The Narrow Therapeutic Window of Glycated Hemoglobin and Assay Variability

Seyed S. Hosseini, Irma Bibler, and M. Arthur Charles

Glycated hemoglobin is measured by a variety of assays, each of which has a unique normal level. Our purpose is to show that among the different assays available in the United States, using the same patient's blood sample, assay results may vary widely and may more or less easily achieve a glycated hemoglobin value within the normal range. The following assays were compared using the same patient's blood sample for each pair of assays: glycohemoglobin affinity assay (GHB Reader; Isolab, Akron, OH) versus gel electrophoresis assay (n = 76); Isolab versus ion capture assay (IMX; Abbott Laboratories, Irving, TX) (n = 57); monoclonal antibody assay (DCA2000; Bayer Diagnostics, Pittsburgh, PA) versus IMX (n = 100); and high-performance liquid chromatography (HPLC) assay (Bio-Rad Variant A_{1c}; Bio-Rad Laboratories, Richmond, CA) versus IMX assay (n = 55). Our analyses indicate that a relative ranking can be established for the ease of achieving a normal glycated hemoglobin level. The ranking indicates that the most stringent or difficult assays for achieving a normal level are the Isolab and DCA2000 assays. The intermediate assays are the IMX and Bio-Rad Variant, and the easiest method for achieving a normal value is the gel electrophoresis assay. Our results indicate that various glycated hemoglobin assays vary widely and are associated with more or less difficulty for an individual patient to achieve a glycated hemoglobin level within the normal range. These results are especially significant with respect to (1) the clinically narrow therapeutic window of glycated hemoglobin values in type 1 diabetes to avoid rapidly advancing severe hypoglycemia rates and chronic microvascular complication rates, and (2) the glycated hemoglobin threshold for rapidly advancing macrovascular disease in both type 1 and type 2 patients.

SINCE THE FORMATION of glycated hemoglobin can be directly proportional to the integrated blood glucose concentration during the prior 40 to 90 days and since chronic diabetic complications are experimentally directly related to glycated hemoglobin levels, not blood glucose levels, there is no other method to determine whether a patient's glucose control is satisfactory.[1-3] Thus, this test is critical for all diabetic patients and their health care providers. Recent reviews have emphasized the importance of standardizing (all assays measuring the same glycated hemoglobin), normalizing (all assay results mathematically corrected to yield similar values), or tracing (mathematically or technically relating assay results to a specific assay used in a clinical trial) all glycated hemoglobin results to reduce the variation created by the several assays currently available.[4,5] This process is under way, but one reason for the slow progress is that less than 15% to 20% of US physicians caring for diabetic patients appropriately utilize glycated hemoglobin levels in their practice.[6,7] In part, this may be due to confusion regarding the target glycated hemoglobin values that patients, allied healthcare providers, and physicians should aim to achieve.

Although there are some relatively unusual caveats regarding the clinical use of glycated hemoglobin values, eg, anemia, renal failure, and hemoglobinopathy, there is another more generalized and poorly understood concern regarding the routine clinical use of glycated hemoglobin levels. This problem relates to the comparison of assays or to the ease or difficulty of a patient's achieving a glycated hemoglobin value within the normal range. One would expect a normal value in one assay to be at least similar to a normal value in another assay regardless of the method or assay used. Each of the many assays has a unique upper-normal level. Some of this variation is due to the molecular species of the glycated hemoglobin measured, and significant variation is due to assay differences even when measuring the same molecular component, eg, hemoglobin A_{1c} (HbA_{1c}). As we will show, these assays are thus not comparable. Detailed studies have described clinically significant variations between assays in the absence of assay normalization.[8,9] Thus, clinical pathology laboratories generally report that for a diabetic patient to have optimal blood glucose control, the value for glycated hemoglobin should be within the normal range for some assays and exceed the upper-normal range for other, more difficult assays; in these latter assays, it is more difficult for a patient to achieve a normal value. It is not clear to clinicians why certain value ranges are used and how these results relate to chronic diabetic complications.

