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TITLE: An update on Hemoglobin A1c analysis and potential interferences

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Hello, my name is Sydney Strickland. I am a Clinical Chemistry Fellow at the University of Virginia. Welcome to this Pearl of Laboratory Medicine on "**An update on Hemoglobin A1c analysis and potential interferences**."

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Ross Molinaro's Pearl on Hemoglobin A1c (Hb A1c) is an excellent source of background information on Hb A1c. I would recommend watching that pearl first as an introduction. I'd like to begin by briefly reviewing what Hb A1c is. Hb A1c is a glycation product of Hb A through a non-enzymatic addition of glucose to the amino group on the N-terminal valine of the beta chain. This glycation is regulated by the mean concentration of glucose within the blood and the amount of time during which Hb is exposed to glucose. Hb A1c is a snapshot of the glycemic control of roughly the past 3 months.

Clinicians use this information for screening patients for diabetes and monitoring glycemic control. Typically, an Hb A1c value greater than 6.5% is diagnostic of diabetes when observed on two occasions. This diagnostic cutoff was established from several large studies, including a 10-year follow-up study in which Hb A1c was measured at baseline and participants were monitored for the prevalence of diabetic retinopathy, nephropathy and neuropathy over that 10 year span. The data indicated that an Hb A1c value of greater than 6.5% was associated with a dramatic increase in the risk to develop diabetic retinopathy in the adult population. Interestingly, these values have not been validated in the pediatric population.

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There are several methodologies used across the country to analyze Hb A1c values. According to proficiency testing documents in 2016 31% of laboratories in the US were using ion exchange HPLC as their methodology for running Hb A1c analysis, while 1% were utilizing a relatively new methodology of capillary electrophoresis or CE. Immunoassays and POC devices still remain the predominant method for Hb A1c analysis by 56% of laboratories due to their high through-put capacity and low technician time required.

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Let’s take a closer look at these methodologies. First, let’s focus on the chromatographic methods. Capillary Electrophoresis or CE separates by both size and charge in a similar fashion to how proteins are separated on an agarose gel in serum protein electrophoresis. Once resolved, these peaks are quantified and the Hb A1c percent is calculated by taking the Hb A1c peak over the Hb A1c plus the Hb A peak. In contrast, the ion exchange HPLC methods separate predominately on charge differences between the Hb species. Once resolved, the Hb A1c percentage is quantified by taking the Hb A1c peak over “total Hb A” which can include Hb A, Hb A1a, Hb A1b, and occasionally Hb F, depending upon the vendor and methodology used.

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Here we have two examples of separation of Hb A1c from Hb A: an electropherogram generated from the Sebia CE software and a chromatogram generated from the Tosoh G8 software. As you can see both methods aim to resolve Hb A1c from Hb A0 in order to accurately quantify both species of Hb A. You can also see due to advances in harmonization, these two methods agree quite well with one another when the same patient sample is run on both as is the case here. Differences in quantification tend to arise when Hemoglobinopathies are present, which are seen on chromatograms or electropherograms as distinct peaks that are not normally present. Quite a few Hb variants are not resolved as distinct peaks by HPLC methods for measurement of Hb A1c methods; CE appears to resolve a very high proportion of the variants.

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As mentioned before, there are several other ways quantify Hb A1c. Boronate affinity chromatography works by utilizing boronate as the capture molecule, where it binds to the cis-diol groups of the glucose molecule. This isolates any glycated proteins, including Hb A1c, but can also include glycated albumin for example and other proteins. Chromatography is then used to resolve the two fractions and Hb peaks are identified spectrophotometrically. The peak ratios are used to estimate the ratio of glycated Hb to total Hb. Although the method measures total glycated Hb (including glycated variants, such as Hb S, if present), results are reported as the expected %Hb A1c based on a derivation equation originally introduced in the late 1980s and early 1990s, prior to some significant work in regards to interferences within Hb A1c testing.

