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Revaluation of biological variation of glycated hemoglobin (HbA_{1c}) using an accurately designed protocol and an assay traceable to the IFCC reference system

Federica Braga [a,b,*], Alberto Dolci [b], Martina Montagnana [c], Franca Pagani [d], Renata Paleari [a], Gian Cesare Guidi [c], Andrea Mosca [a], Mauro Panteghini [a,b]

[a] Centre for Metrological Traceability in Laboratory Medicine (CIRME), University of Milano, Milano, Italy
[b] Clinical Biochemistry Laboratory, 'Luigi Sacco' University Hospital, Milano, Italy
[c] Clinical Biochemistry Laboratory, Verona University Hospital, Verona, Italy
[d] Laboratory Medicine Unit, Poliambulanza Foundation, Brescia, Italy

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ABSTRACT

Background: Glycated hemoglobin (HbA_{1c}) has a key role for diagnosing diabetes and monitoring glycemic state. As recently reviewed, available data on HbA_{1c} biological variation show marked heterogeneity. Here we experimentally revaluated these data using a well designed protocol.
Methods: We took five EDTA whole blood specimens from 18 apparently healthy subjects on the same day, every two weeks for two months. Samples were stored at −80 °C until analysis and assayed in duplicate in a single run by Roche Tina-quant® Gen.2 immunoassay. Data were analyzed by the ANOVA. To assess the assay traceability to the IFCC reference method, we preliminarily carried out a correlation experiment.
Results: The bias (mean ± SD) of the Roche immunoassay was 0.3% ± 0.7%, confirming the traceability of the employed assay. No difference was found in HbA_{1c} values between men and women. Within- and between-subject CV were 2.5% and 7.1%, respectively. Derived desirable analytical goals for imprecision, bias, and total error resulted 1.3%, 1.9%, and 3.9%, respectively. HbA_{1c} had marked individuality, limiting the use of population-based reference limits for test interpretation. The estimated critical difference was ~10%.
Conclusions: For the first time we defined biological variation and derived indices for the clinical application of HbA_{1c} measurements using an accurately designed protocol and an assay standardized according to the IFCC.

1. Introduction

Glycated hemoglobin (HbA_{1c}) is the recommended biomarker for the assessment of glycemic control and prediction of vascular complications in diabetic patients. As the amount of HbA_{1c} is strictly related to blood glucose concentrations, the American Diabetes Association (ADA) has recently recommended the use of HbA_{1c} also for diagnosing diabetes mellitus [1].

There is now an international agreement in defining the HbA_{1c} measurand as "all hemoglobin molecules having a special hexapeptide in common, which is the stable adduct of glucose to the amino-terminal valine of the hemoglobin β-chain (βN-1-deoxyfructosyl-hemoglobin)" [2]. Starting from the measurand definition, the Working Group on Standardization of HbA_{1c} (WG-HbA_{1c}) established by IFCC has developed a reference measurement system, based on the concept of metrological traceability that, if correctly applied, may assure the standardization of HbA_{1c} measurements and result comparability [3]. Once the traceability of commercial HbA_{1c} assays to the reference system is obtained, the establishment of analytical goals that should be fulfilled to make HbA_{1c} testing clinically reliable becomes crucial [4,5]. To this aim, the widely accepted strategy is to derive the desirable analytical performance from the biological variation of the analyte. Thus, robust data on biological variation represent key information. In a recent systematic review, we however demonstrated that available studies designed to experimentally derive data on biological variation of HbA_{1c} show limitations on type of selected subjects, sampling frequency, sample storage, analytical specificity of the employed assay, and in statistical derivation of results [6].

