	No	No	-
Bio-Rad Variant II Turbo 2.0	HPLC	No	No	No	No	No<25% HbF
Ortho-Clinical Vitros	IA	No	No	No	No	$
Roche Cobas c513	RTPCR	No	No	No	No	$
Sebia Capillarys 2 Flex Piercing	CA	No	No	No	No	No<15% HbF
Siemens DCA Vantage	IA	No	Yes↑/No*	Yes↑/No*	No	No<10% HbF
Siemens Atellica	IA	No	No	No	No	$
Siemens Dimension	IA	No	No	No	No	$
Tosoh G8 ver. 5.24, 5.28	HPLC	No	No	No	No	No≤30% HbF
Trinity HPLC	HPLC	No	No	No	No	No≤15% HbF

$ - Without particular method data, it can be expected that HbF levels exceeding~ 10-15% interfere with both immunoassay and boronate affinity procedures.

↑ - Interference causes a higher result.

* Conflicting data in the literature

Table 3: Most frequently used techniques to measure HbA1c and affected interference [124].

Standards for HbA1c

The DCCT completed in 1993, demonstrated that the chronic complications of diabetes are related to glycemic control which can be monitored by HbA1c. DCCT also provided data which shows relation between HbA1c values to mean blood glucose [90]. Earlier the HbA1c test was not being used to its maximum capabilities because HbA1c assay methodologies were not standardised across laboratories; but further research has abundantly demonstrated the benefits and viability of standardising HbA1c assays [69, 137]. In 1996 by the recommendation of the American Association for Clinical Chemistry (AACC) subcommittee the NGSP was initiated. The program's objective was to standardise HbA1c test results so that they could be compared to clinical laboratory results recorded in the DCCT [71]. A working group on HbA1c standardization has been established by the IFCC, and it has created a reference system that will serve as the foundation for the global standardization of all HbA1c assays. In order to attain global uniformity, the IFCC created a Reference Measurement Process (IFCC-RMP). To calibrate the IFCC-RMP, pure HbA1c and HbA0 are combined. Endo proteinase is used to cleave Hb-containing washed and lysed erythrocytes, and the resultant hexapeptides are then quantified using either HPLC-CE or HPLC-electrospray mass spectrometry. Whole blood panels are given values by the IFCC-RMP, which manufacturers use as calibrators. This creates the full quality chain from the IFCC-RMP to the patient. A global network of reference labs in Europe, Asia, and the US includes the IFCC-RMP [137]. While NGSP results can be directly correlated with clinical outcomes and diabetes care objectives, IFCC results are accuracy-based. Although there is a strong correlation between the IFCC and NGSP, the absolute numbers are different, and there is considerable discussion regarding which figures should be presented worldwide.

After assessing the link between the NGSP and IFCC networks, a master equation was built to show the relationship as follows:

NGSP = [0.9148 x IFCC] + 2.152

In 2007, the IFCC recommended that IFCC HbA1c be reported as mmol HbA1c/mol Hb. The primary equation is now,

NGSP = [0.09148 x IFCC] + 2.152

with these updated units. By changing the units, any discrepancy between NGSP and IFCC results is eliminated.

Numerous studies have demonstrated that HbA1c is a measure of average glucose (AG) over the weeks-to-months [106]. The Estimated Average Glucose (eAG) was determined using weighted data from at least two days of continuous glucose monitoring that was carried out up to four times, as well as capillary glucose was self-monitored daily in seven points on at least three days per week.

The association between eAG and HbA1c was determined by linear regression analysis and was as follows:

eAG (mg/dl) = (28.7 x HbA1c) - 46.7, r2 = 0.84

The above interconversions have been tabulated in numerical form below (see table 4).

NGSP HbA1c (%)	*IFCC HbA1c (mmol/mol)*	*eAG (mg/dL)*	*eAG mmol/l*
5.0	31	97	5.4
6.0	42	126	7.0
7.0	53	154	8.6
8.0	64	183	10.2
9.0	75	212	11.8
10.0	86	240	13.4
11.0	97	269	14.9
12.0	108	298	16.5

Table 4: The correlations between NGSP and IFCC HbA1c as well as with eAG (mmol/L and mg/dL) [90].

Glucose Vs HbA1c Measurements

Glucose	*HbA1c*
In glucose assays, certain conditions must be met prior to blood collection for diagnostic purposes,	No such condition needs to be met for HbA1c assays
When testing for glucose assay, blood sampling should be done by a very specific procedure which helps in rapid processing, separation, and storage of serum/plasma at a minimum temperature of 4°C	HbA1c must avoid a situation where the temperature will be above 23°C for more than 12 hours. If not, then it can be stored at 4°C, where the stability will last for at least a week.
Glucose assays test measurement is commonly available worldwide	HbA1c assays measurement are not available worldwide.
Standardized to RMP	Standardized to RMP
Routine calibration for optimal results is required adequately	Routine calibration for optimal results is required adequately
Severe Interferences may increase glucose concentration	Some interferences may reduce red-cell life, and which lead to reduce HbA1c values.
Haemoglobinopathies have little impact on glucose assays unless the patient is ill	HbA1c assays may result in measurement errors.
There are no reported problems regarding haemoglobinopathy traits in glucose assays	In HbA1c assays, most assays are not affected but sometimes deviations can be seen in them
Affordable in most low and middle-income country settings.	In most low and middle-income country, it is unaffordable.

