Invited critical review

Biological variability of glycated hemoglobin

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\textbf{Abstract}

\textbf{Background:} The measurement of glycated hemoglobin (HbA1c) has a pivotal role in monitoring glycemic state in diabetic patients. Furthermore, the American Diabetes Association has recently recommended the use of HbA1c for diabetes diagnosis, but a clear definition of the clinically allowable measurement error is still lacking. Information on biological variability of the analyte can be used to achieve this goal.

\textbf{Methods:} We systematically reviewed the published studies on the biological variation of HbA1c to check consistency of available data in order to accurately define analytical goals.

\textbf{Results:} The nine recruited studies were limited by choice of analytic methodology, population selection, protocol application and statistical analyses.

\textbf{Conclusions:} There is an urgent need to determine biological variability of HbA1c using a specific and traceable assay, appropriate protocol and appropriate statistical evaluation of data.

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\textbf{1. Introduction}

Glycated hemoglobin or \(\beta\text{-N-1-deoxyfructosyl-hemoglobin} \) (HbA\(_{1c}\))\textsuperscript{1} is the product of a nonenzymatic reaction of glycation, namely a condensation between the aldeidic group of glucose and the amino group of the terminal valine in the \(\beta\)-chain of hemoglobin A\(_{0}\). The amount of HbA\(_{1c}\) is strictly related to blood glucose concentration.

Considering the average life span of red cells, the HbA\(_{1c}\) value should mimic the mean glycemic value of the previous 2–3 months. The American Diabetes Association (ADA) recommends HbA\(_{1c}\) determination in patients with diabetes mellitus on therapy in order to monitor the glyco-metabolic status in the medium–long term and thus reduce the risk of vascular complications \cite{1}. Studies performed on non-diabetic subjects showed that even slight elevations of HbA\(_{1c}\) concentration in blood correlated with an increased cardiovascular risk \cite{2,3}. The “Standards of medical care in diabetes” recently published by ADA, recommends the use of HbA\(_{1c}\) as a diagnostic criteria for diabetes, a finding which may further increase the clinical relevance of HbA\(_{1c}\) testing \cite{1}.

Many analytical methods are available for HbA\(_{1c}\) determination \cite{4}. Techniques based on the difference in isoelectric point of HbA\(_{1c}\) and other hemoglobins, and immunochemical methods specifically

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\textsuperscript{1} Nonstandard abbreviations: HbA\(_{0}\), glycated hemoglobin; ADA, American Diabetes Association; NGSP, National Glycohemoglobin Standardization Programme; SI, ‘Sistème International de Mesure’; CV\(_i\), intra-individual biological variation; CV\(_G\), inter-individual biological variation.

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measure HbA\(_1c\); other techniques, such as affinity chromatography, determine all glycated hemoglobins, including molecules with glucose bound to lysines \(17\) and \(166\) and to amino-terminal valines of \(\alpha\)-chain. The disagreement between results obtained with different methods prompted to organize harmonization programmes on a national basis, e.g. the National Glycohemoglobin Standardization Programme (NGSP) \(5\). Despite the improvement obtained by the implementation of these programmes, results of HbA\(_1c\) assayed in different countries were, however, still significantly discordant \(6\).

Therefore, in 1995 the IFCC created a working group with the aim of international standardization of HbA\(_1c\) assays \(7\). Following the theory of metrological traceability, first it was unequivocally defined the measurand HbA\(_1c\) as hemoglobin molecules having a special hexapeptide in common, which is the stable adduct of glucose to the amino-terminal valine of the hemoglobin \(\beta\)-chain (\(\beta\)-N-1-deoxyfructosyl-hemoglobin). Then, a metrological reference system was implemented, including specific reference methods for measuring HbA\(_1c\) as defined, reference materials and a network of laboratories performing the reference measurement procedure in a strictly controlled way \(8\). Expression of HbA\(_1c\) results in mmol \(\beta\)-N-1-deoxyfructosyl-hemoglobin/mol hemoglobin \(\beta\)-chain, in agreement with the ‘Sistéme Internationale de Mesure’ (S1), was also recommended \(9,10\).

Once the traceability of HbA\(_1c\) results by the commercial assays to the reference measurement system is obtained, it is important to define the acceptable uncertainty of HbA\(_1c\), measurements in view of their clinical use \(11\). Lacking outcome-related goals, there is a consensus for using biological variation to formulate analytic variation limits \(12\). In particular, desirable imprecision performance (expressed as CV) should be less than one-half of the intra-individual biological variability (CV\(_I\)) and desirable bias should be \(0.25\sqrt{CV^2 + \text{(inter-individual biological variation (CV\(_G\))}^2}\)} at \(\alpha = 0.05\) \(13,14\).

