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.

Diabetes Care 27:1259–1264, 2004

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.

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Received for publication 24 November 2003 and accepted in revised form 21 February 2004.

Abbreviations: DCCT, Diabetes Control and Complications Trial; HGI, hemoglobin glycation index; MBG, mean blood glucose; UAER, urinary albumin excretion rate.

In a previous study (16), we developed a hemoglobin glycation index (HGI) based on the relationship between observed and predicted HbA_{1c} levels. Predicted HbA_{1c} was calculated based on observed MBG using a multiple regression equation that compared HbA_{1c} and MBG for the studied population. Calculated in this manner, HGI quantifies the magnitude and direction of individual differences in observed HbA_{1c} 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 HbA_{1c} distinct from that attributable to MBG. The accumulated evidence thus strongly suggests that an individual's HbA_{1c} levels are determined by two major components: *1*) MBG and *2*) other individual factors responsible for biological variation in HbA_{1c}.

Biological variation in HbA_{1c} has been linked to both macro- and microvascular pathology. For example, HbA_{1c} 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 HbA_{1c} was a continuous mortality risk factor across the whole population distribution, even in people without diabetes. Biological variation in HbA_{1c} was also associated with nephropathy in a small population of type 1 diabetic patients with chronic hyperglycemia (20). We hypothesized that biological variation in HbA_{1c} 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, HbA_{1c}, 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 HbA_{1c} for each clinical encounter for all participants in the DCCT. We then evaluated the relationship between biological variation in HbA_{1c} 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 HbA_{1c} 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. HbA_{1c} 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 HbA_{1c}

We previously developed (16) a statistical model to assess between-individual variation in HbA_{1c} 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 HbA_{1c} 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 (1° prevention or 2° intervention), and race. The SD of the profile set glucose values used to calculate MBG was found to have negligible influence on HbA_{1c} or HGI and was not included as a covariate in the final model.

This model was used to predict HbA_{1c} from profile set MBG for all encounters in the DCCT. The predicted HbA_{1c} was then used to calculate HGI for each clinic visit as follows: HGI = observed HbA_{1c} − predicted HbA_{1c}, where observed HbA_{1c} is the measured HbA_{1c} for the quarterly clinic visit and predicted HbA_{1c} 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 HbA_{1c} 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 HbA_{1c} 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 HbA_{1c} levels were not statistically significantly different, then the 99% CI around the individual's mean HGI should include zero.

Biological variation in HbA_{1c} 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

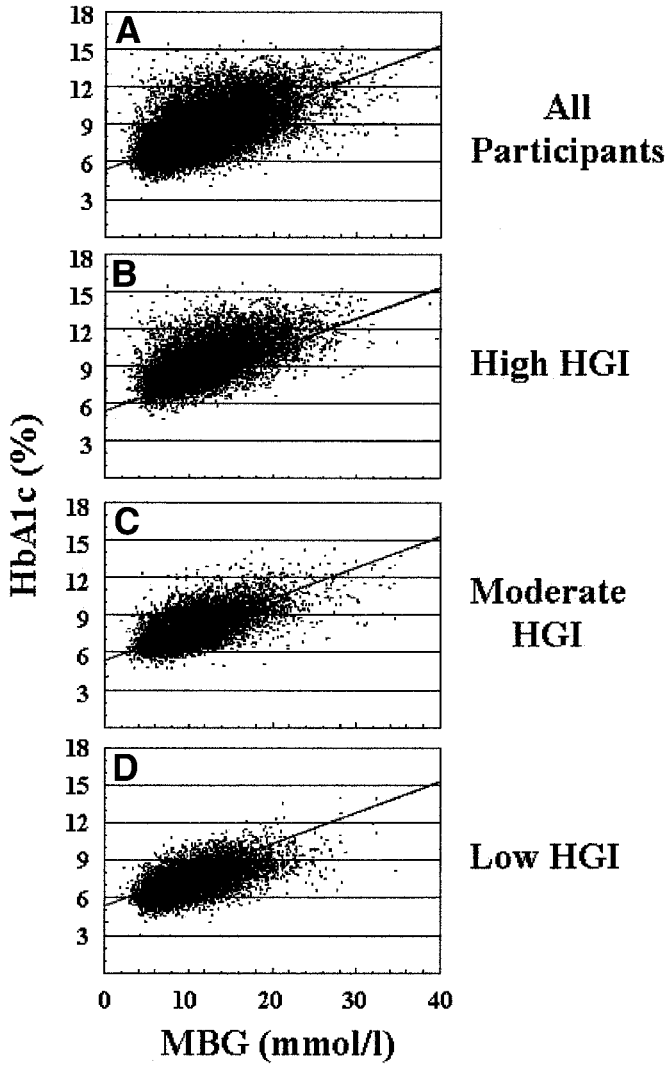

Figure 1—*MBG and HbA_{1c} in the DCCT.* A: *The observed MBG and HbA_{1c} for every participant at each clinic visit during the 9-year study.* B–D: *All observations for participants in the high-, moderate-, and low-HGI groups, respectively. The regression line shown in each panel was derived from the simple regression for HbA_{1c} 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.*