Table 5: Advantages and disadvantage of assays for glucose and HbA1c [140].

Clinical Significance of HbA1c Measurement

Monitoring: To Know the Diabetic state.

The International Diabetes Federation (IDF) estimates that in 2017, 424 million people worldwide had diabetes and by 2045, that number is estimated to rise to 628 million [31]. 122.8 million persons between the age of 65 to 99 are predicted to have diabetes in 2017, with a prevalence rate of 18.8%. If current trends hold, there would be 253.4 million adults over 65 who have diabetes in 2045 [31]. Proper diabetes treatment, which involves controlling glycemia as well as cardiovascular disease risk factors including hypertension and hypercholesterolemia, persons with diabetes can live long, high-quality lives. With the right use of medications, a nutritious diet, and the suggested amounts of physical exercise, this physical disease can be cured [14, 62, 114]. As per World Health Organization (WHO), diabetes affected 108 million people in 1980 and 422 million people in 2014. In low- and middle-income nations, prevalence has been rising more rapidly as compared to high income nations. People with DM frequently use HbA1c testing to monitor their long-term glycemic control because HbA1c is directly aligned with the risk of developing diabetic complications like cardiovascular disease (CVD) [70, 141]. Other than that, elevated HbA1c levels are associated with an increased risk of diabetes-related complications, such as kidney problems, nerve damage, and eye issues [31]. Since HbA1c measures the average glycemia, it provides information on the level of blood glucose, therapeutic response to be taken and risk of developing or aggravating diabetic problems. If no other hypoglycemic medicine is effective in treating pregnancy-related hyperglycemia and type 2 diabetes, insulin is usually recommended. Metformin, sulphonylureas, GLP-1 analogues, and DPP4 inhibitors are among the treatments for type 2 diabetes that are often prescribed [31]. Continuous comprehensive eye exams, treatment of eye complications (retinopathy), assessment and treatment of cardiovascular diseases, measurement of urine albumin and creatinine and estimated glomerular function (eGFR) for kidney health are all necessary components of glycemic control [31].

Diagnosis: Regular health check-ups and symptomatic cases

Due in part to the lack of assay uniformity, the American Diabetes Association (ADA) has not previously advised using HbA1c to diagnose diabetes. However, HbA1c assays are now very standardised, and the outcomes can be applied consistently through time and across populations. HbA1c measures may be used in addition to or in place of the glucose tolerance test and Fasting Plasma Glucose (FPG). Recently, the ADA proposed HbA1c with a cut-point of 6.5% as an alternative to FPG-based criteria (FPG 7.0 mmol/L) for the diagnosis of diabetes [88]. According to epidemiologic research, there is a correlation between HbA1c and the risk of retinopathy that is identical to that for matching FPG and 2-hour post load plasma glucose (2-h PG) criteria. Since fasting is not necessary, the HbA1c is more convenient than the FPG and has fewer daily fluctuations during stressful and unwell times. It also appears to have stronger preanalytical stability. The HbA1c levels and blood glucose levels are found to be correlated. The average blood glucose level of an individual is determined by the haemoglobin that has been glycated by glucose, or HbA1c. The established glucose criteria for the diagnosis of diabetes (FPG and 2-h PG) are still in use. Diabetes can continue to be diagnosed when a random (or casual) PG of <200 mg/dl (11.1 mmol/l) is found. In these situations, it is possible that the medical professional would also perform an HbA1c test as part of the initial evaluation of the diabetes severity and that the result would be higher than the diagnostic cut point. Unless the diagnosis is unequivocal on clinical grounds, such as in a patient with the characteristic symptoms of hyperglycemia or hyperglycemia crisis, a test result that indicate diabetes is repeated to exclude laboratory error, as is the case with most diagnostic tests. Since there is a higher chance of concurrence in this situation, it is recommended to repeat the same test for confirmation. For instance, if the HbA1c is 7.0% and a follow-up result is 6.8%, diabetes is officially diagnosed. The results of two distinct tests, FPG and HbA1c, may be provided for the same patient in some circumstances. In this case, the diagnosis of diabetes is confirmed if the results of the two distinct tests are both above the diagnostic threshold.

