Stability Over Time of Glycohemoglobin, Glucose, and Red Blood Cell Survival in Hematologically Stable People Without Diabetes

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We previously have shown that an affinity, high-performance liquid chromatography (HPLC) method is a highly reproducible and sensitive method for determining percent total glycohemoglobin (tGHb) in people with diabetes. In this study we extended the use of this method to a determination of the correlation of percent tGHb with the fasting plasma glucose concentration in people without known diabetes. We also determined the correlation of the tGHb with the reticulocyte count, as an index of red blood cell (RBC) survival, and with a carbon monoxide (CO) method for determining RBC survival. In addition, the stability of the tGHb, glucose, RBC mass, hemoglobin, and reticulocyte counts over a 1-year period was evaluated. Total glycohemoglobin, overnight fasting plasma glucose concentration, hemoglobin, RBC and reticulocyte count, and the calculated percentage of RBC count represented by reticulocytes were determined monthly for at least 12 months (range, 12 to 26 months) in 48 adults (mean age, 51 years; range, 31 to 82 years). In 37 of the subjects, RBC survival using a CO method also was determined. There was a highly significant linear correlation between the fasting glucose concentration and the tGHb. There was only a weak correlation between the percent reticulocytes or with the RBC survival determined by the CO method. The tGHb, plasma glucose, RBC count, hemoglobin, and percent reticulocytes were very stable over a 12-month or greater period. We conclude that there is a good correlation between the tGHb and plasma glucose concentration in a population without known diabetes. Variations in RBC survival as indicated by a reticulocyte count within the reference range is not likely to have a clinically significant effect on interpretation of tGHb data in the context of an integrated glucose concentration. Nevertheless, this remains to be proven using RBC survival methods that are more precise than those currently available.

A DIRECT COMPARISON between the glycohemoglobin results obtained using a boronate affinity, high-performance liquid chromatography (HPLC) method that measures all glycosylated hemoglobin molecules (tGHb) and a cation-exchange HPLC method used to quantify the hemoglobin A_{1c} (HbA_{1c}) fraction of hemoglobin was reported previously from our laboratory.[1] The HbA_{1c} data were obtained in the central laboratory for the Diabetes Control and Complication Trial (DCCT). The comparative data were obtained by dividing blood samples and analyzing the samples in both laboratories. Blood was obtained from people with and without known diabetes. The major findings from this study were: (1) approximately 40% to 50% of the HbA_{1c} fraction as determined by the method used in the DCCT is not glycosylated hemoglobin in people without known diabetes; (2) the glycosylated hemoglobin present in the HbA_{1c} fraction represents approximately 50% to 60% of the total glycosylated species present; and (3) the tGHb incremental increase is about 25% greater than the incremental increase in HbA_{1c} in samples from people with diabetes.

Overall, differences in glycosylation of hemoglobin, due to differences in blood glucose values integrated over the life span of a red blood cell (RBC), could be determined with greater specificity and precision using an affinity HPLC measurement of tGHb than when the HbA_{1c} fraction was determined by the method used in the DCCT.

These data indicate that the determination of percent tGHb is likely to be a better method for estimating the long-term integrated glucose concentration in people with diabetes. It also may be more useful in surveying populations for the presence of diabetes than is the determination of the percent HbA_{1c}.

Current diagnostic criteria for diabetes do not include glycohemoglobin values.[2] This is due to a lack of uniformity in methods used to determine percent GHb and to a reported lack of sensitivity and specificity.[2-6] In part, the latter may be due to the nonspecificity of the methods used. For example, hemoglobinopathies and the derivatization of hemoglobin by adducts other than glucose affects the interpretation of HbA_{1c} values when determined by the method used in the DCCT and by other methods that depend on a change in charge on the hemoglobin molecule.[1] Neither has been reported to significantly affect tGHb values.

