HbA1c and interference due to hemoglobin disorders
Scientific discussion paper

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Introduction

Hemoglobin A1c (HbA1c) is a powerful tool for monitoring long-term glycemic control. As an elevated concentration of HbA1c is associated with a wide range of diabetic complications, it is invaluable for evaluating and managing people with diabetes mellitus.[1]

Several methods are available to the laboratory for measuring HbA1c and provide the concentration in mmol/mol (International Federation of Clinical Chemistry [IFCC] units) and/or as a percentage (National Glycohemoglobin Standardization Program [NGSP] units).[2,3] However, to correctly interpret the HbA1c results it is important to know exactly what each method measures and what impact interference from, for example, hemoglobin (Hb) variants, may have on the HbA1c result.[1] To assist with this interpretation, the European Reference Laboratory (ERL) in The Netherlands has conducted a Hb variant study supported by Roche. The ERL functions as a reference laboratory for the IFCC, as well as a primary (Queen Beatrix Hospital) and secondary (Isala Klinieken) reference laboratory for the NGSP. The results of this study can be found in the study hand-out inserted at the back of this discussion paper.

This discussion paper provides an in-depth summary of the various methods available for measuring HbA1c and the impact hemoglobin disorders, such as a Hb variant or thalassemia, can have. In general, there are two main categories of methods. The first comprises of methods that are based on the difference in charge of the molecules, such as cation exchange high performance liquid chromatography (CE-HPLC) and capillary electrophoresis. The second category consists of methods based on structural differences, such as affinity high performance liquid chromatography (affinity-HPLC) and immunoassays.[2]

One of the fundamental differences between the method types is that for those based on charge differences, all forms of non-physiologic Hb must be excluded to calculate HbA1c correctly, whereas this is not the case for methods based on structural differences.[1] To do this, methods such as CE-HPLC must be able to identify all Hb variants present and their glycated forms which can be problematic given that all molecules with the same electrical charge will be eluted at the same retention time leading to co-elution.[1] Hence, this can affect the specificity of the test and it is this potential lack of specificity that prevents CE-HPLC being adopted as a reference method for the IFCC reference system and prompted the development of alternative methods.[4]

As methods based on structural differences, such as immunoassays, include all forms of Hb in the HbA1c calculation this specific identification of Hb variants and their glycated forms is not required.[2] In addition, the Tina-quant® HbA1c assay from Roche specifically detects the first four amino acids of the N-terminal end of the β-globin chain and, with this, it detects the real analyte as defined by the IFCC as representing the only valid anchor for HbA1c testing.

Hemoglobin and hemoglobin disorders

The role of Hb is to carry oxygen in the blood from the lungs to the rest of the body, thus preserving vital functions. The molecule consists of two pairs of different globin chains (or subunits), such as one α chain plus a β, γ or δ chain, that are folded into a unique protein structure. At its core, each globin chain contains a heme group comprising an iron ion (charged atom) which is the oxygen binding site.[5,6]

In healthy individuals, Hb consists of ~97 % HbA, <2.5 % HbA2 and 0.5 % HbF.[1] HbA contains two α- and two β-globin chains (α2β2) and while the α subunit is encoded by four genes, the β subunit is encoded by two genes. Other physiologic Hb variants like HbA2 and HbF contain two α- and two δ-globin chains (α2δ2), and two α- and two γ-globin chains (α2γ2), respectively.[3,5,6] In addition to the physiologic Hb variants, there are also mutated forms (non-physiologic) that exist as they confer a heterozygote advantage against certain diseases (e.g., malaria). Inherited Hb disorders are, therefore, among the most common monogenic diseases; they are carried by ~7 % of the world's population and, due to global migration, these mutations now occur worldwide.[5,7] Inherited Hb disorders fall into two main groups:[8]

1) **Hemoglobinopathies: due to structural abnormalities in the globin proteins**

2) **Thalassemias: due to underproduction of normal globin proteins, often due to mutations in regulatory genes.**

To date, 1,585 Hb mutations (hemoglobinopathies and thalassemias) have been reported.[9]

Hemoglobinopathies

Hemoglobinopathies are caused by gene mutations that affect the structure of the globin chains of the Hb molecule. Most hemoglobinopathies are clinically silent. However, others are asymptomatic, but result in abnormal hematologic laboratory findings, while a few cause serious diseases. Most structural abnormalities are caused by a single mutation resulting in the substitution of one amino acid for another, deletion of a portion of the amino acid sequence, abnormal hybridization between two chains, or abnormal elongation of the globin chain.[5,6]

Hemoglobin variants

There are more than 1,100 known human Hb variants and new ones are still being discovered.[9] The majority are rare in terms of prevalence, but numerous in terms of their structural differences and some reach high frequencies in specific population groups.[5] One example of a high frequency variant is HbS, which in the homozygous form causes sickle cell anemia, but is benign when heterozygous (sickle cell trait). HbS is an inherited variant of normal adult HbA and results from a substitution of the amino acid valine for glutamic acid in the sixth position of the β-globin chain (Figure 1).
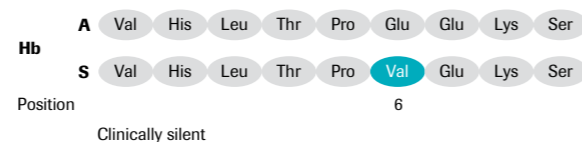

Fig. 1: Hemoglobin (Hb) S in the heterozygous form (sickle cell trait); substitution of valine for glutamic acid in the sixth position of the β-globin chain

HbS is the most commonly encountered Hb variant worldwide and belongs to the so-called common Hb variants. The frequency of the gene for HbS is highest among people of African origin (about 1 in 10), but is also found in people of Mediterranean (Italian, Greek, Turkish) descent.[5,7]

The other common Hb variants are HbE, HbC and HbD which also exist in both the heterozygous and homozygous forms. Table 1 shows the locations of the point mutations causing these variants and their clinical manifestations.[1,5,10,11]

Variant	**Point mutation**	**Clinical manifestation**
HbAS	β6 Glu → Val	No
HbSS	β6 Glu → Val (both genes)	Sickle cell anemia (Sickle cell disease)
HbAC	β6 Glu → Lys	No
HbCC	β6 Glu → Lys (both genes)	Hemolytic anemia (HbCC disease)
HbAE	β26 Glu → Lys	No
HbEE	β26 Glu → Lys (both genes)	Hemolytic anemia (HbE disease)
HbAD (e.g., Los Angeles)	β121 Glu → Gln	No
HbDD (e.g., Los Angeles)	β121 Glu → Gln (both genes)	Hemolytic anemia (HbD disease)
Uncommon Hb variants	Depends on variant	Depends on variant

Table 1: Hemoglobin (Hb) variant mutations and clinical manifestations[1,5,10,11]

Hemoglobin E is the second most common variant after HbS (sickle cell disease). HbE is common in Southeast Asia, where its prevalence is over 50 % in some parts of Thailand, Cambodia and Laos; it is also found in Sri Lanka, North Eastern India, Bangladesh, Pakistan, Nepal, Vietnam, Malaysia and China. HbE can interact with β thalassemia to become HbE/β thalassemia, which is a severe health problem in Asia.[5,10–12]

The HbC trait behaves in a similar way to the HbS trait in that it is benign and causes no clinical manifestations. The prevalence of HbC is as high as 30 % in Sub-Saharan Africa, while in the USA approximately 1 in 1,800 individuals have HbCC, whereas HbSC (sickle cell disease) affects 1 in 1,100.[13]

Finally, HbD is a general term for several Hb variants and is the fourth most common Hb variant. Of these, HbD Punjab (alias HbD Los Angeles) is by far the most common and other HbD variants include HbD Iran, HbD Ouled Rabah and HbD Granada. HbD is most often found in people living in India, Pakistan, England, Ireland, Holland, Australia, China, Iran, Turkey and in their descendants. HbAD is a clinically silent condition, but co-inheritance of HbD with sickle cell or thalassemia produces a clinically significant disease. Homozygous HbDD is a rare and a relatively mild disease.[14]

Methods

Glycated forms of Hb variants

Hemoglobin A1c is a specific glycated Hb resulting from the attachment of a glucose molecule to the N-terminal valine of the Hb β-globin chain. However, this reaction can also occur with all other Hb variants such as HbS, HbE, HbC and HbD. Therefore, two forms of glycated Hb can be found in heterozygous individuals carrying HbA and a Hb variant gene, such as HbE; one is HbA1c and in this example the other form is HbE1c, as shown in Figure 2.[1,2]
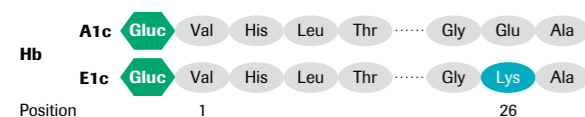
Fig. 2: The two forms of glycated hemoglobin (Hb) that can occur in a heterozygous individual carrying HbA and the variant HbE

Patients who are homozygous for a variant gene (for example those with HbEE) do not have HbA and, therefore, do not have any HbA1c. In these patients only the glycated form of the variant can be found, such as HbE1c.

Thalassemias

Thalassemias are caused by a genetic defect that results in production of an abnormally low amount of a hemoglobin chain.[5,15]

The defect can affect the α-, β-, γ- or δ-globin chain or a combination of the β-, γ- and δ-globin chains in the same patient, but never the α- and β-globin chains together.[6] The imbalance in the number of globin chains means an inadequate number of red blood cells are produced and those that are generated are paler in color (hypochromic) due to the lack of Hb and can be smaller than normal (microcytic). They also contain a surplus of the unaffected chains which can result in the destruction of the red cell in the marrow (ineffective erythropoiesis) or circulation (hemolysis).[8]

Although thalassemia and hemoglobinopathy are usually distinct diseases with different causes, in some cases they may overlap. This would occur when the cause of the globin protein abnormality also affects protein production and therefore some hemoglobinopathies are also thalassemias.

Thalassemias involving the β-globin chain can be divided into two categories: β0 thalassemia and β+ thalassemia. In β0 thalassemia either an abnormal gene is not expressed or, less frequently, there is a gene deletion. In contrast, with β+ thalassemia there is reduced expression of the abnormal gene so that even in the homozygous state there is still some hemoglobin A production. Consequently, there are three phenotypes of β thalassemia depending on the combination of alleles.

β thalassemia minor (or β thalassemia trait) is usually clinically asymptomatic and is a heterozygous state with one deleted or mutated β-globin gene and one normally functioning gene (β+/β or β0/β). β thalassemia intermedia is caused by a homozygous or compound heterozygous β-globin gene mutations resulting in a decrease in β-globin chain production (β+/β+ or β0/β+). Affected individuals can often maintain a normal life but may need occasional transfusions, such as during pregnancy or times of illness, depending on the extent of the anemia. Patients with β thalassemia major or Cooley's anemia are dependent on blood transfusions and are either homozygous or compound heterozygotes for β-globin gene mutations (β0/β0, β+/β+ or β0/β+). Due to the blood transfusions, measuring HbA1c in these individuals does not give accurate information on glycemic control.[16,17]

Different laboratory methods are available to measure HbA1c and, therefore, assess long-term glycemic control (Table 2):

- **Immunoassays (e.g., the Tina-quant® HbA1c assay from Roche)**
- **Boronate affinity-HPLC**
- **Cation exchange HPLC1–3**

Given that each method is based on the physical, chemical or antibody properties of the normal (HbA) Hb molecule, it is important to understand the test principle for each method.

It is also vital to know about any clinically significant interference the different methods may have and how this can affect the accuracy of the HbA1c measurement.[1] As noted previously, any condition that shortens erythrocyte survival, or decreases mean red blood cell age, will falsely lower the HbA1c test result regardless of the assay method used.[2] HbA1c results from patients with HbSS, HbCC and HbSC should, therefore, be interpreted with caution given the pathologic processes, including anemia, increased red cell turnover and transfusion requirements, that adversely impact HbA1c as a marker of long-term glycemic control. Alternative forms of testing, such as glycated serum proteins or glycated albumin, should be considered for these patients.

