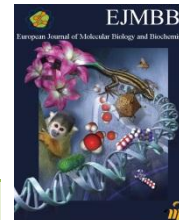




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GLYCATED HAEMOGLOBIN: THE YESTERDAY, TODAY, AND TOMORROW!

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ABSTRACT

Glycated haemoglobin has been in use to monitor control of blood glucose in diabetic patients for about three decade as the 'gold standard' for monitoring glycaemic control , as a predictor of diabetic complications and as a screening tool for diagnosis of DM and metabolic syndrome . It provides an average blood glucose level during preceding 10 - 12 weeks. It is a very convenient blood test, can be done in any clinical setting regardless of prandial state. There were thirty different laboratory methods available to measure glycated haemoglobin with significant variability of results on same sample. IFCC developed a new reference method to measure the glycated haemoglobin, and the method is accepted worldwide as only valid anchor for the measurement of HbA1c. In 2009 International expert committee recommended the use of HbA1c to diagnose diabetes with a threshold 6.5%. IFCC recommended the use of a new unit, i.e. mmol HbA1c/mol of total haemoglobin in place of percentage. Meanwhile a trial was conducted to find out relationship between average blood glucose and glycated haemoglobin, and a linear regression equation was developed to measure average blood glucose from HbA1c. Using the equation one can calculate average blood glucose from glycated haemoglobin in mmol/mol. This average blood glucose will be reported as "eAG" (estimated average glucose) and it will be used to monitor glucose control as eGFR (estimated glomerular filtration rate) is used to monitor renal function in chronic kidney disease patients. How easy or difficult would it be to abandon a term and a unit, in use for three decades and introducing a new unit (mmol/mol) and even a new term (eAG); only time will tell. Health professionals will need to familiarize with new term and units, they will also have to spend more time with their patients to educate them about new developments. This review provides an update on the optimal HbA1c targets, its reliability in control of diabetic complications, limitations of test results and its importance in control of diabetes patients and their complications, various cut-off values obtained in studies performed both in India and worldwide. Diagnostic methods and laboratories are insufficiently standardized for HbA1c in India. The clinician must consider the overall patient profile in addition to a number of local variations and disorders especially hemoglobinopathies /anemias before accepting an abnormal HbA1c value. Supportive or repeat tests may be required leading to increase in cost and delay in diagnosis. In the present Indian scenario, especially the fragmented unorganized health care sector in suburban areas, HbA1c cannot be accepted as a sole and independent test to diagnose diabetes mellitus.

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INTRODUCTION

Measurement of glycated proteins primarily GHb, is widely used for routine monitoring of long-term glycemic status in patients with diabetes mellitus. GHb is used both as an index of mean glycaemia and as a measure of risk for the development of diabetes complications. This test is also being used increasingly by quality assurance programs to assess the quality of diabetes care (e.g., requiring that health-care providers document the frequency of GHb testing in patients with diabetes and the proportion of patients with GHb values below a specified value). The terms glycated hemoglobin, glycohemoglobin, "glycosylated" (which should not be used) hemoglobin, Hb A1 and Hb A1c have all been used to refer to hemoglobin that has been modified by the nonenzymatic addition of glucose residues. However, these terms are not interchangeable.

In order to eliminate this confusing nomenclature, the term "A1c test" has been suggested. As described in the text, most of the clinical outcome data that are available for the effects of metabolic control on complications (at least for the DCCT and UKPDS) used assay methods that quantified Hb A1c. HbA1c has been the most widely used and accepted test for monitoring the glycaemic control in individuals with diabetes. Once a haemoglobin molecule is glycated, it remains in the red blood cell for the rest of its life-span (120 days). As such, it provides information about the degree of long-term blood glucose control. The HbA1c level does not reflect exact mean blood glucose; rather, it is weighted proportionally towards recent levels. The formation of glycated Hb depends upon ambient glucose concentrations in which erythrocytes circulate as well as the duration of exposure. A whole blood sample for glycated Hb is sufficient regardless of prandial state and clinical setting.

HISTORICAL PERSPECTIVE

The story begins in the 1950s with studies of the electrophoretic and chromatographic heterogeneity of haemoglobin in non-diabetic subjects. Several varieties of haemoglobin were found in low concentrations and were proved not to be artefacts. In 1958 Allen et al. published a paper describing the heterogeneity of haemoglobin A. Homquist et al. had published on the β chain N terminally blocking group of HbA1c but the definitive structure was elucidated by Bunn et al. In 1968 Bookchin and Gallop subsequently reported that the largest of these minor fractions, designated Hb A1 had a hexose moiety linked to the N-terminus of the β 3-globin chain. Working independently, Rahbar reported that an abnormal fast moving haemoglobin fraction was present in just two of 1200 patients tested in Tehran; both had diabetes.

Huisman and Dozy had previously attributed an increase in fast moving haemoglobin in four diabetic patients to the tolbutamide they were being treated with. Further studies by Rahbar and colleagues' in 1969 showed that this haemoglobin variant found in abnormally high

concentrations in diabetes was identical to the Hb A1c originally identified by Allen et al. In a study of identical twins concordant and discordant for diabetes, Tattersall et al. showed that the abnormal proportion of fast haemoglobin found in diabetes was an acquired manifestation of metabolic abnormality and not, as had been suggested, an inherited marker for diabetes. The final and crucial observation which led to the use of assays of abnormal haemoglobins as a method of assessing diabetic control was the demonstration by Koenig et al. that Hb A1c concentration was proportional to fasting blood glucose concentration and glucose tolerance. Furthermore, they showed that HbA1c concentrations fell when diabetic control was improved by treatment. The use of haemoglobin A1c for monitoring the degree of control of glucose metabolism in diabetic patients was proposed in 1976 by Anthony Cerami, Ronald Koenig and co-workers. In the 1980s, HbA1c evolved as a better index of glycaemic control in clinical trials. This, along with the other method that emerged by that time, namely, self-monitoring of blood glucose (SMBG) greatly enhanced the achievement of glycaemic control. Regular SMBG had a positive effect on improving glycaemia especially in individuals treated with insulin. SMBG reflects the immediate plasma glucose levels, whereas HbA1c measures long-term glycaemic control. After the data from The Diabetes Control and Complications Trial (DCCT) and UK Prospective Diabetes Study (UKPDS) became available, HbA1c has become an integral part of monitoring the glycaemic control in DM (Diabetes mellitus). The American Diabetic Association (ADA) recommendation of the goal of achieving a HbA1c level of less than 7 as evidence of satisfactory glycaemic control in patients treated for DM revolutionized the significance of HbA1c as a diagnostic test for assessing the adequacy of glycaemic control.

