Study Summary
THIS IS A SUMMARY AND DISCUSSION OF AN ORTHO CLINICAL DIAGNOSTICS STUDY:

An Overview of HbA1c Laboratory Measurement with a Focus on the Novel VITROS® A1c Slides Assay

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HbA1c - Biomarker for Diabetes Mellitus

The significance of detecting and monitoring diabetes

Diabetes is a disease caused by abnormal production or response to insulin, leading to elevated glucose in blood and urine. Globally, diabetes prevalence has nearly doubled since 1980, rising from 4.7% to 8.5% in the adult population.1 Half of the 463 million adults living with diabetes today are unaware that they have the condition, and are therefore at high risk of developing serious diabetes-related complications, either acute or chronic.2 Therefore, early detection and appropriate treatment to ensure glycemic control are essential to prevent disability and death.

Hemoglobin A1c (HbA1c) as a diabetes biomarker

Elevated HbA1c indicates chronic blood glucose elevations (hyperglycemia) and correlates strongly with diabetes complications.3-6 Accurate measurement of patient’s HbA1c is crucial for the optimal management of diabetes and a favorable prognosis. Therefore, clinical guidelines recommend HbA1c biomarker measured with a standardized assay for diagnosing diabetes mellitus (DM) and for monitoring glycemic control in patients with Type 1 or Type 2 DM.7

Glycation: formation of glycated hemoglobin (Hb) and glycated albumin (GA)

Although standardized HbA1c assays are available, numerous factors such as anemia, blood transfusions, and/or hemoglobin variants potentially influence HbA1c measurement.

The addition of glucose to the valine and lysine residues on the Hb α and β chains forms glycated Hb. The amount of glycated Hb generated is directly proportional with the glucose blood concentration. HbA1c is the main glycated Hb form, in which glucose is bound specifically to the N-terminal valine of the hemoglobin β chain on an alpha amino group (Fig 1a).8,9 Proteolytic digestion of HbA1c yields fructosyl valine.9 Glycation refers to a monosaccharide (usually glucose) attaching nonenzymatically to the amino group of a protein and it occurs in both diabetic and non-diabetic individuals (Fig 1b).9 However, in diabetes, hyperglycemia leads to accelerated non-enzymatic glycation. Therefore, the level of glycated proteins is higher in diabetic than in non-diabetic individuals.
Glycation of proteins can interfere with their normal functions by disrupting molecular conformational changes, altering enzymatic activity, impeding protein–protein interactions and functioning of receptors. The increased free radical production and oxidative stress are further responsible of the diabetes complications.

Hemoglobin (Hb) is a tetrameric iron-containing protein within red blood cells (RBC) consisting of four globular protein subunits (Table 1). In normal adults there are three Hb types encountered: adult Hb (HbA) - with its glycated (HbA1) and non-glycated (HbA0) fractions-, fetal Hb (HbF), and HbA2; their normal relative concentrations are represented in Fig. 2.

Hemoglobin A1 can be glycosylated non-enzymatically in both diabetic and non-diabetic individuals. In diabetic patients, the increased glycated HbA1 has three major forms: HbA1a, HbA1b, and HbA1c. HbA1c is produced continuously at a rate dependent on glucose concentration and represents the major glycated Hb species. (Table and Fig. 2).

The extent of hemoglobin glycation is influenced by the concentration of glucose in the blood. Since the life span of erythrocytes is approximately 120 days, HbA1c reflects the average glucose concentration over the preceding 8–12 weeks. Therefore, any cause of shortened erythrocyte survival or decrease in mean erythrocyte age (e.g. recovery from acute blood loss, hemolytic anemia) will reduce exposure of erythrocytes to glucose, affecting the HbA1c result.

In addition to Hb, glycation of the ε-amino group of internal lysine residues Lys199, Lys233, Lys439, and Lys525 of circulating albumin in plasma leads to glycated albumin (GA). (Figure 3).

Proteolytic digestion of glycated albumin produces fructosyl lysine. Compared to HbA1c, glycated albumin reflects the average blood glucose only over the past 2–3 weeks.

Therefore, measurement of GA levels could be used for glycemic control in the case of condition that affects the lifespan of the red blood cells.