Since the reproducibility and specificity of the HPLC affinity method for determining tGHb are excellent, the major variables in interpreting tGHb results are likely to be due to biological variations in circulating glucose concentration and in RBC survival in the same person over time and between individuals at a single time. Therefore, we decided to investigate these variables. The RBC count and percent reticulocytes were used as a surrogate for RBC survival (turnover). To our knowledge, the stability of RBC count, hemoglobin mass, glycohemoglobin, reticulocyte count, and fasting plasma glucose values in the same subjects without known diabetes (or known hematological abnormalities) over an extended period of time have not been reported. It has been reported that the percent HbA_{1c}

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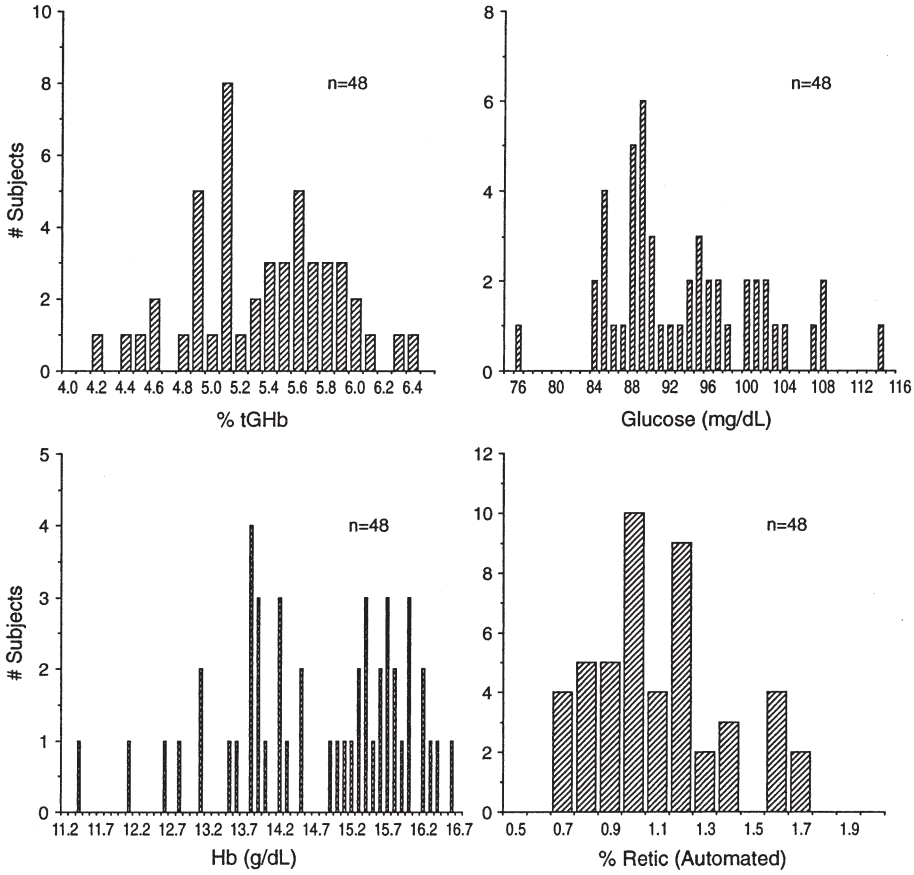

Fig 1. Distribution of means of data obtained monthly for 12 to 26 months in individual subjects.

is rather stable over time in a population without known diabetes.[7]

MATERIALS AND METHODS

Blood samples were obtained between 7 AM and 8 AM after an overnight fast at monthly intervals for at least a 12-month period from 48 subjects with normal fasting glucose values. Twenty-three males and 25 females with a mean age of 50.8 years (range, 31 to 82 years) were studied. All had liver function, thyroid function, and renal function tests within the reference range and were clinically well. Also, none were regularly taking large amounts of acetylsalicylic acid or vitamin C, or were known to drink alcoholic beverages in excess. Most were employees working full time and/or members of their families. None of the subjects were smokers. The mean duration of the study was 15 months (range, 12 to 26 months). The samples were processed for determination of tGHb, plasma glucose, hemoglobin, RBC count, and percent reticulocytes the same day the samples were obtained. In 37 subjects, RBC survival was estimated using a carbon monoxide (CO) method[8] on a single occasion. These subjects were selected only by their willingness to do the test.

Total glycohemoglobin was determined using an automated, HPLC method and an instrument purchased from Primus (Kansas City, MO). Plasma glucose was determined using a Johnson & Johnson (Piscataway, NJ) Vitros glucose instrument. Hemoglobin and RBC counts were determined automatically using a Coulter (Englewood, NJ) GENS apparatus (reference range, 14 to 18 g/dL and 4.6 to 6.2 $\times$ 10^{12}/L, respectively). The absolute reticulocyte count and percent of RBCs present as reticulocytes were determined manually in all subjects. In 22 subjects, they also were determined automatically using a Coulter GENS apparatus for the full duration of the study. In the remaining subjects, automatically determined reticulocyte counts were obtained over a period of at least 3 months. The automated reticulocyte method became available after the study was begun. However, it clearly is a more reproducible method.