METHODS AND MATERIALS

Biochemistry

Glycated haemoglobin is defined as haemoglobin that is irreversibly glycated at one or both N-terminal valines of the beta chains. This definition does not exclude haemoglobin that is additionally glycated at other sites on alpha or beta chains. It is modified haemoglobin, with a stable adduct of glucose (covalently linked) to the N-terminal valine of the β chain. Normal adult haemoglobin consists predominantly of HbA also called HbA0 ($\alpha_2\beta_2$), HbA2 ($\alpha_2\delta_2$) and HbF ($\alpha_2\gamma_2$) (97, 2.5 and 0.5% respectively). About 6% of total HbA is termed HbA1, which in turn is made up of HbA1a1, HbA1a2, HbA1b and HbA1c. The number "1c" represents the order of haemoglobin detection on chromatography. These fractions are defined by their electrophoretic and chromatographic properties, which differ slightly from those of the major component HbA0, despite the amino acid sequences of HbA1 and HbA0 being identical. HbA1c is the most abundant of these fractions and in health comprises approximately 5% of the total HbA fraction.



Structural and chemical investigations elucidated that glucose, in the open chain format, binds to the N-terminal to form an aldimine (Schiff base) before undergoing an Amadori rearrangement to form a more stable ketoamine. This is a nonenzymatic process that occurs continuously *in vivo*.

Non-enzymatic Glycation verses Enzymatic Deglycation:

Most proteins (including haemoglobin) react with sugars to form covalent compounds without the involvement of enzymes. This chemical process is termed non-enzymatic glycation. The resulting accumulation of advanced glycation end products is associated with the progression of the complications of diabetes whereas enzymatic deglycation reverses the process of non-enzymatic glycation and generates free amino groups.⁶ Enzymatic deglycation is a formidable defence system against non-enzymatic glycation in mammalian cells. This system operates using fructosamine-3-kinase (FN3K), phosphorylating fructoselysine residue on glycated proteins and thereby destabilizing the compound, ultimately causing the decomposition of the glycated proteins. This process of enzymatic deglycation is overwhelmed by episodes of extreme hyperglycaemia in individuals with diabetes as nonenzymatic glycation continues unabated. In the long run, it alters the stability of the protein structure, ultimately leading to cellular dysfunction.¹⁰ These Advanced Glycation End products (AGEs) directly and indirectly (via receptors) promotes the development of cardiovascular disease. They accumulate in different parts of the body and interact with receptors for advanced glycation end products (RAGE), induce oxidative stress, increase inflammation and enhance extracellular matrix deposition, thereby accelerating the process of endothelial dysfunction. Consequently, they result in accelerated plaque formation and ultimate atherosclerosis in diabetes. Glycated haemoglobin, intermediary compound is reversible but after some internal rearrangement of the compound, a stable HbA1c is formed. Several glycation sites of the HbA molecule exist; N-terminal valine residue of the β -chain is the predominant glycation site, accounting for 60% of bound glucose. Of the three types of HbA1 namely, HbA1a, HbA1b, and HbA1c. HbA1c represents the most prevalent glycated species.

Other proteins which undergo glycosylation

Albumin, α_2 macroglobulin, antithrombin III, fibrinogen, ferritin, HDL and LDL, transferrin; all of them are short half-life proteins. The glycosylation process of short half- life proteins stops at the formation of the stable ketoamine adduct

Rationale

Traditionally, HbA1c has been thought to represent average glycemia over the past 12 to 16 weeks.

In fact, glycation of hemoglobin occurs over the entire 120-day life span of the red blood cell but within these 120 days recent glycemia has the largest influence on the HbA1c value. Kinetic studies have revealed that glycemia in the recent past influences the GHb values more than the remote past thus; mean blood glucose of past 1 month, 2 months and 3 months contributes 50%, 40% and 10% respectively to the final result. By mathematical modelling the $t_{1/2}$ of HbA1c is estimated to be 35.2 days. This means that half of glycation seen during estimation has occurred in the previous 35.2 days. The advantage that HbA1c can give as an assessment of average plasma glucose can also be perceived as a drawback because it does not give an indication of the stability of glycemic control. Thus, in theory, one patient with wildly fluctuating glucose concentrations could have the same HbA1c value as one whose glucose varies little throughout the day.

ASSAYS TO MEASURE HbA1c

Till 1999, the following assays were being used for measuring HbA1c were boronate affinity chromatography (more than 50% of laboratories), cation or ion-exchange high performance liquid chromatography (HPLC) methods (30%), immunoassay (15%), and electrophoretic methods (<5%). However, presently, cation exchange performed by HPLC is the most widely used assay method. HbA1c accelerates faster in a cation-exchange resin. Ion exchange chromatography takes advantage of the lower isoelectric point that develops when glucose attaches to the α -chain N terminal valine and HbA1c acquires an extra negative charge. The concentration of haemoglobin is measured using a spectrophotometer and quantified by calculating the area under each peak of the chromatogram compared with a calibrated chromatogram.

