How to report HbA_{1c} in presence of hemoglobin variants

Mariarosa Carta[1], Renata Paleari[2], Alessandro Terreni[3], Andrea Mosca[2], on behalf of the Intersociety Study Group (SIBioC-SIPMeL) on Diabetes

[1]Medicina di Laboratorio, ULSS 8 Berica, Vicenza (IT)

[2]Dipartimento di Fisiopatologia medico-chirurgica e dei trapianti, Università degli Studi di Milano (IT)

[3]Centro regionale di riferimento per la Sicurezza e Qualità in Laboratorio, Azienda Ospedaliero-Universitaria Careggi, Firenze (IT)

ABSTRACT

Measurement of glycated hemoglobin (HbA_{1c}) has a key-role in the management of diabetic patients. Clinicians need reliable and accurate measurements, with negligible pre-analytical and post-analytical errors. Among the pre-analytical variables, the presence of hemoglobin variants is a challenge to the laboratorians, both on pre-analytical and analytical phase. The purpose of this document is to give some practical advices on how to report HbA_{1c} values in presence of hemoglobin variants. This is an update of a previously reported document, published in 2011. The list of the most diffused method for measuring HbA_{1c} has been updated, and the most recent enzymatic assays have been included. A new aspect concerns the post-analytical phase, in which we recommend to report the presence of the hemoglobin variant in the final laboratory report.

Keywords: *glycated hemoglobin, hemoglobin variants, post analytical phase*

FOREWORD

In 2011 our study group has developed a document related to this issue (1), which needs to be updated and revised. This new version takes into account methods implemented since then, and promotes new recommendations and operative instructions.

BACKGROUND

Glycated hemoglobin (HbA_{1c}) is a fundamental biomarker used in diagnosis and monitoring of diabetic patients. However, the presence of hemoglobin variants may interfere in the assay, thus producing unreliable or difficult to be interpreted results (2). Furthermore, the presence of a hemoglobin variant may cause a positive or a negative interference, either on the analytical phase or in the pre-analytical/biological phases.

The pre-analytical interference takes usually place when hemoglobin variants are present and not forming stable glucose adducts, or show abnormal glycation kinetics compared to HbA (such as Hb G Coushatta), or cause a reduction in red cell lifespan (such as Hb Zürich). It is generally acknowledged that each interference due to a hemoglobin variant has to be evaluated experimentally, and for all the analytical methods used for HbA_{1c} measurement (3).

Regarding the worldwide diffusion, the most common hemoglobin variants are HbS, HbC, HbD and HbE. In subjects with homozygosis for HbS and for HbC (HbSS, HbCC) or with double heterozygosis (HbSC), HbA is absent, and consequently HbA_{1c} can't be produced, although a glycated component of the variant hemoglobin could be present. These subjects manifest a severe hemolytic anemia. Notably, since the HbA_{1c} production is related to the time of red cell exposure to glucose, it is advisable, under these circumstances, to monitor glycemic control by other methods such as, the various devices for the Continuous Glucose Monitoring (CGM) or by Flash glucose monitoring, or by other parameters not red cell related (glycated albumin, 1,5-anhydroglucitol).

Red cell lifespan, in the carriers of the above-mentioned hemoglobin variants, is generally regarded as normal. Some evidences, although not recent, suggest that red cell lifespan in HbC carriers, can be significatively reduced (4).

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Corresponding Author: Andrea Mosca, Dip. Fisiopatologia MCT Via Fratelli Cervi 93 20054 Segrate (IT), E-mail andrea.mosca@unimi.it

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HbA_{1c} can be therefore measured on these patients, providing that the hemoglobin variant does not interfere in HbA_{1c} assay. Table 1 reports the possible interference related to the most common hemoglobin variants, as seen in several methods for HbA_{1c}. This information has been obtained from the NGSP website (5), to which the reader is referred in order to recover an extensive bibliography on this topic.

