Hemoglobin A1c Testing of Patients with Hemoglobinopathies

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Level of Difficulty: Intermediate

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1. a  b  c  d
2. a  b  c  d
3. a  b  c  d
4. a  b  c  d
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7. a  b  c  d
8. a  b  c  d
9. a  b  c  d
10. a  b  c  d

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   5  4  3  2  1

2. The objectives of this Distance Learning course were met.
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INTRODUCTION

Hemoglobin A1c is the most useful single index of blood glucose control available to diabetics. Increased HbA1c is closely linked to risk of long-term microvascular diabetic complications (1). HbA1c is measured in the laboratory using a variety of methods. The presence of hemoglobinopathies in a patient presents a confounder to HbA1c testing, possibly yielding erroneous laboratory rest results. Beginning with a description of HbA1c and its relationship to blood glucose followed by methods of testing for HbA1c in the laboratory, this course focuses on the problematic aspects of testing for HbA1c in a patient with a hemoglobinopathy using each of these testing methods. General conclusions, limitations, and recommendations for testing are given.

OBJECTIVES:
On completion of this course the participant will be able to
1. describe hemoglobin A1 structure and function
2. discuss the formation of hemoglobin A1c (HbA1c) and its relationship to blood glucose levels
3. give the normal HbA1c range and the recommended percentage for diabetics
4. outline the chronic complications of diabetes due to long term increase in blood glucose
5. list the names of three HbA1c testing methodologies
6. state examples for each of the HbA1c methodologies
7. describe how hemoglobinopathies may give erroneous results in HbA1c testing

DESCRIPTION OF HBA1c

Hemoglobin, found in red blood cells, carries oxygen to the tissues and facilitates removal of carbon dioxide from the body. Hemoglobin is a tetrameric molecule made up of four globin chains attached to four heme groups. The majority of hemoglobin in normal adults is designated as hemoglobin A, or A1, which contains two alpha and two beta chains (Figure 1). Hemoglobin A1c is an in vivo glycosylated form of hemoglobin A1 with a glucose molecule irreversibly attached to the N terminal amino group of the beta chain.
RELATIONSHIP OF GLUCOSE AND HbA1c

In the erythrocytes, the relative amount of HbA1 converted to stable HbA1c increases with the average concentration of glucose in the blood. The conversion to stable HbA1c is limited by the erythrocyte’s life span of approximately 100 to 120 days. The level of HbA1c at any time is contributed to by all circulating erythrocytes, from the oldest to the youngest. As the older RBCs die off, the younger ones contribute more to the level of HbA1c, meaning that the blood glucose levels in the preceding 30 days contribute more to the HbA1c (approximately 50%) than the levels from 90-120 days. Therefore HbA1c reflects the blood glucose level during the preceding two to three months. HbA1c is thus suitable to monitor long-term blood glucose control in individuals with diabetes mellitus (3).

The correlation between HbA1c and mean plasma glucose over the previous two to three months is shown in Table I.

Figure 1: Hemoglobin A1: Structure of the hemoglobin molecule
Adapted from reference 2.
USE OF HbA1c IN MANAGEMENT OF DIABETES

The normal range of HbA1c is 4 to 5.9% of the total hemoglobin. In diabetics the higher the average blood glucose level is over a two to three month period, the higher the percentage of HbA1c. Measuring HbA1c levels gives a view of the blood sugar control over that period of time, whereas day to day glucose levels may fluctuate widely. With HbA1c as a guideline, the physician can better evaluate the diabetic’s glucose control and can make adjustments in treatment.

The American Diabetes Association recommends that diabetics have a goal of HbA1c less than 7.0%. The International Diabetes Federation and the American College of Endocrinology suggest a lower goal of 6.5% (4).

Control of glucose levels is important to help decrease chronic complications of diabetes. These complications are related to blood vessel diseases. These vascular diseases are divided into microvascular diseases and macrovascular diseases.