Diagnosis of diabetes during pregnancy

According to the International Diabetes Federation, hyperglycemia occurred in 21.3 million or 16.2% of live births to women in 2017. Of these, 86.4% were caused by gestational diabetes mellitus (GDM), and 6.2% were caused by diabetes that was discovered before becoming pregnant [31]. Gestational diabetes mellitus (GDM) has long been referred to as any degree of glucose intolerance that begins or is initially noticed during pregnancy. Even though delivery usually resolves most cases, the definition was applicable whether the condition remained after birth, and it did not rule out the potential that undetected glucose intolerance may have developed before or concurrently with the pregnancy. Although its limitations were known for a long time, this definition made it easier to develop a consistent approach for the detection and categorization of GDM. The percentage of pregnant women with undiagnosed type 2 diabetes has increased because of the increasing obesity and diabetes epidemic among women of reproductive age. Mothers having GDM also have a high chance of giving birth babies with type 2 diabetes [15, 25, 35]. For the diagnosis of GDM in pregnant women, HbA1c shown high sensitivity with relatively low specificity and was a possible predictor of Persatuan Diabetes Malaysia (PDM) [5]. Because there is a very low chance of high blood sugar during pregnancy, it is advised to have an oral glucose tolerance test (OGTT) between the 24th and 28th week of pregnancy to distinguish between GDM and other pregnancy symptoms [83].

HbA1c and Covid-19

Currently, several studies have been developed to focus on details of the clinical and virological course of SARS-CoV-2 infection. Accordingly, findings show that those who are older and have chronic conditions such as diabetes and hypertension are more likely to have coronavirus disease 2019 (COVID-19) and have a higher death risk [134, 148, 150]. As we all know, DM is a condition that needs to be taken seriously in terms of public health because of its rising prevalence and link to several illnesses, such as heart disease, renal failure, and stroke [63]. There is evidence that improved glucose control is related to better clinical outcomes in COVID-19 patients [147, 151]. However, it is unclear if COVID-19 contributes to hyperglycemia. A prior study suggested that the pancreas could be a target of coronavirus infection because SARS-CoV was found in the pancreas [28]. Another study discovered that SARS-CoV damaged the endocrine part of the pancreas, suggesting that SARS-CoV may cause acute insulin-dependent diabetes mellitus [145]. Furthermore,

infection causes significant changes in whole-body metabolism, including glucose, lipid, and protein metabolism [79]. Even though several research suggests that diabetes is a significant risk factor for COVID-19. Some recent research has raised concerns about the relevance of testing HbA1c levels in COVID-19 patients upon hospital admission [100]. In COVID-19 patients, a high HbA1c level is linked to inflammation, hypercoagulability, and poor blood oxygen saturation, and patients with diabetes have a higher mortality risk (27.7%) [135]. As a result, it is important that all COVID-19 patients are checked for absolute hyperglycemia at the time of admission so that immediate and effective treatment can be started. Before the infection, the risk of COVID-19 related death may be higher in people with diabetes and poor glycemic management [100].

Life of Erythrocytes vs HbA1c

Erythrocytes are the most abundant cells in the bloodstream and the first to be affected by changes in the composition of plasma. Erythrocyte shape and function are both influenced by chronic hyperglycemia. Erythrocyte related markers can serve as a useful guide for the prevention, diagnosis, and treatment of DM and its consequences. HbA1c can be used as a marker for chronic hyperglycemia and can show an overall index of glycemia during the last 120 days, which is the average life span of a red blood cell. The HbA1c test is being used to identify diabetes, with a cut-off level of 6.5% being recommended as a positive diagnosis [103].

There are some factors affecting HbA1c level, like certain diseases, age, gender and race. Due to these, falsely elevated and lowered HbA1c results are observed (see Table 5) [48, 103]. Any disease that increases the erythrocyte's lifespan or is linked to a reduction in red blood cell turnover falsely elevates the HbA1c levels. For example, in patients with iron deficiency anemia, the HbA1c values were found to be considerably higher, and decrease in level was observed after iron therapy. It was unclear what caused the elevated levels of HbA1c. Like this, any condition that reduces erythrocyte life or is linked to higher red blood cell turnover leads to lowered HbA1c levels. Haemolytic anemia, splenomegaly, acute and chronic blood loss, and other conditions can all provide falsely reduced HbA1c values [48]. Most patients with end-stage renal illness have HbA1c levels that are abnormally low. This is mainly because of the associated chronic anemia with reduced red cell lifespan [97].