Table 1: Hemoglobin Subunits

<table>
<thead>
<tr>
<th>Subunit</th>
<th>ααββ</th>
<th>ααγγ</th>
<th>ααδδ</th>
</tr>
</thead>
<tbody>
<tr>
<td>HbA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HbF</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HbA2</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Figure 2: Hemoglobin Fractions

<table>
<thead>
<tr>
<th>HbF</th>
<th>HbA</th>
<th>HbA2</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5%</td>
<td>97%</td>
<td>2.5%</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>HbA0</th>
<th>HbA1</th>
<th>HbA2</th>
</tr>
</thead>
<tbody>
<tr>
<td>93–95%</td>
<td>5–7%</td>
<td>2.5%</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>HbA1a</th>
<th>HbA1b</th>
<th>HbA1c</th>
</tr>
</thead>
<tbody>
<tr>
<td>4–6%</td>
<td>0.5%</td>
<td>0.5%</td>
</tr>
</tbody>
</table>

Laboratory measurement of HbA1c

The HbA1c result could be influenced by analytical interference (e.g. medications, bilirubin, hemoglobin variants) and/or clinical conditions (e.g. anemia, chronic kidney disease). A falsely elevated or decreased result could lead to incorrect treatment.

Hemoglobinopathies

Hemoglobinopathies are genetic abnormalities of hemoglobin broadly characterized into two groups: thalassemia syndromes - which affect Hb synthesis, and structural hemoglobin variants.
The structural variants are caused by point mutations which modify an amino acid in the protein sequence of the β chain. While most of the mutations are rare, some of the most common are represented in Figure 4.13,14

Worldwide, the prevalence of the Hb variants is between 5-7%.15 The descending order of these most common Hb variants is HbS, HbE, HbC, and HbD with differences by regions. In the United States, HbS is the most prevalent variant, followed by HbC and HbE.16

The clinical significance of these variants depends on the genetic status. While the homozygotes have mutations in both β-globin subunits resulting in disease, the heterozygotes have a combination of variant and normal Hb, resulting in trait status.14

Individuals who are homozygous for a certain Hb variant (e.g. sickle-cell disease or HbSS) or express multiple Hb variants (e.g. HbSC) may suffer from anemia due to shortened RBC lifespan, and may require transfusion.14 Any cause of shortened erythrocyte survival or decrease in mean erythrocyte age (e.g. recovery from acute blood loss, hemolytic anemia) will reduce exposure of erythrocytes to glucose, affecting the HbA1c result. Alternative forms of testing such as glycated serum protein or glycated albumin should be considered for patients with altered RBC lifespan or with clinically symptomatic hemoglobinopathy.

In contrast, individuals who are heterozygous for a certain Hb variant (e.g. sickle-cell trait or HbAS) are often asymptomatic and have a normal red blood cell (RBC) lifespan. Therefore, in these patients, HbA1c assays with no analytical interferences from variants may be used for long-term glycemic control.7,17

The impact of physiological and analytical interferences caused by hemoglobinopathies

Some hemoglobinopathies can generate physiological interference by causing a reduced erythrocyte lifespan; in this case, the HbA1c (or total glycosylated Hb) would be falsely lowered, regardless of the method used.16

In addition to Hb variants, elevated HbF could also cause analytical interference. A major Hb during intrauterine life and at birth, HbF decreases during the first year of life to reach the 1% adult level. Elevated HbF levels (up to 30%) can occur in pathologic conditions (e.g., leukemia, anemia, thalassemia) or a hereditary persistence of fetal hemoglobin and are asymptomatic.14 Therefore, patients and their physicians may be unaware of the existence of the condition.

A falsely elevated HbA1c result may lead to unnecessary and/or excessive treatment and result in hypoglycemia and/or other drug-related side effects. A falsely decreased HbA1c result may expose patients to high risk for diabetes complications (e.g. nephropathy and various cardiovascular disorders) due to inadequate therapy.
Laboratory methods for HbA1c measurement

The methods used for HbA1c testing are based on charge differences such as ion-exchange high performance liquid chromatography and capillary electrophoresis, and methods based on structural differences such as enzymatic, immunoassays, and boronate affinity chromatography.14

Figure 5: HbA1c Methods

High performance liquid chromatography (HPLC):

(A) Ion-exchange HPLC method uses buffers of increasing ionic strength to separate hemoglobin HbA1c from other Hb species by differences in charge. The concentration of Hb in each fraction is quantified by calculating the area under each peak. The chromatograms have to be reviewed to identify the non-physiological forms of Hb, making the method labor-intensive. The main advantage of this method is that it allows a presumptive identification of Hb variants. However, changes or mutations in the hemoglobin molecule affecting the charge will alter the HbA1c results if these variants co-elute with HbA or HbA1c.14,18 Running the test in the decreased chromatographic resolution mode could result in interference from the Schiff base, carbamylated Hb, or Hb variants, which may coelute with the peaks of interest, while running it in the high-resolution chromatography prolongs the turnaround time.19

(B) Boronate affinity HPLC is considered to have the least interference from Hb variants and derivatives. The structural difference between HbA1c and hemoglobin AO resulted from the presence of the glucose group in HbA1c constitutes the basis for this method.19 The m-aminophenylboronic acid specifically interacts with the glucose moiety (cis-diol groups) and binds all glycated Hb to the affinity resin. After elution, all the glycated Hb is measured, and the results are standardized to HbA1c units.14 A caveat of this method is that the end user is not capable to discern a presumptive Hb variant;19 Interference might happen if the capture reagent reacts with non-glycated Hb or if Hb variants are glycated.

