Bimodality of Glycosylated Hemoglobin Distribution in Pima Indians

Relationship to Fasting Hyperglycemia

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SUMMARY

Glycosylated hemoglobin (HbA_1) concentrations were determined in 300 Pima Indians aged 15 yr and older. Frequency distributions of HbA_1 were unimodal in the 15–24-yr-old age group, but were bimodal in those aged 25 yr and over. The bimodality indicated that the subpopulation with diabetes could be identified by the presence of elevated HbA_1 levels. This group was comprised primarily of subjects who also had fasting plasma glucose levels of ≤ 140 mg/dl, but subjects with impaired glucose tolerance without fasting hyperglycemia had HbA_1 levels that were not significantly higher than those with normal glucose tolerance. The prevalence of diabetic retinopathy was much higher in the subgroup with elevated HbA_1 levels and increased with increasing HbA_1 level. HbA_1 levels and triglyceride concentrations showed only a modest association. HbA_1 determinations provided no advantage over fasting or post challenge glucose levels in the diagnosis of diabetes. DIABETES *28*:984–989, November 1979.

Hemoglobin in diabetic subjects is exposed to high concentrations of glucose, which enters the erythrocytes by simple diffusion and occurs there in the same concentration as in the plasma. Hemoglobin becomes glycosylated in proportion to the plasma glucose concentration by a slow, nonenzymatic reaction.[2] Three minor hemoglobins, HbA_{1a}, HbA_{1b}, and HbA_{1c} were recognized chromatographically over 20 yr ago,[3,4] but it was only after Rahbar[5,6] observed an abnormal hemoglobin in diabetic subjects that several minor hemoglobins were found to be elevated in diabetes mellitus. The most abundant of these hemoglobins, HbA_{1c}, is the product of the aldehyde reaction between glucose and the terminal valines of the two beta chains of hemoglobin with subsequent formation of a Schiff base, which rearranges to form a ketoamine.[7,8] HbA_{1a} appears to be a mixture of glucose-6-phosphate and fructose 1-6-diphosphate combined with hemoglobin, but HbA_{1b} is not yet fully characterized.[9,10]

These minor hemoglobins, commonly referred to as glycosylated hemoglobin or HbA_1, are formed continuously during the life span of the red cell. The percentage of glycosylated hemoglobin is generally believed to represent an integrated record of plasma glucose concentrations over a period of several weeks.[2] The concentration is elevated in uncontrolled diabetes[5,6,11,12] and slowly decreases if plasma glucose levels are reduced.[13–16]

We investigated the relation between glycosylated hemoglobin and the concentration of plasma glucose, both fasting and 2 h after a glucose load, in Pima Indians over a wide spectrum of glucose tolerance. Pima Indians have an extraordinarily high prevalence of diabetes. As the frequency distributions of the fasting and postload glucose levels have been demonstrated to be bimodal among the Pima,[17–19] the characteristics of the distributions of HbA_1 concentrations were determined. In addition, the relationships between HbA_1 levels, triglyceride levels, and the prevalence of retinopathy were examined.

METHODS

Blood samples from members of the Gila River Indian Community examined between September 1977 and June 1978 were obtained after an overnight fast and again 2 h after a 75-g oral carbohydrate load (Dexcola, Custom Laboratories, Baltimore, Md.). After mydriasis, ophthalmoscopic examinations were performed by a physician without knowl-

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edge of the subject's glucose level or if diabetes had been previously diagnosed. The presence of microaneurysms or proliferative changes was taken as evidence of retinopathy. Measurements of HbA_1 levels were also made in 38 Caucasians to determine if racial differences existed in hemoglobin concentrations at a given glucose level.

Blood was collected in sodium fluoride for measurement of plasma glucose by the method of Hoffman[20] and in EDTA for triglyceride and hemoglobin determinations. Plasma triglyceride concentrations were measured on fasting samples by the enzymatic method of Bucolo and David.[21]