Immunoassays, on the other hand, utilize two different methods to quantify Hb A1c and total Hb. The first method uses antibodies specific for the β-chain site of glycation to bind Hb A1c and that fraction is quantified through a turbidimetric method where the more Hb A1c that is bound, the more antibody precipitates and the less light is allowed through. The total concentration of Hb is measured by a spectrophotometric method similar to that used for the quantification of Hb on a CBC analyzer. The ratio of these two numbers is taken to quantify a value that is then plotted using linear regression to identify the Hb A1c % as a total of Hb A. Using two methods, immunoassay and spectrophotometry, to quantify Hb A1c % results in an additive effect of the total error and can lead to an increase in bias and therefore a decrease in the assays overall precision.

Unlike the chromatographic and CE methods, these two methods –boronate-affinity and immunoassays-- do not detect Hb variants that may be present within the sample (unless the point mutation is near the glycation site and thus changes the antibodies interaction, for example). Further studies are needed to ascertain if a bias exists within the quantification of Hb A1c in the presence of hemoglobinopathies by boronate affinity. Both of these methods often will report a value even when the chromatographic methods will not; however, the accuracy of these reported values is questionable in the cases where the Hb A1c values could be falsely low due to increased red cell turnover, as is the case in sickle cell disease and sickle cell trait patients. For more information on this, please refer to Strickland, et al (2018).

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A third alternative method to quantify Hb A1c is through an enzymatic process. The premise behind the Abbot system is to quantify the Hb and Hb A1c separately and then take a ratio (in a similar fashion to the measurement procedure of the immunoassay methods). However, in the enzymatic method Hb is measured spectrophotometrically as Met-Hb, which is produced during the assay pretreatment with an oxidizing agent. The second step in the assay is to use a protease to cleave the peptide containing the glycated site from the N-terminus of the β-chain with fructosyl peptide protease. This peptide is then further processed by treatment with fructosyl peptide oxidase producing hydrogen peroxide (H_2O_2). The H_2O_2 reacts with a color reagent to form a color change that can be quantified. This methodology is advantageous as it is high throughput and can be incorporated onto an automated analyzer; however, similar to immunoassays, utilization of both an enzymatic method and a spectrophotometric method to generate a final Hb A1c % will decrease the precision of the assay.

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Immunoassays are the predominant approach used in POC devices; however one boronate affinity method is currently seeking FDA approval. Current guidelines state that a diagnosis of diabetes cannot be made by POC Hb A1c devices, due predominately to the lack of good QC material. To rectify this, laboratories that manage POC programs and clinical offices utilizing POC devices should instate certified non-manufacturer quality assurance programs (QAP) in order to optimize the performance of their analyzers. Recent data has shown that POC devices such as the Afinion and DCA models are capable of meeting QAP goals for trueness and imprecision when QC is utilized as it would be in a laboratory setting. Monitoring of known diabetic patients without Hb variants can be done with POC devices once a diagnosis has been established.

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What can influence the Hb A1c percentages reported from any of these methods? First, the survival of the red blood cells. If the RBCs are in circulation for less time, for example in sickle cell disease in which they have increased turnover, the RBCs are not exposed to the glucose in circulation for the normal amount of time. For example, the red line in the graph represents RBCs that stay in circulation for the normal 120 days. In this particular glucose environment, they reach an Hb A1c percentage of 16%. The blue line represents cells that remain in circulation for half that amount of time, 60 days; in the same glucose environment, they will only reach an Hb A1c percentage of 8%. Those are drastically different numbers and are interpreted very differently by clinicians. Therefore, it is very important that clinicians are aware when there are potential causes of increased RBC turnover when interpreting reported Hb A1c values in their patients.

Other factors that can change the Hb A1c value include iron deficiency anemia and vitamin B12 deficiency which can both lead to increased Hb A1c percentages.

