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# Two-dimensional analysis of glycated hemoglobin heterogeneity in pediatric type 1 diabetes patients

James M. Hempe [a,b,*], Amanda M. McGehee [a], Stuart A. Chalew [a,b]

[a] Research Institute for Children, Children's Hospital, New Orleans, LA 70118, USA
[b] Department of Pediatrics, Louisiana State University Health Sciences Center, New Orleans, LA 70118, USA

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ABSTRACT

Interindividual and ethnic variation in glycated hemoglobin levels, unrelated to blood glucose variation, complicates the clinical use of glycated hemoglobin assays for the diagnosis and management of diabetes. Assessing the types and amounts of glycated hemoglobins present in erythrocytes could provide insight into the mechanism. Blood samples and self-monitored mean blood glucose (MBG) levels were obtained from 85 pediatric type 1 diabetes patients. Glycated hemoglobin levels were measured using three primary assays (boronate-affinity chromatography, capillary isoelectric focusing (CIEF), and standardized DCA2000+ immunoassay) and a two-dimensional (2D) analytical system consisting of boronate-affinity chromatography followed by CIEF. The 2D system separated hemoglobin into five subfractions, four of which contained glycated hemoglobins. Glycated hemoglobin measurements were compared in patients with low, moderate, or high hemoglobin glycation index (HGI), a measure of glycated hemoglobin controlled for blood glucose variation. MBG was not significantly different between HGI groups. Glycated hemoglobin levels measured by all three primary assays and in all four glycated 2D subfractions were significantly different between HGI groups and highest in high HGI patients. These results show that interindividual variation in glycated hemoglobin levels was evident in diabetes patients with similar blood glucose levels regardless of which glycated hemoglobins were measured.



Hemoglobin is a tetrameric red blood cell (RBC)[1] protein consisting of two pairs of α,β globin heterodimers [1,2]. High blood glucose concentrations promote the nonenzymatic posttranslational chemical modification of specific globin amino groups via the Maillard reaction. These spontaneous reactions occur at rates determined by glucose concentration and amino group p$K_a$. Glycation proceeds through a series of intramolecular rearrangements and fragmentations that produce an assortment of early, intermediate, and late glycation products. Glycated hemoglobin is a general term used to describe the heterogeneous set of hemoglobin complexes derived by the Maillard reaction.

Glycated hemoglobin assays can be divided into three general types based on their mode of separation [3]. Boronate affinity chromatography divides hemoglobin molecules into boronate-reactive and boronate-unreactive fractions based on the presence or absence, respectively, of chemical modifiers that contain accessible coplanar vicinal diol groups (two hydroxyl groups on adjacent carbon atoms) [4,5]. In contrast, modern immunoassays use monoclonal antibodies developed against synthetic peptides that mimic the glycated N-terminus of β globin [3,6]. Charge-based assays, such as cation-exchange chromatography and isoelectric focusing, can separate hemoglobin molecules into multiple fractions based on differences in surface charge and isoelectric point (p*I*) [7]. In this report, the abbreviation A1c is used as originally intended [8], to identify a specific low p*I* hemoglobin fraction isolated by charge-based separation techniques.

Glycated hemoglobin assays are used clinically to indirectly monitor blood glucose levels in diabetes patients [9]. Because different glycated hemoglobin assays measure different subsets of chemically modified hemoglobins, they also give different results for the same blood sample. Widespread interassay variation prompted the development of national and international standardization programs to "harmonize" assays used by hospital laboratories and clinics [10,11]. In this report, the abbreviation HbA1c is used to specifically refer to results obtained using standardized glycated hemoglobin assays.

Glycated hemoglobin assay standardization has unquestionably improved the care and outcomes of diabetes patients. New concerns have emerged, however, about the way HbA1c assay results are used to diagnose and manage diabetes [12–15]. At issue is the entrenched practice of using HbA1c as an unbiased estimate of blood glucose concentration. The clinical use of HbA1c first arose

* Corresponding author at: Research Institute for Children, Children's Hospital, New Orleans, LA 70118, USA. Fax: +1 504 896 9413.
*E-mail address:* jhempe@chnola-research.org (J.M. Hempe).

[1] *Abbreviations used:* RBC, red blood cell; HGI, hemoglobin glycation index; PBS, phosphate-buffered saline; CIEF, capillary isoelectric focusing; MBG, mean blood glucose; CBG, capillary blood glucose; GHb, glycated hemoglobin; pI isoelectric point.

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from the need to monitor the effects of lifestyle and pharmacologic interventions that lower blood glucose levels. As blood glucose levels are highly dynamic, direct measurement of average blood glucose over long periods of time was technologically infeasible prior to the advent of personal glucose meters. Early evidence that glycated hemoglobin levels were positively correlated with average blood glucose [16,17] quickly led to the now widespread use of HbA1c as a surrogate for directly measured average blood glucose concentration.

More recently, however, a number of studies have shown that HbA1c and blood glucose concentrations are not quantitatively related the same way in all individuals and ethnic groups in both diabetic and nondiabetic populations [14,15,18–21]. For still unknown reasons, some individuals have consistently lower or higher than average HbA1c levels due to factors other than blood glucose concentration. This biochemical trait appears to be hereditary [22,23] and significantly complicates the recommended uses of HbA1c [24] for the diagnosis and management of diabetes in multiethnic populations.

