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CAT
Critically Appraised Topic

Clinical and analytical interferences with HbA1c assays.

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CLINICAL BOTTOM LINE

Diabetes mellitus is and continues to be a major global health threat with hundreds of million people affected, contributing enormously to worldwide morbidity, mortality and healthcare costs. Correct measurement and interpretation of HbA1c values is critical in diagnosing and managing the disease. Physicians and laboratory professionals should be aware of several factors that can cause problems in correctly measuring and interpreting HbA1c values. Possible analytical interferences include, depending on the method used, hemoglobin variants, elevated fetal hemoglobin, severe hypertriglyceridemia and hyperbilirubinemia. There are also several clinical situations to consider where HbA1c is not a true reflection of glycemic control, including altered red blood cell life span, chronic kidney and liver disease. We suggest a workflow in our lab to help physicians detect increased red blood cell turnover and aid in interpretation of HbA1c in this situation by adding hemoglobin and reticulocyte measurements. We conclude with a retrospective comparison of HbA1c measurements between Tosoh G8 and Alere Afinion in patients with some common hemoglobin variants (HbAS, HbAC, HbAE, HbAD, Hb Riccarton).

CLINICAL/DIAGNOSTIC SCENARIO

Introduction

Diabetes mellitus is and continues to be a major global health threat (1). It is estimated by the IDF (International Diabetes Federation) that 1 in 11 adults aged 20-79 years had diabetes mellitus globally in 2015, which equates to 415 million adults. This is a staggering amount of individuals and this estimate is projected to rise to 642 million by 2040. Over 90% of these cases are type 2 diabetes mellitus (T2DM) (2). The causes of the rising epidemic of diabetes mellitus are multifaceted: aging population, economic development, urbanization, unhealthy eating habits and a sedentary lifestyle. Diabetes mellitus and its long term complications have contributed tremendously to worldwide morbidity and mortality. In addition it bears a major economic burden with an estimated cost of $327 billion in the US in 2017 (3).

Glycosylated hemoglobin, expressed as hemoglobin A1c (HbA1c), is a biochemical marker that is indispensable in the management of diabetes mellitus. This marker allows to monitor longer-term glycemic control (4,5). The Diabetes Control and Complications Trial (DCCT) demonstrated a strong linear correlation between mean glucose and HbA1c values and has conclusively demonstrated that the risk of complications is directly related to glycemic control as measured by HbA1c (4,6).
Because of the improving quality of the test, the HbA1c concentration is also being increasingly applied in the diagnosis of diabetes (7). In 2011 the WHO recommended that "HbA1c can be used as a diagnostic test for diabetes providing that stringent quality assurance tests are in place and assays are standardized to criteria aligned to the international reference values, and there are no conditions present that preclude its accurate measurement" (8).
Correct diagnosis and follow up of diabetes mellitus is thus paramount in reducing worldwide morbidity, mortality and healthcare costs. For laboratory professionals and clinicians alike, knowledge of analytical and clinical influences on HbA1c values is therefore indispensable.

What is Hemoglobin A1c?

Hemoglobin (Hb) consists out of four globin chains. There are three major types of hemoglobin in red blood cells. Adult hemoglobin (HbA) is the most abundant form in most adults and consists of two alpha and two beta globin chains. HbA2 is a minor form of Hb and consists of two alpha and two delta chains. Fetal hemoglobin (HbF) consists of two alfa and two gamma chains.

HbA1c is defined as post-translational irreversibly glycated hemoglobin at one or both N-terminal valines of the beta chains. This does not exclude hemoglobin which is additionally glycated at other sites on the alpha or beta chains (9). HbA1c is formed by a non-enzymatic glycation pathway when hemoglobin is exposed to plasma glucose. The level of HbA1c is directly proportional to the length of exposure and the plasma glucose concentration. The

reaction begins with the condensation of a free primary amine group from hemoglobin with a carbonyl group of glucose, resulting in the formation of a Schiff base. This base is not stable and may dissociate back or undergo Amadori rearrangement to form a stable ketoamine (Figure 1).

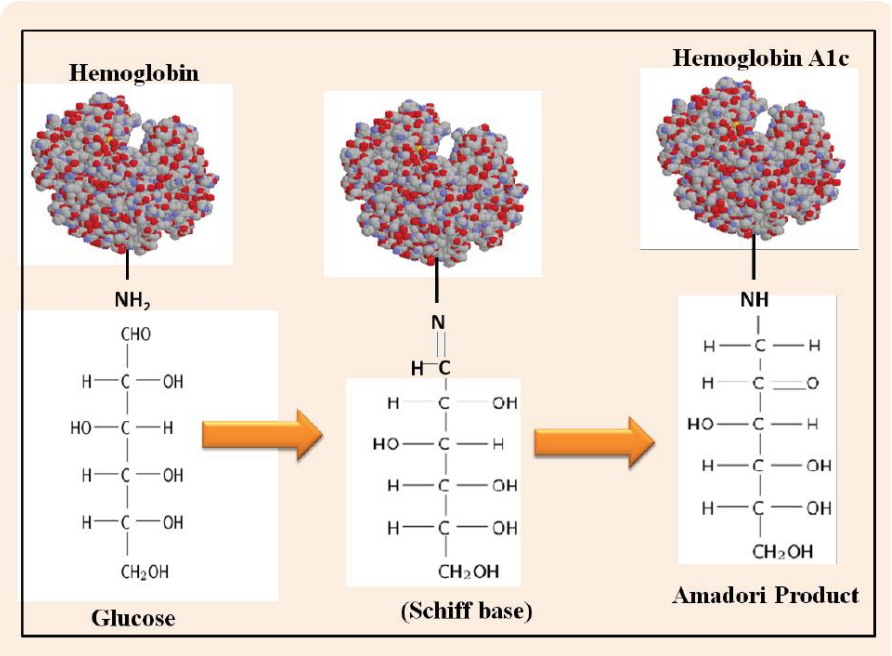


Figure 1: non-enzymatic glycation pathway of the beta chain of HbA (10).

A few other terms are used to describe hemoglobin that has reacted with glucose and terminology can sometimes get confusing for the layman. Glycohemoglobine (GHb) is a general term for glucose bound nonenzymatically to hemoglobin with a ketoamine structure. HbA1c constitutes the major portion of these glycated hemoglobins. Total GHb is a term describing the sum of HbA1c and Hb glycated with glucose at other sites, including the glycated N terminus of the α chain and the glycated ε amino groups of lysine residues. It is used in the context of affinity chromatographic methods, which can measure this total GHb (11).
HbA1 is a historical term used to describe GHb species that are more negatively charged forms of HbA detected by cation-exchange chromatographic and electrophoresis methods. These minor components were named HbAla, HbAlb, and HbAlc in order of their elution from the column (also named 'fast' hemoglobins).