**Microvascular diseases** affect the eyes, kidneys and nerves. High blood glucose causes thickening of capillary walls. As a result the capillary walls become weaker and more permeable.

**Eye Complications—Diabetic Retinopathy:**
Retinopathy occurs in about 13% of diabetic patients after five years; 50% to 80% after ten to fifteen years respectively. Weakened defective capillaries, release of vasoproliferative factors and increased intraluminal pressure cause microaneurysms to form in the retina. Microaneurysms lead to increased vascular permeability and leaking of fluid and red cells, causing macular edema and intraretinal hemorrhages. This condition threatens central vision. The next stage of eye complications is formation of new, brittle blood vessels (neovascularization). Spontaneous bleeding from these vessels leads to vitreous hemorrhages, further impairing vision. Recently injection of Avastin into the vitreous cavity has been successful in decreasing the leakage and the proliferation of blood vessels. Avastin is an anti-vascular endothelium growth factor. Further disease includes retinal scarring and retinal detachment, eventually causing blindness.

**Kidney Damage—Diabetic Nephropathy**
Endothelial damage in the kidney leads to increased glomerular permeability to macromolecules. Further damage results in glomerular sclerosis. The first sign of kidney disease is hypertension, coincident with or shortly followed by microalbuminuria. Later...

### TABLE I. Correlation Between HbA1c and Mean Plasma Glucose

<table>
<thead>
<tr>
<th>HbA1c (%)</th>
<th>mg/dL</th>
<th>mmol/l</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>65</td>
<td>3.5</td>
</tr>
<tr>
<td>5</td>
<td>100</td>
<td>5.5</td>
</tr>
<tr>
<td>6</td>
<td>135</td>
<td>7.5</td>
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<tr>
<td>7</td>
<td>170</td>
<td>9.5</td>
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<tr>
<td>8</td>
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<td>9</td>
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<td>275</td>
<td>15.5</td>
</tr>
<tr>
<td>11</td>
<td>310</td>
<td>17.5</td>
</tr>
<tr>
<td>12</td>
<td>345</td>
<td>19.5</td>
</tr>
</tbody>
</table>
the kidneys lose their ability to cleanse and filter the blood, eventually requiring kidney dialysis or kidney transplant.

**Nerve Damage—Diabetic Neuropathy**
In diabetes the blood flow to the nerves is limited, leading to damage that includes demyelination. Weakness, burning, pain, and diminished sensation occur in the extremities. Eventually complete loss of sensation in the feet may lead to the patient’s being unaware of injuries. Because of poor circulation these injuries may not heal, become infected, and may lead to gangrene, which requires amputation. Nerve damage also affects the ability of men to get an erection. Diabetic neuropathy may affect nerves to the stomach and intestine, causing nausea, weight loss and diarrhea.

**Macrovascular Disease** affects the heart and larger blood vessels. Diabetes accelerates atherosclerosis, leading to coronary heart disease, strokes, and pain in the lower extremities due to decreased blood supply.

**VALUE OF DECREASING BLOOD GLUCOSE LEVELS**
HbA1c is a valuable tool used by the physician and patient to prevent chronic complications of diabetes. Studies have shown a 10% decrease in relative risk for microvascular disease for every 1% reduction in HbA1c. The Diabetes Control and Complications Trial (4) included 1,441 people with type 1 diabetes (formerly called insulin dependent diabetes mellitus or juvenile onset diabetes). Of these subjects half had no retinopathy, normal albumin excretion, and diabetes for less than five years. The other half had mild-to-moderate retinopathy with normal kidney tests or only microalbuminuria. The subjects were randomly divided into conventional or intensive therapy groups. The conventional treatment consisted of no more than two insulin injections a day with blood glucose monitoring twice a day. They were seen every two to three months. The intensive treatment group either had insulin pumps or three or more injections per day. Blood sugar was done three to four times a day. They were seen every month. The conventional group averaged an HbA1c of 9.1%; the intensive group averaged 7.2%. The intensive groups had 70% reduction of retinopathy, 60% less microalbuminuria, and 64% reduction in clinical neuropathy compared to the conventional therapy group. Other studies have corroborated these results.

