Biological Variation in HbA$_{1c}$ Predicts Risk of Retinopathy and Nephropathy in Type 1 Diabetes

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OBJECTIVE — We hypothesized that biological variation in HbA$_{1c}$, distinct from variation attributable to mean blood glucose (MBG), would predict risk for microvascular complications in the Diabetes Control and Complications Trial (DCCT).

RESEARCH DESIGN AND METHODS — A longitudinal multiple regression model was developed from MBG and HbA$_{1c}$ measured in the 1,441 DCCT participants at quarterly visits. A hemoglobin glycation index (HGI = observed HbA$_{1c}$ – predicted HbA$_{1c}$) was calculated for each visit to assess biological variation based on the directional deviation of observed HbA$_{1c}$ from that predicted by MBG in the model. The population was subdivided by thirds into high-, moderate-, and low-HGI groups based on mean participant HGI during the study. Cox proportional hazard analysis compared risk for development or progression of retinopathy and nephropathy between HGI groups controlled for MBG, age, treatment group, strata, and duration of diabetes.

RESULTS — Likelihood ratio and $t$ tests on HGI rejected the assumption that HbA$_{1c}$ levels were determined by MBG alone. At 7 years’ follow-up, patients in the high-HGI group (higher-than-predicted HbA$_{1c}$) had three times greater risk of retinopathy (30 vs. 9%, $P < 0.001$) and six times greater risk of nephropathy (6 vs. 1%, $P < 0.001$) compared with the low-HGI group.

CONCLUSIONS — Between-individual biological variation in HbA$_{1c}$, which is distinct from that attributable to MBG, was evident among type 1 diabetic patients in the DCCT and was a strong predictor of risk for diabetes complications. Identification of the processes responsible for biological variation in HbA$_{1c}$ could lead to novel therapies to augment treatments directed at lowering blood glucose levels and preventing diabetes complications.

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Chronic hyperglycemia as measured by mean blood glucose (MBG) or HbA$_{1c}$ has been linked to the development and progression of microvascular diabetes complications (1,2).

Blood glucose levels are clearly a major determinant of HbA$_{1c}$ levels. Population studies in patients with diabetes have shown that HbA$_{1c}$ is highly correlated with preceding MBG (3–5). However, evaluation of the relationship between HbA$_{1c}$ and MBG among individuals within a population shows that there is considerable variation in HbA$_{1c}$ around the population linear regression line at any given MBG value (6). This variation is often treated as random, but there is considerable evidence that much of it is due to nonrandom, patterned variation of biological origin. Thus, some individuals at the same MBG value have consistently higher HbA$_{1c}$ levels and others consistently lower HbA$_{1c}$ levels than that expected under the hypothesis that HbA$_{1c}$ is solely determined by MBG.

Such biological variation has been demonstrated for many measured clinical analytes and is mediated by physiological and biochemical processes that differ between individuals (7). The existence of biological variation suggests that each person has a different homeostatic set point that determines their particular mean level of an analyte over time. Statistically, biological variation can be detected by the presence of higher between-individual variation in analyte levels compared with within-individual variation, as has been demonstrated for HbA$_{1c}$ in nondiabetic human populations (8,9).

There is also evidence that biological variation in HbA$_{1c}$ is genetically determined, as demonstrated by a recent twin study (10) that showed that genetic effects explained 62% of the population variance in HbA$_{1c}$.

In individuals with normal glucose tolerance, biological variation in HbA$_{1c}$ can be demonstrated in a relatively straightforward manner by repeat measurement of HbA$_{1c}$ over time and comparing between- and within-individual variation (8,9). Studies (11–13) in nondiabetic subjects have shown that between-individual variation in HbA$_{1c}$ was not related to glycemia and that the tendency for some individuals to have higher or lower than average HbA$_{1c}$ levels was consistent over time.

In patients with abnormal glucose tolerance, different approaches must be used to prevent variation in HbA$_{1c}$ caused by MBG from obscuring the effect of biological variation. Madsen et al. (14) and Hudson et al. (15) used a “glycosylation index,” which was calculated as the ratio of HbA$_{1c}$ to blood glucose. Using this index, they documented significant between-individual variation in hemoglobin glycation among type 1 diabetic patients.
In a previous study (16), we developed a hemoglobin glycation index (HGI) based on the relationship between observed and predicted HbA1c levels. Predicted HbA1c was calculated based on observed MBG using a multiple regression equation that compared HbA1c and MBG for the studied population. Calculated in this manner, HGI quantifies the magnitude and direction of individual differences in observed HbA1c from that predicted by the population regression equation while accounting for the influence of MBG. When studied in patients with type 1 diabetes over a 2-year period, we found (16) that HGI was statistically significantly different between individual patients, consistent within individuals over time, and was not related to erythrocyte turnover. We interpreted these results as evidence of biological variation in HbA1c distinct from that attributable to MBG. The accumulated evidence thus strongly suggests that an individual’s HbA1c levels are determined by two major components: 1) MBG and 2) other individual factors responsible for biological variation in HbA1c.