Which glycated hemoglobin level is acceptable is important, since the rates for both chronic diabetic complications and severe hypoglycemia in type 1 diabetes increase as glycated hemoglobin increases or decreases, respectively.[3] The Diabetes Control and Complications Trial (DCCT) describes a narrow therapeutic window of glycated hemoglobin values in type 1 diabetes using the HbA_{1c} assay (Fig 1). Briefly, the absolute number of severe hypoglycemic events increases rapidly with glycated hemoglobin levels less than 7% to 8%, and although chronic complications increase at glycated hemoglobin levels of 6% to 7%, they increase even more rapidly at levels greater than 7% to 8%.[3,10] Because of this narrow therapeutic window for glycated hemoglobin, near 7% to 8% using the DCCT HbA_{1c} assay, clinicians who treat type 1 patients must be aware of the variability between glycated hemoglobin assays.

Similar data also exist for chronic complications in type 2 patients with regard to both microvascular and macrovascular disease.[11,12] In type 2 studies, severe hypoglycemia is much less of a concern. Unfortunately in the Japanese study, HbA_{1c} values were not normalized or traceable to the DCCT or UK Prospective Diabetes Study (UKPDS), making interstudy comparisons to the Japanese study difficult.[11] The DCCT and UKPDS did report HbA_{1c} levels that are comparable, and thus HbA_{1c}

From the Diabetes Research Program and the Departments of Medicine and Pathology, University of California, Irvine, CA.

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Address reprint requests to M. Arthur Charles, MD, PhD, Department of Medicine, Medical Sciences I, Room C250, University of California, Irvine, CA 92697.

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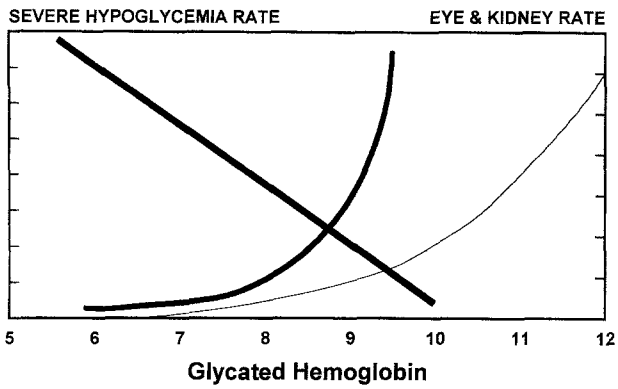

Fig 1. Narrow therapeutic window is illustrated where the straight line indicates the severe hypoglycemia rate, the bold curvilinear line reflects the eye new event rate, and the fine curvilinear line reflects the event rate for increasing microalbuminuria. The Y-axis parameters are plotted against the X-axis DCCT glycated hemoglobin level. All of these curves primarily reflect DCCT data, but also reflect other studies as well. Note the convergence of hypoglycemia rates and complication rates near a glycated hemoglobin level of 8%. This is the narrow therapeutic window for treating type 1 patients, which strongly emphasizes the need to use a DCCT-normalized glycated hemoglobin assay.

standards exist for microvascular complications in type 1 patients, as well as microvascular and macrovascular complications in type 2 patients. This report emphasizes that not all assays available in the United States are traceable to these studies which set the clinical standard for glucose control as measured by glycated hemoglobin.

These considerations are now more significant, since the American Diabetes Association (ADA) has recommended the use of DCCT-traceable glycated hemoglobin levels and has set defined goals.[13] A HbA_{1c} level greater than 8% indicates that the healthcare provider should take action to decrease the blood glucose level, and a HbA_{1c} value less than 7% is the goal. As we describe herein, a value of 7% by a DCCT-traceable assay may vary by 1% to 3% with a non–DCCT-traceable assay. Thus, the provider may falsely assume that a level of 7% is satisfactory, when in fact the DCCT-traceable level could actually be anywhere from 4% to 10%.

SUBJECTS AND METHODS

Subjects

Values for the normal range of each glycated hemoglobin assay were obtained in blood samples from nonmedicated healthy individuals with a normal fasting blood glucose level. Different groups of normals were used for each of the assay analysis, and the glycated hemoglobin values were normally distributed. The age range for normals was 18 to 70 years. Blood samples in diabetic patients were obtained from routine clinical orders by a personal physician. The subjects were both outpatients and inpatients, and none had anemia, hemoglobinopathy, or renal failure. The study was approved by the University of California at Irvine (UCI) Human Subjects Review Board. All data reported herein were determined in our laboratory.