Biological variation is composed by within-subject variation (CV_I), reflecting changes occurring in the same individual over time, and

Abbreviations: HbA_{1c}, glycated hemoglobin; ADA, American Diabetes Association; WG-HbA_{1c}, Working Group on Standardization of HbA_{1c}; IFCC, International Federation of Clinical Chemistry and Laboratory Medicine; CV_I, intra-individual biological variation; CV_G, inter-individual biological variation; CV_A, analytical variation; CIRME, Centre for Metrological Traceability in Laboratory Medicine; SIT, Servizio di Taratura in Italia; JCTLM, Joint Committee for Traceability in Laboratory Medicine; II, index of individuality; CD, critical difference; n, number of specimens required to ensure that the mean result is within ±5% of the individual's homeostatic set point; IH, index of heterogeneity; SD_T^2, total variance of all measurements; SD_S^2, average within-subject total variance; SD_A^2, average within-run analytical variance; SD_I^2, average within-subject biological variance; SD_G^2, between-subject biological variance.

* Corresponding author at: Laboratorio Analisi Chimico-Cliniche, Azienda Ospedaliera 'Luigi Sacco', Via GB Grassi 74, 20157 Milano, Italy. Fax: +39 02 503 19835.
E-mail address: federica.braga@studenti.unimi.it (F. Braga).

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between-subject variation (CV_G), representing the differences between individuals. These two biological variation components, along with the analytical variation (CV_A), compose the total variance of a set of data made up of serial results from a group of subjects [7]. To derive reliable data on biological variation, in addition to use an assay which is traceable to the reference measurement system (in order to assure similar analytical specificity), an accurately designed experimental protocol is needed. In agreement with the approach recommended by Fraser and Harris [8], evaluated subjects should be ostensibly healthy to ensure that any fluctuation of the analyte is not caused by superimposed pathological variations and under controlled conditions that remove pre-analytical sources of variation. Biological samples should be taken at set time intervals, immediately processed and stored frozen. Only when all specimens are available, analysis should be performed, in a single run in duplicate using a traceable and specific assay. This permits to avoid between-run analytical variation and to directly estimate within-run variation from the results of duplicates.

As the current lack of reliable data on biological variation represents a major limitation to the effective application of HbA_{1c} measurement in laboratory medicine, we planned a study to derive ex-novo all biological variation parameters of HbA_{1c} by adopting an accurately designed experimental protocol and performing HbA_{1c} measurements with an assay fulfilling traceability to the IFCC reference measurement system.

2. Materials and methods

2.1. Subjects and specimens

We collected five EDTA whole blood specimens from each of 18 apparently healthy volunteers (9 men and 9 women, ages 26–52 years), who were enrolled among the laboratory staff (all Caucasian subjects) and gave written consent to be tested. The inclusion criteria were that the subjects had no familiarity for diabetes, thalassemia syndrome and other hemoglobinopathies and, for women, have regular menstrual cycles and do not use hormonal contraceptives. None of the subjects took any medication or was smoker; furthermore, fasting plasma glucose and body mass index (BMI) were <6.0 mmol/L and <30.0 kg/m^2, respectively, in all subjects.

Samples were obtained on the same day, every two weeks for two months (October to December). After overnight fasting and without any morning exercises, venous blood was collected between 09.00 and 09.30 a.m. in the sitting position for 1–5 min with minimal stasis by the same skilled phlebotomist, using a 20 G straight needle directly into 3 mL-siliconized vacuum tubes containing K_2 EDTA (Terumo Europe NV, Leuven, Belgium, cat. no. VF-053SDK). Blood specimens were immediately aliquoted and stored at −80 °C until analyzed. When all the specimens were available, they were thawed and assayed.

