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Editorial

Gaps in the Glycation Gap Hypothesis

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Patients with diabetes mellitus are at risk for developing severe debilitating complications, including retinopathy, nephropathy, neuropathy, myocardial infarction, and stroke. Randomized trials have documented that lowering hemoglobin A_{1c} (Hb A_{1c})[4] concentrations significantly reduces the onset and rate of progression of microvascular complications *(1, 2)*. Moreover, Hb A_{1c}, which is formed by the nonenzymatic attachment of glucose (termed glycation) to hemoglobin and reflects the mean glucose concentration over the preceding 8 to 12 weeks *(3, 4)*, correlates with the risk for developing microvascular and cardiovascular complications in diabetes *(5, 6)*. Hb A_{1c} measurement is widely used to monitor glycemic control and to adjust therapy. More recently, its use has been advocated for diagnosing diabetes *(7)*. Although intraindividual variation in Hb A_{1c} values is minimal in nondiabetic persons *(8)*, considerable interindividual variation has been reported *(9, 10)*.

Evidence that has accumulated over the last few years suggests that race influences Hb A_{1c}. Mexican Americans and blacks have higher mean Hb A_{1c} values than whites *(9, 10)*. Similarly, Hb A_{1c} values increase with age *(11)*. In addition to these factors, some investigators have proposed that interindividual variation is due to differences in the glycation rate *(9, 12)*, a premise that is not accepted by all *(13)*. A large retrospective observational analysis that appears in this issue *(14)* provides additional information to consider.

Cohen et al. suggested that a "glycosylation gap," defined as the difference between the Hb A_{1c} concentration and that predicted by the fructosamine concentration, could explain the excess interindividual variation in Hb A_{1c} *(15)*. They also suggested that the glycosylation gap predicted the progression of nephropathy, although Hb A_{1c} in their small cohort was not itself associated with such progression, in contrast to the compelling evidence from the Diabetes Control and Complications Trial (DCCT) *(1, 5)*. Similarly, McCarter et al. proposed the hemoglobin glycation index (HGI) to explain observations that some individuals have Hb A_{1c} values that are higher or lower than expected from measurements of blood glucose *(12)*. The HGI was calculated as the difference between the measured Hb A_{1c} value and that predicted from the mean blood glucose concentration. In analyzing publicly available data from the DCCT, McCarter et al. found that the HGI, which was computed from infrequent glucose measurements, was a statistically significant predictor of retinopathy and nephropathy and inferred that this finding supported the concept that biological variation in glycation contributes to the risk of microvascular complications *(12)*.

Accurate measurement of mean blood glucose is difficult, and surrogate methods have been used. Fructosamine is the marker most widely used in studies that have supported the glycation gap (GG) concept. In addition to hemoglobin, glucose can attach nonenzymatically to free amino groups of other proteins to form ketoamines. The fructosamine assay measures glycated serum proteins, the most common of which is albumin. The half-life of albumin in the blood is 14 to 20 days, and the fructosamine concentration reflects the mean glucose concentration over 10 to 14 days, a much shorter period than that reflected by the Hb A_{1c} assay.

In a study described in this issue of *Clinical Chemistry*, Rodríguez-Segade et al. *(14)* followed 2314 patients with type 2 diabetes for a mean of 6.5 years. Values for the GG, which was calculated as the measured Hb A_{1c} value minus that predicted from fructosamine, were used to divide the patients into tertiles. The authors observed that the risk of progression of nephropathy in the medium- and high-GG groups was significantly greater than that in the low-GG group. In contrast to the study of Cohen et al. *(15)*, Rodríguez-Segade et al. did observe that higher Hb A_{1c} values were significantly associated with the progression of nephropathy. Furthermore, the GG predicted the progression of nephropathy, even after adjustment for Hb A_{1c}.

The DCCT *(13)*, however, demonstrated statistically that the HGI must be strongly correlated with Hb A_{1c} and not with the mean blood glucose concentration, owing to the fundamental relationship between the correlation of a residual from the regression of *y* on

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[4] Nonstandard abbreviations: Hb A_{1c}, hemoglobin A_{1c}; DCCT, Diabetes Control and Complications Trial; HGI, hemoglobin glycation index; GG, glycation gap.

x with y itself or with x itself. The same applies to the GG, likewise a residual obtained from the regression of Hb A_{1c} (y) on fructosamine (x) *(14)*. The GG is strongly positively associated with Hb A_{1c} [see Fig. 2A in *(14)*] but is not correlated with fructosamine [see Fig. 2B in *(14)*]. The DCCT analysis showed that the HGI did not contribute to the risk of progression of retinopathy or nephropathy after adjustment for the Hb A_{1c} value *(13)*.