**HBA1c TESTING METHODOLOGIES (5)**
Currently (July 2012) there are 140 certified commercial tests for HbA1c for use in the laboratory. They are in the following methodologies:

1. **Immunoassay:** An antibody to a specific antigen on a glycated hemoglobin molecule is used to detect the amount of HbA1c. An antibody to the glycated amino terminus of beta chains is one example.
2. **Cation or ion exchange high performance liquid chromatography (HPLC):** Phosphate buffers of increasing ionic strength are used for stepped elution of the hemoglobins, which are detected by absorbance at 415 and 690 nm, enabling calculation of the percentage of HbA1c in the sample.
3. **Boronate affinity chromatography:** The boronic acid reacts with the cis-diol groups of glucose bound to hemoglobin to form a reversible complex, thus selectively holding the glycated hemoglobin on the column. The non-glycated hemoglobin does not bind. Sorbitol is then added to dissociate the complex and elute the glycated hemoglobin. Absorbance of the bound and unbound fractions is used to calculate the percentage of glycated hemoglobin.
4. Enzymatic: Employs an enzyme that cleaves the N-terminal valine.

HEMOGLOBINOPATHIES—A CONFOUNDER OF HBA1c TESTING

Hemoglobinopathies are a group of diseases resulting from a defect in structure of the hemoglobin molecule. Over 700 hemoglobin variants have been discovered to date; most are the result of point mutations in the globin chains.

Hemoglobinopathies are of concern because the presence of some variants will affect the accuracy of HbA1c measurements. This concern is discussed below. The homozygous conditions of HbS, HbC, and HbD may cause severe anemias. HbA1c levels are affected by severe anemias. If patients with these hemoglobinopathies have significant anemia or decreased RBC life span, the HbA1c levels would be low. Therefore it is recommended that other tests be used to estimate glucose control in patients with homozygous HbS, HbC and HbD. Since RBC survival is normal in heterozygotes, HbA1c measurement can be used as long as the Hb variant does not interfere with the assay method or with glucose’s binding to hemoglobin.

There are 16 million diabetics in the United States, of which more than 150,000 have a hemoglobin variant. The most common variants are Hemoglobin S and Hemoglobin C (6). Table II shows interference of heterozygous variants of HbS, C, D, and E and elevated HbF with various HbA1c methods. In the United States HbAS is the most common variant, followed by HbAC, HbAE, and HbAD. In Blacks in the U.S. 10% have either HbAS or HbAC. Since the diabetic rate in Black men is 13% and in Black women is 16.3%, it is important for laboratories to keep in mind the possibility of interference of HbA1c test results in this population. HbE is found primarily in Southeast Asians and is now encountered frequently in the U.S. HbD is found most commonly in the Punjab region of India and occurs in the U.S.

Elevated HbF levels can occur in patients as a result of pathologic conditions (e.g., leukemia, anemia, thalassemia) or as hereditary persistence of fetal hemoglobin (up to 30%), usually asymptomatic and therefore undiagnosed (10).

RESULTS OF TESTING FOR HbA1c IN THE PRESENCE OF A HEMOGLOBINOPATHY

The four methods of testing for HbA1c and their results in the presence of a hemoglobinopathy appear below: Also see Table II, “Interference of Heterozygous Variants S, C, D, E, and Elevated HbF with specific HbA1c Methods”

1. Immunoassay: HbF, HbGraz, and HbRaleigh have been shown to decrease levels of HbA1c. Immunoassay tests use an antibody specific for the glycated amino terminus of β globin. When the amino terminus is altered by an amino acid substitution, the antibody may not recognize the altered structure (Hb Graz and Hb Raleigh). Fetal Hb (HbF) contains γ chains instead of β chains. The amino terminal end, being different from HbA, is not recognized by the antibody.