Once hemoglobin is glycated it remains in this state for the rest of the red blood cell life-span (120 days average). The HbA1c concentration is therefore a reflection of the average glucose concentration in the preceding 2-3 months (6). However it is to be noted that HbA1c is a 'weighted' average, meaning that glucose levels of the preceding 30 days contribute substantially more to the level of HbA1c than do glucose levels 90-120 days earlier. This explains why HbA1c levels can increase or decrease fairly rapidly and you don't have to wait 120 days to see the effect of intensifying treatment or worsening glycemic control.

Different values are recommended for normal reference range, for diagnosis and limits during treatment (Table 1).

Standard interpretation norm*			IFCC (mmol/mol)	NGSP (%)
Normal reference range			20-42	4-6
Decision limits	Monitoring therapy	Target treatment	53	7
		Limit change therapy	64	8
	Diagnosis	Low risk	<40	<5.8
		Increasing risk future diabetes	40-46	5.8-6.4
		Diabetes	>46	>6.4

Table 1: normal reference range, diagnosis and treatment targets for HbA1c in both IFCC and NGSP units (12).

Standardization of HbA1c measurements

The National Glycohemoglobin Standardization Program (NGSP) was established in 1993 to standardize HbA1c results so that clinical laboratory results are comparable to those reported by the DCCT (Diabetes Control and Complications Trial). Up until then, HbA1c methods had not been standardized among laboratories, which was a problem for managing diabetes patients. Without standardization to the DCCT values, HbA1c values were not related to clinical outcomes. NGSP/DCCT HbA1c values are reported as a percentage of total hemoglobin.

The International Federation of Clinical Chemistry and Laboratory Medicine (IFCC) was established in 1995 to achieve a uniform international standardization. It has developed a reference system for HbA1c which ensures traceability to a higher order reference method. All manufacturers of HbA1c analyzers/kits calibrate their methods against this IFCC-reference method. It is important to note that IFCC units are reported in mmol/mol while NGSP units are reported in %.
The relationship between the NGSP network and IFCC networks was evaluated and a master equation was developed to document this relationship. In 2007, the IFCC recommended that IFCC HbA1c be expressed as mmol HbA1c/mol Hb. With these new units, the master equation equates to NGSP = [0.09148 * IFCC] + 2.152 or IFCC = (10.93 * NGSP) - 23.50. This change in units avoids any confusion between NGSP and IFCC results.
The master equation links IFCC results to clinically meaningful HbA1c results from the DCCT and also provides the NGSP with traceability to a higher order reference method.

There are 2 main reasons why it is critical to standardize HbA1c measurements. Firstly patients with diabetes mellitus are monitored with HbA1c over long periods of time, often years or decades. This means it is very important for them for the HbA1c to be comparable between different labs. Secondly, because HbA1c has a narrow target range for monitoring treatment and a set cut-off for diagnosis, an inaccurate measurement will have impact on the treatment and diagnosis of a patient. Therefore it is essential that this parameter meets certain quality requirements:

- Good reproducibility (see Table 2)
- Standardization to the IFCC reference method.

Parameter	Excellent	Goed	Acceptabel	Slecht	Onacceptabel
Afwijking Doelwaarde	<0.2%	0.2 - 0.29%	0.30 - 0.39%	0.40 - 0.49%	≥ 0.50%
Reproduceerbaar-heid (CV)*	<1.4%	1.4 – 1.99%	2.0 – 2.99%	3.0 – 3.99%	≥ 4%
Lineariteit (r)	>0.9970	0.9950-0.9970	0.9900- 0.9949	0.9800-0.9899	<0.9800

Table 2 : Sciensano criteria for EKE evaluation concerning accuracy, CV and linearity (13)

The current practice in most labs in Belgium is to report the HbA1c results in both units: mmol/mol (IFCC) and the derived % (NGSP).

Methods of HbA1c measurements

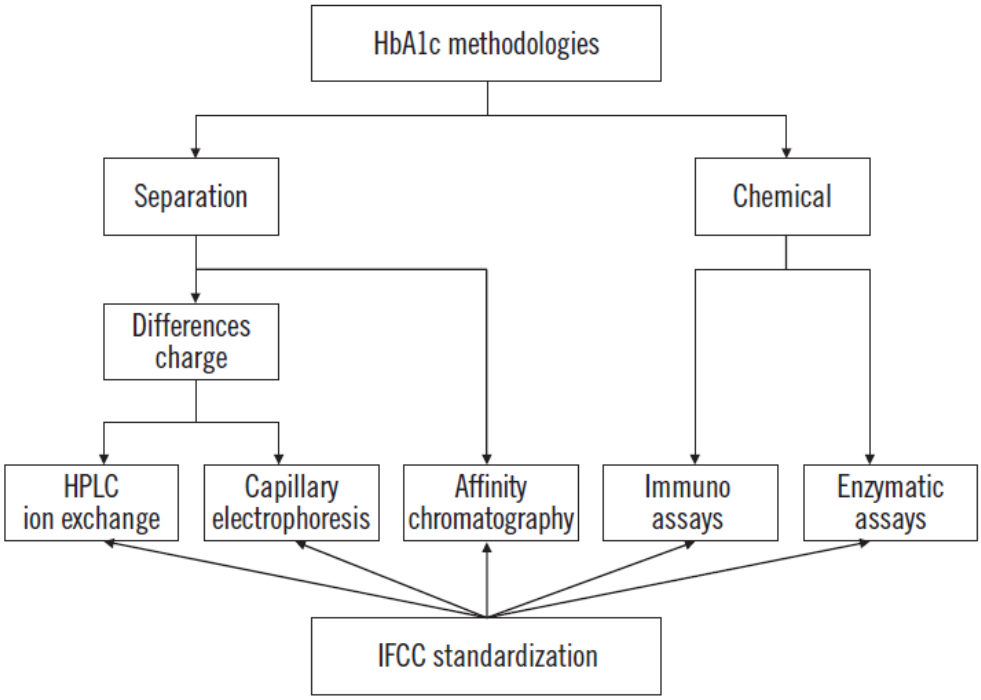


Figure 2: an overview of the different measuring principles for HbA1c measurements. (12)

Several laboratory methods have been developed for measuring HbA1c (figure 2). The two main analytical types rely on the separation of hemoglobin in different fractions (separation methods) or on the difference in chemical structure (chemical methods).
Although different analytes are measured with these methods, assays are standardized according to the Reference Measurement Procedure (RMP) of the IFCC.

Separation methods
Glycated and non-glycated hemoglobin have different chemical properties which allows them to be separated in different fractions.

HPLC (High-performance Liquid Chromatography) ion exchange methods use the difference in polarity to separate the different fractions over a column. The HbA1c measurement is based on the ratio of the HbA1c peak area to the total hemoglobin peak areas. With this technique HbF, carbamylated Hb and genetic Hb variants can be visualized.
Capillary electrophoresis uses the difference in charges to separate the Hb fractions using a high-voltage electrical field and electroosmotic flow (14).
Boronate Affinity Chromatography uses a column packed with particles coated with boronic acid. Glycated hemoglobin molecules have an affinity to boronic acid through their cis-diol bindings formed by stable glucose attachment to Hb. This method measures total glycated GHB, both the HbA1c (glucose bound to the N-terminal valine of the beta-chain) and Hb glycated at other sites. Since only two fractions are present in these methods (glycated and non-glycated), the glycated portion is compared to the total Hb. As these other glycated Hb are formed proportionally to HbA1c, calibration enables the results to be expressed in terms of HbA1c (15). This method is known to show the least interference due to the presence of Hb variants and derivatives (16).