The BioRad Diamat (HPLC cation exchange using Bio-Rex 70 resin; BioRad, Hercules, CA, USA) was the reference method used for the DCCT; the MonoS assay (Pharmacia Biotechnology, Uppsala, Sweden) and KO500 (Japanese Society for (Clinical Chemistry) are also ion-exchange HPLC systems. There is no question that different methods and laboratories yield different HbA1c results. This has caused major comparability problems and the need for standardization which has been emphasized by many diabetes organizations.

The National Glycohaemoglobin Standardization Program (NGSP) was created in 1993 specifically to help laboratories across the United States and internationally report HbA1c results that are traceable directly to the DCCT and UKPDS standards. The NGSP has been remarkably successful in this effort.

Standardization of Glycated Haemoglobin measurement; why it is necessary?

Glycated Hb has been accepted as the gold standard measurement for the assessment of chronic hyperglycaemia for nearly three decades. There are thirty



different laboratory methods available to measure glycated haemoglobin. Various analytical methods based on different assays principles, from ion-exchange chromatography to immunoassay and electrophoresis has been used to measure glycated haemoglobin. Such a lack of standardization resulted in wide variability within results (4.0% to 8.1%) on the same sample making it difficult to compare patients results among laboratories. This disparity has always been a source of anxiety among health care providers. It becomes even more important in this age of heavy economical migration, when people travel long distances and take their native record with them. Therefore having same method and unit to measure HbA1c is need of the day. To overcome this problem, in 1995 the International Federation of Clinical Chemistry (IFCC) took the lead in developing a uniform international standardization of HbA1c. For the calibration of the reference method, mixtures made of pure HbA1c and HbA0 were developed.

A laboratory network was also setup, which use two reference assays that combined reverse-phase high performance liquid chromatography (HPLC) with mass spectroscopy or capillary electrophoresis, using same mixture as calibrators. The IFCC then defined HbA1c as haemoglobin that is irreversibly glycated at one or both N-terminal valines of the betachains. This definition also covers Hb that is additionally glycated at any lysine residue in the β -chain. Prior to the IFCC's definition, HbA1c had been defined as a certain peak in an HPLC system, which obviously did not sound very scientific. Haemoglobin that is only glycated at a lysine site is not included in the measurement of HbA1c. Since the IFCC measurement is too specific, it only measures one molecular species of HbA1c: thus, non-HbA1c components are not included in final results. Consequently HbA1c values obtained by using IFCC method are 1.5 to 2 percentage points lower than the NGSP results traced to DCCT, as well as Swedish and Japanese designated comparison methods. 31 Concerns were raised about the impact of this value change on patient care, which could result in less than desirable control of glycaemia in diabetic patients. To overcome this problem a "master equation" was developed to formulize the relationship between the IFCC reference method and all three designated comparison methods (DCMs) namely, the National Glycohemoglobin Standardization program of US (NGSP), Japanese Diabetes Society/Japanese Society of Clinical Chemistry (JDS/JSCC), and Mono-S in Sweden. The master equation allows for the conversion of the IFCC results to more customary HbA1c results, which could be traced to results from DCCT and United Kingdom Prospective Diabetes Study (UKPDS). In 2004, the American Diabetes Association, European Association for the study of Diabetes, and International Diabetes Federation working group of the HbA1c assay was established to harmonize the reporting systems. It included members from the ADA, IDF, EASD, NGSP and IFCC. In

2007, the IFCC recommended that HbA1c results be expressed as mmol HbA1c/mol Hb instead of an HbA1c percentage. Patients using mmol/l or mg/dl for self-monitoring of day-to-day glucose control find it difficult to understand when their doctors discussed haemoglobin levels in percentages.

To eliminate confusion and streamline these discrepancies, a consensus statement³⁴ on the worldwide standardization of haemoglobin A1c measurement was adopted in May 2007 by the ADA, EASD, IDF and IFCC. It states that new IFCC reference system is the only valid anchor for implementing the standardization of the measurement of HbA1c. In addition, HbA1c results were to be reported worldwide in IFCC units (mmol glycated Hb / mol total Hb) and derived NGSP units (%), using the IFCCNGSP master equation. Thus, the 25 to 42 (mmol/mol) range would indicate non-diabetics, as the similarly derived NGSP units of the non-diabetic range were 2.5 to 4.2% (HbA1c). It was also resolved that if the ongoing "average plasma glucose study" was concluded successfully (i.e. confirmed the relationship between average blood glucose and HbA1c) then the A1c-Derived Average Glucose Equivalent would also be reported as an interpretation of HbA1c results.

RESULTS & DISCUSSION

Diagnosis of Diabetes Mellitus

Historically, the measurement of glucose has been the means of diagnosing diabetes. Type 1 diabetes has a sufficiently characteristic clinical onset, with relatively acute, extreme elevations in glucose concentrations accompanied by symptoms, such that specific blood glucose cut points are not required for diagnosis in most clinical settings. On the other hand, type 2 diabetes has a more gradual onset, with slowly rising glucose levels over time, and its diagnosis has required specified glucose values to distinguish pathologic glucose concentrations from the distribution of glucose concentrations in the non-diabetic population. The diagnosis of diabetes was made when

- 1) Classic symptoms were present;
- 2) The venous FPG was >140 mg/dl (>7.8 mmol/l); or
- 3) After a 75-g glucose load, the venous 2HPG and levels from an earlier sample before 2 h were >200 mg/dl (>11.1 mmol/l). In 1997, the Expert Committee on the Diagnosis and Classification of Diabetes Mellitus [16] re-examined the basis for diagnosing Diabetes. In comparing the relationship between FPG and 2HPG values and retinopathy, it was apparent that the previous FPG cut point of 140 mg/dl (7.8mmol/l) was substantially above the glucose level at which the prevalence of retinopathy began to increase. As a result, the committee recommended that the FPG cut point be lowered to >126 mg/dl (7.0 mmol/l) so that this cut point would represent a degree of hyperglycemia that was "similar" to the 2HPG value and diagnosis with either measure would result in a similar prevalence of diabetes in the population. The 1997 report



also recommended that the FPG level, rather than the 2HPG, be the preferred test to diagnose diabetes because it was more convenient for patients and less costly and time consuming and the repeat-test reproducibility was superior.