2. Cation Exchange Chromatography: Three situations exist. The first situation occurs when the native hemoglobin variant (non-glycated) co-elutes with HbA1c resulting in a gross overestimation of HbA1c values. These excess values may be as high as 54%. Examples include Hb Raleigh (beta 1 Val → Ala) and Hb Sherwood Forest (beta 104 Arg → Thr). The second situation occurs when the glycated hemoglobin variant co-elutes with HbA1c and the non-glycated hemoglobin variant is separated from HbA resulting in an overestimation of HbA1c, but to a lesser degree than the first situation. The third situation occurs when the hemoglobin variant co-elutes with HbA1, whereas the glycated hemoglobin variant separates from HbA1c. This results in an underestimation of HbA1c. Hb D is an example of a hemoglobin variant that causes this.
3. **Boronate affinity**: Boronate affinity has shown the least interference from hemoglobin variants.

4. **Enzymatic**: reported to be unaffected by HbS, HbC, HbD, HbSherwood Forest, HbGraz, HbDpadova.

**Case Study**

A 45-year-old Cambodian male with a five-year history of diabetes had been tested three times a year for HbA1c. The range of measurements by immunoassay technique varied between 5.5 and 6.6%. The laboratory initiated a cation exchange HPLC procedure. His HbA1c by the new method was 7.5%. The HbA1c on the same specimen by the old method was 6.6%. Other blood values on the patient were hematocrit = 40%, MCV = 78 fl, MCH = 25 pg. Because of these low values, the laboratory suspected a hemoglobinopathy. Electrophoresis of the patient’s blood sample revealed 18 % HbF. The cause of this HbF (α2γ2) elevation is most likely due to hereditary persistence of fetal hemoglobin (HPFH). HPFH can be due either to deletion of the δ or β globin genes on chromosome 11 or to point mutations in the promoter of one of the γ-globin genes.

**Discussion**: Immunoassay uses an antibody specific for the glycated amino terminal end of β chain. Since the antibody does not recognize the glycated amino terminal end of the γ chain, the HbA1c level is falsely low. HPLC method determines HbA1c levels by comparing the areas of the HbA1c and HbA peaks in the HPLC chromatogram. The glycated and non-glycated forms of HbF migrate differently from HbA1c and HbA so the assay is not affected by the presence of high levels of HbF. Using the falsely low results of the immunoassay for HbA1c meant that the patient’s diabetes had not been adequately treated or controlled.

**CONCLUSION AND RECOMMENDATIONS**

Based on the above discussion, it appears that boronate affinity chromatograph is the best method for accurately detecting HbA1c. However, there are limitations. Boronate affinity chromatography cannot detect that a variant hemoglobin is present (8). When a hemoglobinopathy is present, HbA1c testing results may not accurately reflect long-term glycemic control (6).

General recommendations regarding testing for HbA1c include evaluating samples with a glycated hemoglobin value of greater than 15%. This includes examining chromatographs manually and obtaining the clinical history of the patient (6). Samples with clinically silent hemoglobin variants should be analyzed by a second method with a different assay principle, preferably boronate affinity (9).
### TABLE II
Interference of Heterozygous Variants S, C, D, E, and Elevated HbF with Specific HbA1c Methods