Immunoassays (e.g., Roche Diagnostics)	**CE-HPLC** (e.g., Tosoh Bioscience)	**Affinity-HPLC** (e.g., Trinity Biotech)
Detection of HbA1c	Detection of fraction of HbA1a, HbA1b and HbA1c	Detection of glycated Hb
Measures real analyte, as defined by the IFCC	Calculated peak	Calculated peak
Including Hb variants and their glycated forms	Excluding Hb variants and their glycated forms	Including Hb variants and their glycated forms

Table 2: Different methods for HbA1c measurement. Affinity-HPLC, affinity high performance liquid chromatography; CE-HPLC, cation exchange high performance liquid chromatography; Hb, hemoglobin; IFCC, International Federation of Clinical Chemistry

Tina-quant® HbA1c assay from Roche

The principle of this assay is that HbA1c in the sample competes with a polyhapten (or agglutinator; a synthetic polymer with multiple copies of the immunoreactive portion of HbA1c) for freely available HbA1c-specific polyclonal antibodies. The HbA1c concentration measured is expressed as a percentage of the total hemoglobin, which is determined in the same sample aliquot and same cuvette as the HbA1c measurement ("twin-test" technology). This "twin-test" technology not only eliminates sources of uncertainty in the percentage HbA1c result, but also allows for excellent precision.[18]

The antibodies used in the Tina-quant® assay are directed against the N-terminal peptide sequence of the amino terminal β-globin chain with its glycated valine group (2-deoxyfructose-valine; Figure 3). In doing so, the Tina-quant® HbA1c assay from Roche specifically detects the first four amino acids of the N-terminal end of the β-globin chain and with this it detects the real analyte, as defined by the IFCC, as representing the only valid anchor for HbA1c testing (Figure 4).[4] Furthermore, this choice of antibody is the reason why Hb variants such as HbS, HbE, HbC and HbD do not affect the HbA1c results given by this test. The mutations resulting in the common Hb variants are not contained within the region detected by the antibody (the first four amino acids from the N-terminal end of the β-globin chain)[1,5,10,11] and hence the amino acid sequence is preserved and recognized regardless of the variant.[18]
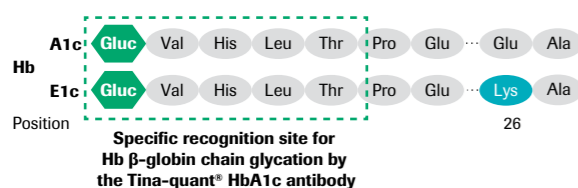

Fig. 3: Epitope used in the Tina-quant® HbA1c assay allows recognition by the antibody regardless of the hemoglobin (Hb) variant

"HbA1c is hemoglobin which is glycated at the N-terminal end of the ß-chain" [ß-N-(1-deoxyfructos-1-yl) hemoglobin]
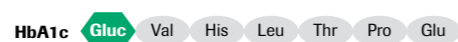

Fig. 4: International Federation of Clinical Chemistry definition of HbA1c – the real gold standard for HbA1c measurement

As the Tina-quant® HbA1c assay includes hemoglobin variants in the HbA1c calculation (see Figure 5) and is based on structural differences, rather than differences in charge, a reliable result is achieved without the need for any post-analytic steps to identify variants. This, therefore, represents a time- and cost-saving when compared with methods such as CE-HPLC.[19]

$$\% \text{ HbA1c} = \frac{\text{HbA1c (incl. HbX1c)}}{\text{Total hemoglobin (incl. HbX)}} * 100$$

Fig. 5: Equation for calculating HbA1c using the Tina-quant® HbA1c assay; hemoglobin (Hb) variants do not need to be identified and excluded from the calculation. HbX and HbX1c represent Hb variants and their glycated form

Of the 1,585 identified Hb mutations (hemoglobinopathies and thalassemias) that affect 7 % of the world's population (~ 500 million people), only 17 non-common Hb variants show a mutation in the first four amino acids. Table 3 shows some of these Hb variants and their occurrence.[9,15]

Hb variant	Ethnicity	Mutation	Occurence
Hb Niigata	Japanese	β1 Val → Leu	Found in a Japanese male
Hb South Florida	Caucasian	β1 Val → Met	Found in one Caucasian family
Hb Doha	Qatari	β1 Val → Glu	Found in oneQatari family
Hb Raleigh	Caucasian, Swedish	β1 Val → Ala	Found in a few Caucasian and Swedish families
Hb Watford	Ashkenazi Jews	β1 Val → Gly	Ashkenazi Jews
Hb Franklin Park	African-American	β2 His → Asn	African-American
Hb Fukuoka	Japanese	β2 His → Tyr	Found in a Japanese female
Hb Marseille	French, American, Austrilian	β2 His → Pro	Found in a French family, three American families, and an Australian family
Hb Deer Lodge	Welsh-Dutch-English, Black	β2 His → Arg	Found in a few Welsh-Dutch-English and black families
Hb Graz	Austrian	β2 His → Leu	Found in several Austrian families
Hb Agrigente	Sicilian	β2 His → Pro	Found in one Sicilian
Hb Okayama	Japanese	β2 His → Gln	Found in a Japanese male
Hb Kamakura	Japanese	β3 Leu → Val	Japanese
Hb Niguarda	Italian	β3 Leu → Met	Italian
Hb Santo Domingo	Dominican	β3 Leu → Gln	Dominican
Hb Jabalpur	Pakistani	β3 Leu → Pro	Pakistani
Hb Benin City	Nigerian	β4 Thr → Pro	Nigerian
Hb Wurzburg	German	β4 Thr → Asn	German

Table 3: There are 1,800+ recorded/reported variants and thalassemias in the word by end of Apr. 2019. The prevalence of some of these rare hemoglobin variants with a mutation in the first four amino acid is lower than 1% 9, 15

Affinity high performance liquid chromatography (affinity-HPLC)

The structural difference between HbA1c and the unglycated form results from the presence of the glucose group in HbA1c. Agents reacting specifically to this glucose moiety can, therefore, be used as the basis for analytic methodology, such as affinity-HPLC. With this method an affinity resin is used which all glycohemoglobins bind to, while all non-glycated Hb do not. As all glycohemoglobins and not just HbA1c bind to the resin the total glycohemoglobin is measured and, therefore, the initial result is higher than for tests that can specifically measure only HbA1c. However, as the formation of glycohemoglobins is proportional, it is possible to standardize the results of affinity-HPLC to the HbA1c units (Figure 6).[19]

$$\% \text{ HbA1c} = \frac{\text{Glyc. Hb}}{\text{Glyc. Hb + non-glyc. Hb}} * 100$$

Fig. 6: Equation for calculating HbA1c when using affinity high performance liquid chromatography. Glyc., glycated; Hb, hemoglobin

Cation exchange high performance liquid chromatography (CE-HPLC)

Proteins such as Hb have different isoelectric points. Hence they can be separated by CE-HPLC, as they elute at different times depending on their charge.[1] Any change (e.g., carbamylated Hb) or variation in the Hb molecule that leads to a change in the charge may alter the normal elution time of that molecule and allow its identification.

The most common charge-based methods for measuring HbA1c utilize CE-HPLC. Consequently, separation is only possible if the target molecule has a different electric charge to the other components in the sample. As non-physiologic forms of Hb must be excluded in the calculation of HbA1c, it is important to ask the following questions to ensure correct HbA1c measurement and result interpretation:

1) Is the non-glycated Hb variant peak well separated from other peaks?

2) Where are the glycated Hb variant peaks and do they impact the HbA1c results?

3) How well can CE-HPLC identify all peaks representing non-physiologic forms of Hb?

The equation for calculating HbA1c using CE-HPLC is shown in Figure 7 and differs considerably from the corresponding equations for affinity-HPLC and immunoassays, due to the fundamental difference in methodologies (charge vs. structural). As can be seen from the equation, if the Hb variant (HbX) or its glycated form (HbX1c) co-elute with HbA or HbA1c the numerator (HbA1c) or denominator (HbA + HbA1c) will be increased and the HbA1c result adversely affected.[1]

$$\% \text{ HbA1c} = \frac{\text{Area (sA1c)} - \text{area (HbX)} - \text{area (HbX1c)}}{\text{Total area} - \text{area (HbX)} - \text{area (HbX1c)}} * 100$$

Fig. 7: Equation for the HbA1c calculation when using cation exchange high performance liquid chromatography

The ability to see Hb variants on a chromatogram is often viewed as a plus. However, for accurate HbA1c measurement it is essential, as exclusion of non-physiologic forms of Hb (HbE, HbE1c, HbS, HbS1c, etc.) is part of the calculation, meaning that they must be identified. Laboratories are required, therefore, to perform the post-analyses necessary to ensure that the reported HbA1c value is free from interference.[14,20] This involves having well-trained staff proficient in identifying the 1,585 different patterns associated with Hb disorders to review all chromatograms. Unfortunately, despite this expertise, visual screening alone may not be sufficient to identify all variants, as some co-elute[1] and further analyses such as gene sequencing have to be undertaken.

Such post-analyses are both labor-intensive and costly and make CE-HPLC a less efficient measurement for HbA1c than other methods from the economic viewpoint.[19] Furthermore, if peaks cannot be separated (due to co-elution) or identified (in the case of rare Hb variants) an accurate HbA1c value cannot be reported and the sample has to be retested using an alternative method, such as by immunoassay. Although CE-HPLC has the ability to determine which Hb variant is present,[21,22] which can lead to further understanding of the patient's condition, the primary aim of HbA1c testing should be to obtain an accurate result.

Hemoglobin variants co-elute with other peaks

Figure 8 shows a Tosoh G8 chromatogram generated as part of the Hb variant study (see study handout for further details; Hemoglobin variant study, Lenters-Westra et al.). No abnormalities are visible (e.g., extra peaks, wrong retention time, etc.) and there are no warnings in the flag field. As this patient was known to have a Hb variant, it highlights the fact that the variant was not identified and that this method is subject to interference.[14,23–25] Had this result been obtained during routine testing the patients would have been deemed to be pre-diabetic based on the HbA1c value of 43 mmol/mol (or 6.07 % [NGSP]).[26] However, in reality, as the Hb variant was not identified it was not excluded within the HbA1c calculation, the equation's denominator was falsely increased (as it included both HbA and the Hb variant) and the final HbA1c value was artificially decreased.[1] Hence, this HbA1c result should not be reported and the sample should be retested using a method not affected by the HbE variant.