In 100 subjects without known hematological abnormalities, the mean percent reticulocytes when counted manually, was 1.58%. The 95% range was 0.50% to 2.60%. The distribution was Gaussian. When determined in the same samples by the automated method, the mean was 1.08% and the distribution was log-normal. The 2.5% to 97.5% range was 0.60% to 2.1%.

In 123 subjects without known diabetes, and with a fasting plasma glucose concentration within the reference range (70 to 110 mg/dL), the mean tGHb was 5.3% with a reference range based on 2 SD of 4.3% to 6.3%. The distribution for both was Gaussian. Data are presented as means $\pm$ SD. Linear relationships were calculated by the Pearson product moment correlation coefficient.[9]

RESULTS

The distributions of the 12-month or greater means obtained for each subject for tGHb, plasma glucose, percent reticulocytes, and hemoglobin over the duration of the study are shown in Fig 1. The mean percent tGHb was 5.3% $\pm$ 0.5%. The mean glucose was 93 $\pm$ 8 mg/dL. The mean hemoglobin was 14.5 $\pm$ 1.2 g/dL. The mean RBC count was 4.7 $\pm$ 0.4 $\times$ 10^{12}/L. The mean percent reticulocytes was 1.7% $\pm$ 0.5% using the manual

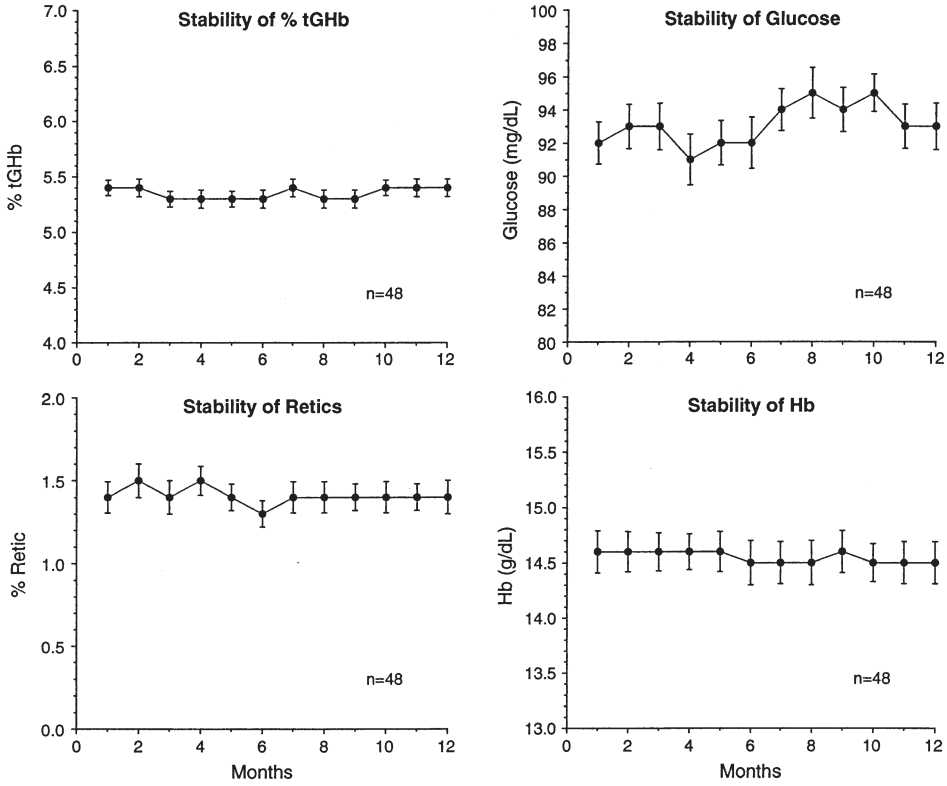

Fig 2. Means ± SEMS for data obtained monthly in all subjects over a 12-month period.

counting method (data not shown). For the automated method it was 1.1% with a range of 0.7% to 1.7%.