Biological variation in HbA1c has been linked to both macro- and microvascular pathology. For example, HbA1c levels have been associated with cardiovascular disease (17) and carotid intimal-medial thickening (18) in nondiabetic subjects. Similarly, data from the European Prospective Investigation of Cancer and Nutrition (EPIC)-Norfolk study (19) showed that HbA1c was a continuous mortality risk factor across the whole population distribution, even in people without diabetes. Biological variation in HbA1c was also associated with nephropathy in a small population of type 1 diabetic patients with chronic hyperglycemia (20). We hypothesized that biological variation in HbA1c might predict risk for microvascular complications of diabetes in the Diabetes Control and Complications Trial (DCCT). The DCCT produced a comprehensive, multiyear dataset containing observations for MBG, HbA1c, and the occurrence of complications in 1,441 patients with type 1 diabetes (2). In the present study, we calculated HGI as a measure of biological variation in HbA1c for each clinical encounter for all participants in the DCCT. We then evaluated the relationship between biological variation in HbA1c and the development or progression of retinopathy and nephropathy.

**RESEARCH DESIGN AND METHODS** — We used publicly accessible data collected by the DCCT and stored in SAS datasets on magnetic tape (National Technical Information Service, Washington, DC). The DCCT was a 9-year study of 1,441 participants with type 1 diabetes conducted to compare the effect of intensive versus conventional blood glucose management on the development and progression of diabetes complications (2). At randomization, all participants in the study were free of advanced micro- or macrovascular complications of diabetes and were stratified into two strata. The primary prevention cohort (n = 726) had no evidence of retinopathy by fundus photography and urinary albumin excretion rate (UAER) <40 mg/24 h (21). The secondary intervention cohort (n = 715) had minimal to moderate retinopathy and UAER <200 mg/24 h. The study participants were also randomized into conventional and intensive treatment groups. Detailed descriptions of the design and outcome of the DCCT have been published elsewhere (2,21,22).

**Calculation of MBG**

A 1-day, seven-sample glucose profile set and a blood sample for HbA1c were collected quarterly over the course of the DCCT from each participant (5,23). Each glucose profile set consisted of seven capillary blood samples drawn before and 90 min after main meals (breakfast, lunch, and dinner) and at bedtime. Profile set data were available for 95% of the scheduled pre- and postmeal time slots and for 92% of the bedtime slots. The protocol also called for 3:00 A.M. glucose measurements, but these were available for <1% of the profile sets. HbA1c levels and the glucose concentrations of the profile set blood samples were determined at a central laboratory (23). For the present study, MBG was calculated quarterly for each participant as the arithmetic mean of the glucose concentrations of the profile sets, excluding 3:00 A.M. measurements.

**Calculation of HGI and assessment of biological variation in HbA1c**

We previously developed (16) a statistical model to assess between-individual variation in HbA1c in diabetic patients. A similar approach was applied to data collected by the DCCT. Briefly, a longitudinal linear response model was developed from all measured HbA1c and the corresponding MBG measured at the same clinic visit using results from all participants, at all clinic visits, during all of the years of the DCCT. The appropriateness of a linear model was confirmed by a spline-fitting algorithm that made no prior assumptions regarding the shape of the relationship. Akaike’s Information Criterion (24) indicated that a random intercept provided the best fit for the data. The model variance was adjusted to account for the correlation between data on the same individual. Other covariates in the model were age, diabetes duration, sex, treatment (intensive versus conventional), stratum (1st prevention or 2nd intervention), and race. The SD of the profile set glucose values used to calculate MBG was found to have negligible influence on HbA1c or HGI and was not included as a covariate in the final model.

This model was used to predict HbA1c from profile set MBG for all encounters in the DCCT. The predicted HbA1c was then used to calculate HGI for each clinic visit as follows: HGI = observed HbA1c − predicted HbA1c, where observed HbA1c is the measured HbA1c for the quarterly clinic visit and predicted HbA1c is the value mathematically derived by inserting the profile set MBG for the same quarterly visit into the population regression equation. The presence of between-individual variation in HbA1c was assessed by analysis of HGI as previously described in detail (16). First, a likelihood ratio test was used to determine whether mean HGI was statistically significantly different among individuals in the DCCT. Second, t statistics were used to separately evaluate each individual’s HGI set and determine whether that individual’s set of observed HbA1c values were significantly different from the values predicted by the population regression equation. This approach was based on the assumption that if an individual’s observed and predicted HbA1c levels were not statistically significantly different, then the 99% CI around the individual’s mean HGI should include zero.