Glycohemoglobin report

Name	%	Time	Area
FP	0.00	0.00	0.00
A1A	0.76	0.25	11.21
A1B	0.99	0.32	14.57
F	1.37	0.40	20.25
LA1C+	1.73	0.49	25.50
SA1C	6.07	0.59	73.69
A0	90.14	0.90	1,327.52
		Total area	1,472.74

IFCC 43 mmol/mol
HbA1 7.82 %

HbA1c 6.07 %
HbF 1.37 %

The **HbE** variant is present but co-elutes (please refer to the piano chromatogram on the next page)
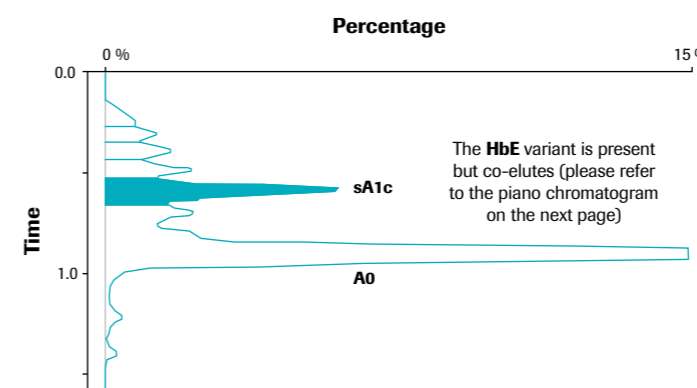
Fig. 8: Tosoh G8 chromatogram containing a hemoglobin variant without an associated warning in the flag field. This appears to be a normal chromatogram, but the variant HbE is present
Re-drawn from a Tosoh chromatogram generated as part of the ERL Hb variant study.
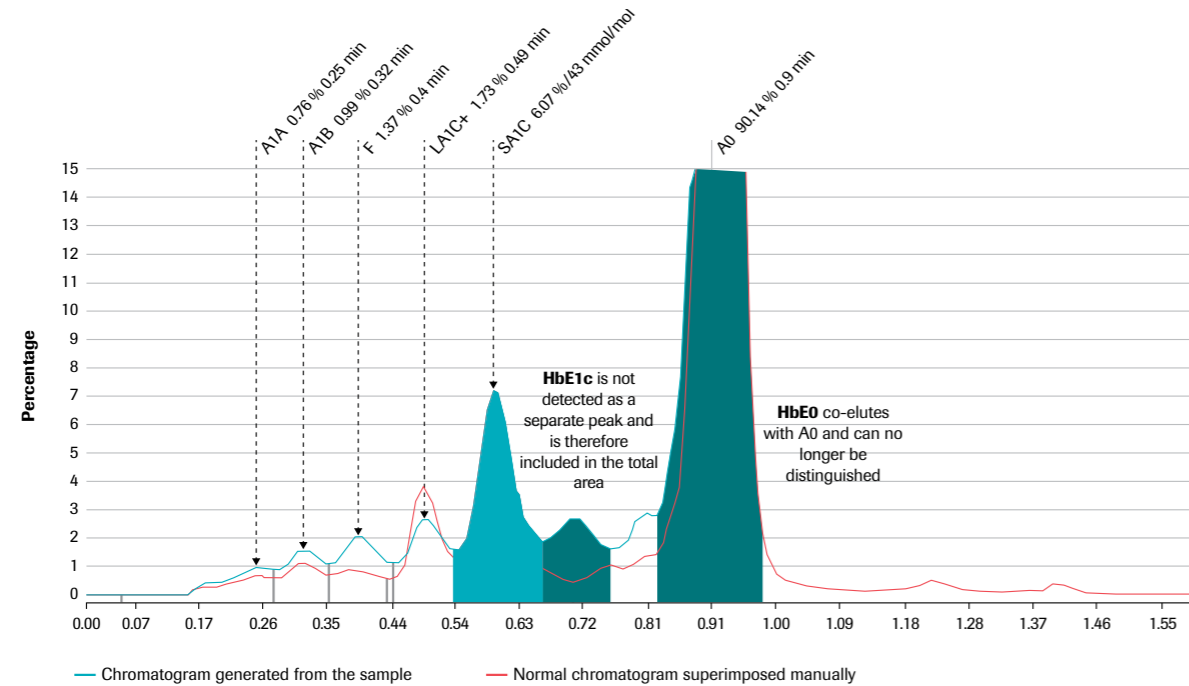
Fig. 9: Piano chromatogram of Fig. 8 generated as part of ERL Hb variant study. HbE1c and HbE0 are not detectable by visual inspection as HbE1c is included in the total area while HbE0 is co-eluting with HbA0

Figure 9 shows the same chromatogram as illustrated in Figure 8, but displayed as a piano chromatogram to show in more detail where the Hb variant is positioned. Neither the HbE1c, nor the HbE0, can be identified as separate peaks; HbE0 has co-eluted with HbA0 and HbE1c is included in the total area. As co-eluted peaks cannot be separated by this method, the Tosoh system is unable to display either the variant or a warning in the flag field. This lack of specificity means that following CE-HPLC testing using the Tosoh G7 and G8 systems, some patients with diabetes and the HbE variant may be deemed to be "healthy" (due to the underestimation of the percentage HbA1c value), as has been reported previously by the NGSP.[27]

Additional results from the Hb variant study showed inference of HbE and HbJ with the Tosoh G8 system. **It should be noted, however, that the problem of co-elution is not limited to Tosoh G7 and G8 and that other CE-HPLC systems, such as the Bio-Rad and Menarini/Arkray systems, are also subject to Hb variant interference.**[14,20,21,24,25] In one study, the results of 298,276 samples tested over a 16-year period using the Bio-Rad Variant Classic and Bio-Rad Variant II Systems are presented.
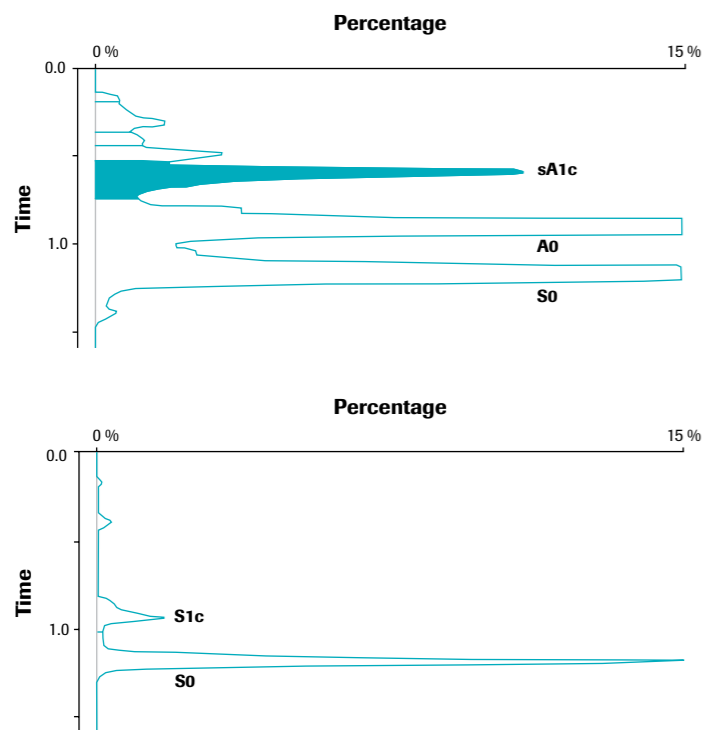
These data clearly show that HbE co-eluted with HbA2 and, furthermore, 26 other Hb variants (e.g., Zurich) also co-eluted with HbA2, while 12 Hb variants (e.g., HbD Iran) sometimes co-eluted with HbA2.[21] In addition, the literature confirms that HbE1c frequently elutes as a shoulder to HbA1c, in most CE-HPLC systems. As Figure 10 emphasizes, if HbE1c cannot be identified, it cannot be excluded during the calculation as required by the test principle, leading to a false decrease in the HbA1c value.[28,29]

$$\% \text{ HbA1c} = \frac{(\text{Area sA1c} - \text{area HbE1c})}{(\text{Total area} - \text{area HbE})} * 100$$

It is not possible to calculate HbA1c, due to the co-elution of HbE with HbA0 and the undetected E1c peak

Fig. 10: Equation for calculating HbA1c – HbE1c and HbE cannot be excluded in the equation as required by the cation exchange high performance liquid chromatography test principle

Each chromatogram needs to be visually checked to verify the result, but if peaks are co-eluting this may not identify all variants and further analyses can be required, making the procedure labor-intensive and costly.

As much of the available interference data relate to HbE1c, it is possible that this is an exceptional case and CE-HPLC can identify other glycated Hb variants as clear peaks on the chromatogram. However, this was also investigated as part of the Hb variant study and the investigators found that other glycated Hb variants could not be identified.

On a Tosoh chromatogram, HbS can normally be identified by an extra peak after the A0 peak as shown on the left hand side of Figure 11; the area relating to HbS can therefore be excluded in the HbA1c calculation.

To determine the position of the HbS1c peak, the chromatogram from a HbSS (homozygous) individual (Figure 11 right hand side) was compared with that of a HbAS (heterozygous) individual (Figure 11 left hand side). As the HbSS chromatogram does not have peaks relating to HbA1c or HbA0, because both globin genes have the "S" mutation, it allows the HbS1c peak to be seen. As the position corresponds to that of the HbA0 peak this suggests that the two co-elute and that in a heterozygous individual the HbA0 peak masks the HbS1c peak. This artificially increases the denominator in the equation (see Figure 12) because the A0 area also contains glycated HbS1c resulting in a decrease in the HbA1c value.

Glycohemoglobin report

Heterozygous HbAS chromatogram				Homozygous HbSS chromatogram			
Name	**%**	**Time**	**Area**	**Name**	**%**	**Time**	**Area**
FP	0.00	0.17	3.33	FP	0.00	0.17	7.17
A1A	0.00	0.00	0.00	A1A	4.02	0.25	10.35
A1B	2.01	0.32	24.97	A1B	0.00	0.00	0.00
F	0.88	0.41	10.92	F	12.59	0.40	32.40
LA1C+	2.33	0.50	28.91	LA1C+	3.87	0.50	9.96
SA1C	10.98	0.60	117.91	SA1C	0.00	0.00	0.00
A0	84.88	0.90	1,053.97	A0	73.30	0.94	188.65
H-V1	30.85	1.18	553.94	H-V1	79.34	1.20	988.41
		Total area	1,795.68			Total area	1,245.79

IFCC 97 mmol/mol   HbA1c 10.98 %
HbA1 12.99 %   HbF 0.88 %

IFCC – – – mmol/mol   HbA1c – – – %
HbA1 – – – %   HbF – – – %

Percentage

0 % 15 %

0.0

Time

1.0

sA1c

A0

S0

Percentage

0 % 15 %

0.0

Time

1.0

S1c

S0

By comparing the chromatogram from a HbAS individual with that of a HbSS individual, it can be seen that S1c co-elutes with HbA0

P00	0.41	1.40	5.06

P00 indicates an unknown variant is present

P00	6.22	1.06	16.02
RET	TIJD LAAG		
TP	TE LAAG		
221	A1B	NOT DETECT	10:00
221	SA1C	NOT DETECT	10:00

Fig. 11: Tosoh G8 HbAS (heterozygous) chromatogram with separated A0 and S0 peaks (top) and Tosoh G8 HbSS (homozygous) chromatogram that does not contain A1c and A0. The comparison of both shows where S1c normally runs in a chromatogram. Re-drawn from a Tosoh chromatogram generated as part of the ERL Hb variant study

$$\% \text{ HbA1c} = \frac{\text{Area sA1c}}{\text{Total area} - \text{area (HbS1c)} - \text{area (HbS0)}} * 100$$

It is not possible to exclude HbS1c in the calculation of HbA1c as it co-elutes with HbA0

Fig. 12: Equation for calculating HbA1c – HbS1c cannot be excluded in the calculation due to co-elution with HbA0

An additional issue with interpreting CE-HPLC derived HbA1c values is highlighted by Figure 11. In the heterozygous chromatogram in Figure 11 (left hand side), a small peak at the end of the chromatogram can also be seen. 'P00' is reported as an unknown variant that elutes at 1.40 (time) and has an area of 5.06. Based on the overall principle for calculating HbA1c, all abnormal Hb variants should be excluded and all physiologic Hb variants included. In this case,

however, although the system does not know which variant P00 represents (and, therefore, whether it is a physiologic or non-physiologic Hb variant) it has still calculated the various areas (Table 4) and a HbA1c value. If P00 represents an abnormal Hb variant, the equation denominator would be increased, thereby falsely decreasing the HbA1c value. Therefore, further analyses are required to identify the unknown variant (P00) before the results can be interpreted correctly.

Name	Area
A1A	0
A1B	24.97
F	10.92
LA1c	28.91
SA1c	117.91
A0	1,053.97
H-VI	553.94
= Total area (without P00)	1,790.52
+ P00 (unknown variant)	5.06
= Total area on chromatogram	1,795.68

P00, mathematically, leads to an increased denominator, because it is included in the calculation of the total area.

Further evaluation is required to clarify whether P00 represents a physiologic or non-physiologic Hb variant before an accurate HbA1c value can be reported. HPLC is not able to do this.