Assays

Glycohemoglobin affinity assay (GHB Reader; Isolab, Akron, OH). This assay is based on a borohydride affinity column that binds glycated hemoglobin but not nonglycated hemoglobin. Glycated hemoglobin is eluted from the column and measured spectrophotometrically. The value for the upper-normal level is 6.9% and was obtained by adding 2 SD to the mean. The interassay and intraassay coefficients of variation (CVs) are 6.2% and 4.1%, respectively. The values are reported as the percentage of total hemoglobin that is glycated. This assay result is not normalized to the DCCT HbA_{1c} assay.

Ion capture assay (IMX; Abbott Laboratories, Irving, TX). This assay uses a polyanion boronate affinity reagent that binds glycated hemoglobin, creating a polyanion–glycated hemoglobin complex. This complex binds to a cationic ion capture glass fiber matrix. The matrix is then washed and fluorophore 4-methylumbelliferone is added to measure fluorescent quenching due to glycated hemoglobin captured on the matrix. The upper-normal level reported herein for total glycated hemoglobin is 7.2% and was obtained by adding 2 SD (1.5%) to the mean value (5.7%) obtained in 60 normal subjects. The interassay and intraassay CVs are 8.4% and 3.7%, respectively, and values are reported as a percentage. The assay result can also be transformed to HbA_{1c} mathematically and the reported HbA_{1c} value can be normalized to the DCCT assay in the commercially available assay kit, but this was not done in the present study.

Gel electrophoresis assay. HbA_{1c} is separated by electrostatic charge differences using a negatively charged agar gel. Glycated hemoglobin is quantified using color densitometry after protein staining.[14] The upper-normal level was obtained by adding 2 SD to the mean, resulting in a value of 7.9%. The interassay and intraassay CVs are 10.3% and 5.9%, respectively. Values are reported as percent HbA_{1c}. This assay is not normalized to the DCCT assay.

Monoclonal antibody assay (DCA2000; Bayer Diagnostics, Pittsburgh, PA). This HbA_{1c} method is based on an immunoassay using antibody-latex particles which, when agglutinated, have increased absorbance at 531 nm. When HbA_{1c} is present, it competes for a limited number of antibody-latex binding sites, resulting in less agglutination and reduced absorbance at 531 nm. This solution assay kit uses 1 μL blood and contains a spectrophotometer that measures the change in light absorbance. The upper-normal level of 5.5% was obtained by adding 2 SD to the mean. The interassay and intraassay CVs are 4.3% and 2.3%, respectively. The reported value is a percentage and the assay assessed in this study is not normalized to the DCCT assay. The DCA2000 result can also be normalized to the DCCT, but this was not done in the present study.

High-performance liquid chromatography assay (Bio-Rad Variant A_{1c}; Bio-Rad Laboratories, Diagnostic Group, Richmond, CA). This assay is a high-performance liquid chromatography (HPLC) method using an automated spectrophotometric method to measure HbA_{1c}. The value for the upper-normal limit is 6.0% and was obtained by adding 2 SD (0.7%) to the normal mean (5.3%) in 60 normal subjects. The interassay and intraassay CVs are 2.5% and 1.4%, respectively. The reported values are the percent HbA_{1c}, and these data are normalized to the DCCT glycated hemoglobin assay for this report.

Statistics

The equality of probabilities in multinomial trials for overlapping outcomes (McNemar's test) was used to statistically validate the differences between the various glycated hemoglobin assays. Linear regression correlation analysis was used to compare assays. The Irwin-Fischer test was used where indicated.

RESULTS

To determine if the gel electrophoresis glycated hemoglobin assay is different from the Isolab affinity column assay, identical samples from patients were tested using both assays. These data were then subjected to linear regression analysis (Fig 2). Seventy-six individuals were evaluated, and the number of individuals with a gel electrophoresis glycated hemoglobin

value higher than normal for that assay was 39, whereas the number with a supranormal value for the Isolab affinity column assay was 71. The number of individuals in whom the gel electrophoresis and Isolab assay results were higher than normal was 39. Since a significantly greater number of individuals had values above the normal range for the Isolab assay compared with the gel electrophoresis assay, it is more difficult to achieve a normal value using the Isolab assay ($P = 2.3 \times 10^{-10}$). More than 3 glycated hemoglobin percentage units separate the upper-normal levels of these two assays (Fig 2).