**Biological variation in HbA1c and risk of microvascular complications**

The DCCT data were used to evaluate the relationship between HGI and risk for the development or progression of retinopathy and nephropathy. Severity of retinopathy was measured by the 25-point Early.
Diabetic Retinopathy Treatment Study interim score (2,22). Development or progression of retinopathy was defined as a sustained change from baseline of three steps in this score at any retinal examination during the DCCT. Development or progression of nephropathy was defined as the occurrence of advanced microalbuminuria, i.e., UAER ≥100 mg/24 h in subjects with UAER <100 mg/24 h at baseline or UAER ≥300 mg/24 h in subjects in the secondary intervention cohort with microalbuminuria at baseline (21).

To assess the relationship between biological variation in HbA1c and microvascular complications, participants in the DCCT were divided by tertiles (33%) into low-, moderate-, and high-HGI groups based on mean HGI during the course of the study. After confirming the validity of the proportional hazards assumption, a Cox regression model was used to compare the risk for development or progression of retinopathy and nephropathy by HGI group over time. The model statistically controlled for the effects of age, diabetes duration, sex, treatment, and stratum. MBG was included in the model as a time-dependent covariate; thus, estimates of risk were adjusted for differences in MBG between individuals in the HGI groups. Statistical analysis was performed using the “stcox” and “sts graph” procedures in STATA-6 (25).

To evaluate the influence of HGI on complications in individuals with high or low blood glucose levels, participants were also subdivided by tertiles into low-, moderate-, and high-MBG groups (n = 480) based on mean MBG over the course of the study. Cox regression analysis was then used to compare the risk of retinopathy by HGI group for individuals in the low- and high-MBG groups. Because of the lower incidence of nephropathy, there were insufficient outcomes to perform a similar assessment for risk of nephropathy.

**RESULTS**

**HGI and biological variation in HbA1c**

MBG was highly correlated with HbA1c ($r = 0.71, P < 0.0001$). Sufficient data for inclusion in the model were available from 1,439 of 1,441 subjects. Mean participant HGI calculated from all available clinic visits (before and after enrollment) were normally distributed, with a mean of 0.00 and a SD of 1.65. Cutoff points for subdivision of the DCCT participants into low-, moderate-, and high-HGI groups were: low, $<0.38$; moderate, $0.38$ to $0.42$; and high $>0.42$. The assumption that HbA1c was solely determined by MBG was not supported by the data. The hypothesis that there were no between-individual differences in HGI was strongly rejected by the likelihood ratio test ($P < 0.0001$). Evaluation of within-individual $t$ statistics for HGI showed that 816 of the 1,439 DCCT participants (57%) had mean HGI that was significantly different ($P < 0.01$) from zero, the value expected if HGI were determined by MBG except for random error.

To further assess biological variation in HbA1c, we plotted the relationship between MBG and HbA1c for all participants at each clinic visit during the 9-year study. B–D: All observations for participants in the high-, moderate-, and low-HGI groups, respectively. The regression line shown in each panel was derived from the simple regression for HbA1c and MBG from the population shown in A. The population regression line bisected the data points in the plots for the population and the moderate-HGI group. In contrast, the vast majority of observations in the high-HGI group (74%) were above the regression line, whereas the vast majority of observations in the low-HGI group (79%) were below the regression line.

![Figure 1 — MBG and HbA1c in the DCCT. A: The observed MBG and HbA1c for every participant at each clinic visit during the 9-year study. B–D: All observations for participants in the high-, moderate-, and low-HGI groups, respectively. The regression line shown in each panel was derived from the simple regression for HbA1c and MBG from the population shown in A.](image-url)

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Risk for development or progression of nephropathy for up to 7 years.

The development or progression of retinopathy measured by the 25-point Early Diabetic Retinopathy Treatment Study interim score (A) was strongly related to HGI and was significantly higher in the high-HGI group (P < 0.0001). The development or progression of nephropathy (B), measured as the occurrence of advanced microalbuminuria, was also strongly related to HGI and was significantly higher in the high-HGI group (P < 0.0001).

Conclusions — This study demonstrates the presence of between-individual biological variation in HbA1c, distinct from that attributable to MBG in the DCCT. As measured by HGI, many individuals had observed HbA1c levels that were significantly higher or lower than predicted by the population regression equation. Individual tendency toward higher or lower than predicted HbA1c was distinct from MBG and was consistent over time in repeat clinic visits for up to 9 years. In fact, for many individuals, all of their quarterly HbA1c levels during the entire 9-year study were above or below that predicted by the population regression equation. This evidence of between-individual variation in HbA1c among patients in the DCCT is consistent with the findings of a number of other studies that evaluated both diabetic and nondiabetic populations.