Table 4: Calculation of the total area (corresponding to the heterozygous chromatogram on the left hand side of Fig. 11). P00 leads to an increased denominator because it is included in the calculation of the total area

Summary

To correctly interpret HbA1c results, it is important to know the method used to generate the result. Methods relying on a difference in charge (e.g., CE-HPLC and capillary electrophoresis) must be able to detect all Hb variants to accurately report the HbA1c result.[1] This is because all forms of non-physiologic Hb must be excluded to calculate HbA1c correctly. If not all Hb variants are correctly identified by CE-HPLC (such as when two molecules have the same charge and co-elute at the same retention time) the chromatogram may look normal, but the reported HbA1c will be artificially increased or decreased.[1] As a result of this lack of specificity, each chromatogram must be visually inspected[14], which can be time-consuming and costly. Furthermore, if the chromatogram looks abnormal (e.g., when the peaks are not well separated) an incorrect result could be generated and the HPLC instrument should provide a flag in the warning field and/or not generate the HbA1c result.[1] Additional investigations, such as gene sequencing, may help to characterize the Hb variant, but if a HbA1c value is required the sample should be retested for confirmation using an alternative method that is not affected by the Hb variant.[28] However, these additional analyses also incur extra costs for the laboratory.

Given these potential issues, an alternative method based on structural differences rather than charge may be better suited for HbA1c testing. The Tina-quant® HbA1c assay provides an accurate and fast HbA1c measurement and, in contrast to CE-HPLC, includes Hb variants in the calculation of the HbA1c value and does not require any post-analytic assessments.[18] On May 23, 2013 the Tina-quant®

HbA1c assay from Roche was FDA-cleared for use as an aid in the diagnosis of diabetes and in identifying patients who may be at risk of developing diabetes. This is the first test globally to gain such approval and these indications are in addition to its established use for monitoring glycemic control. Approval was granted following extensive testing of the assay. The data generated included results from a precision study (coefficient of variance <1.4 %) as well as from a Hb variant interference study that showed no significant interference with this assay from HbS, HbC, HbE, HbD and HbA2. The Tina-quant® HbA1c assay, therefore, conforms to the requirement by the FDA that performance testing must demonstrate that there is little or no interference from common Hb variants, including HbS, HbC, HbE, HbD and HbA2.[30]

The Tina-quant® HbA1c assay uses antibodies against the first four amino acids in the N-terminal peptide sequence of the amino terminal β-globin chain, as well as the glucose bound to the N-terminal valine. This area is generally conserved and therefore is not affected by the mutations causing the common Hb variants.[1,5,10,11] In fact, of the 1,585 Hb mutations identified to date, only 17 are located within this region.9 This explains why Hb variants such as HbS, HbE, HbC and HbD do not affect the performance of the Tina-quant® HbA1c assay, whereas they can interfere with CE-HPLC tests (Table 5).[18] Hence the Tina-quant® assay efficiently measures HbA1c irrespective of the majority of known Hb mutations (hemoglobinopathies and thalassemias)[9] carried by ~7 % of the world's population (round 500 million people).[5,7]

Hemoglobin variant	Tina-quant® HbA1c	CE-HPLC (e.g., Tosoh)	Affinity-HPLC (e.g., Primus)
HbAS (sickle cell trait)	Not affected	Can be affected*	Not affected
HbAE	Not affected	Can be affected*	Not affected
HbAD	Not affected	Can be affected*	Not affected
HbAC	Not affected	Can be affected*	Not affected
Rare variants	In general not affected (only 17 variants are located within the target for the assay)	Can be affected* (more than 1,100 Hb variants exist)	In general not affected (except e.g., Hb Himeji)

Thalassemia	Tina-quant® HbA1c	CE-HPLC (e.g., Tosoh)	Affinity-HPLC (e.g., Primus)
β thalassemia minor (β+/β or β0/β)	Not affected	Not affected	Not affected
β thalassemia major (β+/β+, β0/β0 or β+/β0)	No medical information**	No medical information**	No medical information**
β thalassemia (minor) and Hb variant (double heterozygosity, e.g., HbS/β+)	Not affected as long as the Hb variant is not located in the first four amino acids of the N-terminal end of the β-globin chain	Not affected as long as the Hb variant does not co-elute with other peaks	Not affected as long as the Hb variant does not co-elute with other peaks

Table 5: Percentage HbA1c results obtained using the Tina-quant® HbA1c assay are not affected by the presence of hemoglobin (Hb) variants

*Dependent on Hb variant, method and the separation quality (co-elution and co-migration);

**Anemia leads to a reduction in the lifespan of the red blood cells and, therefore, less time for glycation; HbA1c should not be used.

CE-HPLC, cation exchange high performance liquid chromatography; Affinity-HPLC, affinity high performance liquid chromatography

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Hemoglobin variant study
Turbidimetry versus HPLC
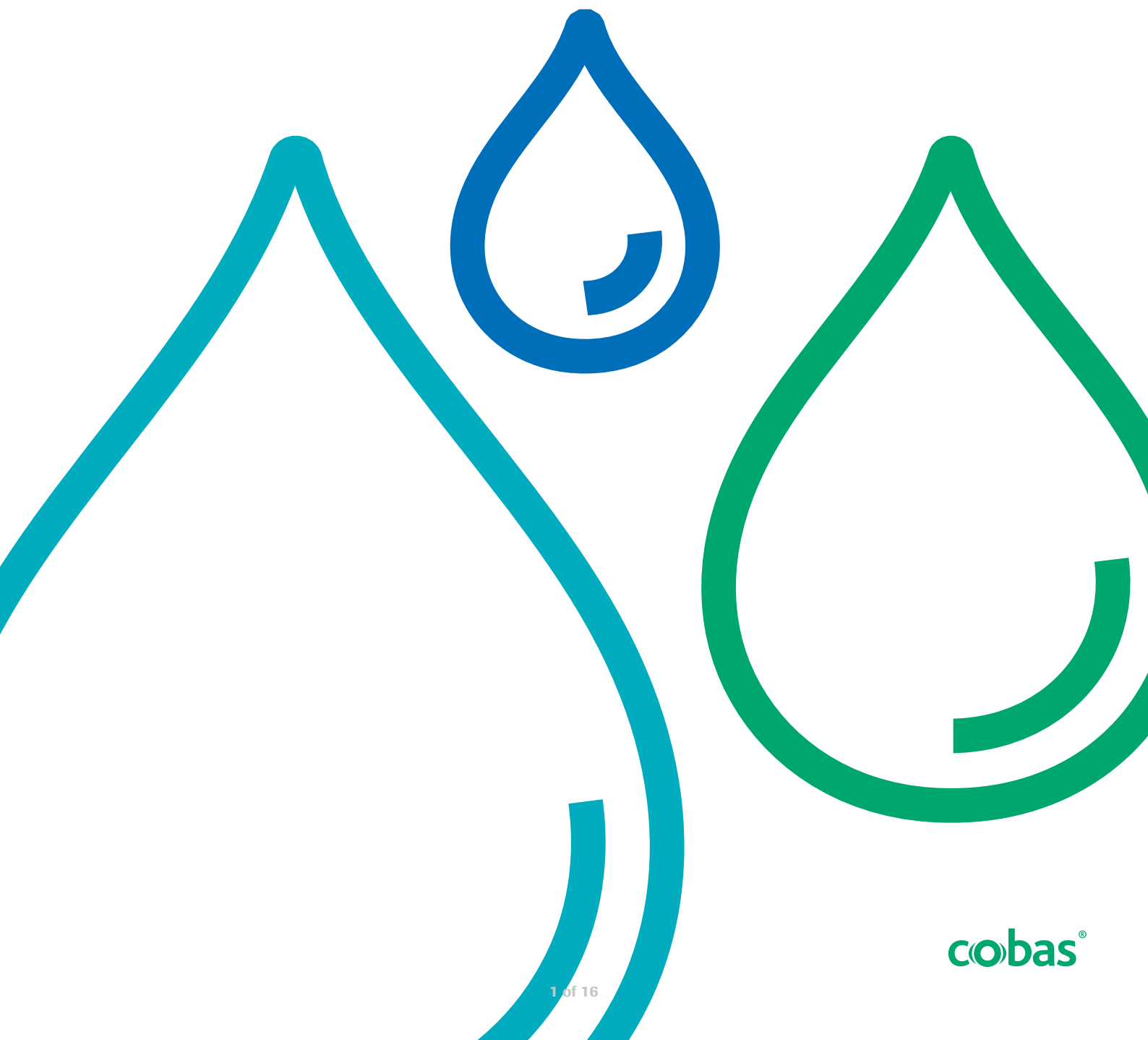
# Part 1: Hemoglobin variant head-to-head method comparison study

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Introduction

Ideally, a patient who has been diagnosed with diabetes would also be screened for hemoglobinopathies and thalassemia. This information could then be used to choose the right method to reliably measure hemoglobin A1c (HbA1c) and also for genetic counseling to avoid Hb major in newborns. Unfortunately, however, this is still not common clinical practice.

The most common remark from technicians using cation exchange high performance liquid chromatography (HPLC) to measure HbA1c, as opposed to immunoassays, is that they want to know if there is a variant present or not. In most cases, a variant will result in an abnormal chromatogram, whereas variant identification is not possible with immunoassays. However, this means every chromatogram should be checked manually for abnormalities prior to reporting the HbA1c result. While this process may enable the identification of potential interference from some Hb variants, more than 1,175 different variants exist. Furthermore, some Hb variants co-elute with HbA generating a normal chromatogram. In these cases, the HbA1c result would be artificially low or high, depending on which Hb variant is present. Figure 1 shows the complexity of HbA1c measurements using cation exchange HPLC. If this flow chart is followed, cation exchange HPLC is a method for determining HbA1c in the presence of Hb variants.

When using an immunoassay, technicians are often concerned that the test gives no indication of whether or not a variant is present. However, an immunoassay does not need to identify individual Hb variants because all forms of Hb are included for the HbA1c calculation. As long as there are no mutations in the epitope for the antibody used in the assay the HbA1c measurement is not affected.

Fig. 1: Complexity of HbA1c measurement with cation exchange HPLC in the presence of Hb variants
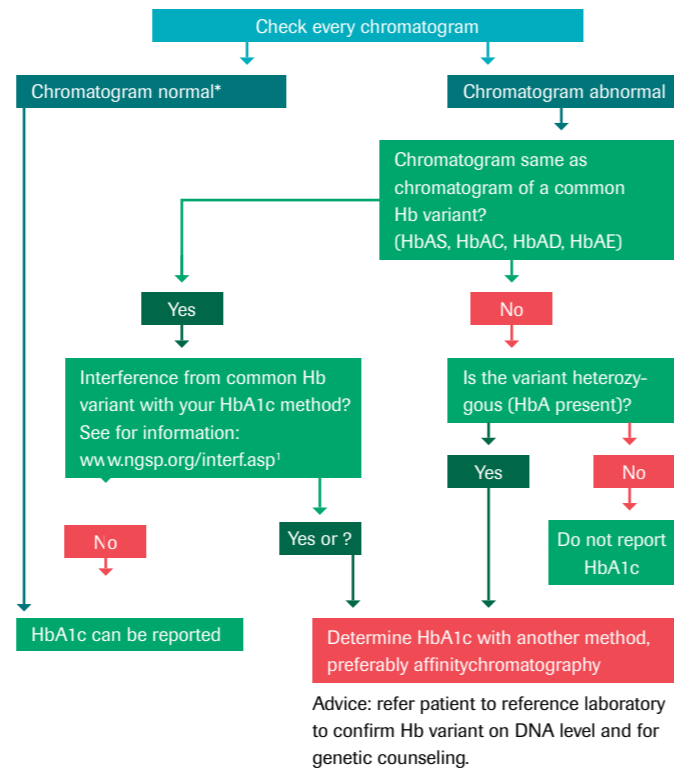
*A normal chromatogram does not guarantee that there is no Hb variant present. If a variant is present the HbA1c value will be falsely low or high, depending on the variant.