2.2. Analytical procedure and measurement protocol

HbA_{1c} was measured on the Integra 400 automated system (Roche Diagnostics, Mannheim, Germany) by a dedicated homogenous competitive immunoassay (Tina-quant® HbA_{1c} Gen.2). The method is based on a two steps approach: in a first step, the total hemoglobin is determined by a colorimetric method and in a second step HbA_{1c} is immunoturbidimetrically quantified. HbA_{1c} value is calculated as HbA_{1c} to total hemoglobin ratio [9]. To determine HbA_{1c}, hemoglobin obtained from hemolysis is cleaved proteolytically by pepsin in order to make the amino-terminal group of its β-chain more accessible to immunological reaction. A monoclonal antibody of the assay, conjugated to latex particles, specifically recognizes glycated amino-terminal groups. Once the reaction is completed, antibodies in excess are agglutinated by a polymer carrying synthetic peptides mimicking the amino-terminal group of the β-chain. The solution turbidity, measured at 552 nm, is in inverse proportion to the amount of natural glycopeptides bound to antibody. The Roche Diagnostics Traceability and Uncertainty document (March 2008) provides a statement that the assay calibrator (C.f.a.s. HbA_{1c}) is traceable to the IFCC reference measurement procedure with an expanded uncertainty of 0.00633 g/L at HbA_{1c} concentration of 0.268 g/L (2.36%).

All analyses were performed in a single run in duplicate, using the analytical system in accordance with the manufacturer's instructions and checking its alignment before the run by PreciControl HbA_{1c} materials (norm and path) according to the manufacturer's established validation range.

2.3. Verification of traceability of laboratory assay

To verify the traceability of results obtained with the employed HbA_{1c} assay, we carried out an "ad-hoc" experiment. Four EDTA whole blood samples with HbA_{1c} concentrations ranging 20 to 42 mmol/mol (the expected range in healthy persons) and urea concentrations <8.5 mmol/L were analyzed in duplicate with both immunoassay and IFCC reference measurement procedure [10], the latter performed in the reference laboratory of the Centre for Metrological Traceability in Laboratory Medicine (CIRME), University of Milano, accredited as calibration laboratory by the National Calibration Service (SIT) (accreditation no. 217) and listed in the Joint Committee on Traceability in Laboratory Medicine (JCTLM) database [11]. Plasma urea concentrations were measured to exclude the presence of carbamylated hemoglobin, a potential interferent of the reference method [10]. Whole blood samples were also checked to exclude hemoglobin variants by dedicated HPLC technique.

The correlation of Roche Integra immunoassay system for the HbA_{1c} to the IFCC reference procedure was evaluated by Passing and Bablok regression analysis and its alignment by estimating the average percentage bias. We postulated that if assay results were not perfectly aligned with the expected values assigned by the reference laboratory, the obtained results could have been used to recalibrate the comparative system and assure the optimal traceability to the IFCC reference procedure.

2.4. Statistical analysis

After testing observations for outliers by Cochran's test [8], the components of variation were calculated by nested ANOVA from replicate analyses. Formulae used to estimate these variables are listed in Table 1. SD_A^2 was estimated from the duplicate results of every specimen, SD_I^2 from the total within-subject variance (referred to as the variance of the mean of duplicate assays) minus one-half the

Table 1
Symbols and formulae for the variance components and other statistics used in this study.

SD_T^2	Total variance of all measurements ($SD_A^2 + SD_I^2 + SD_G^2$)
SD_S^2	Average within-subject total variance
SD_A^2	Average within-run analytical variance
SD_I^2	Average within-subject biological variance ($SD_S^2 - 1/2SD_A^2$)
SD_G^2	Between-subject biological variance ($[(2kr-1)/2\,k(r-1)]\,\{SD_T^2 - SD_A^2 - [(2kr-2)/(2kr-1)]\,SD_I^2\}$, where k is the number of specimens per subject and r is the number of subjects)
CV_S	Within-subject total coefficient of variation
CV_A	Analytical coefficient of variation
CV_I	Within-subject biological coefficient of variation
CV_G	Between-subject biological coefficient of variation
II	Index of individuality (CV_I/CV_G)
IH	Index of heterogeneity ($CV_S/[(2/k-1)^{1/2}\times 100]$, where k is the number of specimens per subject)
CD	Critical difference ($2.77\ (CV_A^2 + CV_I^2)^{1/2}$)
n	Number of specimens required to ensure that the mean result is within ±5% of the individual's homeostatic set point [$1.96^2\ (CV_A^2 + CV_I^2)/25$]