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 HbA_{1c} 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 HbA_{1c}

MBG was highly correlated with HbA_{1c} ($r = 0.71$, $P < 0.0001$). 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 HbA_{1c} 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 HbA_{1c}, we plotted the relationship between MBG and HbA_{1c} for all participants in the DCCT and separately for each HGI group (Fig. 1). We reasoned that if the variation in the relationship between MBG and HbA_{1c} within the population was solely random, then the distribution of data points above and below the regression line should be similar in each plot. This was not the case, however, since approximately three-quarters of all observa-

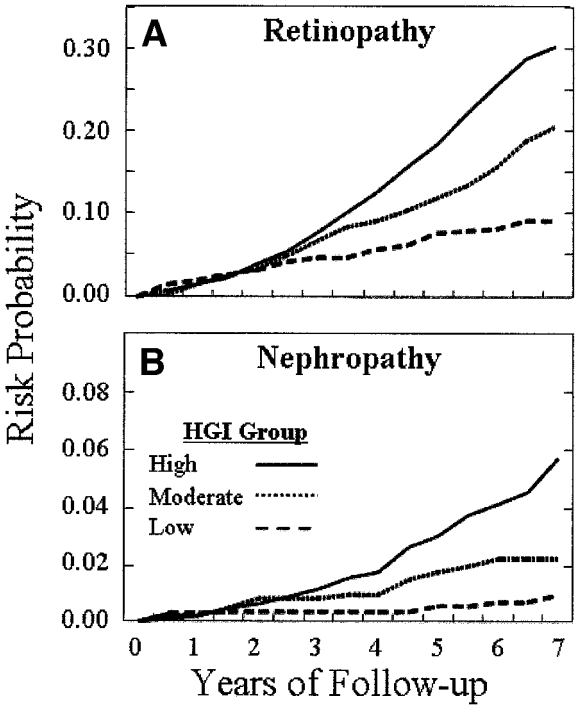

Figure 2—*Biological variation in HbA_{1c} and risk of retinopathy and nephropathy. Cox regression analysis was statistically adjusted for the influence of MBG and used to compare risk of retinopathy and nephropathy in DCCT participants subdivided by mean HGI into low-, moderate-, and high-HGI groups. 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* ($P < 0.0001$).

tions for patients in the high- and low-HGI groups were above or below the population regression line, respectively. Disproportionate distribution of data points around the population regression line in the different HGI groups indicates that much of the variation observed in the relationship between HbA_{1c} and MBG in the DCCT population was related to HGI and was not random.

Biological variation in HbA_{1c} and microvascular complications

Data were available to evaluate the relationship between HGI and risk of retinopathy and nephropathy for up to 7 years. Risk for development or progression of retinopathy with MBG held constant was significantly different ($P < 0.0001$) among patients in the low-, moderate-, and high-HGI groups (Fig. 2*A*). After 7 years, patients in the high-HGI group had three times greater risk of retinopathy (30%) compared with those in the low-HGI group (9%). Risk for development or progression of nephropathy was also significantly different ($P < 0.0001$) in the low-, moderate-, and high-HGI groups (Fig. 2*B*). After 7 years, patients in the high-HGI group had six times greater risk of nephropathy (6%) compared with those in the low-HGI group (1%).

The influence of HGI on risk for development or progression of retinopathy was also evaluated at extremes of blood glucose by subdividing the participants into low-, moderate-, and high-MBG groups. Despite relatively good glucose control based on MBG during the study (low MBG) (Fig. 3*A*), patients within this well-controlled subgroup in the high-HGI group still had a 30% risk for developing retinopathy, whereas patients in the moderate- and low-HGI groups had only a 2–4% risk of retinopathy (significantly lower than in the high-HGI group, $P < 0.006$). A similar influence of HGI on risk of retinopathy was observed in patients in relatively poor glucose control (high MBG) (Fig. 3*B*). Patients in the high-HGI group had a 35% risk of retinopathy, whereas those in the moderate- and low-HGI groups had significantly lower ($P < 0.001$) risk levels of 23 and 7%, respectively.

CONCLUSIONS — This study demonstrates the presence of between-individual biological variation in HbA_{1c} distinct from that attributable to MBG in the DCCT. As measured by HGI, many individuals had observed HbA_{1c} levels that were significantly higher or lower than predicted by the population regression equation. Individual tendency toward higher or lower than predicted HbA_{1c} 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 HbA_{1c} 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 HbA_{1c} among patients in the DCCT is consistent with the findings of a number of other studies that evaluated both diabetic and nondiabetic populations (8,9,11–16,20). Although these studies differed from the DCCT by having fewer participants, shorter periods of observation, and different analytical methodologies, the results collectively indicate that individual factors in addition to MBG play a major role in determining HbA_{1c} levels.