For the vast majority of the more than 1,175 Hb variants identified to date,[2] the mutation is not in the first four amino acids of the Hb protein and therefore does not interfere with the Tina-quant® HbA1c Gen.2 and Gen.3 assays from Roche; the mutation is in this region for only a very small number of variants (17/1,175).[2]

Furthermore, it is important to note that the prevalence of these 17 Hb variants is very low, and often they only occur in certain individuals (e.g. Hb Okayama in Japanese men) or in a few families, e.g. (Hb Graz in Austria) globally. Figure 2 shows the complexity of HbA1c measurement with an immunoassay and Table 1 lists some of the rare Hb variants that can interfere with the Tina-quant® HbA1c Gen.2 and Gen.3 assays.
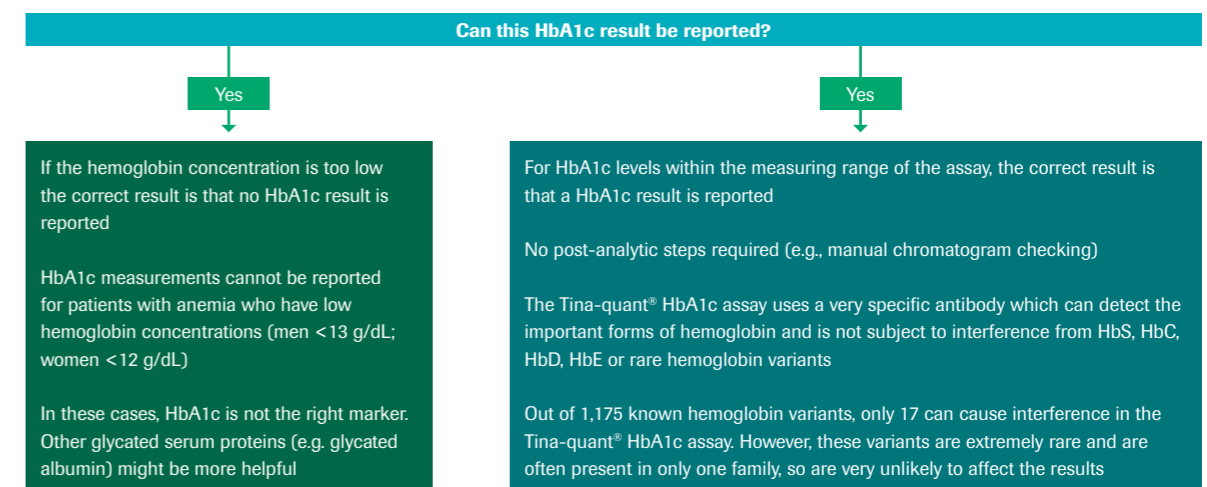

Fig. 2: Complexity of HbA1c measurement with an immunoassay in the presence of hemoglobin variants

Hb variant	Substitution of amino acid
Hb Raleigh	β1 Val → Ala
Hb Niigata	β1 Val → Leu
Hb Deer Lodge	β2 His → Arg
Hb Okayama	β2 His → Gln
Hb Graz	β2 His → Leu
Hb Agrigente	β2 His → Pro
Hb Fukuota	β2 His → Tyr

Table 1: Hemoglobin variants that can interfere with the Tina-quant® HbA1c Gen.2 and Gen.3 assays and potentially also with cation exchange HPLC2

Study design

The study objective was to investigate the potential interference of different Hb variants with different analytical methods for measuring HbA1c.

Samples were analyzed at two sites using the following methods:

Site 1: IFCC/NGSP Reference Laboratory (European Reference Laboratory, Isala, Zwolle, NL)
- Tina-quant® HbA1c Gen.2 on the COBAS INTEGRA® 800 analyzer; IFCC and NGSP certified immunoassay (Roche)
- Trinity Ultra2, IFCC and NGSP certified affinity chromatography HPLC (Trinity Biotech)
- Tosoh G8; IFCC certified cation-exchange HPLC (Tosoh Bioscience)

Site 2: IFFC/NGSP Reference Laboratory (European Reference Laboratory, Queen Beatrix Hospital, Winterswijk, NL)

- Tina-quant® HbA1c Gen.2 on the **cobas c** 501 module; immunoassay (Roche)
- Tina-quant® HbA1c Gen.3 on the **cobas c** 501 module; immunoassay (Roche)
- Menarini HA-8180V; IFCC and NGSP certified cation exchange HPLC (A. Menarini Diagnostics)

The IFCC reference system is currently the only valid method for standardizing HbA1c measurements.[3] All instruments and methods were calibrated using the IFCC secondary reference material (batch Berlin). The IFCC calibrators were run as samples and IFCC offline calibrated values calculated retrospectively. The final data evaluation to determine any potential interference of the variant was based on the IFCC offline calibrated results for all methods.

As the majority of Hb variants are considered not to interfere with Trinity Ultra[2] affinity chromatography, this method served as a comparator and all samples were analyzed in duplicate using this method.

For the other methods investigated, if 10 or more samples of one variant type were available, samples were measured individually, otherwise samples were tested in duplicate. All samples were investigated over 5 runs on 5 different days of about equal length.

Sample material consisted of whole blood from single donors. HbF samples comprised a mixture of blood from adults and neonates.

For variants Hb Volga and HbSS, no results could be reported. In the case of HbF, the mean value of the Menarini HA-8180V and the Tosoh G8 was taken as the reference method.

Significant differences between each method and the comparator, and recoveries between normal HbAA samples and samples containing variant Hb were calculated using a Deming regression at a target value of 42 mmol/mol and 75 mmol/mol, respectively. The maximum expected deviation was ~5% and a deviation >10% was considered to be significant. The results were also plotted in a graph and if the results of samples with Hb variants fell within the dispersion of the normal HbAA samples the variant was considered not to interfere with the method.

Results and discussion
Results using Tina-quant® HbA1c assay from Roche
The study shows that the Hb variants HbAS, HbAC, HbAD, HbAE, HbA2, HbAJ, HbAG and the rare variants do not interfere with the Tina-quant® HbA1c Gen.2

and Gen.3 assays run on the **cobas c** 501 module or the Tina-quant® HbA1c Gen.2 assay run on the Advice: refer patient to reference laboratory to confirm Hb variant on DNA level and for genetic counseling. COBAS INTEGRA® 800 analyzer (Table 2, Figures 3 – 5, 8 – 11, 13 – 15, 17 – 19 and 21 – 23). Only HbF >8% and the Hb Okayama variant were found to interfere with the Tina-quant® HbA1c Gen.2 assay (Figures 26 – 28).

The effect of Hb Okayama can be explained by the fact that the antibodies used in these assays target the four amino acids and the glucose at the N-terminal end of the hemoglobin β-globin chain. In the case of Hb Okayama, there is a substitution of the second amino acid (β-2 His ➔ Gln) and therefore this variant will not be recognized by the antibodies in the assay. HbF does not glycate as fast as HbAA, but is included in the measurement of total Hb, which explains why the HbA1c result is falsely low. It is very difficult to get samples from patients with different HbF and HbA1c values and for this reason the HbF samples tested comprised mixtures of blood from adults and neonates. The literature confirms that most of the common HbA1c methods are affected when HbF is >15%.[4,5] In these studies samples from adults were used instead of mixtures of blood from adults and neonates and this could account for the fact that the current study observed interference at HbF >8% rather than HbF >15%.

Results using Menarini HA-8180V
The Menarini HA-8180V did not produce a HbA1c result for samples containing HbAD, HbAE, HbAJ, HbAG and most of the rare variants. However, reporting no result in the case of an abnormal chromatogram, where the Hb variant is not completely separated from the A0 peak, is the correct outcome. The Menarini HA-8180V did produce a result for samples containing HbAS and HbAC and elevated A2. The HbS and HbC peaks are completely separated from the A0 peak in the chromatogram and similarly the HbS1c and HbC1c peak are completely separated from the HbA1c peak. The software can therefore accurately subtract the area of the variant peak from the total area and calculate the HbA1c value correctly and these variants do not cause interference (Figures 6 and 11). All investigated variants except Hb Indonesia and Hb Hopkins-2 gave abnormal chromatograms with the Menarini HA-8180V and, in most cases, gave no HbA1c result (Table 2). Using this HPLC technique, the HbF peak is separated from the total area and therefore a true HbA1c result is obtained.

Results using Tosoh G8
Table 2 and Figures 7, 12, 16, 20 and 25 show that the Tosoh G8 produced a result for samples containing HbAS, HbAC, HbAD, HbAE, elevated A2, HbAG and HbAJ. However, interference due to HbAE and HbAJ was observed, with false low values obtained in samples containing these two variants (Figures 20 and 25). Figure 7 shows lower results obtained with the Tosoh G8 when samples contained the variant HbAS and the deviation from the HbAA samples was borderline (9.5% deviation at 42 mmol/mol and 8.5% at 75 mmol/mol).

cobas c 501 module with Tina-quant® HbA1c Gen.2 assay

	Linear regression	Deming regression	R²	42 mmol/mol	Significant difference	75 mmol/mol	Significant difference
AA	y = 1.07x - 3.67	y = 1.09x - 4.42	0.978	41		77	
AS	y = 1.04x - 2.91	y = 1.05x - 3.26	0.987	41	No	75	No
AC	y = 1.02x - 1.29	y = 1.03x - 1.64	0.985	42	No	76	No
AD	y = 0.96x + 0.37	y = 0.97x - 0.05	0.983	41	No	72	No
AE	y = 0.99x - 0.04	y = 1.00x - 0.44	0.958	42	No	74	No
HbF					> HbF 8%		
A2					No		
Rare variants					Hb Okayama		

cobas c 501 module with Tina-quant® HbA1c Gen.3 assay

	Linear regression	Deming regression	R²	42 mmol/mol	Significant difference	75 mmol/mol	Significant difference
AA	y = 1.08x - 3.01	y = 1.09x - 3.70	0.980	42		78	
AS	y = 1.10x - 4.38	y = 1.10x - 4.68	0.990	42	No	78	No
AC	y = 1.01x - 0.77	y = 1.03x - 1.59	0.965	42	No	76	No
AD	y = 0.97x + 1.99	y = 0.98x + 1.41	0.976	43	No	75	No
AE	y = 1.08x - 2.89	y = 1.10x - 3.78	0.971	42	No	78	No
HbF					> HbF 8%		
A2					No		
Rare variants					Hb Okayama		

COBAS INTEGRA® 800 analyzer with Tina-quant® HbA1c Gen.2

	Linear regression	Deming regression	R²	42 mmol/mol	Significant difference	75 mmol/mol	Significant difference
AA	y = 1.08x - 4.28	y = 1.08x - 4.56	0.992	41		77	
AS	y = 1.07x - 4.40	y = 1.08x - 4.72	0.989	41	No	76	No
AC	y = 1.00x - 0.72	y = 1.01x - 1.07	0.980	41	No	75	No
AD	y = 0.98x - 0.52	y = 0.99x - 0.82	0.988	41	No	73	No
AE	y = 0.99x - 0.47	y = 1.00x - 0.89	0.984	41	No	74	No
HbF					> HbF 8%		
A2					No		
Rare variants					Hb Okayama		

However, it should be noted that the Deming regression lines calculated for the common variants were based on 20 samples compared with 50 samples for the normal HbAA and that the distribution of HbA1c over the clinically important range for HbA1c samples containing the common variants (HbAS, HbAC, HbAD and HbAE) was not always optimal. It is debatable, therefore, whether the method for calculating the deviation from normal samples is correct, because Figure 7 clearly shows a difference from normal HbAA samples. This phenomenon of lower results with HbAS variants using the Tosoh G7/G8 has been observed in previous studies (not published) and, as yet, there is no explanation given that the chromatograms show that the S0 peak is completely separated from the A0 peak, and hence a correct result would be expected. All investigated variants except Hb Indonesia and Hb Hopkins-2 gave abnormal chromatograms with the Tosoh G8 and,

in most cases, gave no HbA1c result (Table 2). Using this HPLC technique, the HbF peak is separated from the total area and therefore a true HbA1c result is obtained.

Conclusion

The perfect method for measuring HbA1c still does not exist and which method is chosen will depend on the laboratory's priorities. Some will choose to use an immunoassay to obtain a fast and reliable HbA1c result regardless of the presence or absence of a Hb variant. Other laboratories, however, will want to know if a variant is present and opt for cation exchange HPLC plus (hopefully) the associated checking algorithm to avoid incorrect results. Hence, the advantages/disadvantages of the different HbA1c methods will be viewed differently by different laboratories.