Chemical methods
The chemical HbA1c methods are based on a specific reaction with glucose bound to the N-terminal valine of the beta chain. Total Hb is measured in parallel with photometry to calculate the HbA1c level.

In immunoassays, antibodies recognize the structure of the N-terminal glycosylated valine on the β-chain of the Hb. An excess of antibodies is added to a hemolyzed sample and after binding to HbA1c, the excess antibodies agglutinate. The turbidity of the resulting immunocomplexes is measured photometrically using a turbidimeter or nephelometer (17).
The currently available enzymatic method measures HbA1c using an enzyme that specifically cleaves the N-terminal valine. This protease cleaves the beta-chain to liberate peptides. Peptides, usually dipeptides, react with fructosyl peptide oxidase, and the resulting hydrogen peroxide is used to quantify HbA1c (18).

Alternative biochemical markers of long term glucose control

Glycated albumin (GA) and fructosamine

Albumin is the most abundant protein in our plasma, accounting for 60-70% of plasma proteins. Because of its high sensitivity to glycation this protein can also be used as a biomarker for hyperglycemia (19). The term fructosamine typically refers to all ketoamine linkages that result from glycation of serum proteins. Because albumin is the most abundant of serum protein, fructosamine is predominantly a measure of GA. Although other circulating proteins such as glycated lipoproteins and glycated globulins may contribute to determine the total concentration of fructosamine. Both fructosamine and glycated albumin levels increase in states of abnormally high glucose concentrations such as diabetes. Because non immunoglobin serum proteins have a much shorter half-life (14-21 days) than hemoglobin (90-120 days in red blood cells), they provide information on glycemic control of a short to intermediate time frame (2 weeks). Another major difference is that the rate of glycation is 9- to 10-fold higher than in hemoglobin (20).

The measurement of glycated albumin and fructosamine seems useful as an alternative index of glycemic control in conditions where HbA1c measurement is unreliable, as we will discuss later.

Estimated HbA1c

With the increasing use of continuous glucose monitoring (CGM) systems in patients with type 1 diabetes mellitus (e.g. Abbott's Freestyle Libre, Roche's Dexcom), it is important to understand how CGM metrics, namely estimated A1c (eA1c), correlates with and can be used as a metric instead of or in addition to HbA1c. An approximate HbA1c level based on the average CGM-measured glucose levels is calculated based on CGM readings from a population of individuals (21). However, randomized trials are needed to examine the relationship between these new glycemic metrics and hard endpoints, such as retinopathy, nephropathy or cardiovascular outcomes. With these GCM systems other derived parameters like 'time in range' are becoming more important to help clinicians personalize diabetes management.

In some patients, the results of a laboratory-measured HbA1c and eA1c are approximately the same, but in others, the eA1c may be either higher or lower than the measured HbA1c. It is important to investigate discrepancies further as they may be a sign of analytical/clinical interferences with the HbA1c measurement, as will be discussed later.

QUESTION(S)

1) *What are important clinical and analytical influences that should be taken into account when interpreting HbA1c values?*
2) *Can reticulocytes help interpret HbA1c values? Should reticulocytes always be determined when determining HbA1c?*
3) *What is the analytic influence of some common hemoglobin variants on the HbA1c values determined with Tosoh G8 in UZ Leuven? What is the preferred method for follow up for each variant?*

SEARCH TERMS

1) *MeSH Database (PubMed): MeSH term: "hba1c reticulocyte, hba1c review, hba1c hemoglobin variants impact, hba1c interferenc, hba1c interpretation, hba1c anemia, hba1c kidney"*
2) *PubMed Clinical Queries (from 1966; http://www.ncbi.nlm.nih.gov/entrez/query.fcgi): Systematic Reviews; Clinical Queries using Research Methodology Filters (diagnosis + specific, diagnosis + sensitive, prognosis + specific)*
3) *Pubmed (Medline; from 1966), SUMSearch (http://sumsearch.uthscsa.edu/), The National Institute for Clinical Excellence (http://www.nice.org.uk/), Cochrane (http://www.update-software.com/cochrane)*
4) *International organizations: e.g. National Committee for Clinical Laboratory Standards (NCCLS; http://www.nccls.org/), International Federation of Clinical Chemistry (IFCC; http://www.ifcc.org/ifcc.asp), American Diabetes Association (ADA; http://www.diabetes.org/home.jsp)*
5) *UpToDate Online*

RELEVANT EVIDENCE/REFERENCES

1) *Guidelines and Recommendations (most recent topics on top))*
2) *Systematic Reviews and Meta-analyses*
3) *Reviews*
4) *Original Articles*
5) Reference Works, Handbooks and Databases
6) *Posters, "grey literature", presentations*

See "bibliography"

1. What are important clinical and analytical influences that should be taken into account when interpreting HbA1c values?

Analytical factors that Interfere with HbA1c Measurement

Analytical Interferences							
Method	**Manufacturer/ Analyzer**	**HbAS**	**HbAC**	**HbAE**	**HbAD**	**HbF**	**CarbHb**
Ion-exchange HPLC	Tosoh G8 Bio-Rad Variant II Menarini ADAMS A1c	No	No	No*	No	No <30%	No
Boronate Affinity	Alere Afinion Trinity Hb9210	No	No	No	No	No <15%	No
Immuno-assays	Roche Cobas c513	No	No	No	No	No<10-15%	-
Immuno-assays	Siemens DCA 2000	No	Yes↑	Yes↑	No	No<10%	No
Enzymatic-assays	Abbot Architect c Diazyme Direct enzymatic	No	No	No	No	-	-
Electrophoresis	Sebia Capillarys 2	No	No	No	No	No<15%	No

*no interference as of software version 5.24 on Tosoh G8 (22)
**Generally assumed interference when HbF >10-15%, causes a clinical significant low bias (23)
- not yet evaluated
Table 3: an overview of analytical factors that interfere with HbA1c measurement and its impact (based on ngsp.org)