Total glycosylated hemoglobin [HbA_1 (a + b + c)] was measured by modifications of the methods of Gabbay et al.[14] and Trivelli et al.[12] One-milliliter samples of whole blood were washed with cold saline in a total volume of 15 ml and centrifuged at 325 × *g* at 4°C. The supernatant was removed and the red cells washed and separated two additional times with the same volume of saline. The last centrifugation was at 900 × *g* to pack the cells. The washed erythrocytes were hemolyzed in 1 ml of distilled water and 0.5 ml of toluene with frequent vortexing for at least 60 min. When the hemolysate was centrifuged at 900 × *g*, it was clear and all red cell membrane fragments were at the interface between the aqueous and toluene layers. The hemolysate was dialyzed overnight at 4°C in cellophane tubing in a tube containing 40 ml of cyanide-phosphate buffer, pH 6.7 (#6 developer of Schnek).[22] Duplicate 300-μl aliquots of the dialysate were chromatographed on Bio-Rex 70 resin (200–400 mesh) columns in 12-ml syringes. HbA_1 was eluted with the cyanide-phosphate buffer, pH 6.7, pumped at the rate of 1.2 ml/min by a Technicon pump, and 100 ml collected in a volumetric flask. HbA_{II} was then eluted with 50 ml of phosphate buffer, 0.3 M, pH 6.4. The absorbance of the two eluates was measured in a spectrophotometer (Perkin-Elmer Coleman) at 415 nm and the percentage of the total hemoglobin in each fraction was calculated. The columns were regenerated immediately by pumping at least 100 ml of the cyanide-phosphate buffer through each column and, after standing overnight, with an additional 60 ml of this buffer just before reuse.

The percent recovery of total hemoglobin from the column was 100.6 ± 3.4 (mean ± SD, N = 40). The major portion of the HbA_1 came off the column in the first 50 ml of eluate (83 ± 2.6%, mean ± SD, N = 24 columns), while 9.4 ± 1.5% and 8.0 ± 1.4% were obtained in the two additional 25-ml volumes of eluate. The coefficient of variation of replicates of the HbA_1 concentrations from the same hemolysate was 2.5 ± 0.1%, N = 250. When blood was drawn from five nondiabetic subjects on multiple days, the HbA_1 level was stable with a coefficient of variation of 2.2 ± 0.6%.

A standard for HbA_1 was prepared by treating the hemolysate with potassium ferricyanide to convert all the hemoglobin to methemoglobin, followed by concentration and dialysis against the cyanide-phosphate buffer and storage in liquid nitrogen as recommended by Evan Hadley using his adaptation of van Assendelft's method[23] (personal communication). Aliquots were thawed periodically and chromatographed in the usual manner. Five of these determinations performed over an 84-day period had a mean ± SD of 15.1 ± 0.32%, indicating that the various columns gave reproducible results over a prolonged period.

Frequency distributions of the logarithms of fasting and 2-h plasma glucose levels and of arithmetic values of glycosylated hemoglobin levels were plotted for each sex in groups aged 15–24 and 25 yr and above. These distributions were bimodal in subjects 25 yr and older. The distributions were then examined using an iterative maximum likelihood procedure to determine the parameters of a model of two overlapping Gaussian distributions.[17] Optimal cutoff points to separate the components of the distributions and probabilities of misclassifying a subject in one component as belonging to the other were estimated using this model.[24]

RESULTS

Of the 300 Pima Indian subjects examined, 77 (26%) carried a previous diagnosis of diabetes. Of these, 72 (38%) were aged 25 yr and over and 5 (5%) were aged 15–24 yr. There were also 18 subjects who had 2-h postchallenge glucose concentrations of ≥200 mg/dl, 11 of whom had fasting plasma glucose levels of ≥140 mg/dl and were considered new diabetics. Sixty-two (81%) of the known diabetics and 9 of the 11 new diabetics with fasting hyperglycemia had HbA_1 concentrations ≥14%, whereas 7 of the 9 with postchallenge hyperglycemia, but with fasting levels <140 mg/dl, had HbA_1 concentrations below this level. In spite of receiving treatment for diabetes, the majority of the known diabetics had elevated HbA_1 levels as did those with untreated fasting hyperglycemia.

Frequency distributions of HbA_1 and glucose concentrations. The characteristics of the frequency distributions of HbA_1 levels in the Pima Indians were examined (Figure 1). The distribution of HbA_1 levels in males, aged 15–24 yr, was continuous and unimodal. In females the distribution was also unimodal, but a few outlying values suggested the presence of a small number of diabetics in this age group. In contrast, in subjects 25 yr and older, the HbA_1 distributions of both males and females were clearly bimodal and fit the model of two overlapping Gaussian distributions. This indicated the presence of two components, suggesting the presence of nondiabetic and overtly diabetic subpopulations.

The mean and standard deviation of the HbA_1 concentrations of these two subpopulations were determined using

FIGURE 1. Histograms and superimposed composite curves to describe the HbA_1 concentrations for males and females aged 15–24 and 25+ yr.