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Some of these variants are very common like Hb C, D, E, F and S and have been studied extensively. Most of the major manufacturers of Hb A1c chromatographic methodologies have taken these variants into account when designing their assays and can typically report a Hb A1c percentage even in the presence of these common variants. However, resolving the peaks is only half of the problem; it does not remove the effect these Hb variants have on Hb A1c in vivo. For example, if the Hb variant is less stable, that will influence the RBC half-life and can influence the overall Hb A1c. In the immunochemical, boronate, and enzymatic methods, we are also assuming that all the Hb variants glycate at the same rate as Hb A, but we don’t actually know that. Some recent literature suggests that there are in fact differences in the degrees of glycation of Hb S, which could lead to confounding problems for assays that don’t discriminate the glycated versions of Hb A and variant Hbs.

Of the more rare Hb variants, there are currently over 1,000 that have been classified, often named by the city or region in which the initial case or family was identified.

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In most cases, the Hb A1c value reported by any of the analyzer methods will be clinically useful. However, as we have discussed there are certain scenarios where this is not the case. In those situations, other tests can be utilized to monitor the glycemic control in patients with diabetes. The first alternative analyte is fructosamine, which is a measurement of other glycated proteins within the plasma, most of which is glycated albumin. While Hb A1c looks at a snapshot of 3 months, fructosamine's window is shorter at only 3 weeks. The disadvantages of using fructosamine as a marker of glycemic control is that interferences, such as fluctuations of immunoglobulins (particularly IgA) and high concentrations of reducing substances (bilirubin, vitamins, etc.), as well as, pre-analytical variables like temperature changes can greatly influence the fructosamine value.

The second alternative analyte is 1,5-anhydroglucitol, marketed as GlycoMark. This analyte assess the glycemic control over the previous 2-14 days. 1,5-anhydroglucitol is freely filtered by the kidney and completely reabsorbed by the renal tubule in healthy patients (without diabetes or kidney disease). In patients with diabetes, when glucose levels exceed 180 mg/dL for extended periods of time, the glucose outcompetes the 1,5-anhydroglucitol at the receptor in the renal tubule to be reabsorbed and thus excessive 1,5-anhydroglucitol is excreted in the urine. In patients with diabetes this marker will be low with poor glycemic control, indicating excess days of plasma glucose levels exceeding 180 mg/dL. One disadvantage of this assay as a marker of hyperglycemia is that it cannot be used in patients with kidney disease (CKD 4/5) as the levels could be falsely low due to poor reabsorption function of the kidney.

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The third alternative analyte is glycated albumin (GA), which is ten times more sensitive to glycation than Hb. GA assesses glycemic control over a 2-3 week timespan and is based upon the half-life of albumin, which is 2 weeks. In a Japanese population study, GA correlated well with Hb A1c values for the diagnosis of diabetes when compared across 1,575 individuals. Previous GA methods used in the US were subject to interferences and most institutions no longer run the assays as Hb A1c and fructosamine have become more prevalent; however, a recent enzymatic assay from a Japanese company has been developed and shows high accuracy, less interference from bilirubin and other endogenous compounds, and is standardized.

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To conclude, there are five main approaches to quantification of Hb A1c: ion-exchange HPLC, CE, boronate-affinity chromatography, immunoassay, and enzymatic). Each of these methods has its advantages and disadvantages and arguments can be made for the proper place for each methodology.

There is currently no consensus on how to calculate Hb A1c and thus different methods utilize different numbers in order to quantify the Hb A1c percentage. However, harmonization has helped to alleviate several of these differences within the normal population. Within the hemoglobinopathy populations, issues still arise depending upon which species of Hb are used to calculate Hb A1c.

Hb A1c is still one of the most helpful tools that clinicians have in order to monitor glycemic control of their patients as it is proportional to the negative outcomes of diabetes such as diabetic retinopathy.

Slide 14: References

Slide 15: Disclosures

Slide 16: Thank You from www.TraineeCouncil.org

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