analytical variance, and SD_G^2 from the total variance of data minus the analytical and intra-individual components. All the components of variance were then transformed to the relevant CV using the overall means. Student's unpaired *t*-test was used to compare the mean HbA_{1c} values and the F-test was applied to assess the difference in intra-individual variances from men and women. The index of individuality (II) yielding information about the utility of conventional population-based reference intervals [12]; the critical difference (CD) (also reported as 'reference change value'), that is, the minimal significant difference ($p \leq 0.05$) between two consecutive measurements of a quantity in the same patient [13]; and the number of specimens (n) that should be collected to estimate the homeostatic set point of an individual [14] were estimated. To study the heterogeneity of within-subject variation, we estimated the index of heterogeneity (IH) that is the ratio of the observed CV of the set of individual variances (including analytical variance) (SD_S^2) to the theoretical CV, which is $[2/(k-1)]^{1/2}$ where k is the number of specimens collected per subject. The SD of the difference between this ratio and its expected value of unity (under the hypothesis of no heterogeneity of true within-subject variances) is $1/(2k)^{1/2}$. A significant heterogeneity is present if the ratio differs from unity by at least twice this SD [8]. In our study, with five data for each subject, an IH <0.632 indicates that the within-subject data are homogeneous. Finally, optimal, desirable and minimum analytical goals for imprecision, bias and total error for HbA_{1c} determination were obtained from biological variability components according to Fraser et al. [15].

3. Results

3.1. Analytical specificity and traceability of the immunoassay

When compared with the IFCC reference procedure, the bias (mean ± SD) of the Roche Integra immunoassay system was 0.3% ± 0.7% (regression equation: Roche = 1.05 IFCC − 1.63 mmol/mol HbA_{1c}, with a correlation coefficient of 0.998). Thus, considering the currently suggested analytical goals for allowable bias (desirable ≤±1.5% and optimal ≤±0.8%) [16], the employed immunoassay fell well within the optimal performance. As the overlap between the commercial system and reference procedure values was complete, there was no need of any data correction.

3.2. Biological variation and derived indices

The study involved collection of 90 specimens, each assayed in duplicate yielding 180 analytical results. No observations were removed as statistical outliers (Cochran's statistic value, 0.071; $p > 0.05$). Fig. 1 shows the individual mean and absolute range of values for HbA_{1c}, whereas Table 2 gives the overall mean value and the estimated average analytical and biological variation (as CV) for all subjects, and separately for men and women. II, CD and n are also reported.

As expected, there was no difference in HbA_{1c} mean values between men and women ($p = 0.74$). Although the intra-individual variation tended to be lower in men, the difference between genders was not significant ($p = 0.09$). As CD is generally applicable only if all individuals have the same within-subject variation, we evaluated the possible heterogeneity of intra-individual variation of HbA_{1c}. The calculated IH (0.958) did not fulfill the homogeneity condition and, although the CD documented in Table 2 may be used as a simple single figure to guide clinical decision making, it is not ubiquitously valid.

3.3. Analytical goals

Minimum, desirable, and optimal analytical goals for imprecision, bias, and total error for HbA_{1c} determination, derived from our biological variation data, are shown in Table 3.