Menarini HA-8180V

	Linear regression	Deming regression	R^2	42 mmol/mol	Significant difference	75 mmol/mol	Significant difference
AA	y = 1.03x -1.54	y = 1.03x - 1.90	0.989	41		76	
AS	y = 1.06x - 5.91	y = 1.07x - 6.31	0.989	39	No	74	No
AC	y = 1.02x - 0.46	y = 1.03x - 1.08	0.973	42	No	76	No
AD					No result		
AE					No result		
HbF							
A2					No		
Rare variants					All investigated rare variants except for Hb Indonesia and Hb Hopkins-2		

Tosoh G8

	Linear regression	Deming regression	R^2	42 mmol/mol	Significant difference	75 mmol/mol	Significant difference
AA	y = 1.02x - 0.37	y = 1.02x - 0.67	0.990	42		76	
AS	y = 0.95x - 1.60	y = 0.96x - 1.97	0.984	38	No	70	No
AC	y = 1.02x - 1.95	y = 1.04x - 2.91	0.960	41	No	75	No
AD	y = 0.83x + 8.41	y = 0.85x + 7.19	0.935	43	No	71	No
AE	y = 0.69x + 0.80	y = 0.70x + 0.29	0.957	30	Yes	53	Yes
HbF							
A2					No		
Rare variants					All investigated rare variants except for Hb Indonesia and Hb Hopkins-2		

Table 2: Medical decision points calculated with Deming regression lines. A deviation >10% compared with HbAA samples was considered to be significant

Lower results obtained with the Tosoh G8 when samples contain the variant HbAS
Hemoglobin S (% variant: 31 – 42 % S)
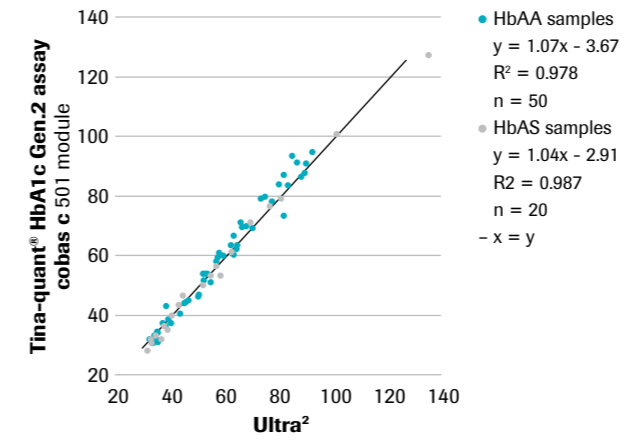
HbAA samples
y = 1.07x - 3.67
R^2 = 0.978
n = 50
HbAS samples
y = 1.04x - 2.91
R2 = 0.987
n = 20
– x = y

Fig. 3: Results from the Tina-quant® HbA1c Gen.2 assay on the **cobas c** 501 module in the presence of HbAS. HbA1c: 30 – 100 mmol/mol

HbAA samples
y = 1.08x - 3.01
R^2 = 0.980
n = 50
HbAS samples
y = 1.10x - 4.38
R2 = 0.990
n = 20
– x = y

Fig. 4: Results from the Tina-quant® HbA1c Gen.3 assay on the **cobas c** 501 module in the presence of HbAS. HbA1c: 30 – 100 mmol/mol

HbAA samples
y = 1.08x - 4.28
R^2 = 0.992
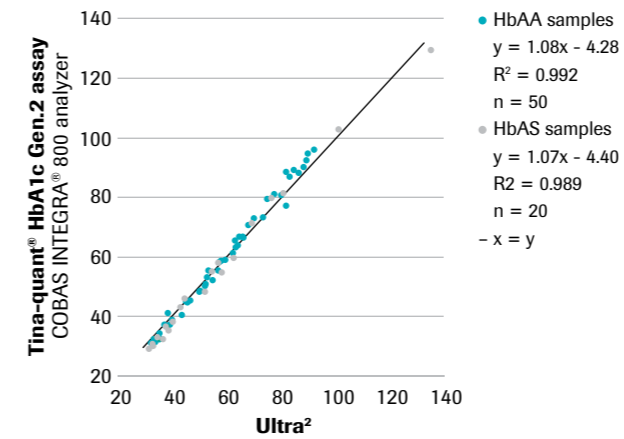n = 50
HbAS samples
y = 1.07x - 4.40
R2 = 0.989
n = 20
– x = y

Fig. 5: Results from the Tina-quant® HbA1c Gen.2 assay on the COBAS INTEGRA® 800 analyzer in the presence of HbAS. HbA1c: 30 – 100 mmol/mol
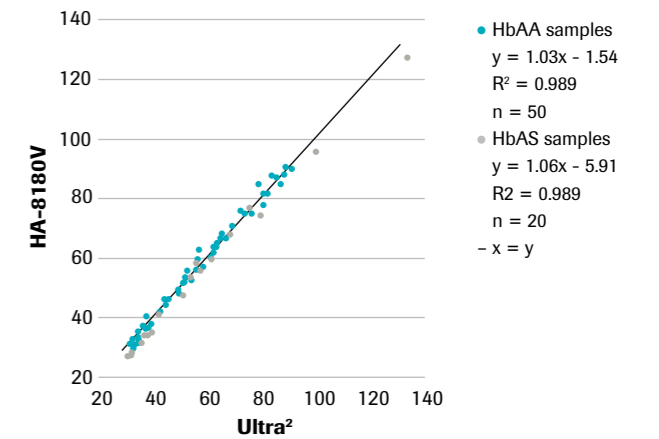

HbAA samples
y = 1.03x - 1.54
R^2 = 0.989
n = 50
HbAS samples
y = 1.06x - 5.91
R2 = 0.989
n = 20
– x = y

Fig. 6: Results from the Menarini HA-8180V in the presence of HbAS HbA1c: 30 – 100 mmol/mol

HbAA samples
y = 1.02x - 0.37
R^2 = 0.990
n = 50
HbAS samples
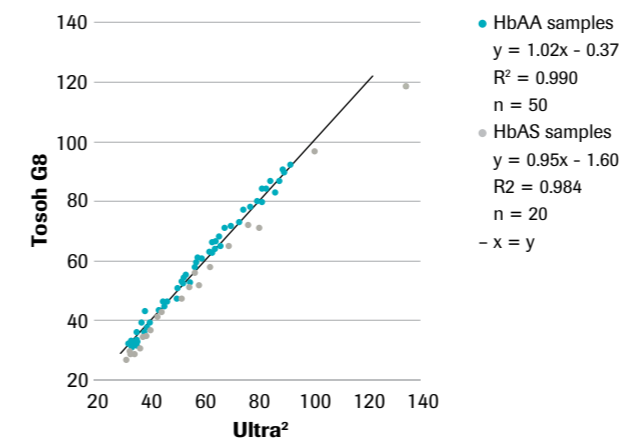y = 0.95x - 1.60
R2 = 0.984
n = 20
– x = y
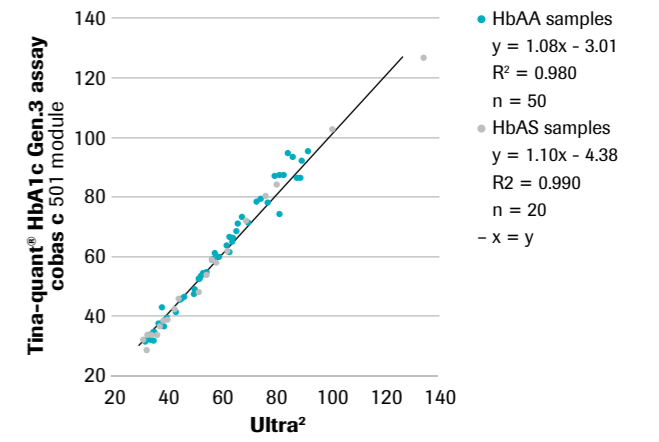
Fig. 7: Results from the Tosoh G8 in the presence of HbAS HbA1c: 30 – 100 mmol/mol

All methods free from interference from HbAC

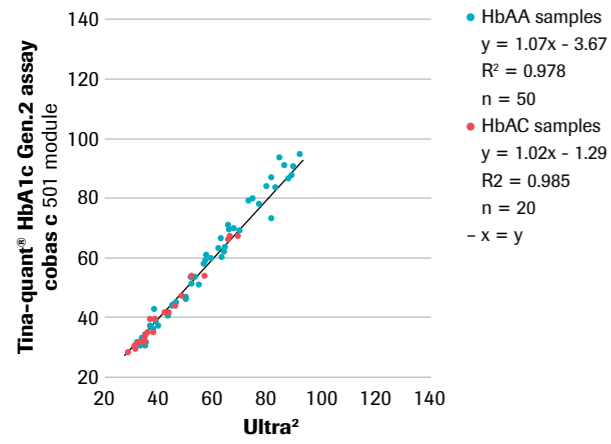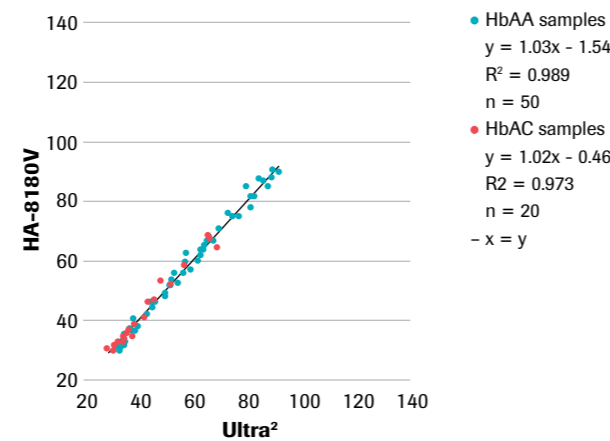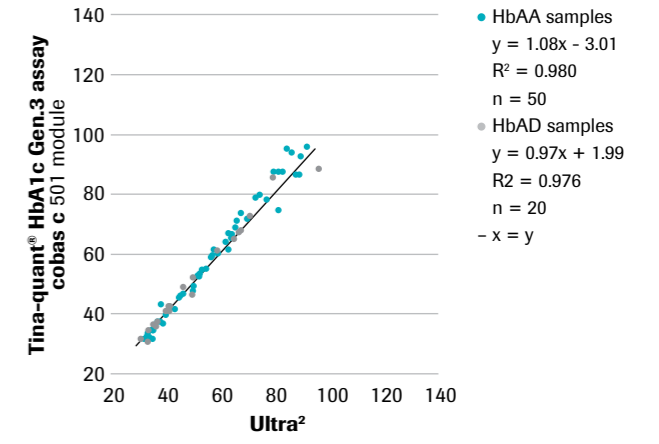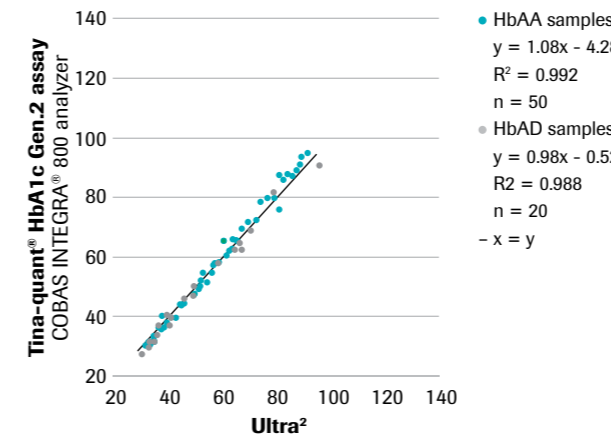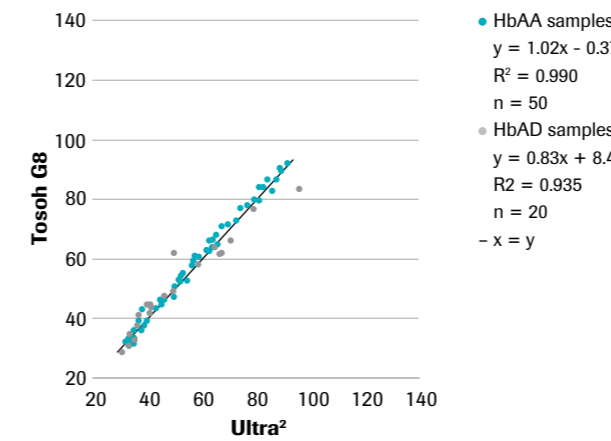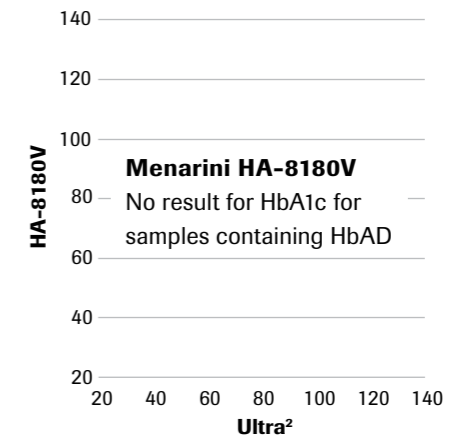Hemoglobin C (% variant: 36 – 42 % C)