Genetic Hemoglobin variants
The most common Hb variants worldwide in descending order of prevalence are HbS, HbE, HbC, and HbD. The presence of any of these four variants affects the ionic charge of the Hb molecule, which may cause interference with ion-exchange methods, depending on how well the variant Hb is separated from HbA. Since boronate affinity chromatography separates total glycated Hb from nonglycated hemoglobin, regardless of the Hb species, there is generally no interference from most Hb variants, including HbS, C, E, and D (24).
Because the HbS and HbC variants are close to the N terminus on the β chain, some (but not all) immunoassays are affected by the presence of these variants. The presence of HbE or HbD, however, with mutations much further away on the β chain, generally does not affect immunoassay methods.
Special care has to be taken with measuring HbA1c in patients with HbAE on HPLC analyzers. Historically HPLC-analyzers have had a hard time with the HbE peak on chromatograms, which led to falsely lowering HbA1c results. Recent software updates on these analyzers has alleviated most of this problem and recent studies confirm that modern HPLC-analyzers can be used to correctly measure HbA1c in patients with HbAE (22).
Because erythrocyte survival is normal in people with heterozygous variants, HbA1c can be used as long as the Hb variant does not interfere with the test method itself (for a summary of published studies see http://www.ngsp.org) (25).
In homozygous HbSS, HbCC, HbDD, or HbEE, it is recommended that other tests be used to estimate glycemic control (fructosamine, glycated albumin) because of reduced erythrocyte lifespan (see clinical factors). In compound heterozygotes it depends on the erythrocyte lifespan. In Hb C disease (HbSC), anemia is milder than in sickle cell disease (HbCC) but glycated albumin or fructosamine should still be used instead of HbA1c (26).
Besides these common variants there are thousands more much rarer variants and each variant has the potential to interfere with HbA1c measurement depending on its chemical properties and method used. If a rare variant is identified with separation based methods/genetics, a literature study should be done on how the variant impacts HbA1c measurements with the method in question. An advantage of using separation methods is that these variants can be identified on the chromatogram. Especially in patient groups with a high prevalence of hemoglobin variants this is important. With immune- or enzymatic essays, there is no way of picking up a variant and thus you do not know if the HbA1c result is impacted unless the value is extremely high or low.

Elevated fetal hemoglobin (HbF)
In the past, many of the ion-exchange column methods and electrophoresis methods could not separate HbF from HbA1c; HbF comigrated or coeluted with HbA1c, which caused a falsely low value. Most of the current ion-exchange HPLC methods separate normal levels of HbF into a separate peak. Some of these methods can also separate HbF when HbF levels are elevated. Still most methods are impacted when HbF levels are too high, generally above 15-30% which for example occurs in patients with hereditary persistence of fetal hemoglobin (HPFH), very young patients or patients treated with hydroxyurea.

Chemically modified derivatives of hemoglobin
Carbamylated Hb (carbHb) is formed by non-enzymatic condensation of cyanate with the N-terminal valine of hemoglobin. In chronic renal failure carbHb is increased due to abnormal urea concentration. This urea dissociates to yield cyanate ions. Some reports suggested HbA1c methods, especially those based on charge separation (e.g., ion-exchange HPLC), may have interference from carbHb that would be expected to falsely increase HbA1c results, but many of these methods are no longer in use. Subsequent reports evaluated newer ion-exchange HPLC assay methods which showed an improved separation of the HbA1c fraction from other hemoglobin adducts and therefore did not show interference from carbHb (27).

Serum indices
Severe hypertriglyceridemia and severe hyperbilirubinemia may also interfere with HbA1c measurement with immuno- and enzymatic essays (28–30). For example very high levels of triglycerides and cholesterol (≥ 15.28 and ≥ 8.72 mmol/L, respectively) led to falsely low HbA1c concentrations on Roche c501 (30). Separation methods are not impacted.

Clinical factors that affect interpretation of HbA1c Results

Clinical Interferences						
	Iron deficiency	**Shortened Erythrocyte Survival**	**Increased Erythrocyte Survival**	**Chronic Kidney Disease**	**Age**	**Liver Disease**
Influence on HbA1c	Increase	Decrease	Increase	Variable	Increase	Decrease

Table 4: An overview of clinical factors that interfere with HbA1c measurement and their impact

Shortened erythrocyte survival
Any clinical situation that increases erythrocyte turnover or enriches the erythrocyte pool with younger cells (hemolytic anemia, recovery from acute blood loss, recent transfusion) will lower the level of HbA1c for any given level of average blood glucose regardless of the assay method used (11,26,31,32).

Increased erythrocyte survival
Splenectomy or hypersplenism will increase mean erythrocyte age. Hemoglobin molecules are exposed to blood glucose for longer and thus falsely elevate HbA1c test results regardless of the method used (31).

Iron deficiency anemia (IDA)
A major public health problem both in developing and developed countries, iron deficiency anemia is associated with higher HbA1c and fructosamine levels (32–34). Son et al demonstrated that IDA in the patients with prediabetes defined by glucose levels had a mean HbA1c of 6.4% vs 6.1% (46 mmol/mol vs 43 mmol/mol) in controls (35). This is sufficient to re-categorise some patients from prediabetes to diabetes. Consistent with these observations, iron replacement therapy lowers both HbA1c and fructosamine concentrations in diabetic and non-diabetic individuals. HbA1c, but not glycated albumin, is increased in late pregnancy in nondiabetic individuals owing to iron deficiency. Insight into the mechanism was recently obtained by the observation that malondialdehyde, which is increased in patients with iron deficiency anemia, enhances the glycation of hemoglobin. Alternative measures of glycemic assessment (e.g., glucose monitoring) must be used in the presence of significant iron deficiency anemia, at least until the iron deficiency has been successfully treated. Iron status must be considered during the interpretation of the HbA1c concentrations in Diabetes mellitus. Iron replacement therapy is thus especially important in diabetic patients with iron deficiency, as it would also increase the reliability of the HbA1c determinations (36).

Chronic renal failure
Renal failure develops in many diabetic patients. The role of glycemic control and the value of HbA1c in diabetic subjects with renal disease are controversial. While interference from carbamylated Hb can be evaluated, the role of renal anemia, erythropoietin intake, and other factors in chronic renal failure is more difficult to evaluate.

Several studies propose the use of glycated albumin (GA) measurement in place of HbA1c as a more accurate assessment of glycemic control in patients with renal disease. One study showed that GA was a better predictor of risk of death and hospitalization in these patients, compared to HbA1c (37). Serum GA levels were also shown to be better correlated with average glucose than HbA1c (27,38).
Recent reports suggest HbA1c underestimates glycemic control in diabetic patients on dialysis and that glycated albumin is a more robust indicator of glycemic control. Further studies are needed to clarify the role of HbA1c in diabetic patients with chronic renal failure (39).

Liver disease
Chronic liver disease may also cause a false, low-level of HbA1c. In the presence of cirrhosis, and particularly decompensated cirrhosis HbA1c is not an accurate representation of chronic glycemia (40). Lower HbA1c for corresponding glucose levels has also been reported even among patients with chronic liver disease even in the absence of cirrhosis (41,42). The mechanisms responsible are still uncertain but several types of anemia (macrocytic, hemolytic anemia) and hypersplenism commonly occurring in liver disease probably play a role (43,44). In patients with liver disease HbA1c levels should probably only be evaluated in context with all liver function parameters as well as red blood cell parameters. Further investigations are needed to clarify the exact cause of the interference in patients with liver disease .

Age and race
The impact of age and race is currently under discussion. Some studies show that the HbA1c concentration increases by approximately 1 mmol/mol (0.1%) per decade. Other studies suggest that the HbA1c concentration is higher in US African Americans and Hispanic populations than in Caucasians, but there are no definite conclusions. It is also unclear whether this would have clinical significance. Although it is assumed that reference ranges and decision limits for Asians are similar to those for Caucasians, this has not been confirmed in reliable epidemiological studies in Indo-Asian and Sino-Asian populations (12).