We developed the hemoglobin glycation index (HGI) to assess and study variation in HbA1c controlled for blood glucose concentration in human populations [18]. HGI is the calculated difference between a subject's observed (measured) HbA1c and a predicted HbA1c based on his or her measured blood glucose concentration. Individuals with low or high HGI have lower or higher than predicted HbA1c, respectively. Using data from the Diabetes Control and Complications Trial we showed that type 1 diabetes patients with high HGI had threefold greater risk for development or progression of retinopathy and sixfold greater risk for nephropathy [25]. HGI is normally distributed in human populations, significantly different between individuals and ethnic groups, and quantitatively consistent within individuals over time and over a wide range of blood glucose concentrations [18,19,26,27]. HGI is also positively correlated with the glycation gap [28], another index of biological variation in HbA1c that is similarly associated with risk for diabetes complications [29–31].

The mechanism responsible for population variation in HbA1c independent of variation in blood glucose concentration remains unclear. RBC life span, glucose transport, pH, phosphate levels, antioxidant status, and other factors have been associated with high glycated hemoglobin levels or have been implicated as agents of variation in hemoglobin glycation kinetics [7,32]. Nuttall [33] suggested that population variation in A1c analytical fractions might be associated with variation in the types or proportions of hemoglobin complexes present in RBCs. This hypothesis was premised on his earlier finding that A1c fractions isolated using Bio-Rex 70 cation-exchange chromatography were heterogeneous and contained nearly equal amounts of boronate-reactive and boronate-unreactive hemoglobin complexes [5]. Information about the types and amounts of the various hemoglobin complexes and how they relate to blood glucose concentration in diabetes patients could help identify mechanisms responsible for the quantitative biochemical trait measured by HGI. To test this hypothesis, we first developed a two-dimensional (2D) analytical system that separates hemoglobin molecules based on both chemical affinity and charge properties. This 2D system and three different primary glycated hemoglobin assays were then used to determine if the set of hemoglobin complexes inside RBCs is quantitatively or qualitatively different in individuals with low, moderate, or high HGI.

## Materials and methods

### *Subjects and blood samples*

Children and adolescents ($n = 85$) with type 1 diabetes were recruited with informed consent at Children's Hospital of New Orleans. Blood samples were collected using EDTA as the anticoagulant. Samples were centrifuged (1500*g*, 5 min, ~25 °C) to isolate RBCs, which were then washed twice in 10 volumes of phosphate-buffered saline (PBS; 137 mmol/L NaCl, 2.7 mmol/L KCl, 10 mmol/L $Na_2HPO_4{\cdot}7H_2O$, 1.8 mmol/L $KH_2PO_4$, pH 7.4). Glucose-depleted RBCs were prepared by incubating 100 μl of washed RBCs in 1 ml of PBS for 6 h at 37 °C. Patients with abnormal hemoglobin variants determined by CIEF were omitted from the study. This project was approved by the Institutional Review Board at the Louisiana State University Health Sciences Center (New Orleans, LA, USA).

### *Blood glucose*

Blood glucose data were downloaded from patient meters at diabetes clinic visits as previously described [26]. Mean blood glucose (MBG) was calculated using all blood glucose measurements recorded during the previous 60 days. A random capillary blood glucose (CBG) measurement was also obtained at each clinic visit (Accu-Chek Inform, Model 2001201, Roche Diagnostics, Basel, Switzerland).

### *HbA1c immunoassay*

HbA1c was measured as part of routine patient care by Children's Hospital Laboratory using a DCA2000+ Analyzer (Bayer, Inc., Tarrytown, NY, USA). Results are reported in National Glycohemoglobin Standardization Program (NGSP) equivalents [34].

### *Boronate-affinity chromatography*

Hemoglobin molecules were separated into boronate-reactive and boronate-unreactive affinity fractions using Glyco-Tek affinity columns and proprietary kit reagents as described by the manufacturer (Helena Laboratories, Beaumont, TX, USA). Hemolysates (1:8) were prepared by adding 25 μl of glucose-depleted RBCs to 200 μl of Glyco-Tek hemolyzing reagent. Chromatography was performed using 50 μl of hemolysate with gravity elution at room temperature. Quantification of hemoglobin in the boronate-reactive affinity fraction was based on absorbance at 415 nm (relatively specific for heme compounds) measured using a DU 800 spectrophotometer (Beckman Coulter, Fullerton, CA, USA). Glycated hemoglobin levels measured by boronate-affinity chromatography (GHb) are reported in nonstandardized units as a percentage of total hemoglobin. The between-run coefficient of variation for a control sample containing 11.3% GHb was 7.1% in 20 runs performed over a 2-year period that included these experiments.

### *Capillary isoelectric focusing*

Hemoglobin in glucose-depleted RBCs was separated by CIEF with pressure mobilization as previously described [35,36] with minor modifications. Hemolysates (1:20) were prepared by adding 5 μl of glucose-depleted RBCs to 100 μl of hemolyzing reagent (10 mmol/L KCN; 5 mmol/L EDTA). Hemolysates were stored at −70 °C prior to analysis. The CIEF system consisted of an internally coated 50-μm-i.d. × 30-cm-long (~20 cm to detector) DB-1 capillary (Agilent Technologies, Santa Clara, CA, USA) and an MDQ capillary electrophoresis instrument with UV detector (Beckman Coulter). Ampholyte solution was 2% (v/v) Pharmalyte pH 6.7 to 7.7 in methylcellulose solution (0.375% w/v in deionized water). Cathode solution was 80 mmol/L borate adjusted to pH 10.25 with sodium hydroxide. Anode solution was 100 mmol/L phosphoric acid in methylcellulose solution. The capillary was conditioned before each analytical run for 5 min at 20 psi with NaOH (100 mmol/L) and then 5 min with 50% methanol in deionized water. Between