HbA1c in the diagnosis of DM

Chronic hyperglycemia sufficient to cause diabetes-specific implications is the hallmark of diabetes. Common sense would dictate that laboratory measures that capture long-term glycemetic exposure should provide a better marker for the presence and severity of the disease than single measures of glucose concentration. Studies consistently demonstrated a strong correlation between retinopathy and A1C but a less consistent relationship with fasting glucose levels [20]. The correlation between A1C levels and complications has also been shown in the setting of controlled clinical trials in type 1 and type 2 diabetes, and these findings been used to establish the widely accepted A1C treatment goals for diabetes care. Large volume of data from diverse populations has now established an A1C level associated with an increase in the prevalence of moderate retinopathy and provides strong justification for assigning an A1C cut point of >6.5% for the diagnosis of diabetes. This cut point should not be construed as an absolute dividing line between normal glycemia and diabetes; however, the A1C level of 6.5% is sufficiently sensitive and specific to identify individuals who are at risk for developing retinopathy and who should be diagnosed as diabetic.

The A1C level is said to be least as predictive as the current FPG and 2HPG values. In selecting a diagnostic A1C level >6.5%, the International Expert Committee balanced the stigma and costs of mistakenly identifying individuals as diabetic against the minimal clinical consequences of delaying the diagnosis in someone with an A1C level >6.5%. An International Expert Committee, after an extensive review of both established and emerging epidemiological evidence, recommended the use of the A1C test to diagnose diabetes, with a threshold of >6.5%, and ADA affirms this decision. The diagnostic A1C cut point of 6.5% is associated with an inflection point for retinopathy prevalence, as are the diagnostic thresholds for FPG and 2-h PG. The diagnostic test should be performed using a method that is certified by the National Glycohemoglobin Standardization Program (NGSP) and standardized or traceable to the Diabetes Control and Complications Trial (DCCT) reference assay. Point-of-care A1C assays are not sufficiently accurate at this time to us for diagnostic purposes.

ADA 2010 Criteria for the diagnosis of diabetes:

1. A1C >6.5%. The test should be performed in a laboratory using a method that is NGSP certified and standardized to the DCCT assay.* OR
2. FPG >126 mg/dl (7.0 mmol/l). Fasting is defined as no caloric intake for at least 8 h.* OR

3. 2-h plasma glucose >200 mg/dl (11.1 mmol/l) during an OGTT. The test should be performed as described by the World Health Organization, using a glucose load containing the equivalent of 75 g anhydrous glucose dissolved in water.*OR

4. In a patient with classic symptoms of hyperglycemia or hyperglycemic crisis, a random plasma glucose >200 mg/dl (11.1 mmol/l).

*In the absence of unequivocal hyperglycemia, criteria 1–3 should be confirmed by repeat testing.

CLINICAL PROFILE OF HbA1c

The ADA recommends measurement of HbA1c (typically 3-4 times per year for type 1 and poorly controlled type 2 diabetic patients, and 2 times per year for well-controlled type 2 diabetic patients) to determine whether a patient's metabolic control has remained continuously within the target range.

Reference Values

> or =18 years: 4.0-6.0%

Reference values have not been established for patients who are <18 years of age.

Interpretation

Diagnosing diabetes American Diabetes Association (ADA)

-Hemoglobin A1c (HbA1c) >6.5%

Therapeutic goals for glycemic control (ADA)

-Adults:

- Goal of therapy: <7.0% HbA1c
- Action suggested: >8.0% HbA1c

Pediatric patients:

- Toddlers and preschoolers: <8.5 % (but >7.5%)
- School age (6-12 years): <8%
- Adolescents and young adults (13-19 years): <7.5%

The 2009 ADA recommendations for clinical practice suggest maintaining a HbA1c value closer to normal yields improved microvascular outcomes for diabetics. Target goals of <7% may be beneficial in patients such as those with short duration of diabetes, long life expectancy, and no significant cardiovascular disease. However, in patients with significant complications of diabetes, limited life expectancy, or extensive comorbid conditions, targeting a <7% goal may not be appropriate.

Since the HbA1c assay reflects long-term fluctuations in blood glucose concentration, a diabetic patient who has in recent weeks come under good control may still have a high concentration of HbA1c. The converse is true for a diabetic previously under good control who is now poorly controlled.

HbA1c results <4.0% are reported with the comment: "Falsely low HbA1c results may be observed in patients with clinical conditions that shorten erythrocyte life span or decrease mean erythrocyte age. HbA1c may



not accurately reflect glycemic control when clinical conditions that affect erythrocyte survival are present. Fructosamine may be used as an alternate measurement of glycemic control. Practitioners must consider an individual patient's health, his/her risk of hypoglycemia, and his/her specific health risks when setting a target A1C level. Patients at high risk of microvascular complications may gain further benefits from reducing A1C below 7%. Because patients are responsible for averting or responding to their own hypoglycemic episodes, the patient's input and the doctor's assessment of the patient's self-care skills are also important. The approximate mapping between HbA1c values and eAG (estimated average glucose) measurements is given by the following equation:

$$eAG(\text{mg/dl}) = 28.7 \times A1C - 46.7$$

$$eAG(\text{mmol/l}) = 1.59 \times A1C - 2.59$$

The American Diabetes Association guidelines are similar to others in advising that the glycosylated hemoglobin test be performed at least two times a year in patients with diabetes that are meeting treatment goals (and that have stable glycemic control) and quarterly in patients with diabetes whose therapy has changed or that are not meeting glycemic goals

Relationship between Mean Blood Glucose and HbA1c:

Attempts to define a true relationship between average plasma glucose and HbA1c level have been made for some times, but studies had limited utility due to fewer measurements of glucose values and the limited number of participants involved. This method is error prone, with no night time samples collected, therefore, not a true representative of 24 hour glycaemia. Nathan et al. used continuous glucose monitoring, which measures interstitial glucose levels every 5 minutes, for 3 months in both nondiabetics and diabetics with relatively stable glycaemia. They reported a mathematical relationship between HbA1c and mean blood glucose, meaning HbA1c could be expressed in an equivalent mean glucose level (i.e., in the same units as patients' self-monitoring units). However this study is limited due to extremely small sample pool. A retrospective analysis of data from DCCT also identified a linear correlation between HbA1c and average blood glucose; however, the study population consisted of T1DM only, and DCCT was not designed to determine such a relationship.