<table>
<thead>
<tr>
<th>Manufacturer</th>
<th>Method</th>
<th>Interference from</th>
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<tbody>
<tr>
<td>Immunoassay</td>
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<tr>
<td>Abbott</td>
<td>Architect/Aeroset</td>
<td>Yes ↑</td>
<td>Yes ↑</td>
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<tr>
<td>Bayer (Metrika)</td>
<td>A1cNOW</td>
<td>Yes ↑</td>
<td>Yes ↑</td>
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<tr>
<td>Beckman</td>
<td>Synchron System</td>
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<td>No</td>
</tr>
<tr>
<td>Dade</td>
<td>Dimension</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Olympus</td>
<td>AU system</td>
<td>Yes ↑</td>
<td>Yes ↑</td>
</tr>
<tr>
<td>Ortho-Clinical</td>
<td>Vitros</td>
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<td>No</td>
</tr>
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<td>OmniScientific</td>
<td>Hba1c on Modular P</td>
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<tr>
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<td>Cobas Integra</td>
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<tr>
<td>Siemens (Bayer)</td>
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<td>No</td>
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<tr>
<td>Ion-exchange HPLC</td>
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<tr>
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<td>Variant II A1c</td>
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<td>Variant II Turbo A1c</td>
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<td>Yes ↑</td>
</tr>
<tr>
<td>Menarini</td>
<td>HA8140 (diabetes mode)</td>
<td>Yes ↑</td>
<td>No</td>
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<tr>
<td>Menarini</td>
<td>HA8160 (diabetes mode)</td>
<td>No</td>
<td>Yes ↑</td>
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<tr>
<td>Menarini</td>
<td>HA8160 (TP mode)</td>
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<tr>
<td>Tosoh</td>
<td>AIC 2.2 Plus</td>
<td>No</td>
<td>Yes ↓</td>
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<tr>
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<td>G7</td>
<td>No</td>
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<td>G8</td>
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<td>No</td>
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<tr>
<td>Boronate affinity</td>
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</tr>
<tr>
<td>Axis-Shield</td>
<td>Afinion</td>
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<tr>
<td>Prumus</td>
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<tr>
<td>Other</td>
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</tr>
<tr>
<td>Diazyme</td>
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</table>

a An ↑ or ↓ indicates an artificial increase or decrease in results.
b Not evaluated.
c HbF levels above 15% cause a clinically significant low bias.
d Offline manual recalculation must be performed if the HbF peak is mislabeled as labile HbA1c (LA1C).

From: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2769887/

### REFERENCES
REVIEW QUESTIONS
Course #DL-973
Choose the one best answer

1. HbA1c is used to
   a. monitor long-term blood glucose control in individuals with diabetes mellitus
   b. monitor the course of a hemoglobinopathy
   c. monitor an individual’s erythrocyte life span
   d. monitor long-term blood fructose control in individuals with a pathologic condition

2. HbA1c is produced when
   a. hemoglobin loses one of its globin chains
   b. glucose attaches to the N-terminal amino group of the beta chain of hemoglobin
   c. hemoglobin binds oxygen to its four heme groups
   d. there is a defect in the structure of the hemoglobin molecule

3. A patient has HbAE. Which of the following A1c methods include tests that interfere with the results?
   a. Immunoassay
   b. Boronate affinity
   c. Ion-exchange HPLC
   d. Enzymatic

4. In the U.S. the most common heterozygous Hb is
   a. HbAE
   b. HbAC
   c. HbAD
   d. HbAS

5. HbA1c immunoassay methods are inaccurate in which of the following hemoglobinopathies?
   a. HbGraz
   b. HbAE
   c. HbSherwood Forest
   d. HbAD

6. A patient has a Hemoglobin D hemoglobinopathy. Which of the following would not be an appropriate HbA1c test?
   a. boronate affinity
   b. cation exchange chromatography
   c. immunoassay
   d. electrospray mass spectrometry
7. The American Diabetes Association recommends that HbA1c be below
   a. 6.5%
   b. 7.5%
   c. 5.5%
   d. 7.0%

8. Microvascular diseases associated with long term increased glucose levels include all the following organs except
   a. kidney
   b. brain
   c. eye
   d. nerves

9. HbF is not recognized in immunoassay because
   a. there is an amino acid substitution at the amino terminal end of the beta globin
   b. the antibody is directed against the alpha chain terminus
   c. HbF has gamma chains instead of beta chains
   d. the folding of HbF hides the amino terminal end of the globin chain

10. Testing for HbA1c reflects the mean blood glucose for the previous
    a. two to three weeks
    b. 100 to 120 days
    c. six months
    d. two to three months