The methods most frequently used for the determination of HbA_{1c} can be classified, based on analytical principles, in the following groups:

- Separative techniques, based on electrical charge difference (HPLC, capillary electrophoresis).
  HbA_{1c} is separated from other hemoglobins according to the difference in the isoelectric points. All variants with an electrical charge different than HbA, could appear, in theory, in the chromatogram/electropherogram and become a possible interference. When using HPLC methods, the HbA_{1c} value is calculated, when no hemoglobin variants are present, by the following formula:

$$[HbA_{1c}]\% = [HbA_{1c}]/[Hbtotal] \times 100$$

If the hemoglobin variant (HbX) and its glucose adduct (HbX_{1c}) elute or migrate separately from HbA and HbA_{1c}, their presence have no effect on the determination of HbA_{1c}, since most of the systems calculate HbA_{1c} according to the following equation:

$$[HbA_{1c}]\text{corrected},\% = [HbA_{1c}]/([HbA] + [HbA_{1c}]) \times 100\ ([a])$$

When, HbX and/or HbX_{1c} do not appear to be separated from HbA and HbA_{1c}, HbA_{1c} could be under- or over- estimated. HbC and HbS, are adequately separated by the most recent HPLCs (5). HbE and HbD, when present, can cause under- or over-estimation with the majority of HPLC methods (6).

- Immunochemical methods.
  Various monoclonal antibodies, recognizing the 4-10 aminoacids of N-terminal β chain have been developed, and employed in several assays. Hemoglobin variants presenting mutations within these sequences (such as HbS and HbC) could produce an interference on HbA_{1c} measurement (5). However, the most recent II and III generation methods do not suffer from such interferences. Remarkably, the presence of variants with mutations outside the epitopes of these antibodies (such as HbE and HbD) do not cause problems in HbA_{1c} measurement (7).

Table 1

Effect of hemoglobin variants (HbS, HbC, HbD and HbE traits) on 16 most often used methods to measure HbA_{1c} (adapted, from ref. 5). More comprehensive information regarding HbA_{1c} assay interference is reported in a sub-table of the NGSP website.

Manufacturer	Analyzer (principle of the method)	Interference (Yes/No)			
		HbAS	HbAC	HbAD	HbAE
Abbott	Architect c (enzymatic)	No	No	No	No
Alere	Afinion (affinity chromatography)	No	No	No	No
Arkray	Adams Ha-8180V (HPLC)	No	No	Yes[§]	Yes[§]
Beckman	DxC 700 AU (immunochemistry)	No	No	No	No
Beckman	Synchron (immunochemistry)	No	No	No	No
Bio-Rad	D-10 (HPLC)	No	No	Yes↑	Yes↑
Bio-Rad	D-100 (HPLC)	No	No	No	No
Bio-Rad	Variant II turbo 2.0 (HPLC)	No	No	No	No
Ortho-Clinical	Vitros (immunochemistry)	No	No	No	No
Roche	Cobas c513 (immunochemistry)	No	No	No	No
Sebia	Capillarys 2FP (capillary electrophoresis)	No	No	No	No
Siemens	DCA Vantage (immunochemistry)	Yes↑	No	No	Yes↑
Siemens	Atletica (enzymatic)	No	No	No	No
Siemens	Dimension (immunochemistry)	No	No	No	No
Trinity - Menarini	Hb9210 premier (HPLC)	No	No	No	No
Tosoh	G8 *versus* 5.24, 5.28 (HPLC)	No	No	No	No

↑interference causing a higher HbA_{1c} result (≥6% compared to the non-carrier, at the HbA_{1c} value of 42 mmol/mol);
§HbA_{1c} not quantified.

([a])This correction is operative when hemoglobin variants in HPLC elute after HbA, but generally is not active if the hemoglobin variant is eluted before HbA. This formula is, as a matter of fact, always used in the capillary electrophoresis systems.

- Affinity chromatography.
  These methods are based on the use of aminophenylboronic acid, which is a powerful binding molecule for glucose bound to human hemoglobin. A possible interference from fetal hemoglobin (HbF) could be present, if HbF % is above 10-15 % (5).
- Enzymatic methods.
  Some proteolytic enzymes digest human hemoglobins, producing a mixture of peptides that can be oxidized by other ketoamino oxidases. The ketoamine bounds are oxidized by a Trinder reaction, resulting in color development which can be quantified by spectrophotometry.

Generally, the last three methods are considered "blind" to hemoglobin variants interference, in relation to the analytical phase. They provide just a numerical value, but the interference from hemoglobin variants has to be kept in mind as a possibility occurring either on the analytical or in the pre-analytical phase.