HbAA samples
y = 1.07x - 3.67
R² = 0.978
n = 50
HbAC samples
y = 1.02x - 1.29
R2 = 0.985
n = 20
– x = y

Fig. 8: Results from the Tina-quant® HbA1c Gen.2 assay on the **cobas c** 501 module in the presence of HbAC. HbA1c: 26–104 mmol/mol

HbAA samples
y = 1.08x - 3.01
R² = 0.980
n = 50
HbAC samples
y = 1.01x - 0.77
R2 = 0.965
n = 20
– x = y

Fig. 9: Results from the Tina-quant® HbA1c Gen.3 assay on the **cobas c** 501 module in the presence of HbAC. HbA1c: 26–104 mmol/mol

HbAA samples
y = 1.08x - 4.28
R² = 0.992
n = 50
HbAC samples
y = 1.00x - 0.72
R2 = 0.980
n = 20
– x = y
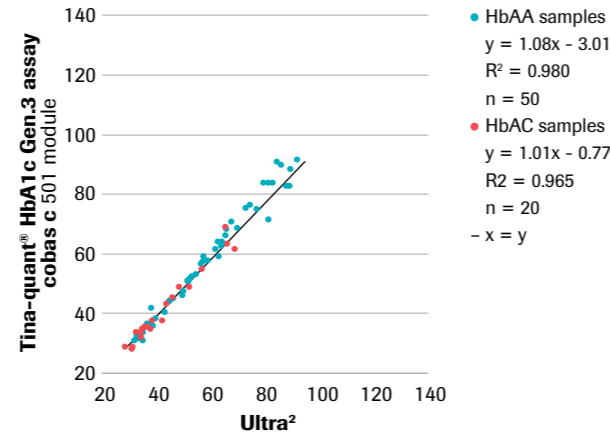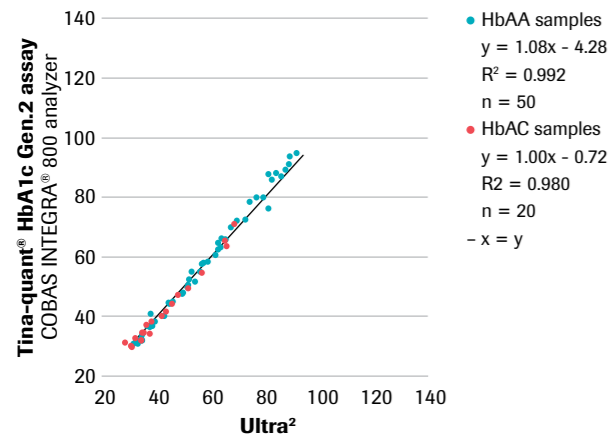
Fig. 10: Results from the Tina-quant® HbA1c assay Gen.2 on the COBAS INTEGRA® 800 analyzer in the presence of HbAC HbA1c: 26–104 mmol/mol

HbAA samples
y = 1.03x - 1.54
R² = 0.989
n = 50
HbAC samples
y = 1.02x - 0.46
R2 = 0.973
n = 20
– x = y

Fig. 11: Results from the Menarini HA-8180V in the presence of HbAC HbA1c: 26–104 mmol/mol

HbAA samples
y = 1.02x - 0.37
R² = 0.990
n = 50
HbAC samples
y = 1.02x - 1.95
R2 = 0.960
n = 20
– x = y
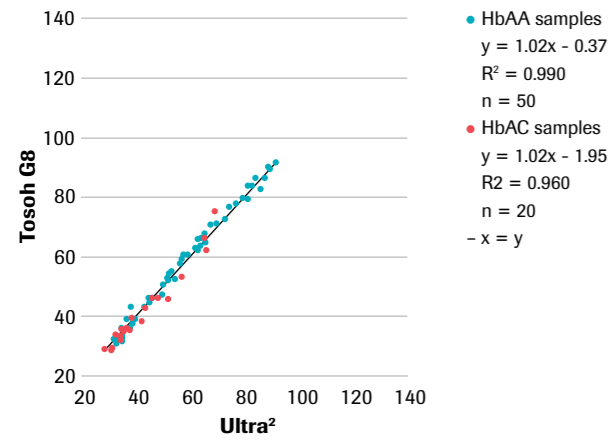
Fig. 12: Results from the Tosoh G8 in the presence of HbAC HbA1c: 26–104 mmol/mol

Presence of HbAD causes no results on Menarini HA-8180V

Hemoglobin D (% variant: 37 – 42 % D)
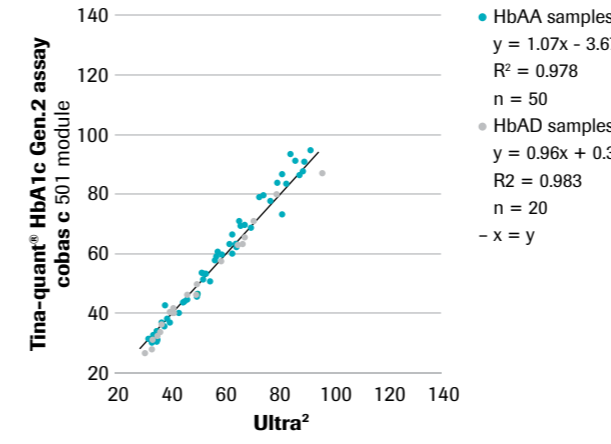
HbAA samples
y = 1.07x - 3.67
R² = 0.978
n = 50
HbAD samples
y = 0.96x + 0.37
R2 = 0.983
n = 20
– x = y

Fig. 13: Results from the Tina-quant® HbA1c Gen.2 assay on the **cobas c** 501 module in the presence of HbAD. HbA1c: 30–96 mmol/mol

HbAA samples
y = 1.08x - 3.01
R² = 0.980
n = 50
HbAD samples
y = 0.97x + 1.99
R2 = 0.976
n = 20
– x = y

Fig. 14: Results from the Tina-quant® HbA1c Gen.3 assay on the **cobas c** 501 module in the presence of HbAD. HbA1c: 30–96 mmol/mol

HbAA samples
y = 1.08x - 4.28
R² = 0.992
n = 50
HbAD samples
y = 0.98x - 0.52
R2 = 0.988
n = 20
– x = y

Fig. 15: Results from the Tina-quant® HbA1c Gen.2 assay on the COBAS INTEGRA® 800 analyzer in the presence of HbAD. HbA1c: 30–96 mmol/mol
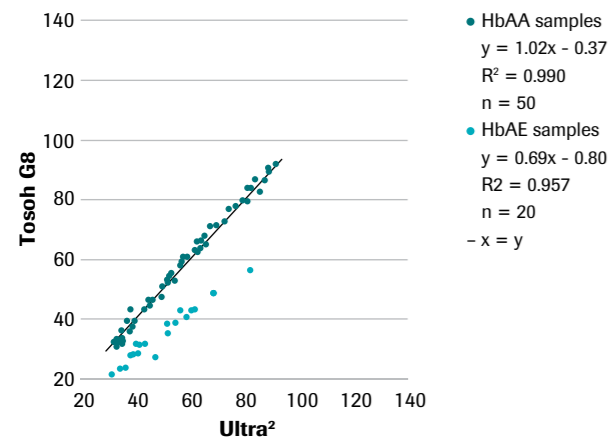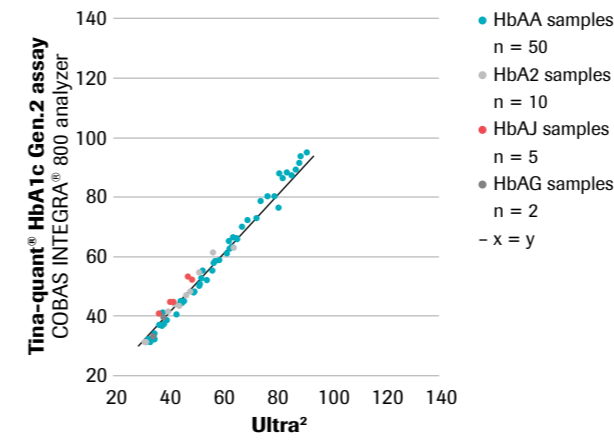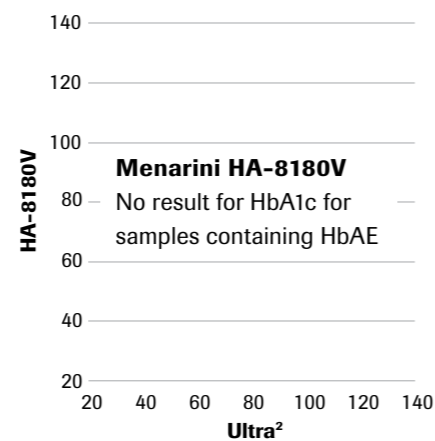

Menarini HA-8180V
No result for HbA1c for samples containing HbAD

HbAA samples
y = 1.02x - 0.37
R² = 0.990
n = 50
HbAD samples
y = 0.83x + 8.41
R2 = 0.935
n = 20
– x = y

Fig. 16: Results from the Tosoh G8 in the presence of HbAD HbA1c: 30–96 mmol/mol

No results on Menarini HA-8180V and false low values on Tosoh G8 in samples containing HbAE

Hemoglobin E (% variant: 27–33 % E)
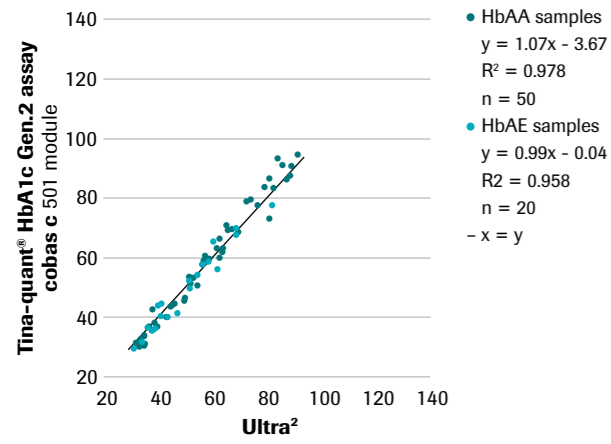
Fig. 17: Results from the Tina-quant® HbA1c Gen.2 assay on the **cobas c** 501 module in the presence of HbAE. HbA1c: 38–81 mmol/mol
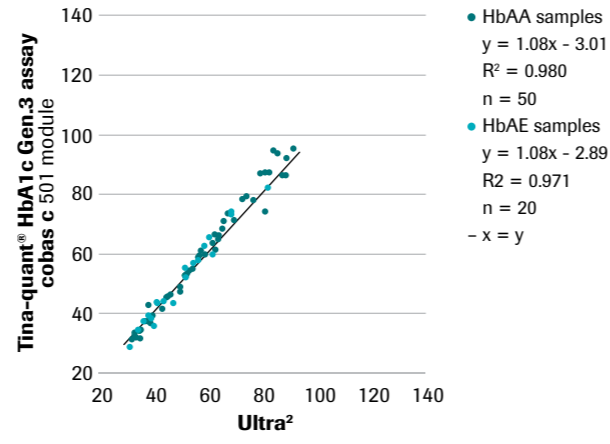
Fig. 18: Results from the Tina-quant® HbA1c Gen.3 assay on the **cobas c** 501 module in the presence of HbAE. HbA1c: 38–81 mmol/mol
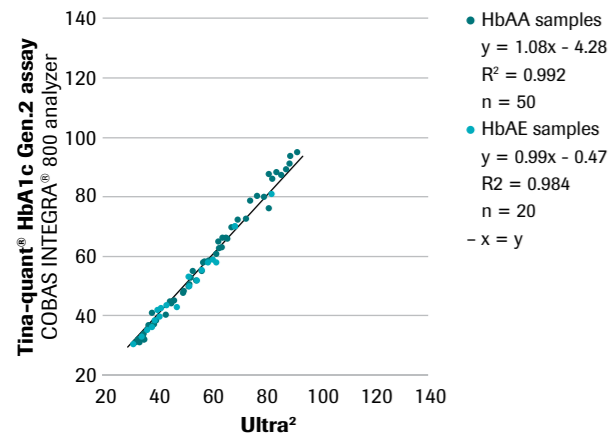
Fig. 19: Results from the Tina-quant® HbA1c Gen.2 assay on the COBAS INTEGRA® 800 analyzer in the presence of HbAE. HbA1c: 38–81 mmol/mol