Correlation with Mean Blood Glucose Levels

A single fasting blood glucose measurement only gives an indication of the patient's immediate past (last 1 to 2 hours) condition, and may not represent the true status of blood glucose regulation. In contrast, the level of glycated hemoglobin is directly related to the average glucose concentration over the life-span of the hemoglobin in the circulation.

Various formulae have been proposed to demonstrate the correlation between the mean blood glucose (MBG) and Hemoglobin A1c (HbA1c).

The following is one from Nathan, et al, N Engl J Med (1984).

$$MBG = 33.3 * HbA1c - 86$$

To verify the correlation, the mean blood glucose level for each patient was obtained as the average of up to 4 daily determinations over a period of 2 months (the average of over 200 glucose readings). Hemoglobin A1c was determined by ion-exchange HPLC at the end of the two month period. Note that the mean blood glucose value is the average glucose level over the past 60 days, and not the glucose value of the specimen obtained at the same time as the HbA1c

CLINICAL USE OF HbA1c

The world is facing an escalating epidemic of diabetes. More than 220 million people worldwide have been diagnosed with diabetes, although the actual number of people with diabetes is likely to be higher because of the insidious onset of Type 2 diabetes. Moreover, many people who have impaired glucose tolerance remain outside the diagnosed community of patients. The increasing life expectancy combined with the emergence of T2DM in children has resulted in phenomenal increase in diabetes related complications, becoming one of the major causes of disability and death worldwide. Type 2 diabetes accounts for 90% to 95% of all cases of diabetes. Furthermore, T2DM significantly increases the risk of heart disease and stroke; indeed, 50% of people with diabetes die of cardiovascular disease.¹⁴ In 2009 The International Expert Committee recommended the use of HbA1c to diagnose diabetes mellitus with a threshold > 6.5%. However the diagnostic test should be standardized to Diabetes Control and Complication Trial (DCCT) reference assay or a method certified by National Glycohaemoglobin Standardization Programme (NGSP). The use of HbA1c as a test went through nearly three decades of detailed scrutiny before being accepted as a diagnostic test for diabetes. Researchers had long been searching for test of glycaemia that could be used to screen and diagnose diabetes as well as monitor the chronic glycaemic control; such as test, may also be able to predict the onset of complications. Glycated haemoglobin acquires importance as a test for glycaemia because it has less intraindividual variation and is a better predictor of cardiovascular complications compared to fasting plasma glucose (FPG) and oral glucose tolerance test (OGTT). In addition, it is used for glucose monitoring of diabetic patients. In another study HbA1c and FPG showed continuous relationship with cardiovascular disease.¹⁸ While screening for diabetes, a relatively common condition, it is more important for the test to specifically identify patients than to be so sensitive as to diagnose many false positives, thereby making screening counterproductive as this approach would put an extra burden on resources, especially in less privileged population of under developed countries. Glycated haemoglobin has been extensively



investigated by clinical trials, In 1979 P.J. Dunn et al suggested that HbA1c is highly reproducible and responsive to changes in glucose tolerance; as such, it could be used to monitor the control of glycaemia. In 1991, Mulkerrin et al reported very poor sensitivity (36%) and predictive value (44%) in their elderly sample (mean age 76 years old), thereby making HbA1c neither useful in screening nor beneficial for diagnosing diabetes. However, in acutely ill hospitalized patients HbA1c > 6% could reliably diagnose diabetes and level 6.0% and impaired fasting glucose (> 100mg/dl) or random plasma glucose of 130- 199mg/dl should lead to further diagnostic workup and closer follow-up. By using these recommendations we may identify a high proportion of individuals with undiagnosed diabetes, who would otherwise only be diagnosed once they developed end organ damage.

HbA1c AND DIABETES COMPLICATIONS

The clinical utility of HbA1c as a tool to assess the risk of diabetes complications was proposed in the publication of the results of the DCCT and also UKPDS47 these studies set out to establish the effect of intensive (as compared with conventional) glycaemic control on the development of microvascular complications in type 1 and type 2 DM patients respectively.

Microvascular complications

The microvascular complications of DM comprise retinopathy, nephropathy and neuropathy. Patients with DM who develop these conditions constitute a large proportion of all subjects who develop blindness, renal failure and/or require limb amputation. The DCCT46 found that when 1441 patients with type 1 DM were randomized to "intensive" rather than "conventional" treatment, their median HbA1c was 7.3% compared with 9.1% throughout the 6.5 years average follow-up period. The subsequent risk of developing retinopathy in the intensively treated group was reduced by 76%, the risk of developing proteinuria was reduced by 54%, and the risk of clinical neuropathy was reduced by 60%. The risk of microvascular complications in both the patient groups rose exponentially as the HbA1c value increased.