Other factors
Ingestion of large amounts of vitamins C or E (e.g., > 1 g/day) also has been reported to lower GHb values, perhaps by blocking glycation (45,46).

2. Can reticulocytes help interpret HbA1c values and should they routinely be determined?

Following our literature review it was obvious that a high red blood cell turnover would provide a false interpretation of the HbA1c result. A physician might not always know if his patient suffers from this condition. We used reticulocytosis as a proxy for red blood cell turnover and also correlated hemoglobin levels with reticulocyte levels in order to determine if a HbA1c order should be co-ordered with reticulocytes/hemoglobin.

We performed a query in our LIS system LWS to investigate how many HbA1c assays were determined together with a reticulocyte count with a time range of 3 months (01-09-2021 to 10-01-2022). Of the 11617 HbA1c assays performed 340 included a reticulocyte count. This means that during this period only 2.9% of all HbA1c requests were accompanied by a reticulocyte count.

As demonstrated in figure 3, rectangle 1 values of HbA1c (higher than 12%) don't seem to occur when there is a reticulocytosis higher than approximately 2%. With reticulocytosis higher than 4%, HbA1c values higher than 8% also rarely occur as we can see in rectangle 2. Rectangle 3 shows that for reticulocyte counts under 2% (normal range), HbA1c values higher than 8 and 12% occur more frequently than in the higher reticulocyte groups. Note that there already is a trend of lower HbA1c values with a reticulocyte count between 2 and 4%. Finally in rectangle 4 we see that HbA1c values lower than 4% are often accompanied by high reticulocyte counts.
This data suggests that we cannot trust the HbA1c % to be a true reflection of glycemic control in cases where reticulocytosis is markedly elevated.

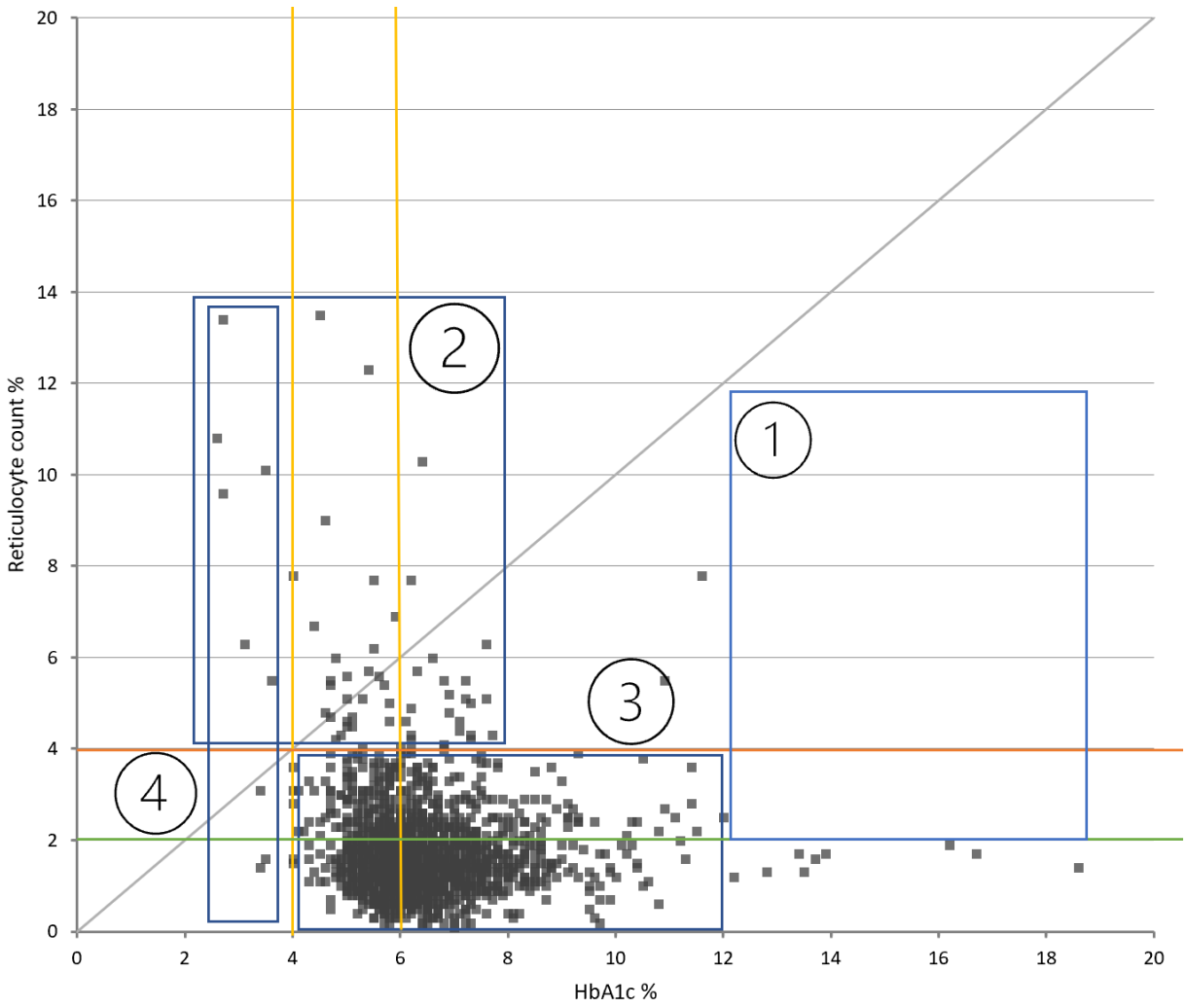


Figure 3 : Reticulocytes correlation with HbA1c (n=1782), yellow lines indicate normal range for HbA1c, green line end of normal range reticulocytes (2%), orange line marks 4% reticulocytes threshold, blue boxes are used to zoom in on specific reticulocytes and HbA1c ranges as referred to in text above

A correlation between Hb values and reticulocyte count showed that lower Hb values clearly correlate with high reticulocyte count (Figure 4). This can help to identify patients with high reticulocyte counts more easily as a hemoglobin measurement is an established, cheaper test that many physicians will easily incorporate in their lab request. Currently approximately 14% of all HbA1c orders are accompanied by a Hb measurement in our hospital (UZ Leuven, Belgium), based on a query spanning a 4 month period (01-01-2022 to 26-04-2022).