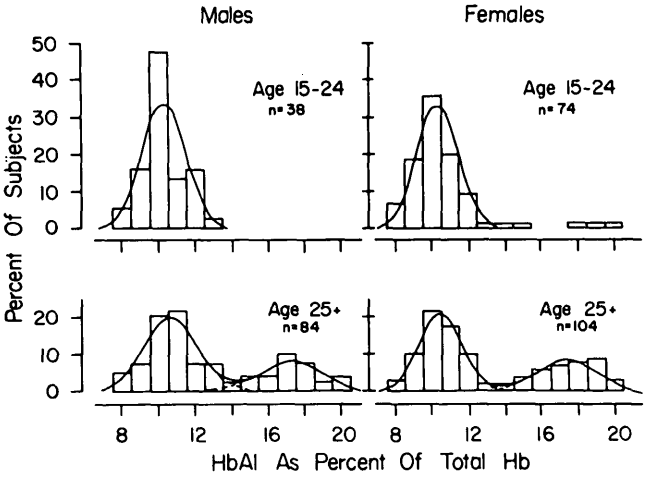


TABLE 1
Characteristics of bimodal distribution of HbA_1 in Pima Indians and optimum cutoff points

Age (yr)	N	Component: Normal (mean ± SD)	Component: Diabetic (mean ± SD)	Estimated cutoff
Males				
15–24	38	10.3 ± 1.16	—	13.7*
25+	84	10.6 ± 1.38	17.2 ± 1.62	14.0
Females				
15–24	74	10.1 ± 1.12	—	13.5*
25+	104	10.4 ± 1.16	17.4 ± 1.67	13.5
Both sexes				
15–24	112	10.2 ± 1.13	—	13.6*
25+	188	10.5 ± 1.26	17.3 ± 1.67	13.7

* Mean of distribution ± 3 SD.

maximum likelihood methods[17] (Table 1). The mean HbA_1 levels in the lower component (nondiabetic) ranged from 10.1 to 10.6%, whereas the upper or diabetic component had mean HbA_1 levels ranging from 17.2 to 17.4%. The optimal HbA_1 levels to divide the two subpopulations ranged from 13.5 to 14.0%. In the Caucasians, the mean HbA_1 concentrations were 10.5 ± 0.95% in 24 nondiabetics and 16.5 ± 2.5% in 12 diabetics.

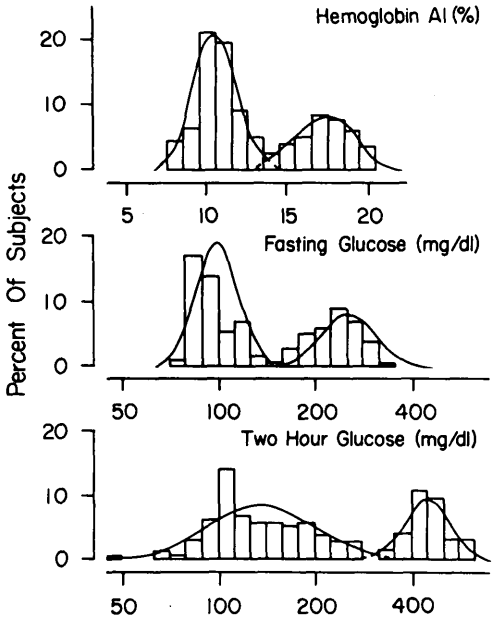
Figure 2 shows histograms of frequency distributions of the fasting and 2-h plasma glucose and HbA_1 concentrations in the Pima Indians aged 25 yr and over and the "best fit" models of the distribution. All three variables are bimodally distributed. When the optimal cutoff or antimodal points are used to classify subjects as nondiabetic or diabetic,[24] 0.9% would be misclassified using the level of HbA_1, while 0.4 and 0.6% would be misclassified using the fasting and 2-h plasma glucose levels, respectively.

HbA_1 and fasting plasma glucose concentrations. Scattergrams of HbA_1 and fasting plasma glucose concentrations for subjects aged 15–24 and 25 yr and older are shown in Figure 3. The 15–24 age group (Figure 3A) is composed mainly of subjects with normal fasting glucose concentrations. One hundred and five of the 112 subjects had fasting glucose levels <115 mg/dl with a mean (± SD) of 90 ± 8 mg/dl; their mean HbA_1 was 10.0 ± 1.1% with a range of 7.8–12.9%. In these subjects the correlation between HbA_1 and fasting glucose levels was 0.18 (NS).