The mean longitudinal data by month are shown in Fig 2. All of the values were surprisingly stable over time. This stability also was demonstrated when individual data were analyzed. For example, the data obtained on samples from the senior author and from the person with the lowest fasting glucose are shown in Table 1.

There was a highly significant correlation between the 12-month or greater mean fasting glucose concentrations and mean

Table 1. Longitudinal Stability of Data in Two Subjects

Subject No. 1					Subject No. 2				
Time Point (mo)	tGHb	Glucose	% Retic	Hb	Time Point (mo)	tGHb	Glucose	% Retic	Hb
1	5.7	100	1.2	15.4	1	4.7	77	0.7	15.0
2	5.8	91	1.2	14.7	2	4.7	78	0.9	15.7
3	5.8	95	1.1	14.9	3	4.7	58	0.9	15.7
4	5.6	88	1.1	15.1	4	4.7	62	1.0	15.4
5	5.5	104	1.2	14.5	5	4.6	67	0.8	15.4
6	5.7	91	1.0	15.4	6	4.4	82	1.2	15.4
7	5.9	92	1.6	15.3	7	4.4	69	0.5	15.5
8	5.9	100	1.2	15.6	8	4.4	80	0.8	15.3
9	5.6	94	1.6	15.3	9	4.5	88	0.8	15.9
10	5.6	95	1.1	14.6	10	4.8	72	0.8	15.6
11	5.9	96	1.6	15.3	11	4.5	89	0.8	16.0
12	6.0	93	1.2	15.4	12	4.6	82	0.9	16.0
13	5.9	91	1.1	15.2	13	4.6	84	0.8	15.1
14	6.0	94	1.1	15.8	14	4.8	76	0.7	15.6
15	6.0	96	1.2	14.8	15	4.6	70	0.9	16.2
No.	15	15	15	15		15	15	15	15
Mean	5.8	95	1.2	15.2		4.6	76	0.8	15.6
SD	0.17	4.2	0.20	0.38		0.14	9.14	0.15	0.34
%CV	2.9	4.4	16.0	2.5		3.0	12.0	19.0	2.2

Abbreviations: Retic, reticulocytes; Hb, hemoglobin; CV, coefficient of variation.

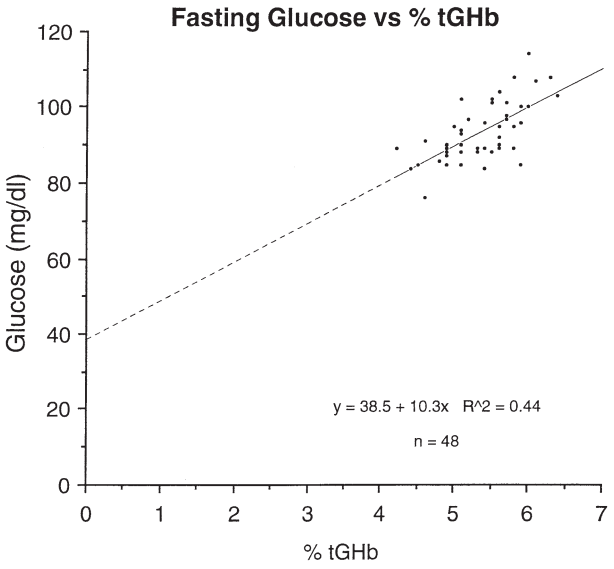

Fig 3. Correlation of 12- to 26-month mean fasting glucose concentration with the corresponding mean percent tGHb in 48 nondiabetic subjects.

percent tGHbs (correlation coefficient, 0.66; $P < .001$) (Fig 3). The variance in glucose values could account for approximately 44% of the variance in percent tGHb.

There was a slight, and nonstatistically significant, correlation between the percent reticulocytes and the tGHb when the reticulocytes were counted either manually (data not shown) or automatically (Fig 4). The correlation coefficient for the latter was 0.12 ($P < .2$). The variance in percent reticulocytes could only account for 1.5% of the tGHb variance.

There also was essentially no correlation between the percent tGHb and the RBC survival calculated using the CO method ($r = 0.39$, $P > .5$) (data not shown).

There was a correlation between the RBC survival determined by the CO method and the percent reticulocytes, but the correlation was not very robust (correlation coefficient, 0.39; $P < .01$) (data not shown). The variance in RBC survival determined by the CO method also was rather large (mean, 129 ± 26.5).