The accurate estimate of the biological variability of an analyte is, therefore, a fundamental prerequisite for the definition of the allowable measurement error, permitting reliable application of laboratory measurements in clinical setting. In this study, we systematically reviewed the published papers on biological variation of HbA\(_1c\), with special focus on the suitability of data, their concordance and possible causes of disagreement, with the aim to ascertain whether such existing studies are valid and scientifically sound enough to be used for attaining analytical goals for HbA\(_1c\).

### 2. Materials and methods

A PubMed search was performed, without any time limit, using as keywords: “Biological variation & HbA\(_1c\)” , “Intra-individual biological variation & HbA\(_1c\)” and “Inter-individual biological variation & HbA\(_1c\)” . As unique inclusion/exclusion criterion, recruited papers should have as explicit aim of the study the experimental assessment of HbA\(_1c\) biological variability components. We therefore ruled out papers reporting biological variation data obtained from literature, even because quoted references in such papers always referred to the already selected papers.

In evaluating published data, special attention was paid to the strategy of data collection and assembly, by considering selection and type of enrolled subjects, study duration, frequency and number of blood sampling, samples storage until analysis, analytical specificity of employed methods and statistical evaluation of data. These features were compared with the theory of biological variation as reported by Fraser and Harris \(15\). In particular, Table \(1\) lists the optimal features for an experimental protocol for accurate estimate of biological variability and Table \(2\) summarizes the recommended statistical approach for quantifying biological variability components.

### 3. Results and discussion

#### 3.1. Selected studies

Table \(3\) shows the main characteristics of selected studies on HbA\(_1c\) biological variability \(16–24\). While all nine studies estimated HbA\(_1c\) CV\(_I\) only five did HbA\(_1c\) CV\(_G\).

#### 3.2. Definition of the measurand and influence of method specificity

According to the theory of metrological traceability, the standard-ization of HbA\(_1c\) measurement is based on the definition of the ‘measurand’. If the adopted methodologies have different analytical specificities, assaying thus a different measurand rather than HbA\(_1c\) (like total glycated hemoglobins, also including hemoglobins glycated on other sites differing from amino-terminal valine of the \(\beta\)-chain), changes in biological variability, that strictly depends from the characteristics of the measurand, can be expected \(25\). Techniques such as ionic exchange or affinity chromatography on microcolumns as well as those based on electroendosmosis, used in the three oldest papers \(16–18\), had specificity markedly different from the IFCC reference measurement system. Godsland and Howey et al. correctly defined the measurand as ‘HbA\(_1c\)’, meaning the whole set of glycated hemoglobin fractions (HbA\(_1c\)+HbA\(_1b\)+HbA\(_1c\)) \(16,17\). On the other hand, affinity chromatography methods used by Phillips and Phillips \(18\) and, more recently, by Rohlfing et al. \(22\) are based on the reaction of cis-1,2-diol groups of glucose (and other sugars)-hemoglobin adduct with immobilized boric acid, thus producing boronated esters. Such methods, therefore, measure total glycated hemoglobins, including the glucose bound to lysines and amino-terminal valines of \(\alpha\)-chains.

Four reported studies used HPLC systems with ion-exchange columns (BioRex 70, Pharmacia Mono S, or Tosoh 2.2) \(19–21,24\). These methods show more specificity for HbA\(_1c\) than the above-mentioned techniques, even if correlations with the IFCC reference measurement procedure still show a significant constant bias witnessing some differences in terms of analytical specificity \(26,27\). The remaining study by Trapé et al. \(22\) used a competitive immunoturbidimetric assay (Tina-quant HbA\(_1c\), Roche Diagnostics) applied on an automatic analyzer of a different company (Synchron CX7, Beckman Coulter). This method is known to display the same specificity for HbA\(_1c\) as the reference method and this study is indeed the only one which is suitable from this point of view \(28\).

#### 3.3. Type of studied subjects

In the enrollment protocol of the studies the limitation most frequently found was the recruitment of diabetic patients. Indeed, to select apparently healthy people is required by the theory of biological variability, which aims to define the physiological fluctuation of analyte

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Main characteristics of an experimental protocol for accurate estimate of the biological variability of an analyte. Adapted from ref. (15).</th>
</tr>
</thead>
<tbody>
<tr>
<td>— Select apparently healthy subjects, using conditions that minimise pre-analytical variables*</td>
<td></td>
</tr>
<tr>
<td>— Specimens taken at set time intervals</td>
<td></td>
</tr>
<tr>
<td>— Specimens processed and stored frozen at (-80) °C</td>
<td></td>
</tr>
<tr>
<td>— When all specimens are available, analysis of all samples in a single run in duplicate</td>
<td></td>
</tr>
</tbody>
</table>

* Usual life styles, no drugs or alcohol, phlebotomy by the same person at the same time of day, optimal protocol for sample transport, processing, and storage.