The publication of the UKPDS in 1998 confirmed that a relationship between HbA1c and microvascular complication risk existed. There was still a 25% reduction in microvascular risk even though the difference in HbA1c between the intensive and conventional treatment groups was not as large as in the DCCT (HbA1c 7.0% Vs. 7.9% over 10 years). Later analysis has shown that when the two treatment groups are combined, a similar exponential relationship between rising HbA1c and rising microvascular risk could be discerned in UKPDS as in the DCCT. After the end of the DCCT, 96% of the patients in the original study agreed to continue to be followed up in a new study known as the Epidemiology of Diabetes Interventions and Complications study. However, the patients were were no longer in two separate treatment

groups. Following the outcomes of the DCCT, it was recommended that all patients follow an intensive treatment regime. It was therefore interesting that, out of a clinical trial scenario, the HbA1c of the previously intensively treated patients increased to an average of about 8%, while that of the conventionally treated group tightened up to a similar value. Long-term followup of these patients has shown that the benefits of improved glycaemic control during the DCCT on the risk of microvascular complications are maintained in the long-term despite the convergence of glycaemia at the end of the original DCCT trial.

This observation that glycaemia from several years previously influences subsequent long-term complication risk has since been termed "metabolic memory" and has reinforced the importance of good glycaemic control as soon as possible after the diagnosis of DM in order to avoid subsequent problems.

Macrovascular disease

Large vessel (macrovascular) disease remains the major cause of morbidity and mortality in patients with DM, with those having type 1 DM being at as high a risk as those with type 2 DM. In type 1 DM, the DCCT found an excess of macrovascular events in the conventional compared with the intensive group (40 versus 23), although this just failed to reach statistical significance ($p=0.08$).^{58,59} Detailed analysis showed it was the mean HbA1c value during the DCCT that explained a large part of this beneficial effect on cardiovascular risk. In the UKPDS, the event rate among the patients with type 2 DM was higher than in the DCCT, but the HbA1c separation between the two groups was less marked. Nevertheless, there was a suggestion of more myocardial infarctions among conventionally treated patients ($p=0.052$). In a subsequent analysis, where the two treatment groups were combined, there was an overall relationship between rising HbA1c and increasing risk of myocardial infarction

OTHER APPLICATIONS

Recently, other applications of HbA1c have been described. HbA1c may predict incident cardiovascular events, even in individuals without DM, HbA1c may be used as a predictor for fasting hyperglycaemia and metabolic syndrome. Increasing HbA1c was associated with increasing cardiovascular risk factors, so that HbA1c has been studied as a predictor of future development of cardiovascular risk.

FACTORS AFFECTING HbA1c MEASUREMENT

As the level of HbA1c depends upon the lifespan of erythrocyte, the most important factor that influences the HbA1c level is erythrocyte turnover rate. Therefore, longer the erythrocyte circulation time, the more glycosylated its haemoglobin becomes. Falsely elevated HbA1c concentrations can be encountered when there is increased circulating erythrocyte life span (i.e., decreased red cell



clearance) or impaired reticulocyte production. In older erythrocytes, haemoglobin is likely to have had a longer period of exposure to hyperglycaemia and this result in the formation of higher HbA1c levels. Some of the well documented causes for elevated HbA1c include alcoholism, iron deficiency, renal failure, and hyperbilirubinaemia. Any condition that shortens the life span of erythrocytes is likely to decrease HbA1c levels, since the average erythrocyte is younger, lasting for lesser time in circulation to be glycated. Falsely decreased HbA1c values are seen in conditions with a reduced erythrocyte life span (i.e., increased haemoglobin turnover) or where a large number of reticulocytes are produced. These younger erythrocytes have less time exposure to ambient glycaemia. Well known causes that result in this condition include acute or chronic blood loss, sickle cell anaemia, thalassaemias, glucose-6-phosphate dehydrogenase (G6PDH) deficiency, haemolytic, aplastic anaemias, and splenectomy. Pregnancy may falsely increase or decrease HbA1c, suggesting that this investigation is not to be considered appropriate for the diagnosis of gestational diabetes mellitus. In case of aplastic anaemias, cessation of erythropoiesis leads to an increase in the number of circulating aged red cells resulting in a progressive rise in HbA1c.

Effect of abnormal haemoglobins

The normal phenomenon is the glycation of adult HbA0 to form HbA1c. However, when abnormal haemoglobins are present, the person is likely to form other glycated products such as HbS1c, HbC1c, and so on, either in addition to or instead of HbA1c. In some persons persistence of foetal haemoglobin can cause considerable problem in measuring HbA1c as they would co-migrate or co-elute with the HbA1c fraction leading to an overestimation of the HbA1c levels.

Effect of anaemia

Iron deficiency anaemia has been known to cause a rise in HbA1c of up to 2% and this has been shown to be reversed with iron supplementation. Given that iron deficiency anaemia is a common finding, especially in premenopausal women, caution should be exercised while interpreting HbA1c results in these patients. Haemolytic anaemia has the opposite effect to iron deficiency and a reduction in HbA1c is observed in affected individuals. This occurs due to reduced red cell survival, meaning a reduction in the availability of haemoglobin for glycation.

Effect of drugs and health conditions

Any drug which gives rise to haemolytic anaemia will result in lowering of HbA1c levels. High-dose aspirin, by forming acetylated haemoglobin, can lead to spurious rise in HbA1c when certain methods are used for estimation, but the effect is usually only apparent at doses (4 g/day) that are well in excess of that prescribed normally. Chronic kidney disease (CKD) can have

complex influences on HbA1c formation and measurement. Patients with CKD can be iron deficient, exhibit haemolytic anaemia and have altered red cell survival, all of which influence HbA1c level.

Compounding the problem is the fact that ureaderived isocyanate can lead to the formation of carbamylated haemoglobin, which can be indistinguishable from HbA1c when using certain HbA1c assay methods.

Differences in HbA1c can also occur due to potential racial and ethnic differences. African Americans have higher HbA1c levels than Caucasian whites. This difference accentuates as glucose intolerance worsens. In these ethnic groups, these significant limitations affect all the possible applications of HbA1c, i.e., to screen for glucose intolerance, to assess the risk for complications, to measure quality of care, and to evaluate disparities in health. If the measured value of HbA1c is > 15% or if a large change in HbA1c coincides with a change in laboratory HbA1c methods, then the presence of hemoglobin variant should be considered

These effects may be summarized below.