Those aged 25 yr and older (Figure 3B) showed two clusters of values, 116 subjects with fasting plasma glucose levels <140 mg/dl and a mean HbA_1 of 10.5 ± 1.39% and 70 subjects with fasting glucose levels >140 mg/dl and a mean HbA_1 of 16.7 ± 2.26%. These clusters had correlation coefficients of 0.4 and 0.5, respectively, compared with 0.9 for the entire group.

The close correspondence between elevation of fasting plasma glucose and HbA_1 levels is shown in Figure 4. The percent of subjects with fasting plasma glucose >140 mg/dl is shown as a continuous line superimposed on the histogram of the frequency distribution of HbA_1 levels. Less than 10% of the subjects with fasting plasma glucose ≥140 mg/dl had HbA_1 levels of 13% or less and all of those had HbA_1 levels between 10 and 13%. Ninety-nine percent of subjects with HbA_1 levels above 14.5% had fasting plasma glucose exceeding 140 mg/dl.

FIGURE 2. Histograms and superimposed composite curves to describe HbA_1 concentrations in the combined males and females aged 25 yr and over. For comparison, histograms and curves for fasting and 2-h glucose concentrations are shown in the same subjects plotted on a logarithmic scale.



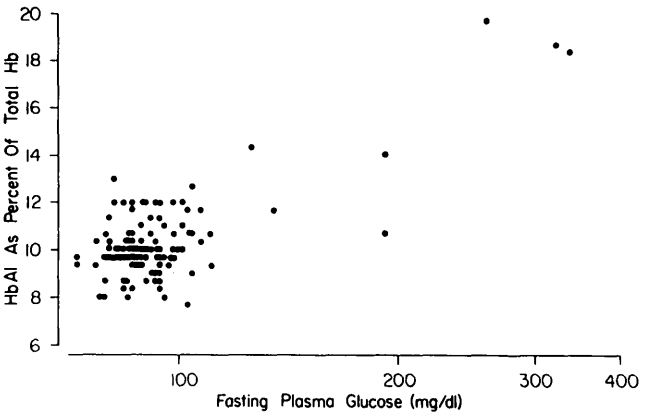
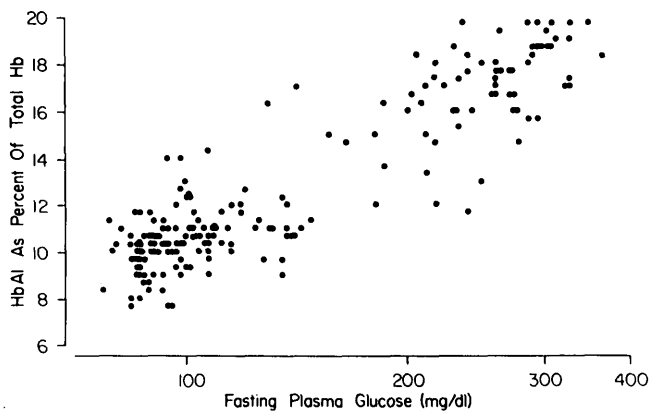


FIGURE 3A. Scattergram of HbA_1 concentrations in subjects aged 15–24 yr plotted against fasting plasma glucose concentrations. Histograms of these data separated for males and females are shown in Figure 1.

FIGURE 3B. Scattergrams of HbA_1 concentrations in subjects aged 25 yr and older. These data are the levels for histograms in Figure 2.



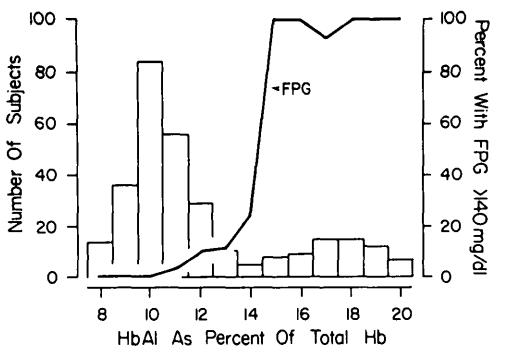


FIGURE 4. Histograms of HbA_1 concentrations in subjects aged 15 yr and over with superimposed line showing percent of subjects with fasting plasma glucose levels ≥140 mg/dl.