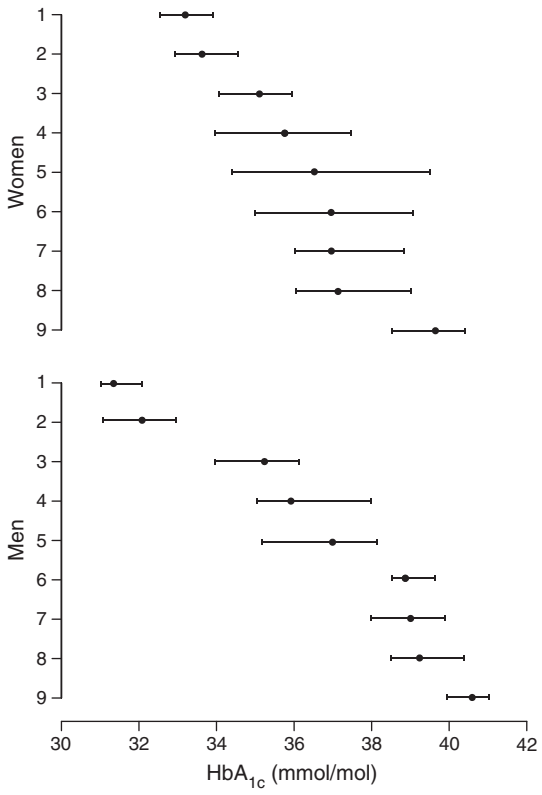

Fig. 1. Individual parametric mean and absolute range of HbA_{1c} values in studied subjects.

4. Discussion

In a recent paper [6], we systematically reviewed the available literature on HbA_{1c} biological variation, concluding that there is still a lack of robust data. Consequently, we claimed the urgent need of a more accurately designed study in order to produce definitive information useful for deriving allowable analytical goals for HbA_{1c} determination. Particularly, there is a need for studies that rigorously comply in format of the model identified by Fraser and Harris [8] and also use analytical techniques that can be traced back to the reference measurement system for HbA_{1c}. Here we present the results of such a study, designed and carried out strictly following the mentioned recommendations.

One of the major limitations most frequently found in previous studies on HbA_{1c} biological variation was the recruitment of diabetic patients [17–20]. The presence of disease, mainly if unstable and not well controlled, may significantly amplify the fluctuation of HbA_{1c} concentrations in blood around the set point, markedly modifying both components (intra- and inter-individual) of biological variation [6]. In addition to the enrolment of healthy nondiseased people, our study was also designed by paying special attention to the overall duration, considering that biological variability estimate is strictly dependent on this factor. As the variability study should not be related

Table 2
Mean values, estimated average variance components and indices derived from data on biological variation of HbA_{1c}.

Group	HbA_{1c}, mmol/mol	CV_A, %	CV_I, %	CV_G, %	II	CD, %	*n*
All	36.3	2.4	2.5	7.1	0.35	9.5	2
Men	36.5		1.9	8.9	0.21		
Women	36.1		3.2	5.1	0.62		

CV_A, CV_I, CV_G, II, CD and n as explained in Table 1.

Table 3
Analytical goals for HbA_{1c} measurement derived from data on biological variation.

Quality level	Imprecision, %	Bias, %	Total error, %
Optimal	≤0.6	≤±0.9	≤±2.0
Desirable	≤1.3	≤±1.9	≤±3.9
Minimal	≤1.9	≤±2.8	≤±5.9

to time periods different from those used in clinical practice for the measurements of HbA_{1c}, we collected blood specimens in a two-month period, a time span corresponding to the advised frequency of the assay in unstable diabetes monitoring [21] and consistent with the previous period of average blood glucose showing an established relationship with the current HbA_{1c} value [22].

From the analytical point of view, the determination of HbA_{1c} was carried out by an immunoassay providing the same specificity for the analyte as defined by the IFCC and an assay calibrator traceable to the IFCC reference measurement system for HbA_{1c} with a stated uncertainty. This is a relevant feature of our study, looking for the first time at the method specificity as per HbA_{1c} measurand definition and at the result traceability. None of the previous studies on HbA_{1c} biological variation experimentally validated the traceability of the employed assay. In fact, Trapé et al. used the 1st generation of the competitive immunoturbidimetric assay adopted in this study, but without verifying its traceability to the HbA_{1c} reference measurement system, not available yet at the time of the study [20]. Other studies assayed a different measurand, like total glycated hemoglobins, also including hemoglobins glycated on other sites differing from amino-terminal valine of the β-chain [17,18,23,24]. If assays are measuring different constituents, the biological variation might not be the same and changes in derived parameters can be expected. To make as accurate as possible the study design, we wanted to experimentally confirm the traceability of the assay results by estimating the bias between the employed immunoassay and the IFCC reference procedure performed in our accredited reference laboratory through an "ad-hoc" protocol. In terms of method alignment, the immunoassay showed a negligible bias, making the derived data on HbA_{1c} biological variability highly reliable.