To determine if the Isolab affinity column assay is different from the IMX ion capture assay for total glycated hemoglobin, simultaneous analyses were again performed and the results are expressed as a linear regression (Fig 3). For this comparison, the total number of individuals with identical samples measured using the two glycated hemoglobin assays was 57. The number of individuals with IMX assay levels higher than normal was 49, whereas for the Isolab assay it was 52. The number of individuals with IMX and Isolab values greater than normal was 47. Thus, with respect to the IMX and Isolab assays, there were no significant differences for the difficulty of achieving a value within the normal range ($P = .23$).

To determine if the IMX ion capture assay is different from the DCA2000 assay, 100 individuals were simultaneously tested using both assays. The linear regression is shown in Fig 4. The number of individuals tested with the IMX assay who had values higher than normal was 52, whereas for the DCA2000 assay the number was 94. The number of individuals with values greater than normal for both the IMX and DCA2000 assays was 47. Thus, it is more difficult to achieve a glycated hemoglobin value within the normal range using the DCA2000 assay compared with the non–DCCT-normalized IMX assay ($P = 6.4 \times 10^{-10}$).

To determine if the IMX ion capture assay for total glycated hemoglobin is different from the Bio-Rad Variant assay for HbA_{1c}, simultaneous analyses were performed and the results are expressed as a linear regression (Fig 5). In this comparison, the total number of individuals with identical samples measured using the two assays was 55. The number of individuals with

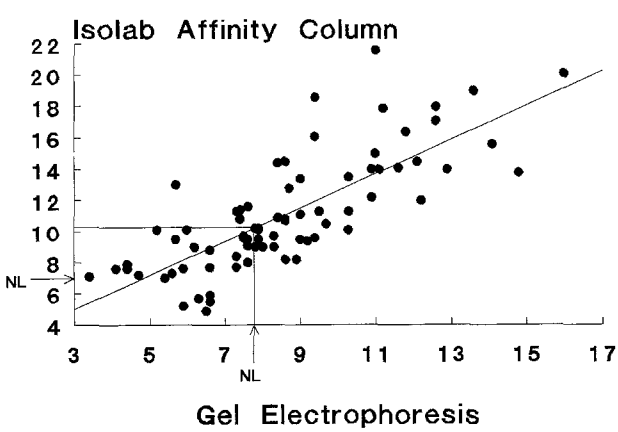

Fig 2. Glycated hemoglobin value (●) used in the identical blood sample measured by gel electrophoresis and Isolab affinity column assays (n = 76, $r = .774$, $P < .0001$). NL, upper-normal value for the respective assay.

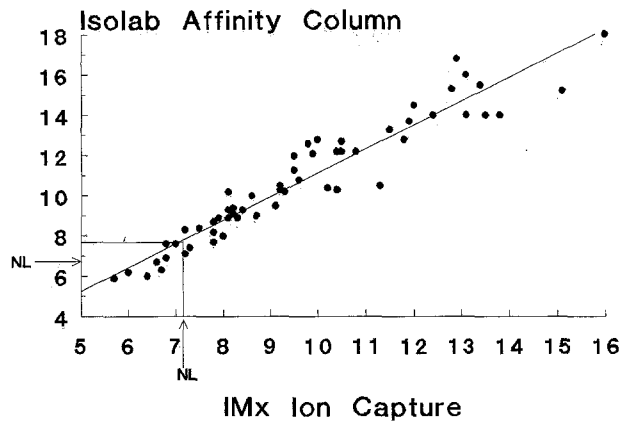

Fig 3. Glycated hemoglobin value (●) used in the identical blood sample measured by IMX ion capture and Isolab affinity column assays (n = 57, $r = .96$, $P < .0001$). NL, upper-normal value for the respective assay.

IMX assay levels higher than normal was 34, and for the Bio-Rad Variant assay, it was 38. The number of individuals with IMX and Bio-Rad Variant values greater than normal was 41. Thus, these two assays were nearly equal with respect to achieving a value within the normal range ($P = .35$).

To estimate if the Isolab affinity column is different from the DCA2000 assay, the Irwin-Fisher test was used since simultaneous values did not exist for these assays. The IMX assay was the common assay used to compare the Isolab and DCA2000 assays. This analysis shows that the Isolab assay was not different with respect to the difficulty of achieving a value within the normal range when compared with the DCA2000 assay ($P = .36$).