<table>
<thead>
<tr>
<th>Table 2</th>
<th>Recommended statistical approach for quantifying biological variability of an analyte. Adapted from ref. (15).</th>
</tr>
</thead>
<tbody>
<tr>
<td>Using all sample data, the components of variance should be dissected out as follows: (\sigma^2_{\text{total}} = \sigma^2_{\text{G}} + \sigma^2_{\text{I}} + \sigma^2_{\text{A}}).</td>
<td></td>
</tr>
<tr>
<td>The analytical ((\sigma_{\text{A}})), intra-individual ((\sigma_{\text{I}})), and inter-individual ((\sigma_{\text{G}})) variations are calculated by analysis of variance:</td>
<td></td>
</tr>
<tr>
<td>— The analytical variation is estimated from the duplicate results for each specimen,</td>
<td></td>
</tr>
<tr>
<td>— The intra-individual variation is estimated from the serial results for each subject,</td>
<td></td>
</tr>
<tr>
<td>— The inter-individual variation is estimated from the total variance ((\sigma^2_{\text{total}})) of data minus the analytical and intra-individual components.</td>
<td></td>
</tr>
</tbody>
</table>

All the components of variation are then transformed to the relevant CVs using the corresponding means.
concentrations in a biological fluid around its homeostatic set point [15]. The presence of disease, mainly if unstable and not well controlled, may markedly modify the obtained information by amplifying such fluctuation. Fig. 1 clearly shows the difference between CVi values obtained in healthy volunteers (from 0.7% to 1.9%) and in diabetic patients (from 2.4 to 9.8%) with no overlap between the two groups. The same is also evident for CVi in the same control programme, sometimes obtained on non-commutable materials displaying a different behaviour from patient samples. Second, HbA1c measurements along the study were often performed on fresh samples the same day of blood collection, spreading them on several analytical runs, instead of performing measurements for all appropriately stored samples only in a single batch and, consequently, exclude from the total variability calculation the effect of between-run analytical variation, so difficult to be correctly assessed.

To perform all the determinations in duplicate on the same analytical run represents the ideal experimental model for a biological variability study, limiting to within-run variability the influence of the analytical variability on total variability of results, with less chance of error in the subsequent estimate of biological variability, obtained by subtraction (Table 2). Unfortunately, the only study which correctly included sample freezing in order to perform all HbA1c determinations on the same batch at the end of blood collection phase, did not assay samples in duplicate and got the information on analytical variability from the results obtained on control materials [21]. This did not allow an accurate definition of CVi as total variability of experimental results was less than the analytical variability alone obtained from quality control data, that was obviously overestimated [21].

In some studies, blood samples were not frozen and were, therefore, assayed in different runs, estimating analytical variability from results obtained on control materials [18,20,24]. Other studies obtained the analytical variability from literature data [19]. The extreme case of such approximation can be found in the study by Rohlfing et al. [23], in which, in addition to the lack of mentioning sample storage conditions, singlicate measurements of samples made it necessary to subtract to total experimental variability of results the “within-day” analytical variability, obtained from quality control data, so overestimated that only an approximate and possibly underestimated CVi was reported as probably <1%.

Homogeneity of collection and in the number of samples drawn from each individual is another critical procedural point; the availability of too many samples from the same subject may imply the technical limitations and the study duration. Such duration should be neither too short (a few days) nor too long (years), so that the variability study may not be related to periods different from those used in clinical practice for the measurements of specific analyte nor influenced by additional causes (e.g. seasonal variation) [21,30]. It seems, therefore, reasonable to adjust the duration of studies evaluating biological variability of HbA1c between 1 and 6 months, which corresponds to the advised frequency of the assay in different clinical situations [1]. If the majority of studies apply this criterion, the choice by the other authors to extend the duration beyond 6 months appears questionable [18,19,22,24]. It is indeed likely that an evaluation lasting for more than 6 months would include variability sources beside the biological one. For instance, Kolatkar et al. [19] attributed to an intensive treatment regimen the decrease in variability of HbA1c concentrations in diabetic patients found along the wide study period (6 years), wrongly interpreting this result as a reduction of the HbA1c CVi. The variation observed in these subjects may undoubtedly be utilized for clinical decision making, but certainly not to estimate the biological variability and the reference change value of HbA1c, which must be assessed on a healthy population for a suitable time period to be adopted as the basic criterion for interpretation of HbA1c results and evaluation of glycemic control in diabetic patients over time.