DISCUSSION

The correlation between fasting glucose or the glucose value 2 hours after ingestion of a standardized amount of glucose and the percent glycohemoglobin has been reported to be rather poor in people without known diabetes regardless of the glycohemoglobin method used.[3,10-13] However, in none of these studies was an automated, boronate affinity HPLC method used.

Using the HbA_{1c} method used in the DCCT, Meigs et al[7] have reported little or no correlation between mean percent HbA_{1c} in quintiles of fasting glucose extending from quintile I (fasting glucose, 60 to 87 mg/dL) to quintile V (fasting glucose, 102 to 140 mg/dL) in more than 2,000 subjects with normal glucose tolerance. Earlier, others[14] did report a correlation between fasting glucose in 165 glucose-tolerant subjects using the same HbA_{1c} method, but the correlation was less ($r^2 = 0.21$) than in the present study.

Several reasons for the lack of correlation have been considered, including assay variability, biological variability, and the rather narrow range of glucose values in a nondiabetic population.[13] The latter makes distinguishing differences difficult unless the methodology for measuring each variable is very precise.

The biological variability may be due to variations in glucose concentration or RBC survival values over time in the same individual and between individuals. It also may be due to an inherent difference in the rate at which globin molecules in hemoglobin became derivatized by glucose at the various glucose binding sites on the globin molecules. Alternatively, it may be due to variance in the rate at which the attached glucose undergoes an Amadori rearrangement in order to produce an essentially irreversible fructosyl adduct on the globin protein, ie, the product measured as glycohemoglobin. Conceptually, people have been referred to as fast and slow glycosylators, although data to support such a concept are lacking.

The present study has addressed several of these issues. First, the method used to determine glycated hemoglobin has a very small coefficient of variation (~1.5% to 2.0% at values within the reference range).[1] Thus, assay variability is not a significant issue. Second, tGHb values were obtained over an extended period of time in order to assess inter- and intrabiological variation. The biological variance in tGHb in subjects with glucose values within the reference range determined monthly over at least 12 months was small both individually (Table 1) and as a group (Fig 2). Third, RBC survival or turnover, at least as estimated by hemoglobin, RBC count, and percent reticulocytes, also was very stable over this extended period of time (ie, over at least 4 RBC turnover times).

The variance in reticulocyte counts was rather large, but the mean percent reticulocytes over the duration of the study was very stable (Fig 2). These data suggest that RBC survival

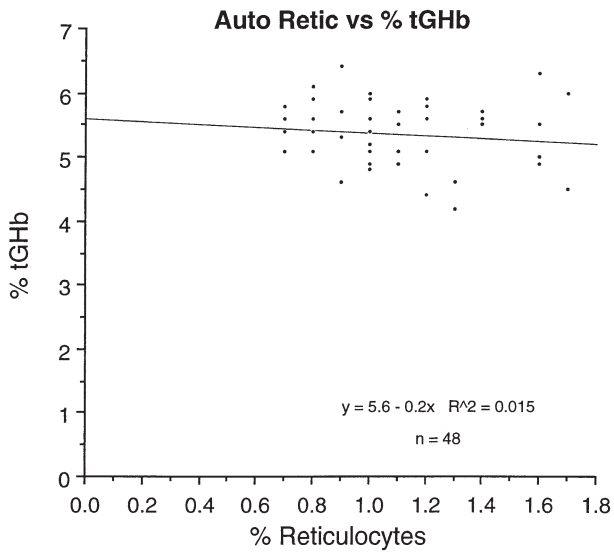

Fig 4. Correlation of 12- to 26-month mean percent reticulocytes with the mean percent tGHb in the same subjects.

variance within each individual is not likely to be an important factor in interpreting the tGHb data in the context of that individual's fasting glucose values. However, the RBC survival in each individual needs to be known in order to very accurately interpret the tGHb as an index of an integrated blood glucose value.

In the present study, an indirect method for estimating RBC survival (percent reticulocytes) correlated weakly with the tGHb values and cannot account for a significant amount of intersubject variance (Fig 4). Theoretically, use of reticulocyte counts adjusted for their residence time in the circulation when taken in the context of a time-stable hemoglobin or RBC count should be an excellent method for estimating RBC turnover. In the present study reticulocyte residence time was not determined and could account, at least in part, for the poor correlation with the tGHb values.