The diagnostic accuracy of HbA1c for diabetes was reduced by the increase in age, which was brought on by a decline in RBC count. Because of their biologically lowered RBC count, older adults cannot be diagnosed with diabetes using HbA1c [142]. HbA1c shows positive correlation with age and the level of HbA1c in male is significantly higher than female. The HbA1c level of male in age group 30-39 years and 40-49 years is higher than female while in age group 50-59 and 60-70 years no significant difference is observed for both sexes and their HbA1c level significantly increased with the age [48]. According to age and gender, different HbA1c cut-point values should be used to diagnose diabetes [48]. The effect of age and on HbA1c level is currently under discussion. According to some research, the HbA1c level rises by about 1 mmol/mol (0.1%) per decade [152]. There are conflicting results from other research suggesting African Americans and Hispanics in the US have greater HbA1c concentrations than Caucasians. Additionally, it is unknown if this would have therapeutic significance [110].

Condition	***Effect on HbA1c***
Anaemias linked to decreased red blood cell turnover, such as iron insufficiency, vitamin B12 deficiency, and folate deficiency anaemias [13, 16, 24, 126]	False Increase
Asplenia (Increased erythrocyte lifespan) [64]	False Increase
Uremia (Formation and detection of carbamyl-haemoglobin) [46]	False Increase
Severe hypertriglyceridemia (When level > 1,750 mg/dL) [30]	False Increase
Severe hyperbilirubinemia (When level > 20 mg/dL) [138]	False Increase
Chronic alcohol consumption (Formation of acetaldehyde-HbA1 compound) [44, 130]	False Increase
Chronic opioid ingestion [105, 130]	False Increase
Chronic salicylate ingestion [130]	False Increase

Lead poisoning [71]	False Increase
Anemia from acute or chronic blood loss includes haemolytic anemia [92]	False Decrease
Splenomegaly (Decreased erythrocyte lifespan) [92]	False Decrease
Pregnancy*(Decreased erythrocyte lifespan) [40, 67, 76]	False Decrease
Vitamin E ingestion (Reduced glycation) [20, 138]	False Decrease
Ribavirin and interferon alpha (Possibly due to haemolytic anemia) [34, 36]	False Decrease
Red blood cell transfusion¥ [121] High glucose concentration in storage medium (False elevated) Dilutional effect (False lowered)	False Increase or False Decrease
Haemoglobin variants (Depends on method and assay used A1c generally reliable for heterozygous variants, but not homozygous variants) [17, 89, 113]	False Increase or False Decrease
Vitamin C ingestion [26] May increase A1c when measured by electrophoresis. Due to competitive inhibition of glycosylation, levels that are measured by chromatography may decrease	False Increase or False Decrease

Table 6: Conditions Associated with Falsely Elevated or Lowered HbA1c.

Discussion

HbA1c has long been used to measure glycemic control in people with diabetes. It can be used to calculate long-term average glycemia and create a diabetes mellitus dosage regimen. The HbA1c test has substantially improved since the major clinical trials (DCCT, UKPDS) revealed the association between glycemic control, HbA1c, and diabetic complications. The HbA1c test is precise, simple to perform, and provides immediate results. It can be an efficient method for diagnosing diabetes, particularly in low- and middle-income nations. Nowadays the HbA1c concentration is a reliable test and an important tool for both the routine care and diagnosis of diabetes. However, suitable assays that are globally accessible and traceable to the IFCC-RMP have not yet been achieved, particularly in developing nations. Although the IFCC-RMP has been adopted as the only legitimate anchor for standardization, HbA1c concentrations are still reported in multiple units, making universal reporting challenging. Although the HbA1c concentration is more routinely employed for diagnosis, it still has to be determined to what extent biological variance restricts its use. It is necessary to generate more precise reference values and clinical decision boundaries relating to patient groups, age, and ethnicity. Rapid and accurate HbA1c laboratory diagnosis is required using several laboratory methods. So far, HPLC, immunoassay, and enzymatic reactions have been used for such testing, but each has limitations. Only a small number of devices are capable of meeting the required performance standards, and it is uncertain how well the test will function when administered by non-experts. Although a higher number and better tools for the management of diabetes are now available. Clinicians, patients, and laboratory workers all needed a lot more training in using such tools to provide improved sensitivity in patient care.

Conclusion

For the detection of HbA1c, several POC instruments are widely used. It has various advantages over other HbA1c techniques and is easy to operate for non-laboratory staff. Even though HbA1c has been approved for diabetes diagnosis, several testing methodologies and cut-off limits are still being debated in most countries throughout the world. The combination of FGT and HbA1c, on the other hand, greatly improves the diagnostic accuracy of these separate tests. However, measuring HbA1c as a sole tool for diagnosing diabetic status cannot be utilized therapeutically because the level of glycated Hb (GHb) depends on a variety of factors, as mentioned in this review, and might produce false results. To ensure that GHb values are correctly interpreted, hematological status should always be considered. As the diabetes epidemic spreads over the world, the HbA1c test may continue to be used as a diagnostic and predictive tool, resulting in better patient treatment and successful clinical outcomes.

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Author Contributions

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Ethics Approval

This article does not contain any studies with human participants performed by any of the authors.

Conflict of interest

The authors declare that they have no conflict of interest.

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