If the patient has a previous diagnosis of a hemoglobinopathy, it is mandatory to evaluate if such variant may or may not interfere with the HbA_{1c} method. Similarly, if the hemoglobin variant is responsible of a hemolytic anemia (such in the case of the unstable hemoglobins) the interpretation of the HbA_{1c} result has to be carefully evaluated, because of a possible reduction of red cell lifespan.

Generally, the presence, if any, of a hemoglobin variant is only occasionally detectable. It is worth to mention that several hemoglobin variants cannot be evidenced regardless of the principle of the method. Their presence could be only supposed after evaluation of other parameters (such as blood cell count and related parameters). The use of high performing analytical methods allows the detection of hemoglobin variants. However, nowadays, the research and characterization of a hemoglobin variant is achieved by DNA analysis which guarantees a rapid and accurate approach.

RECOMMENDATIONS

I. The users of separative assays to quantify HbA_{1c} should carefully inspect their chromatograms/electropherograms before issuing the final report. If an abnormal pattern is found, because of the presence of a possible hemoglobin variant, there are two possibilities:
   i. the laboratory professional cannot perform additional investigations. In such a case, the HbA_{1c} result should not be reported. A comment suggesting further investigations is needed in the final report;
   ii. the laboratory professional can perform additional investigations. Under these circumstances, the HbA_{1c} result can be reported, provided that the hemoglobin variant is identified as S, C, D or E in heterozygosis, and it can be well separated from HbA so that it does not affect the method (Table1).

II. The users of any method need to remember that extreme HbA_{1c} values (such as either very low, or very high), should arise the possibility of assay interference. The same holds true if a marked discordance between HbA_{1c} and plasma glucose is found.

Table 2
Examples of comments to be added to the laboratory report in case of presence, in the heterozygous state, of a hemoglobin variant.

Hb variant	Comment
HbS	Patient with heterozygosis for HbS. The HbA_{1c} result has been corrected for the presence of HbS.
HbC	Patient with heterozygosis for HbC. The HbA_{1c} result has been corrected for the presence of HbC.
HbD	Patient with heterozygosis for HbD. The HbA_{1c} result has been corrected for the presence of HbD.
HbE	Patient with heterozygosis for HbE. A possible microcytic anemia is often associated to this condition. Even if the HbA_{1c} result has been corrected for the presence of HbE, the phenotypes could be very different, mostly in association with a β-thalessemia trait.
Unidentified Hb variant	HbA_{1c} not measurable due to the presence of an unknown hemoglobin variant. Other tests useful for the evaluation of the glycemic control are suggested, as well as further investigations in order to characterize the hemoglobin variant.

III. The presence of the hemoglobin variant should be reported in the final report. Some examples of additional comments are shown in Table 2.
IV. In all the cases where HbA_{1c} cannot be reported, it is advisable to use plasma glucose tests to monitor glucose control, avoiding hemoglobin-based tests (i.e. continuous glucose monitoring, flash glucose monitoring, glycated albumin).

ADDITIONAL CONSIDERATIONS

Some additional considerations may be added:

- The measurement of HbA_{1c} in subjects with homozygosis or double heterozygosis for a number of hemoglobin variants, as well as in subjects with double heterozygosis for a β hemoglobin variant in association with β-thalassemia, should not be ordered or performed.
- When an unknown hemoglobin variant is found, it is important to use a separative method with a system dedicated to the hemoglobinopathies.
- In several Italian Regions globin gene sequencing is routinely performed in at least one of the Italian National Health System Regional reference laboratories. Gene sequencing is not strictly necessary when clinical symptoms related to possible hemoglobin defects are absent. Further investigations by Next Generation Sequencing could be also considered, since this technique is somewhere also applied to the study of hemoglobin defects.

Finally, the interpretation of an HbA_{1c} result in a patient carrying a newly characterized hemoglobin variant, not belonging to those mentioned so far (S, C, D, E), has to be performed by specifically competent personnel, given the possible molecular interactions between the different hemoglobins present. For an in-depth study on this topic, the reader is referred to an excellent recent review (8).

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CONFLCT OF INTEREST

None.

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