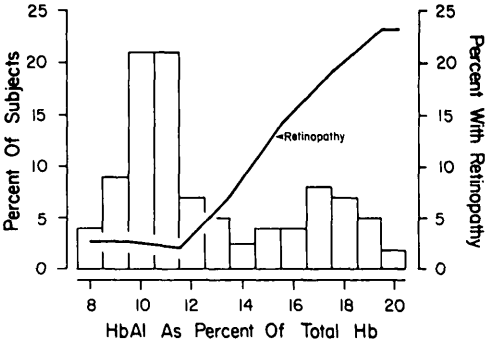


FIGURE 5. Histograms of HbA_1 concentrations with superimposed line showing percent of subjects with retinopathy.

Since both fasting and postchallenge glucose levels are frequently used for the diagnosis of diabetes,[25,26] HbA_1 levels were evaluated in subjects classified as having normal, impaired, or diabetic fasting and 2-h glucose levels (Table 2). The mean (±SD) HbA_1 level in 116 subjects with fasting plasma glucose <140 mg/dl was 10.5 ± 1.39%. The mean HbA_1 levels in 47 of these subjects with impaired glucose tolerance or equivocal fasting glucose levels, i.e., those with 2-h glucose levels of 140–199 mg/dl and fasting glucose levels <140 mg/dl and/or those with fasting plasma glucose levels of 115–139 mg/dl, did not differ significantly from those with completely normal glucose levels. In contrast, the levels in subjects with diabetes, defined by fasting plasma glucose levels ≥140 mg/dl and 2-h plasma glucose levels ≥200 mg/dl, were clearly elevated (mean 16.8 ± 0.27) and differed significantly from levels in subjects with normal or impaired glucose tolerance ($P < 0.01$). Thus, distinct elevations of HbA_1 levels did not occur except in subjects with both fasting and postchallenge hyperglycemia.

HbA_1 levels and prevalence of retinopathy. The prevalence of retinopathy in subjects 25 yr of age and older is shown in relation to the frequency distribution of HbA_1 in Figure 5. The majority of subjects with retinopathy had HbA_1 levels of at least 14%; above this level the frequency of retinopathy increased as glycosylated hemoglobin levels increased. Four individuals with retinopathy had HbA_1 levels below 14%. All had diabetes of long duration (12–22 yr documented by review of medical records): three were receiving insulin or oral hypoglycemic agents, and one, a known diabetic of 12-yr duration with renal failure treated by hemodialysis, had a HbA_1 of 7.6%, probably secondary to a shortened red cell half-life.

TABLE 2
Relation of HbA_1 levels (mean ± SD) to fasting and 2-h plasma glucose concentrations in subjects 25 yr and older

Fasting glucose (mg/dl)	2-H glucose (mg/dl)		
	<140	140–199	≥200
<115	10.3 ± 1.39 (69)	10.5 ± 1.05 (27)	11.0 ± 0.92 (2)
115–139	10.7 ± 1.25 (3)	11.3 ± 1.14 (3)	11.6 ± 1.85 (12)
≥140	—	13.8 ± 3.3 (2)	16.8 ± 2.20 (68)

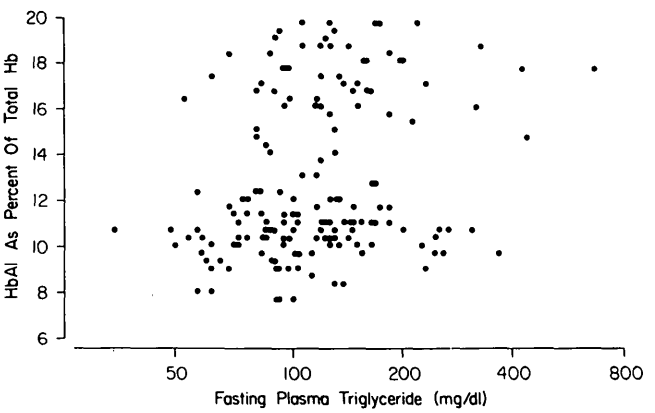
HbA_1 levels and fasting plasma triglyceride concentrations. The HbA_1 levels are plotted against the plasma triglyceride concentrations in Figure 6. Among the 162 subjects studied, there were weak but positive correlations between triglyceride and HbA_1 levels ($r = 0.24$), similar to those found between triglyceride and fasting ($r = 0.26$) and 2-h plasma glucose levels ($r = 0.28$).