Enzymatic methods: Uses an enzyme which cleaves the N-terminal valine.10 Lysed blood samples are subjected to proteolytic digestion which releases the substrate for fructosyl valine oxidase. The produced hydrogen peroxide is measured using a horseradish peroxidase-catalyzed reaction with a chromogen.15 These methods are unaffected analytically by the presence of Hb variants but the user cannot discern the presence of a Hb homozygous variant.19,20

Immunoassays: Use antibodies which target usually the 4-10 aminoacids at the N-terminal end of the Hb β-chain to specifically measure HbA1c.14 Assay design is variable (e.g. immunoturbidimetry, latex-enhanced competitive immunoturbidimetry). In case the antibodies target the amino acids where mutations occur, the Hb measurement may be affected by the Hb variants.18

Immunochemical assays (enzymatic, immunoassays) are not affected by problems related to electrical charge and can be adapted easily in the routine medical laboratory.20

Capillary electrophoresis (CE): CE separates Hb molecules by charge and mass. Charged molecules are highly resolved by their electrophoretic mobility and their separation depends on electrolyte pH and electro-osmotic flow. CE can identify Hb variants and does not have analytical interference from common Hb variants.

The advantages and disadvantages of these methods are summarized in Table 2.19

Enzymatic methods: Uses an enzyme which cleaves the N-terminal valine. Lysed blood samples are subjected to proteolytic digestion which releases the substrate for fructosyl valine oxidase. The produced hydrogen peroxide is measured using a horseradish peroxidase-catalyzed reaction with a chromogen. These methods are unaffected analytically by the presence of Hb variants but the user cannot discern the presence of a Hb homozygous variant.

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The advantages and disadvantages of these methods are summarized in Table 2.
### Table 2: Advantages and Challenges of HbA1c Methods

<table>
<thead>
<tr>
<th>Method</th>
<th>Principle</th>
<th>Advantages</th>
<th>Challenges</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enzymatic</td>
<td>Measures HbA1c using an enzyme that specifically cleaves the N-terminal valine</td>
<td>No analytical interference from Hb variants</td>
<td>Unable to detect Hb variants</td>
</tr>
<tr>
<td>Immunoassay</td>
<td>Uses antibody targeted against the glycated N-terminus of the b chain</td>
<td>No analytical interference from the most common Hb variants using newer-generation assays</td>
<td>Unable to detect Hb variants; newer-generation antibodies still susceptible to interference from rare Hb variants</td>
</tr>
<tr>
<td>Boronate affinity</td>
<td>Glycohemoglobin binds affinity resin while nonglycated hemoglobins pass through the column</td>
<td>Minimal analytical interference from Hb variants</td>
<td>Measures all glycated Hbs, not just HbA1c; unable to detect Hb variants</td>
</tr>
<tr>
<td>Ion-exchange HPLC</td>
<td>Separates Hb species based on charge</td>
<td>Ability to detect the most common Hb variants</td>
<td>Prone to interference by Hb variants that coelute with peaks of interest</td>
</tr>
<tr>
<td>Capillary electrophoresis</td>
<td>Separates Hb species based on charge and hydrodynamic volume</td>
<td>High chromatographic resolution and resulting ability to detect many Hb variants</td>
<td>Throughput</td>
</tr>
</tbody>
</table>

Table 3 is an example of various methods and their interferences - not an exhaustive list. For additional methods and information on interferences, please refer to the NGSP website where data from independent studies is also available, see section on More Comprehensive Information Regarding HbA1c Assay Interferences.21