Factors affecting HbA1c measurement

a) Erythropoiesis

Increased HbA1c: iron, vitamin B12 deficiency, decreased erythropoiesis.

Decreased HbA1c: administration of erythropoietin, iron, vitamin B12, reticulocytosis, chronic liver disease.

b) Altered Haemoglobin

Genetic or chemical alterations in haemoglobin: haemoglobinopathies, HbF, methaemoglobin, may increase or decrease HbA1c.

c) Glycation

Increased HbA1c: alcoholism, chronic renal failure, decreased intraerythrocyte pH.

Decreased HbA1c: aspirin, vitamin C and E, certain haemoglobinopathies, increased intra-erythrocyte pH.

Variable HbA1c: genetic determinants.

d) Erythrocyte destruction

Increased HbA1c: increased erythrocyte life span: Splenectomy.

Decreased A1c: decreased erythrocyte life span: haemoglobinopathies, splenomegaly, rheumatoid arthritis or drugs such as antiretrovirals, ribavirin and dapsone.

e) Assays

Increased HbA1c: hyperbilirubinaemia, carbamylated haemoglobin, alcoholism, large doses of aspirin, chronic opiate use.

Variable HbA1c: haemoglobinopathies.

Decreased HbA1c: hypertriglyceridaemia

LIMITATIONS OF USING HbA1c: THE INDIAN SCENARIO

The most important limitation in India is the cost of providing the assay for its routine use. Second, any condition that changes red cell turnover, such as



haemolytic anemia, chronic malaria, major blood loss, glucose-6-phosphate dehydrogenase deficiency, sickle cell anemia or blood transfusions, will lead to spurious A1C results. These conditions including the thlassaemias are highly prevalent in certain parts of India. Besides, Hereditary persistence of fetal Hb, renal insufficiency, malignancy, iron deficiency anemia, vitamin B 12 and folate deficiency, splenectomy also show increased values. Some studies have shown that alcoholism, lead poisoning, opiate addiction, excessive use of salicylate and pregnancy can lead to falsely elevated HbA1c. Age and regional differences do exist in values of HbA1 which have not been studied widely in India. We do not have sufficient data on whether Indians are high glycaters or low glycaters. HbA1c assay results cannot be trusted in certain rare clinical settings, such as rapidly evolving type 1 diabetes, where the A1C level will not have had time to “catch up” with the acute elevations in glucose levels.

HbA1c test are performed using different methods like High performance liquid chromatography, affinity chromatography, cation exchange chromatography, isoelectric focussing, radioimmunoassay, spectrophotometric assay, electrophoresis and electrospray mass spectrometry. Tests to diagnose diabetes should be performed using clinical laboratory equipment using a method that is NGSP certified and standardized to the DCCT assay. Point-of-care (POC) instruments have not yet been shown to be sufficiently accurate or precise for diagnosing diabetes. Looking at the enormous variation in the health care system in India, labs and methods used for estimation appear to be far from standardized. With dearth of accredited labs and limited resources, the routine use of HbA1c is questionable. It would not be practical to have HPLC as the only method for HbA1c assessment to be used for diagnostic puposes. Also according to Rancho Bernardo study, the HbA1C cut point of 6.5% had a sensitivity/specificity of 44/79%. In their cohort of older adults, the suggested HbA1C cut point of 6.5% had relatively low sensitivity and specificity for type 2 diabetes diagnosis in all age-groups and in both sexes. They concluded that the limited sensitivity of the A1C test may result in delayed diagnosis of type 2 diabetes, while the strict use of ADA criteria may fail to identify a high proportion of individuals with diabetes by HbA1C 6.5% or retinopathy Also in another study by Cavagnoli et al HbA1c > 6.5% mmol/mol) showed limited sensitivity to diabetes diagnosis, although with high specificity. The results suggest that this cut-off point would not be enough to diagnose diabetes. They concluded that Its use as the sole diabetes diagnostic test should be interpreted with caution to assure the correct classification of diabetic individuals [43].The decision about which test to use to assess a specific patient for diabetes should be at the discretion of the health care professional, taking into account the availability and practicality of testing an individual patient or groups of patients. As with most diagnostic tests, a test result diagnostic of diabetes should

be repeated to rule out laboratory error, unless the diagnosis is clear on clinical grounds, such as a patient with classic symptoms of hyperglycemia or hyperglycemic crisis. It is preferable that the same test be repeated for confirmation, since there will be a greater likelihood of concurrence in this case. In case of non confirmation by repeat testing the healthcare professional should opt to follow the patient closely and repeat the testing in 3– 6 months. Clinicians should continue to use the previously recommended approaches to diagnose diabetes based on glucose measurements. The decision to change to A1C assays as the means of diagnosing diabetes should take into account the performance of local A1C assays and the local prevalence of conditions that may interfere with the assay. Clinicians must be aware of these conditions, particularly in populations in which they are more prevalent. If A1C testing is not possible owing to patient factors that preclude its interpretation (e.g., hemoglobinopathy or abnormal erythrocyte turnover) or to unavailability of the assay, previously recommended diagnostic measures (e.g., FPG and 2HPG) and criteria should be used. Mixing different methods to diagnose diabetes should be avoided. The diagnosis of diabetes during pregnancy, when changes in red cell turnover make the A1C assay problematic, continues to require glucose measurements.

The risk for diabetes based on levels of glycemia is a continuum. Therefore; there is no lower glycemic threshold at which risk clearly begins. Those with A1C levels below the threshold for diabetes but > 6.0% should receive demonstrably effective preventive interventions. Those with A1C below this range may still be at risk and, depending on the presence of other diabetes risk factors, may also benefit from prevention efforts. The A1C level at which population-based prevention services begin should be based on the nature of the intervention, the resources available, and the size of the affected population.