Of the comparisons made, it is possible to rank the various assays for ease or difficulty of achieving a value within the normal range. Thus, the most difficult assays are the total glycated hemoglobin Isolab affinity column assay and the HbA_{1c} DCA2000 assay, followed by the total glycated hemoglobin IMX ion capture assay and the HbA_{1c} Bio-Rad Variant assay. The easiest assay to achieve a normal glycated hemoglobin level in this comparison is the HbA_{1c} gel electrophoresis

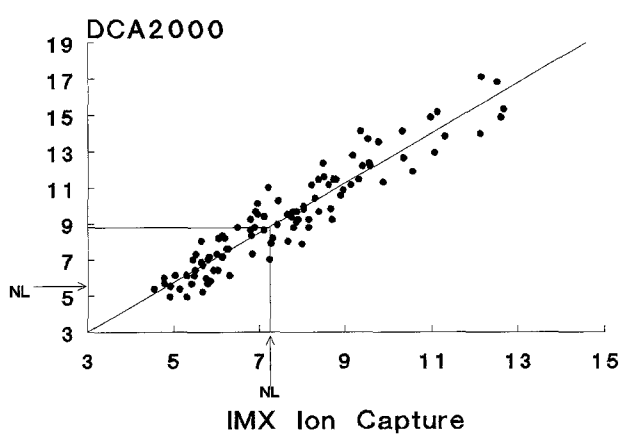

Fig 4. Glycated hemoglobin value (●) used in the identical blood sample measured by IMX ion capture and DCA2000 assays (n = 100, $r = .95$, $P = .004$). NL, upper-normal value for the respective assay.

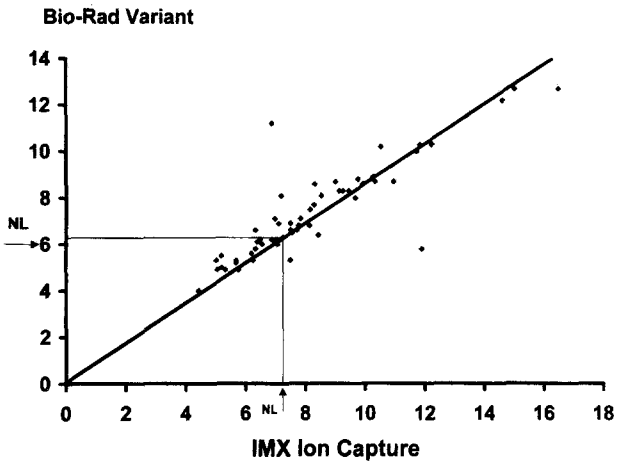

Fig 5. Glycated hemoglobin value (●) used in the identical blood sample measured by IMX ion capture and Bio-Rad Variant HPLC assays (n = 55, $r = .86$, $P \leq .0001$). NL, upper-normal value for the respective assay.

assay. In an attempt to rank the IMX and Bio-Rad assays, the following analysis was performed. Since the IMX total glycated hemoglobin assay is linear to the IMX HbA_{1c} transformation, and since the latter HbA_{1c} assay is normalized to the DCCT and the HbA_{1c} result is about 1.5% lower than the IMX total glycated hemoglobin, the DCCT-normalized HbA_{1c} assay more easily achieves a value in the normal range.

DISCUSSION

Our results are the first to show, using statistical analyses, that some glycated hemoglobin assays vary such that they are not clinically comparable and are associated with more or less difficulty for an individual patient to achieve a glycated hemoglobin level within the normal range. These results emphasize that defining the clinically ideal value is difficult without using a DCCT-traceable, -standardized, or -normalized assay. This is a significant caveat for the use of glycated hemoglobin assays in clinical diabetes, since it is a common clinical belief that the normal values for glycated hemoglobin assays are similar. Our data show that in certain assays, a "normal" glycated hemoglobin level may in fact not be required to avoid rapidly advancing chronic diabetic complications. These concepts are supported by other similar reports of assay variability.[4,8,9]