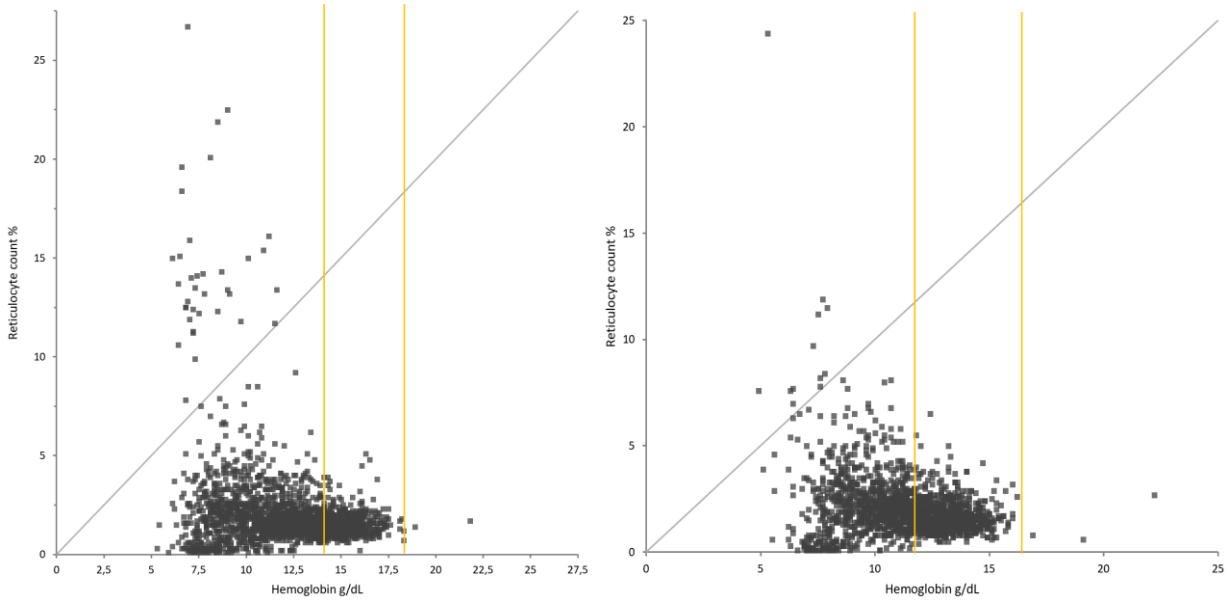


Figure 4: Hb correlation with HbA1c in male (left graph, n=2106) and female (right graph, n=1893) adult patients. Yellow lines indicate normal reference range (>= 14 to <= 18 g/dL in males and >=12 to <= 16g/dL in females).

Conclusion

This data led us to three propositions in our lab to help physicians interpret HbA1c values:

1. A comment on the laboratory report when reticulocytes are elevated (>2%): 'correlate with glycemic profile/estimated HbA1c'.

2. Suggest that all HbA1c measurements should be accompanied by a hemoglobin measurement. When hemoglobin levels are decreased (below 14 g/dL in males and below 12 g/dL in females), reflex order a reticulocyte count. Inform the clinician that they should also order a reticulocyte count when there is clinical suspicion of increased red blood cell turnover (including other hemolysis parameters as haptoglobin, bilirubin, LDH).

3. For HbA1c values lower than 4%, reflex order a reticulocyte count.

3. What is the analytic influence of some common hemoglobin variants on the HbA1c values determined with the Tosoh G8 in UZ Leuven?

We compared HbA1c measurements by the Tosoh G8 (HLC-723G8, Tosoh Bioscience, Tokyo, Japan) system with the Alere Afinion (Afinion™ AS100, Abbott, Illinois, USA) boronate affinity method. Because we assume that boronate affinity methods are resistant to interference by most hemoglobin variants, a good correlation between the two methods means that the Tosoh G8 measurement is not significantly influenced by the variant. Good correlation was defined on the Sciensano criteria of EKE evaluation (Table 2, see above), with a deviation from reference method (Afinion) of maximum 0,4% absolute difference being acceptable. We retrospectively compared results of both the G8 measurement and the Afinion measurement on the same patient sample, by performing a Bland-Altman/Passing-Bablok analysis (Table 5).

Hemoglobin variant	n	mean abs difference % (NGSP)	95% CI % (NGSP)	correlation coefficient	Intercept 95% CI	Slope 95% CI
Hb S	27	0,164	-0,240 to 0,569	0,961	-0,98 to 0,82	0,89 to 1,20
Hb C	7	-0,113	-0,490 to 0,264	0,975	-6,84 to 1,79	0,63 to 2,00
Hb D	12	-0,19	-0,067 to 0,447	0,993	-1,45 to 0,25	1 to 1,29
Hb E	21	0	-1,071 to 1,072	0,619	0,40 to 2,84	0,45 to 0,90
Hb Riccarton*	20	0,2	0,264 to 0,667	0,988	-0,47 to 1,26	0,80 to 1,09
Hb J-Baltimore	5	0,764	-1,532 to 3,060	0,184	-1,23 to 8,69	-0,56 to 1,63
Hb G-Siriraj	3	-1,063	-2,035 to -0,092	0,789	-1,48 to 2,13	0,47 to 1,11

* Only HbA1c profiles with full integration of shoulder fraction

Table 5: Bland-Altman and Passing Bablok analysis of comparison between G8 and Afinion HbA1c measurement

HbAS

With a correlation coefficient r=0,952 and a mean absolute difference of 0,16% (95% CI of -0,240 to 0,569) it seems that the Tosoh G8 is acceptable for measuring HbA1c in patients with HbAS. This data supports the current practice in UZ Leuven to use the Tosoh G8 for measuring HbA1c in patients with HbAS.

We excluded 4 data points in our correlation. One patient had a high level of HbF(>30%) which is known to interfere on the Tosoh G8. Three other patients didn't have any identifiable factor for interference.

HbAC

With a correlation coefficient r=0,975 and a mean absolute difference of -0,11% (95% CI of -0,490 to 0,264), there is no interference of HbA1Con the Tosoh G8 measurement. Current practice is to use Tosoh G8 for measuring HbA1c in patients with known HbAC and it seems reasonable to continue doing that.

HbAD

With a correlation coefficient r=0,993 and a mean absolute difference of -0,19% (95% CI -0,067 to 0,447), we have an excellent correlation with the Afinion and we will continue to use the Tosoh G8 in patients with known HbAD as is current practice in UZ Leuven. Three outliers were excluded from the analysis, without any directly identifiable factors for interference.

HbAE

With a correlation coefficent r=0,619 and a mean absolute difference of 0% (95% CI -1,071 to 1,072) it seems that the Tosoh G8 is influenced by the presence of heterozygous HbAE and should be measured with an alternative method (Afinion), as is current practice in UZ Leuven. However recent literature suggests that from software version 5.24, the Tosoh G8 is no longer influenced by the presence of HbAE (47). As the data points in this comparison date back approximately 2 years it is possible that earlier results were not run on the latest software version. As of now UZ Leuven uses software version 5.28 on the Tosoh G8, which also should not give interference by the presence of HbAE. We will investigate this further and in the meantime continue to use the Afinion for patients with HbAE.

Hb Riccarton

Hb Riccarton has been described to affect HbA1c measurements on HPLC analyzers (48). We performed a retrospective evaluation of HbA1c measurements for patients with suspected Hb Riccarton (n=30) Suspected Hb Riccarton was defined as a visible shoulder to the right of the sA1C fraction on the G8-chromatogram in combination with a broader hemoglobin A fraction on capillary zone electrophoresis (Minicap, Sebia, Paris, France). All G8-chromatograms were inspected for full integration of the shoulder fraction.