We considered the possibility that between-individual variation in the relationship between HbA1c and MBG in the DCCT might be an artifact of measurement error. The likelihood that the observed results were due to analytical error was minimal because all glucose and HbA1c measurements were determined by a central laboratory. Thus any error associated with the analysis of glucose concentrations or HbA1c levels should be random among individuals, and although random error could obscure the presence of between-individual variation in HbA1c, it cannot be the source of it. Only nonrandom measurement bias that produced consistently false differences between observed and predicted HbA1c levels could cause the observed between-individual variation in HGI. Compared with HbA1c, MBG is relatively susceptible to sampling error. Timed blood sample collections before and after meals limit, but do not eliminate, the possibility of sampling bias. Thus, unmeasured (e.g., nocturnal) glucose values that were markedly different from measured values could lead to un-
showed that HbA1c levels are not mean-
error. Biological variation and not measurement
served in the DCCT is attributable to
extremely different from the measured
blood glucose values would have to be
under- or overestimation of MBG. For this to
explain the results of our study, however, un-
der- or overestimation of MBG would have to be
remarkably consistent within individuals over all 9 years of the study.
Furthermore, examination of data from
individual DCCT patients with extremes
of HGI showed that the unmeasured
blood glucose values would have to be
extremely different from the measured
values and in many cases biologically ab-
surd. Consistent individual differences in
unmeasured glucose values could be
casued by consistent differences in glu-
cose fluctuations during the day. How-
ever, we evaluated the SDs of the glucose
values used to calculate MBG and deter-
mined that glycemic variation had negli-
gible influence on HbA1c or HGI. A recent
study (26) of 256 diabetic patients also
showed that HbA1c levels are not mean-
ingfully affected by glycemic variation.
We conclude, therefore, that the be-
tween-individual variation in HbA1c ob-
served in the DCCT is attributable to
biological variation and not measurement
error.

Figure 3—Biological variation in HbA1c, and risk of retinopathy in individuals with good or poor
glucose control. Cox regression analysis was used to compare risk of retinopathy in DCCT
participants with high or low blood glucose levels after subdivision by mean HGI into low-, moder-
ate-, and high-HGI groups. Despite low glucose levels during the course of the study, pa-
tients with low MBG and high HGI had a significantly greater risk of retinopathy compared with
patients in the low- and moderate-HGI groups (A). Risk of retinopathy among patients with high
glucose levels during the course of the study were also significantly different in the low-, moder-
ate-, and high-HGI groups (B).

Nonenzymatic glycation of proteins, including hemoglobin, occurs via the
Maillard reaction. This reaction between reducing sugars and terminal or ε-amines
is also an important step in the formation of advanced glycation end products. Ad-
vanced glycation end products are a heter-
ogeneous class of compounds that have
been implicated in the pathophysiology of
diabetes complications, aging, and Alz-
heimer’s disease (27). Evidence of a link
between biological variation in HbA1c and
microvascular complications in the
DCCT suggests that factors responsible
for biological variation in nonenzymatic
hemoglobin glycation may also influence
individual susceptibility to diabetes com-
plications. Nonenzymatic hemoglobin
glycation is a function of intracellular glu-
cose and factors that influence glucose
binding to hemoglobin. The latter in-
cludes intracellular pH and 2,3-
bisphosphoglycerate concentrations and
the levels or activities of glycolytic or deg-
laying enzymes (13–15,28,29). Al-
though HbA1c levels can be affected by
erythrocyte age, we previously showed
(16) that HGI was not related to erythro-
cyte turnover rates based on creatine levels.

The important novel finding of this study
is that biological variation in HbA1c
is an important predictor for the develop-
ment and progression of diabetes com-
lications. This suggests that there are two
important components of risk for the mi-
crovascular complications of diabetes.
The first is the well-recognized effect of
chronically elevated blood glucose. The
second component is the less-recognized
and poorly understood effect of factors
other than glucose that are responsible for
biological variation in HbA1c. The exis-
tence of these two components of risk
suggests that two therapeutic approaches
also exist for the prevention of diabetes
complications. One is the current ap-
proach to diabetes management, where
treatment largely depends on pharmaco-
logic and lifestyle interventions to lower
blood glucose levels as near as possible to
the physiological range. As our data show,
however, reducing blood glucose levels
alone may not be sufficient because even
patients in the DCCT with relatively low
blood MBG levels had elevated risk of re-
tinopathy if they belonged to the high-HGI
group (Fig. 3A). Consequently, a second
therapeutic approach may be needed, one
that involves in the currently unknown
mechanisms that mediate biological vari-
ation in HbA1c. Elucidation of these
mechanisms could promote the develop-
ment of novel therapeutic interventions
and individually customized manage-
ment programs. HGI or other indexes of
biological variation in HbA1c could thus
prove clinically important for identifying
high-risk patients and monitoring the ef-
cacity of therapy.

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Variation in HbA1c and diabetes complications