Apart from the employed assay, the experimental protocol was designed for further control analytical features impacting on reliability of results. To estimate the analytical variance, all the determinations were performed in duplicate on the same analytical run, limiting to within-run variability the influence of the analytical variance on total variance of results, with less chance of error in the estimate of biological variability obtained by subtraction. Nevertheless, the HbA_{1c} measurement with the Roche Tina-quant® showed an SD_A^2 accounting for almost one third of total variance in a subject. As a consequence, also under optimal conditions of analytical variance, our mean CV_A (2.4%) was higher than even the minimum goal for imprecision (≤1.9%) reported in Table 3, showing that, from this point of view, the reliability of the analytical system should be improved.

The mean CV_I for HbA_{1c} obtained in our group of subjects (2.5%) is lower than that (3.4%) reported by Ricòs et al. [16], while the CV_G (7.1%) is slightly higher when compared to the listed one (5.1%). This results in more stringent goals for imprecision and total error and relatively larger goals for bias. Values of biological variability components for HbA_{1c} listed by Ricòs et al. are apparently obtained from the mean of results available in the literature, taken as valid by the authors of the compilation. However, we previously showed the fallacy of determining a single value of CV_I and CV_G simply using the mean of available results [6].

The II provides information about the utility of using reference intervals for test interpretation [12]. Particularly, if the II is <0.6 the use of population-based reference intervals is of very limited value in the detection of unusual results for a particular individual and may be misleading [12]. For HbA_{1c} the II was between 0.2 (men) and 0.6 (women), confirming that classical reference intervals have little use in the interpretation of test results in both sexes. This justifies the adoption of diagnostic cutoff points independent of value distribution in the reference population and based upon relative risk of vascular complication, as recently recommended by the ADA [1].

As the major clinical application of HbA_{1c} determination is for long-term assessment of glycemic state in diabetic patients, the knowledge of CD is of pivotal importance for the critical evaluation of the significance of changes in results obtained from analysis of serial samples in the same individual. From our data, an average CD of ~10% can be assumed as a figure to guide clinical decision making, even if, given the heterogeneity of within-subject variation of HbA_{1c}, this information cannot be extrapolated to all patients. Furthermore, it is obvious that CD shown in Table 2 cannot be used by other laboratories working with different analytical imprecision.

From the data obtained in this study, it is also possible to estimate the number of specimens (n) required to determine the individual's homeostatic set point using the rearrangement of the usual standard error of the mean formula given in Table 1. Particularly, two samples are required to estimate an individual's HbA_{1c} value to within 5% of the true mean value. The importance of this concept is evident in the use of HbA_{1c} in the diagnosis of diabetes where the clinical decisions should be based upon comparison of an analytical result with strict numerical criteria, that is, the established decision limit [48 mmol/mol (6.5%)], and has been implemented with relation to HbA_{1c} by ADA that correctly recommended the use of two test results as a guide to decision [1]. Moreover, these data should be helpful in deciding which are the most appropriate analyses to screen the population for diabetes. Indeed, the measurement of a relatively constant marker in an individual, such as HbA_{1c}, tends to be a more accurate estimate of its true mean value in that subject than the measurement of a more variable factor, such as plasma glucose. The CV_I of plasma glucose is actually much higher (5.7%) [16] and, consequently, a higher number of samples (n = 5) would be required to establish the individual's homeostatic set point.

In conclusion, in this study we defined for the first time biological variation components of HbA_{1c} and derived indices for the correct clinical application of the test using an accurately designed protocol and an assay traceable to the IFCC reference measurement system. This has permitted to obtain reliable calculation of analytical goals for imprecision, bias and total error that can now be safely utilized in the performance evaluation of routine HbA_{1c} assays [25].

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