A New Term to Replace HbA1c:

The A1c-derived average glucose study was conducted in 10 different locations in North America, Europe, and Africa. The two largest countries namely, India and China with huge diabetes population were left out, leaving it less representative. The study population comprised of 507 patients, 268 T1DM and 159 T2DM patients, and 80 nondiabetic subjects.

The researchers sought to examine the relationship of average blood glucose with HbA1c across a wide range; (i.e. between HbA1c 5% to 13%). They collected approximately 2,700 blood glucose readings from each participant over 3 month period, the highest number of blood glucose readings per person to date in a single study. The goal of the study was to report glycated haemoglobin results not in the usual HbA1c percentage format but as A1c-derived averages in the same units used in self-monitoring, (i.e., mg/dl or mmol/l). The study concluded that the estimated average glucose (eAG) can now be calculated from HbA1c using a linear regression equation.



This eAG will now be used to monitor glycaemia in diabetic patients as the estimated glomerular filtration

rate (eGFR), which is used to monitor chronic kidney disease, from the measurement of serum creatinine.

Table 1. WHO Criteria for diagnosis of DM

Condition	2hr glucose (PP)	Fasting glucose	HbA1c	
	mmol/l (mg/dl)	mmol/l (mg/dl)	mmol/mol	DCCT %
Normal	< 7.8 (<140)	<6.1(<110)	<42	<6.0
Impaired fasting glucose	<7.8(<140)	>6.1(>110) & 7.0(<126)	42-46	6.0-6.4
Impaired glucose Tolerance	>7.8(>140) & < 11.1(<200)	<7.0(<126)	42-46	6.0-6.4
Diabetes Mellitus	>11.1(.200)	>7.0(126)	>48	>6.5

Table 2. A comparison of glycated Hb in different measuring units

NGSP HbA1c %	IFCC mmol/mol	eAG mg/dl	eAG mmol/L
4	20	68	3.8
5	31	97	5.4
6	42	126	7.0
7	53	154	8.6
8	64	183	10.2
9	75	212	11.8
10	86	240	13.4
11	97	269	14.9
12	108	297	16.5

CONCLUSION

HbA1c levels are used to monitor glycaemic control throughout the world, and all major clinical trials including DCCT in T1DM and the United Kingdom prospective diabetes study (UKPDS) in T2DM have used it as a tool to monitor glycaemic control among the study population. Indeed, it did not happen overnight; valuable time and resources have been spent to familiarize patients and health care providers with it. Yet after more than three decades of use, today only 25% of patients in a cross-sectional study were able to report their correct recent HbA1c, and 66% did not know their last HbA1c.38 Daily self blood testing, measured in mmol/L or mg/dl and HbA1c measurement in percentage are somewhat confusing. Given the narrow range of percentages, it is sometimes difficult for patients to comprehend the consequences of even a percent increase or decline in HbA1c. Patients and their caretakers are used to the idea that the HbA1c level should be less than 7% in diabetic patients: a higher reading indicates that the glycaemic control is getting out of hand. Now the IFCC results will be provided in mmol HbA1c per mol haemoglobin. Keeping the NGSP results in percentages along with IFCC results will make the change less confusing. With the introduction of the new term "estimated average glucose " (eAG).eAG will be reported along with HbA1c results in the interim. Many physicians feel it would be easier to discuss eAG than haemoglobin in a diabetes out-patient. Others argue that the term eAG will only serve to confuse patients with diabetes: these experts also call for further research,

especially among different ethnic groups and special circumstances (e.g., children and pregnant women). How soon all these controversies are resolved, culminating in this term being accepted in clinical use and entering into the lexicon, remains to be seen The major fraction of the healthcare system in India is a fragmented and unorganized private sector, ranging from corporate hospitals to small clinics and private practitioners Very few laboratories performing the tests have been standardized . After the ADA 2010 recommendation, there has been a gradual increase in acceptance of HbA1c as a diagnostic test for diabetes mellitus. But the clinicians prescribing and interpreting the tests results are likely to miss the numerous limitations, precautions and variations of using HbA1c for diagnosis of diabetes mellitus. Simply speaking every single HbA1c report must be correlated with the method and lab used. Any disorder of red blood cells or haemoglobin must be excluded and all local interfering factors discussed above must be taken into account.

Besides a repeat HbA1c testing or plasma glucose estimation is usually recommended before abnormal values can be accepted. All this involves an increased cost and delay in diagnosis. This might not be a limitation in large organized and standardized city hospitals.

But in the present Indian scenario Glycated haemoglobin, HbA1c cannot be accepted as a sole and independent test to diagnose diabetes mellitus.

TAKE HOME MESSAGE

- Elevated HbA1c even without a diagnosis of diabetes is independent risk factor for cardiovascular disease



- HbA1c > 6.5% is diagnostic for the Diabetes mellitus.
- The HbA1c target is 7.0% in most treated patients with diabetes
- The ideal target of HbA1c is as long as it does not result in life threatening hypoglycaemia.
- The worldwide standardization of HbA1c was necessary given the significant mobility of today's world population.
- The equivalent of the current HbA1c target 6.5% is 48 mmol/mol in the IFCC unit.
- eAG would be used in diabetic patients as eGFR is used in patients with chronic kidney disease.
- every single HbA1c report must be correlated with the method and lab used
- This assay is not useful in determining day-to-day glucose control and should not be used to replace daily home testing of blood glucose

- It provides an assessment of chronic hyperglycemia, no fasting is necessary and Intraindividual variability is very low (critical value of <2%)
- A single test could be used for both diagnosing and monitoring diabetes
- When using HbA1c to diagnose diabetes, an elevated HbA1c should be confirmed with a repeat measurement, except in those individuals who are symptomatic and also have an increased plasma glucose >200 mg/dL. In India there are infra-structural limitations to use HbA1c.

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