There has been extensive discussion related to standardizing glycated hemoglobin assays.[4,5,8,9] This approach could resolve the problem we describe, since if all assays were standardized, especially to the DCCT and UKPDS assays, then all glycated hemoglobin assay results would be identical and a specific value encompassing all standardized assays could be assigned as a clinical target to avoid chronic complications. If clinical assays were standardized or values normalized to the UKPDS or the DCCT, then a clinically defined, realistic, and targeted glycated hemoglobin level could be emphasized, eg, as for hypertension or low-density lipoprotein levels. Standardization and/or normalization of glycated hemoglobin assays are further required if the potential for these assays is to be realized for the diagnosis and appropriate treatment of diabetes. The definition of a clinically ideal value for glycated hemoglobin should not necessarily be a value within the normal range for a given assay, but rather a value defined in an assay that is calibrated correctly to reduce variance and is traceable or normalized to the DCCT and UKPDS assays. The fact that calibration is important is shown in this report, since there is significant scatter or unacceptably elevated assay CVs for most of the assays shown. High CVs create inaccuracies when determining the number of glycated hemoglobin values within the normal range. Thus, the variance between assays can exaggerate differences between glycated hemoglobin levels. Currently, the Internet provides access for healthcare providers and clinical pathologists attempting to resolve these problems, by listing certified assays that not only are carefully calibrated but also have their results normalized such that they are traceable to the DCCT (http://www.missouri.edu/~diabetes/ngsp/).

In clinical research trials comparing the rate of chronic complications and/or severe hypoglycemic events, standardized or normalized assays are essential for intertrial comparisons. This was recently illustrated during attempts to compare the several Scandinavian trials with the DCCT, where even among HPLC assays measuring HbA_{1c}, there was nearly a 2% glycated hemoglobin difference.[9] Similar problems arise when comparing severe hypoglycemic events in patients using implantable, programmable insulin infusion devices, where markedly lower hypoglycemia rates are observed when compared with other forms of intensive insulin treatment.[15] In the two major US trials of implantable pumps, one study used an assay similar to the DCCT; thus, comparisons between severe hypoglycemic events are reasonable from the glycated hemoglobin perspective.[16] Unfortunately, the standardization of glycated hemoglobin assays may require a significant amount of time, as would be predicted by (1) the rate at which glycated hemoglobin assays are used by physicians,[6,7] and (2) the knowledge base related to modern diabetic care among physicians treating diabetic patients in the United States.[17] These problems can be resolved by educating the healthcare community, which was the motivation for this study.

The glycated hemoglobin target level must be individualized for each patient's clinical circumstances. The ADA goals appear generally reasonable.[13] Since achieving a value in the normal range for the DCCT or UKPDS was extremely difficult, such a value in the general US diabetic population is not a reasonable clinical goal, not only because of the difficulty but also because of early increased rates of severe hypoglycemia in type 1 patients.[3,18] Severe hypoglycemia rates were much more prevalent in the glycated hemoglobin range less than 7% to 8%, when in fact the chronic complication rates for advancing retinopathy were relatively low but nevertheless increasing.[3,10] The goals would be especially important during the first 2 years of intensive insulin treatment in type 1 patients.[18] Using the DCCT glycated hemoglobin data would suggest that a value greater than 8.0% would ideally be a critical level to avoid, since markedly accelerating rates of diabetic retinopathy and microalbuminuria occur.[10] Thus, the safe and practical therapeutic window of glycated hemoglobin for type 1 diabetic patients to achieve to avoid both acute and chronic complications is narrow and near 7% to 8% (Fig 1). For type 2 patients, hypoglycemia rates are much lower and are not of the same magnitude of

concern.[12] Perhaps the correct national target glycated hemoglobin level is more important when viewed as (1) individualized for each patient, (2) optimally avoiding acute and chronic diabetic complications, and (3) incorporating the concept that methods for glucose control in both type 1 and type 2 diabetes are currently neither ideal nor easy for the provider or the patient. In specialized diabetes clinics, a more reasonable glycated hemoglobin goal would be less than 6.0%. Support for this goal comes from the UKPDS, which suggests that for type 2 patients with macrovascular disease risk, the glycated hemoglobin level should be less than 6.2%.[19] The DCCT also described the first statistical retinopathy increase at 6.0%.[20] It is fortunate that these two sets of data are comparable, since the UKPDS and DCCT used a traceable HPLC HbA_{1c} assay.[21] Finally, there is strong evidence that macrovascular diseases are related to blood glucose levels within or slightly above the normal range.[22,23]

In summary, our data emphasize the need for standardizing or normalizing glycated hemoglobin assays or values, respectively, and until then, physicians should try to restrict themselves to using assays that are directly comparable or traceable to the DCCT and UKPDS assays.

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