The mean absolute difference of HbA1c assays (%) and correlation coefficient between both methods of Hb1Ac assays are shown in table 1. Poor correlation was found in samples with only partial integration of the shoulder fraction.

Hemoglobin variant	n	Mean abs difference % (NGSP)	95% CI % (NGSP)	correlation coefficient
Hb Riccarton (all)	30	0,03	-0,6 to 0,7	0.967
Full integration	20	0,20	-0,26 to 0,66	0.988
Partial integration	10	-0,31	-0,78 to 0,16	0.948

Table 5. Passing Bablok/Bland-Altman analysis of comparison between G8 and Afinion HbA1c measurement for Hb Riccarton

In patients with suspected Hb Riccarton the Tosoh G8 can be used for accurate HbA1c monitoring after visual chromatogram inspection for full integration of sA1C shoulder. When full integration fails the boronate affinity assay with Afinion has shown to be a useful alternative.

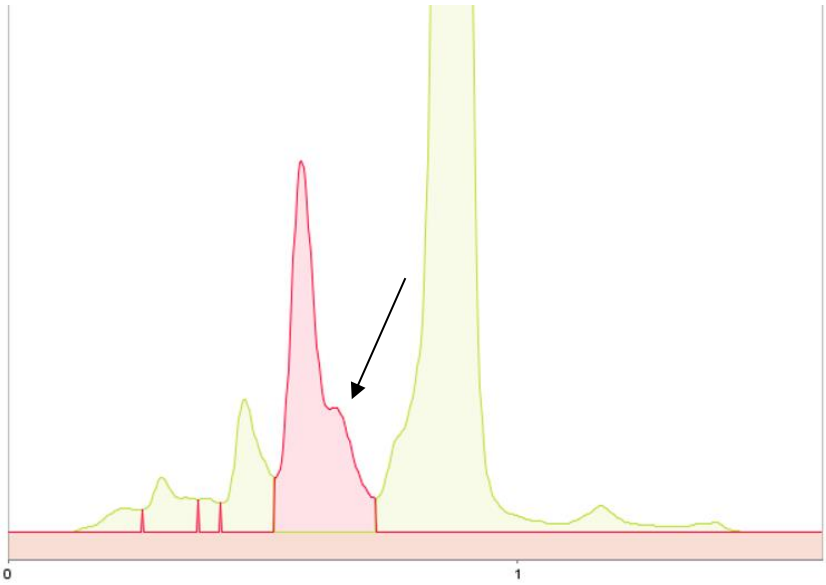


Figure 4: G8 chromatogram showing a visible shoulder to the right of the sA1C fraction, suggestive for the presence of Hb Riccarton variant

Hb J-Baltimore

With a correlation coefficient r=0,184 and a mean absolute difference of 0,76% (95% CI -1,532 to 3,060) it is not possible to use TosohG8 for patients with Hb J-Baltimore. We will use the Afinion in patients with known Hb J-Baltimore. This evaluation was only performed on a limited amount of samples (n=5).

Hb G-Siriraj

With a correlation coefficient r=0,789 and a mean absolute difference of -1,06% (95% CI -2,035 to -0,092), there is too big of an absolute difference. Because this evaluation was performed on a very limited amount of samples (n=3), we cannot draw any meaningful conclusions.

Hb Raleigh

In literature it is suggested that the Val→Ala substitution at the beta chain terminus produces substantial acetylation, preventing glycation at this position and thus falsely lowering results for immunoassay and likely boronate affinity methods (49). We probably should use alternative methods like fructosamine or glycated albumin in these patients. We have no comparative data for UZ Leuven patients between Tosoh G8 and Alere Afinion.

Conclusion

We will continue to use Tosoh G8 for follow-up of HbA1c measurements in patients with HbAS, HbAC and HbAD. For HbAE we will continue to use Afinion for now until we have more real world data with the software update. For Hb Riccarton we will continue to use G8, but inspect the chromatograms to see if they are properly integrated. Where full integration fails, Afinion will be used instead. For Hb J-Baltimore and Hb G-Siriraj, Afinion will be used. For patients with Hb Raleigh both methods are inadequate and alternative methods like fructosamine should be used.

The first time a patient with a heterozygote Hb variant gets a HbA1c measurement in our lab we use both methods to pick up any large discrepancies and can decide for individual patients to keep on using the Afinion for follow-up

HbA1c measurements if the difference is too big (>0,4% absolute difference). This is something we recommend if you have a boronate affinity method available in your lab as well.

COMMENTS

TO DO/ACTIONS

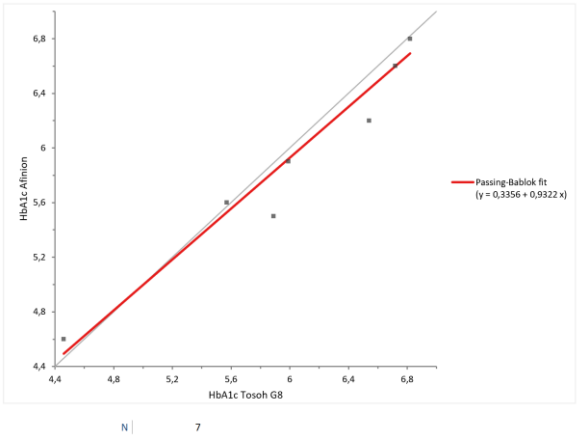
1) Add a comment to the lab rapport when reticulocytes are above 2% 'correlate with glycemic profile/estimated HbA1c'.

2) Discuss these findings with the endocrinology department : what is their clinical assessment when estimated A1c values from GCM show discrepancies with lab measured HbA1c and how do they feel about adding Hb or reticulocytes to their HbA1c lab request.