To our knowledge, information on the mean and range of reticulocyte residence time before maturation to a mature RBC is extremely limited. In any regard, our results indicate that correction of the percent tGHb data by adjusting for the percent reticulocytes, even using an automated method, did not improve the precision of the percent tGHB as an index of the integrated blood glucose value.

In addition, using an independent, CO method for estimating RBC survival, we also found little correlation with the percent tGHb. Methodological problems in accurately determining RBC survival may have affected the results using each method.

In this regard, others[15] using a 51-chromium method for determining RBC survival and a different, but unspecified method for determining percent glycohemoglobin, reported a log-linear relationship between RBC survival and percent GHb in subjects with hemolytic and other anemias. These data suggest that a major shortening in RBC survival beyond the usual reference range is necessary for a significant reduction in percent GHb to be recognized, ie, variations in RBC survival within the reference range should have only a modest effect on the percent GHb. As indicated above, the data obtained in the present study are compatible with this concept. We have obtained additional data in a separate study (unpublished data) that reinforces this concept.

In the 48 subjects in whom repeated automated reticulocyte counts were obtained in the present study in order to reduce intraindividual variance, the median percent reticulocytes for the group was 1.05, with a range of 0.7 to 1.7 (Fig 1). These data indicate a mean RBC survival of approximately 95 days and a range of 59 to 143 days using this method. However, the calculated RBC survival estimate also assumes a linear disappearance rate of RBCs from the circulation, ie, only the oldest RBCs are being removed. Available data indicate that it is largely the case but some randomness in RBC removal also is likely to be present.[16]

A considerable variation in RBC survival between people has been reported previously using a number of different methods.[8] At present a completely satisfactory and readily available method for determining RBC survival is not available.[17]

There was a highly significant correlation between the fasting glucose and percent tGHb (Fig 3). Indeed, the correlation coefficient was 0.66. Nevertheless, a potential limitation of the present study is the lack of 24-hour integrated glucose values in the subjects while ingesting their usual diet.

If one extrapolates linearly the relationship of the mean tGHb to zero glucose concentration, it intersects the Y axis at approximately 38 mg/dL glucose. This may be interpreted as indicating that for a considerable period of each day the glucose concentration is greater than the morning fasting value. In normal subjects, this is unlikely.

We have previously calculated that meal induced increases over the morning glucose value contributes approximately 3% in women and 7% in men (mean, 5%) to the mean 24-hour integrated glucose concentration in normal young subjects when eating a standard diet similar to that of the general population.[18] Data obtained in normal young males by others[19] also indicate that the 24-hour integrated glucose concentration was only about 4% greater than the initial morning glucose value. The subjects in the present study were older and are likely to be more insulin-resistant. Thus, the post-meal fraction of the total 24-hour integrated glucose concentration could have been greater. However, data obtained by others[20,21] suggest that it is not likely to be much greater. At most, it is likely to increase the 24-hour integrated glucose concentration by approximately 9% over the determined fasting value. In subjects with mild to moderately severe type 2 diabetes, ingestion of food also only contributes a small percentage (~3% to 10%).

Another factor to consider is the kinetics of glucose binding to hemoglobin. The binding of glucose to amino groups on the globin protein is relatively slow (hours) and the conversion to a fructosyl adduct is exceedingly slow ($t_{\frac{1}{2}}$ = ~5 days).[22] Thus, very transient increases in glucose concentration may have a smaller effect on the tGHb than quantitative 24-hour glucose data may indicate.

An explanation for the higher than expected glucose values when the tGHb data are extrapolated to zero tGHb (Fig 3) may be that the tGHb to glucose correlation becomes very curvilinear at these low glucose concentrations. There is an ordering of affinities for glucose attachment to various sites in the globin molecule of hemoglobin.[23] Thus, a difference in binding affinity could explain the results. This remains to be determined, but it would require very large differences in affinity and is unlikely. Thus, the reason for the Y intercept being greater than approximately 5 to 10 mg/dL remains unknown.

In summary, the present data suggest that moderate variations in RBC survival are not likely to significantly affect the clinical interpretation of the tGHb data, if the percent of reticulocytes is within the reference range and the subjects are hematologically normal. It also is not likely to significantly affect the interpretation of tGHb data in population-based studies of the prevalence of diabetes. It is the fasting glucose concentration that is the major determinant of percent tGHb in people without known diabetes.

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