### 3.5. Sample analysis and assessment of analytical variability

Apart from the method employed, in the retrieved studies two further limitations can be found when compared to the ideal protocol mentioned in Table 1. First, HbA1c measurements were often performed in duplicate. Duplicate measurement permits a direct evaluation of the within-run analytical variability, and that is better than deriving it from the results of the laboratory internal quality control programme, sometimes obtained on non-commutable materials. It is indeed likely that an evaluation lasting for more than 6 months, wrongly interpreting this result as a reduction of the HbA1c CVi, which may not be related to periods different from those used in clinical practice for the measurements of specific analyte nor influenced by additional causes (e.g. seasonal variation) [21,30]. It seems, therefore, reasonable to adjust the duration of studies evaluating biological variability of HbA1c between 1 and 6 months, which corresponds to the advised frequency of the assay in different clinical situations [1]. If the majority of studies apply this criterion, the choice by the other authors to extend the duration beyond 6 months appears questionable [18,19,22,24]. It is indeed likely that an evaluation lasting for more than 6 months would include variability sources beside the biological one. For instance, Kolatkar et al. [19] attributed to an intensive treatment regimen the decrease in variability of HbA1c concentrations in diabetic patients found along the wide study period (6 years), wrongly interpreting this result as a reduction of the HbA1c CVi. The variation observed in these subjects may undoubtedly be utilized for clinical decision making, but certainly not to estimate the biological variability and the reference change value of HbA1c, which must be assessed on a healthy population for a suitable time period to be adopted as the basic criterion for interpretation of HbA1c results and evaluation of glycemic control in diabetic patients over time.

### 3.4. Study duration and sampling frequency

It is well known that a biological variability estimate is strictly dependent on the time interval between consecutive sample collec-
impossibility to perform all measurements in the same run. A range from a minimum of 11 to a maximum of 27 weekly blood samples obtained in subjects during the same study means to estimate the biological variability in a period from 3 to 6 months, making incorrect to approximate individual data to a mean CVI value [16].

3.6. Statistical analysis of data

The statistical method used to assess variability components was not specified (totally or partially) in three papers [16,19,22]; the remaining studies correctly employed analysis of variance (ANOVA). CVG estimate was added to CVI estimate in five studies [17,20,21,23,24], three of them carried out in groups of apparently healthy subjects (CVG values from 3.3% to 6.8%) [20,21,23].

4. Conclusions

A clear message from this systematic review of available literature is the obvious lack of robust data on biological variability of HbA1c concentrations in blood (Table 4). Despite all limitations and remarks reported in the present paper, it should, however, be noted that the information currently available on HbA1c biological variability is generally taken as definitive in most scientific and professional circles. The most popular informations on biological variability of biochemical and hematological analytes, employed in daily practice worldwide, are those compiled by Ricòs et al., freely available at www.westgard.com/biodatabase1.htm. Values of biological variability components for HbA1c listed in this database (CVI 3.4%, CVG 5.1%) are apparently obtained from the (weighed?) mean of results available in the literature, taken as valid by the authors of the compilation. It is, however, impossible to understand the criterion (if one) of study evaluation and selection of data, so that some doubts appear justified. The observation reported in the present study and, particularly, the finding of a substantial difference of variability results obtained in healthy individuals and diabetic patients (Figs. 1 and 2) show the fallacy of determining a single value of CVI and CVG simply using the mean of available results. Despite all reported limitations, keeping into account the available data obtained in healthy subjects [16,20], the CVI of HbA1c should approximately be 1.8–1.9%, much lower than that reported by Ricòs et al. in the mentioned database. It is, however,
evident the urgent need of more accurately designed studies, producing robust information to be used for attaining analytical goals for HbA1c determination.

Acknowledgment

We thank Prof. Sandro Eridani (Department of Science and Biomedical Technology, University of Milano. I) for assistance in revising the English language.

References


Table 4

Summary of the characteristics of studies on biological variability of HbA1c, evaluated in this systematic review.

<table>
<thead>
<tr>
<th>Study no.</th>
<th>Method specificity as per HbA1c measured definition</th>
<th>Recruitment of healthy subjects</th>
<th>Optimal study duration</th>
<th>Optimal protocol of sample analysis</th>
<th>Statistical analysis described</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>No</td>
<td>Yes</td>
<td>±</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>2</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>3</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>4</td>
<td>±</td>
<td>No</td>
<td>No</td>
<td>No</td>
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<tr>
<td>5</td>
<td>±</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>6</td>
<td>±</td>
<td>Yes (F only)</td>
<td>Yes</td>
<td>±</td>
<td>Yes</td>
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<tr>
<td>7</td>
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<td>No</td>
<td>No</td>
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<td>8</td>
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<td>Yes (M only)</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>9</td>
<td>±</td>
<td>No</td>
<td>No</td>
<td>No</td>
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