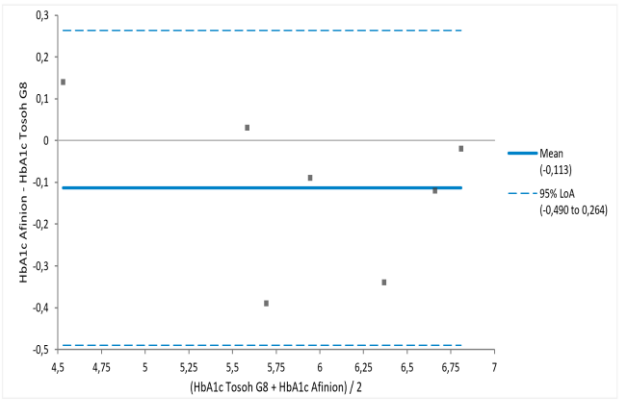
ATTACHMENTS

Attachment 1

Passing Bablok and Bland-Altman in patients with HbAC

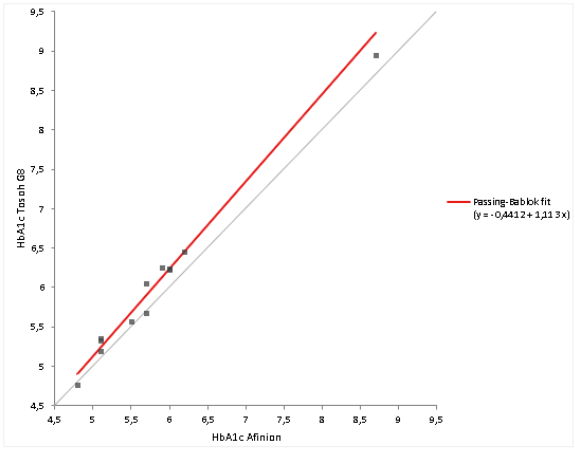


N 7

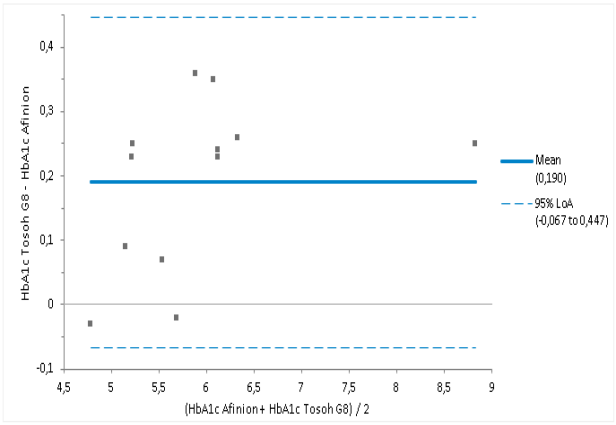


N 7

Passing Bablok and Bland-Altman in patients with HbAD

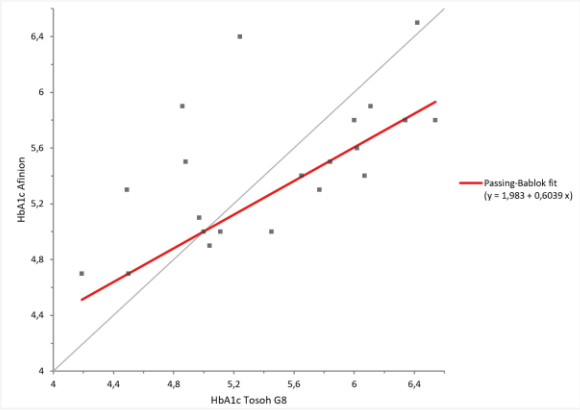


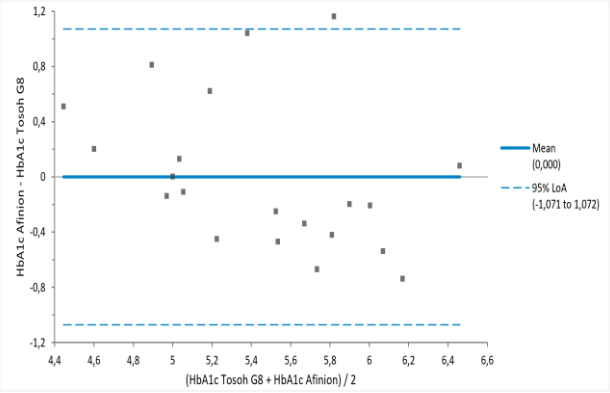
N 12



N 12

Passing Bablok and Bland-Altman in patients with HbAE

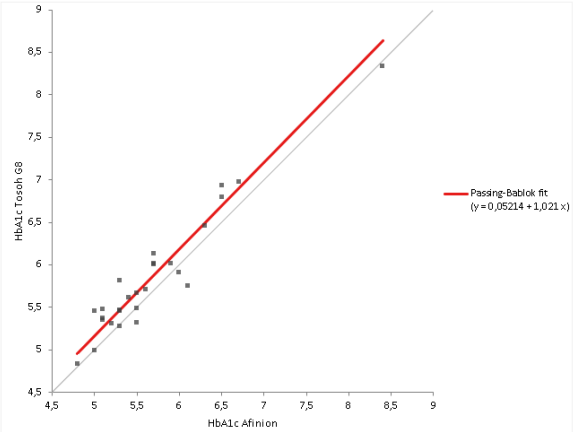


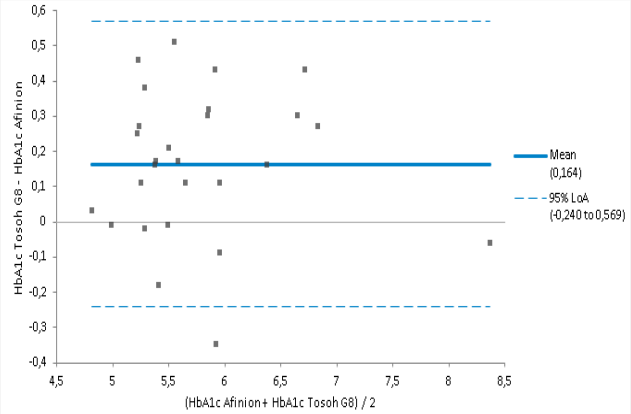


N 21

N 21

Passing Bablok and Bland-Altman in patients with HbAS

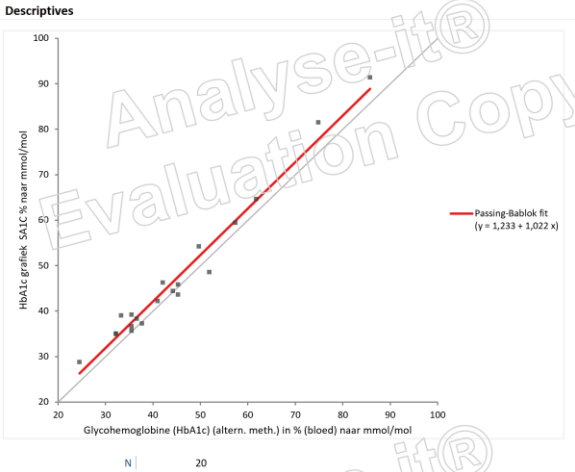


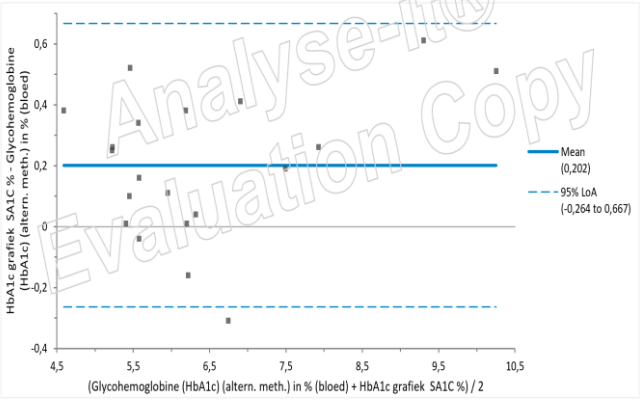


N 27

N 27

Passing Bablok and Bland-Altman in patients with Hb Riccarton (only full integration chromatograms)





N 20

N 20

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