DISCUSSION

The present study demonstrates that HbA_1 concentrations are bimodally distributed in Pima Indians above 24 yr of age. Pimas of this age have an extraordinarily high prevalence of diabetes. Frequency distributions of fasting, 1-, and 2-h postload plasma glucose levels have been previously shown to be bimodally distributed, as was confirmed in this study.[17,18] The vast majority of subjects with high HbA_1 levels are the same subjects who have elevated fasting and 2-h plasma glucose levels. They constitute the hyperglycemic component of the frequency distributions, which were shown previously to be characterized by the presence of a high frequency of the specific microvascular complications of diabetes.[19] The observation of bimodality in HbA_1 frequency distributions provides an independent confirmation of the fact that a hyperglycemic, or diabetic, subpopulation may be identified.

While the overall correlation between HbA_1 levels and both fasting and 2-h plasma glucose levels is high, within the nondiabetic and diabetic subpopulations the correlations between HbA_1 and the corresponding glucose levels is much lower. Those with impaired glucose tolerance, de-

FIGURE 6. Scattergram of HbA_1 concentrations plotted against the concentration of fasting plasma triglyceride on a logarithmic scale.



fined as having 2-h postload plasma glucose levels of 140–199 mg/dl or fasting glucose levels between 115 and 139 mg/dl, had HbA_1 levels that were only slightly higher than subjects with unequivocally normal glucose levels. The measurement of glycosylated hemoglobin levels, therefore, failed to discriminate between those with mild impairments of glucose tolerance and those with unequivocally normal glucose values. This agrees with reports of Dolhofer et al.[27] and Lev-Ran and Vanderlann,[28] who found considerable overlap in HbA_1 levels between subjects with normal and slightly impaired glucose tolerance.

In contrast, subjects with diabetic glucose levels (fasting plasma glucose levels equal to or greater than 140 mg/dl and 2-h postload plasma glucose levels equal to or greater than 200 mg/dl) almost invariably had elevated HbA_1 levels. Subjects with diabetes could be equally well identified by the presence of elevations in HbA_1 fasting or 2-h postload glucose levels.

HbA_1 concentrations in Pima Indians with normal glucose tolerance (10.4 ± 1.3%) were somewhat higher than those reported in other groups of normal subjects (6.5% by Trivelli et al.,[12] 7.45% by Gonen et al.,[15] 8.2% by Graf et al.[29]), although the differences between diabetic and nondiabetic subjects in our study were similar to those found in other reports. This was not secondary to racial differences in glycosylation of hemoglobin, because we found similar levels in Caucasians and Indians. These differences in reported HbA_1 levels may reflect variation in recovery of hemoglobin from red cell membranes and/or differences in chromatographic separation of glycosylated hemoglobin. Such differences emphasize the need for standardization of HbA_1 determinations and the necessity of having appropriate controls to enable interpretation of HbA_1 data.

Data in the present report permit only a preliminary assessment of the relationship of HbA_1 levels to the complications of diabetes. Retinopathy was confined almost entirely to the population that had elevated HbA_1 levels and the percent of subjects with retinopathy increased with increasing HbA_1 concentration within this group. This relationship is similar to the one we reported concerning fasting and 2-h plasma glucose levels.[18] Complications, however, are also a function of the duration of diabetes. Prospective studies of the relationship of HbA_1 level of the incidence of microvascular complications will be necessary to determine the usefulness of HbA_1 concentrations in predicting the development of complications. Nevertheless, it is intriguing that retinopathy was rare in those who had well-controlled diabetes with HbA_1 levels within the nondiabetic range at the time of the study.

While diabetes is associated with elevated triglyceride concentrations,[30] HbA_1 and triglyceride concentrations in the Pima Indian population showed only a modest (but statistically significant) correlation. This result contrasts with the high correlation reported by Peterson et al., who measured HbA_1 levels before and after normalization of glucose levels in 10 diabetics.[31]

The determination of glycosylated hemoglobin concentration provides no great advantage over the measurement of fasting and/or postchallenge glucose levels for the diagnosis of diabetes. The demonstration of bimodality in the distribution of glycosylated hemoglobin levels, however, has provided independent confirmation of the existence and possible importance of bimodality in glucose levels in the Pima Indians. Those who fall in the hyperglycemic component of the population are characterized by elevated levels of glycosylated hemoglobin and perhaps other proteins, whereas those with lesser degrees of glucose intolerance are not. Since the Pima Indians without elevated HbA_1 levels have a low prevalence of retinopathy, measurement of HbA_1 levels may prove useful to assess the role of hyperglycemia in the development of complications of diabetes.

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