We considered the possibility that between-individual variation in the relationship between HbA_{1c} 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 HbA_{1c} measurements were determined by a central laboratory. Thus any error associated with the analysis of glucose concentrations or HbA_{1c} levels should be random among individuals, and although random error could obscure the presence of between-individual variation in HbA_{1c}, it cannot be the source of it. Only nonrandom measurement bias that produced consistently false differences between observed and predicted HbA_{1c} levels could cause the observed between-individual variation in HGI. Compared with HbA_{1c}, 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-

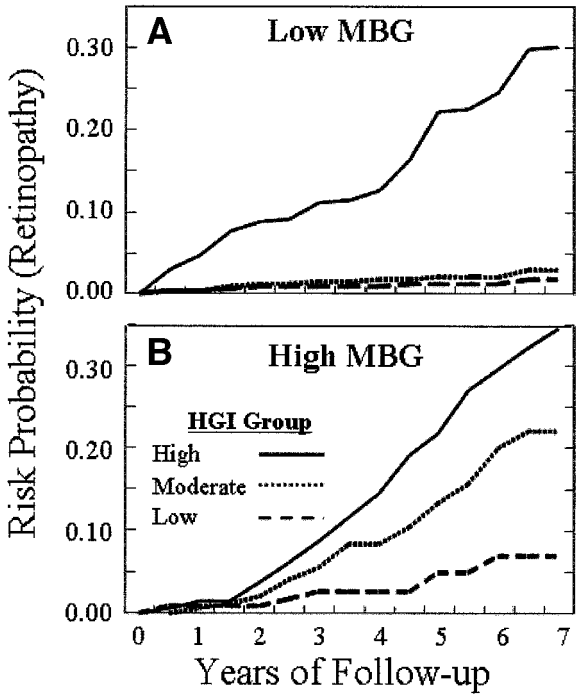

Figure 3—*Biological variation in HbA_{1c} and risk of retinopathy in individuals with good or poor glucose control. Cox regression analysis was used to compare risk of retinopathy in DCCT participants with high or low blood glucose levels after subdivision by mean HGI into low-, moderate-, and high-HGI groups. Despite low glucose levels during the course of the study, patients 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-, moderate-, and high-HGI groups* (B)

der- or overestimation of MBG. For this to explain the results of our study, however, under- 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 absurd. Consistent individual differences in unmeasured glucose values could be caused by consistent differences in glucose fluctuations during the day. However, we evaluated the SDs of the glucose values used to calculate MBG and determined that glycemic variation had negligible influence on HbA_{1c} or HGI. A recent study (26) of 256 diabetic patients also showed that HbA_{1c} levels are not meaningfully affected by glycemic variation. We conclude, therefore, that the between-individual variation in HbA_{1c} observed in the DCCT is attributable to biological variation and not measurement error.

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. Advanced glycation end products are a heterogeneous class of compounds that have been implicated in the pathophysiology of diabetes complications, aging, and Alzheimer's disease (27). Evidence of a link between biological variation in HbA_{1c} and microvascular complications in the DCCT suggests that factors responsible for biological variation in nonenzymatic hemoglobin glycation may also influence individual susceptibility to diabetes complications. Nonenzymatic hemoglobin glycation is a function of intracellular glucose and factors that influence glucose binding to hemoglobin. The latter includes intracellular pH and 2,3-bisphosphoglycerate concentrations and the levels or activities of glycolytic or deglycating enzymes (13–15,28,29). Although HbA_{1c} levels can be affected by erythrocyte age, we previously showed (16) that HGI was not related to erythrocyte turnover rates based on creatine levels.

The important novel finding of this study is that biological variation in HbA_{1c} is an important predictor for the development and progression of diabetes complications. This suggests that there are two important components of risk for the microvascular 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 HbA_{1c}. The existence of these two components of risk suggests that two therapeutic approaches also exist for the prevention of diabetes complications. One is the current approach to diabetes management, where treatment largely depends on pharmacologic 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 retinopathy if they belonged to the high-HGI group (Fig. 3*A*). Consequently, a second therapeutic approach may be needed, one that intervenes in the currently unknown mechanisms that mediate biological variation in HbA_{1c}. Elucidation of these mechanisms could promote the development of novel therapeutic interventions and individually customized management programs. HGI or other indexes of biological variation in HbA_{1c} could thus prove clinically important for identifying high-risk patients and monitoring the efficacy of therapy.

Acknowledgments— This research was supported in part by the Foundation for the Louisiana State University Health Sciences Center, New Orleans, Louisiana (to J.M.H. and S.A.C.), and by a grant from the Research Institute for Children, New Orleans, Louisiana (to J.M.H.).

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