### Table 3: Hb Variant Interference by Manufacturer HbA1c Methods

<table>
<thead>
<tr>
<th>Vendor</th>
<th>Device Name</th>
<th>Methodology</th>
<th>Variant Interference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abbott Laboratories²²</td>
<td>ARCHITECT c8000 System Hemoglobin A1c</td>
<td>Enzymatic</td>
<td>HbS, HbC, HbD, HbE, HbA2</td>
</tr>
<tr>
<td>Siemens²³</td>
<td>ADVIA® Chemistry Enzymatic Hemoglobin A1c (A1c_E) Assay</td>
<td>Enzymatic</td>
<td>No</td>
</tr>
<tr>
<td>Ortho Clinical Diagnostics²⁴</td>
<td>VITROS® Chemistry Products A1c Slides</td>
<td>Enzymatic</td>
<td>No</td>
</tr>
<tr>
<td>Diazyme Laboratories²⁵</td>
<td>Diazyme Direct HbA1c Assay</td>
<td>Enzymatic</td>
<td>No</td>
</tr>
<tr>
<td>Ortho Clinical Diagnostics²⁶</td>
<td>VITROS Chemistry Products HbA1c Reagent</td>
<td>Quantitative turbidimetric, inhibition immunoassay</td>
<td>No</td>
</tr>
<tr>
<td>Roche²⁷</td>
<td>Cobas® INTEGRA 800 Tina-quant HbA1cDx Gen.2 assay</td>
<td>Quantitative turbidimetric, inhibition immunoassay</td>
<td>No</td>
</tr>
<tr>
<td>Roche²⁸</td>
<td>Cobas c 513 Analyzer 3 Tina-quant HbA1cDx Gen.3 Assay</td>
<td>Quantitative turbidimetric, inhibition immunoassay</td>
<td>No</td>
</tr>
<tr>
<td>Beckman Coulter²⁹</td>
<td>DxC700 AU HbA1c Advanced</td>
<td>Quantitative turbidimetric, inhibition immunoassay</td>
<td>No</td>
</tr>
<tr>
<td>Siemens²¹</td>
<td>ADVIA Chemistry Hemoglobin A1c Assay</td>
<td>Quantitative, latex agglutination inhibition method</td>
<td>No</td>
</tr>
</tbody>
</table>
The Novel Whole Blood VITROS A1c Slides

**VITROS A1c Slides use an enzymatic method to determine HbA1c**

The VITROS A1c Slides assay is a multilayered, analytical element coated on a polyester support. A drop of whole blood patient sample is deposited on the slide where a surfactant lyses the red blood cells releasing glycated hemoglobin. A second surfactant denatures the glycated hemoglobin to provide access to proteolytic cleavage sites.

The assay is based on an enzymatic method using the enzyme fructosyl amino acid oxidase (FAOD) or fructosyl peptide oxidase (FPOX). These oxidases (FAOXs/FPOXs) are FAD-containing enzymes that catalyze the proteolysis of HbA1c and the subsequent oxidation of the liberated fructosyl valyl histidine (substrate). FAODs/FPOXs catalyze the oxidation of the C-N bond linking the C1 of the fructosyl moiety and the nitrogen of the amino group of fructosyl amino acids. The reaction proceeds through an intermediate, which hydrolyzes to produce glucosone and the valyl histidine amino acid. (Figure 6 and Figure 7). The reduced FAD is then reoxidized by molecular oxygen with the release of hydrogen peroxide.

In the VITROS A1c Slides, the final reaction is catalyzed by horseradish peroxidase and involves the oxidation of a chromogen (leuco dye) by hydrogen peroxide. The resulted colorimetric signal is read by reflectance spectroscopy at 670 nm (Figure 7). The concentration of glycated hemoglobin is directly proportional to the reflectance density of the dye formed.
VITROS A1c Slides assay design is robust to analytical interference from common Hb variants and other glycated amino acids.

The substrate for the reaction (fructosyl-alpha-valyl-histidine) is produced by cleavage of the first two amino acids located far from the sites where the common Hb variants occur (Figure 8).

The specificity of the oxidase enzyme for this substrate ensures no analytical interference from the common Hb variants or from other glycated amino acids.

This was confirmed with testing of samples containing various concentrations of variants: samples containing hemoglobin variants HbA2 up to 7%, HbS up to 41%, HbC up to 36%, HbD up to 41%, HbE up to 40% and HbF up to 15% of the total hemoglobin concentration do not interfere. In addition, this method was tested with samples treated with 50 mg/dL Acetaldehyde, 150 mg/dL Urea, and 1500 mg/dL Glucose for four hours at 37°C and found unaffected by the presence of acetylated hemoglobin, carbamylated hemoglobin, and labile glycated hemoglobin.

Conclusion

In summary, a multitude of variants have been described, however, the most common are HbS, HbC, HbD, HbD, and HbE. While the Hb variant traits do not alter the RBC lifespan significantly, they can still interfere analytically with some HbA1c assays due to mutations at key sites. The enzymatic method used by the VITROS A1c Slides assay employs an oxidase with high specificity for a substrate not affected by common Hb variant mutations. Therefore, no analytical interference with Hb variants is expected with the VITROS A1c Slides assay.

Conditions altering RBC lifespan will result in physiological interference which will affect all HbA1c assays. Therefore, HbA1c results must be interpreted in the clinical context and in case of discrepant results, a hemoglobin variant or other type of analytical interference should be considered. In case of known conditions resulting in chronic anemia, glycated albumin and/or fructosamine (glycated serum protein) are recommended as measures of more rapid changes in blood glucose.
REFERENCES


26. Instructions For Use VITROS Chemistry Products HbA1cPub. No. 355871_EN Version 5.1


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