In contrast, the results of Rodríguez-Segade et al. *(14)* show that their GG is significantly associated with the risk of progression of nephropathy, even after adjustment for the Hb A_{1c} value. The authors interpret this finding to demonstrate that nonglycemic factors may determine, in part, the Hb A_{1c} value. In fact, however, the models that use the GG in addition to Hb A_{1c} are examining the effect of fructosamine when added to the Hb A_{1c} value, because the GG itself is a linear function of the Hb A_{1c} and fructosamine concentrations, namely the regression residual. Thus, an alternative interpretation is that the fructosamine concentration, which reflects the mean blood glucose concentration in the extracellular space over the previous 10 to 14 days, is independently associated with the risk of nephropathy when added to the Hb A_{1c} value, which reflects the mean blood glucose concentration over the last 8 to 12 weeks.

The analyses of these interrelationships are complex. The DCCT provided systematic annual measurements of albumin excretion within a narrow window with frequent (every 3 months) measurements of Hb A_{1c}, to which a discrete-time Cox proportional hazards model applies. Whether the follow-up by Rodríguez-Segade et al. *(14)* is sufficiently systematic to justify either a continuous-time or a discrete-time proportional hazards model is not clear. Variation in the timing of the nephropathy and the measurements of Hb A_{1c} and fructosamine could have affected the point estimates of the hazard ratios and their significance levels.

It is also curious that the high-, medium-, and low-GG groups differed significantly when also adjusted for both the updated mean Hb A_{1c} and updated mean fructosamine values over time [see Fig. 3 in *(14)*]. Because the GG is defined as the Hb A_{1c} (y) minus a linear function of fructosamine ($a + bx$), with x being the fructosamine concentration, the expected GG value is a constant for given values of Hb A_{1c} and fructosamine. Thus, no difference between the high-, medium-, and low-GG groups would be expected after statistical adjustment for Hb A_{1c} and fructosamine values. The fact that the GG groups remain significant after such adjustment suggests that elements other than GG are at play.

One possibility is that the GG as computed violates one of the assumptions of the Cox proportional hazards model, a basic principle of which is that all covariates, including any time-varying covariates, must reflect only the history of each study participant at each time during follow-up. The updated mean Hb A_{1c} and fructosamine values satisfy this principle, whereas the GG, as computed, does not. The GG is the mean of all residuals over the entire follow-up period for each participant. It is then used to construct groups with high, medium, and low GG values. These groups are then entered into the model as fixed covariates, i.e., as though the GG were measured at baseline. GG was not a baseline measure, however, and this procedure could have led to biased results.

Rodríguez-Segade et al. suggest using the GG, presumably calculated from the results of fructosamine and Hb A_{1c} assays, in the management and risk stratification of persons with diabetes *(14)*. Accordingly, it is worth considering the characteristics of the 2 assays. Automated assays for fructosamine have been commercially available for >25 years, but the initial assay was nonspecific. The improved second-generation assay has values approximately 90% lower than those for the first-generation assay *(16)*. Although Hb A_{1c} is expressed as a fraction of the total hemoglobin concentration, there is disagreement as to whether fructosamine values should be corrected for protein concentrations. Nevertheless, changes in the serum protein concentration or half-life may alter the fraction of protein that is glycated, and it is accepted that fructosamine should not be measured when the serum albumin concentration is <30 g/L. Unfortunately, Rodríguez-Segade et al. *(14)* did not report albumin values for their patients. Another concern is that the fructosamine method was changed during the study. Unlike the Hb A_{1c} assay, the fructosamine assay is not standardized across instruments, nor is the accuracy monitored. Rodríguez-Segade et al. *(14)* initially used a nitroblue tetrazolium assay. Under alkaline conditions, fructosamine reverts to the eneaminol form, which reduces nitro blue tetrazolium to a formazan that can be quantified by measuring the absorbance *(17)*. In 2001, Rodríguez-Segade et al. instituted the GlyPro assay, which uses ketoamine oxidase to oxidize the ketoamine bond to release H_2O_2, which is quantified photometrically *(17)*. The authors used a regression equation derived from their analysis of a subset of samples to convert the values from one assay to the other. The initial characterization of the GlyPro assay, however, showed that the data from male and female individuals were distributed differently (with different reference intervals) and that separate, sex-specific regression equations were needed to convert results obtained with the nitro blue tetrazolium assay to values consistent with

the other assay *(18)*. Rodríguez-Segade et al. did not do this.

Despite rigorous standardization, the measurement of Hb A_{1c} is subject to some limitations as well. Any change in the erythrocyte life span will alter Hb A_{1c} concentrations. Moreover, hemoglobin variants may interfere with some Hb A_{1c} assays *(19)*. The interferences are usually method specific, however, and Hb A_{1c} can be measured accurately in the vast majority of individuals, if an appropriate method is used.

Several articles have proposed the concept of variable glycation; however, the data demonstrating clearly different rates of glycation between individuals have been minimal. On the other hand, high-quality epidemiologic studies and clinical trials have documented Hb A_{1c} to be the strongest predictor of the risk of developing microvascular complications. Given the ambiguities in the computation and interpretation of the GG, we encourage clinicians to continue to use Hb A_{1c} in managing patients with diabetes.

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