Fig. 20: Results from the Tosoh G8 in the presence of HbAE
HbA1c: 38–81 mmol/mol

Interference from HbAJ and HbAG is observed with Menarini HA-8180V and samples containing HbAJ gave false low results on Tosoh G8

Hemoglobin A2, AJ and AG (% variant: 4–7 % A2, 49–51 % AJ, approximately 18 % AG)
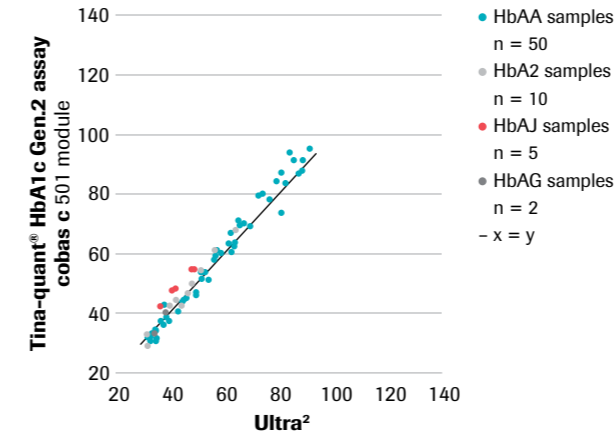
Fig. 21: Results from the Tina-quant® HbA1c Gen.2 assay on the **cobas c** 501 module in the presence of HbA2, HbAJ and HbAG
HbA1c: 30–70 mmol/mol A2, 36–54 mmol/mol AJ, 34–43 mmol/mol AG
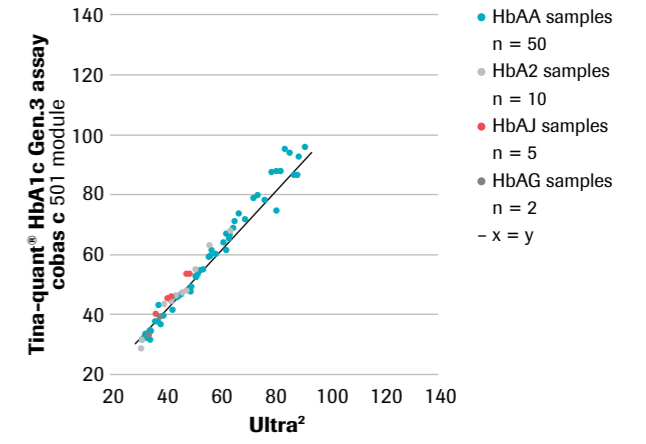
Fig. 22: Results from the Tina-quant® HbA1c Gen.3 assay on the **cobas c** 501 module in the presence of HbA2, HbAJ and HbAG
HbA1c: 30–70 mmol/mol A2, 36–54 mmol/mol AJ, 34–43 mmol/mol AG

Fig. 23: Results from the Tina-quant® HbA1c Gen.2 assay on the COBAS INTEGRA® 800 analyzer in the presence of HbA2, HbAJ and HbAG
HbA1c: 30–70 mmol/mol A2, 36–54 mmol/mol AJ, 34–43 mmol/mol AG
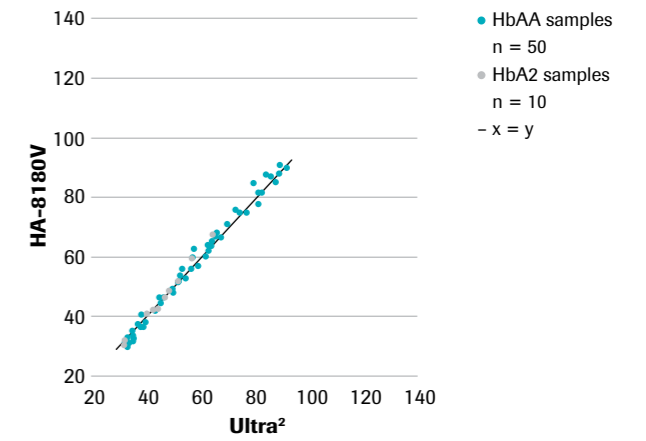
Fig. 24: Results from the Menarini HA-8180V in the presence of HbA2
HbA1c: 30–70 mmol/mol
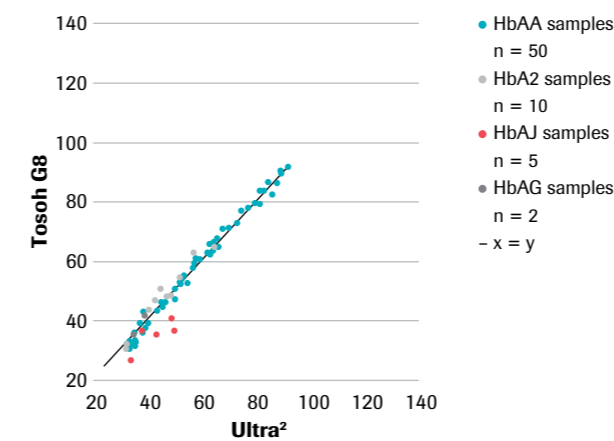
Fig. 25: Results from the Tosoh G8 in the presence of HbA2, HbAJ and HbAG
HbA1c: 30–70 mmol/mol A2, 36–54 mmol/mol AJ, 34–43 mmol/mol AG

All investigated rare variants except for Hb Indonesia and Hb Hopkins-2 gave an abnormal chromatogram and no HbA1c result with Menarini HA-8180V and Tosoh G8
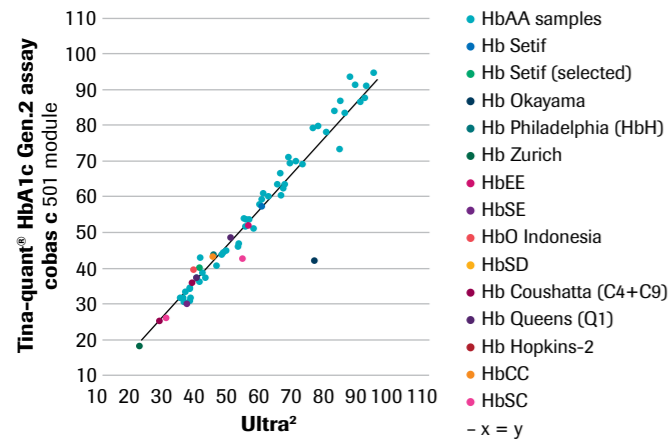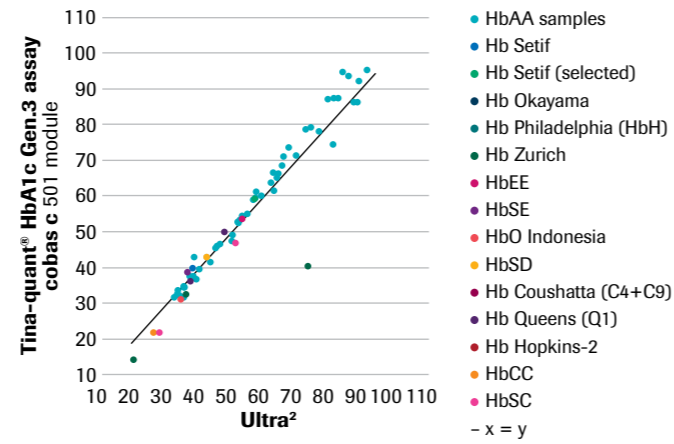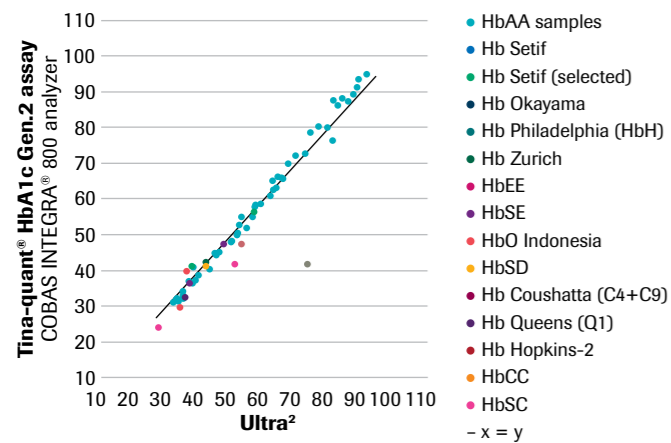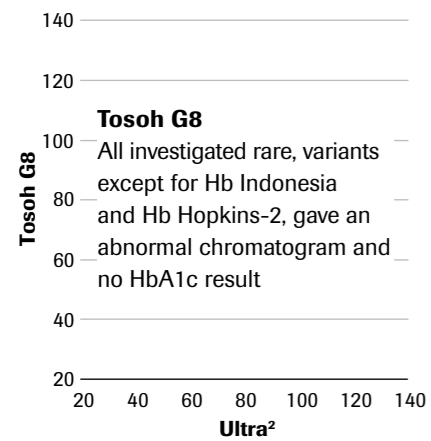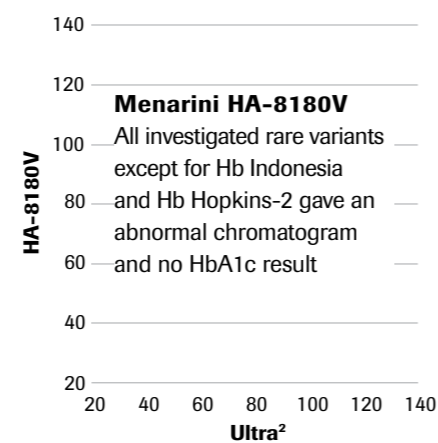Rare variants

Legend:
- HbAA samples
- Hb Setif
- Hb Setif (selected)
- Hb Okayama
- Hb Philadelphia (HbH)
- Hb Zurich
- HbEE
- HbSE
- HbO Indonesia
- HbSD
- Hb Coushatta (C4+C9)
- Hb Queens (Q1)
- Hb Hopkins-2
- HbCC
- HbSC
- – x = y

Fig. 26: Results from the Tina-quant® HbA1c Gen.2 assay on the **cobas c** 501 module in the presence of rare hemoglobin variants

Legend:
- HbAA samples
- Hb Setif
- Hb Setif (selected)
- Hb Okayama
- Hb Philadelphia (HbH)
- Hb Zurich
- HbEE
- HbSE
- HbO Indonesia
- HbSD
- Hb Coushatta (C4+C9)
- Hb Queens (Q1)
- Hb Hopkins-2
- HbCC
- HbSC
- – x = y

Fig. 27: Results from the Tina-quant® HbA1c Gen.3 assay on the **cobas c** 501 module in the presence of rare hemoglobin variants

Legend:
- HbAA samples
- Hb Setif
- Hb Setif (selected)
- Hb Okayama
- Hb Philadelphia (HbH)
- Hb Zurich
- HbEE
- HbSE
- HbO Indonesia
- HbSD
- Hb Coushatta (C4+C9)
- Hb Queens (Q1)
- Hb Hopkins-2
- HbCC
- HbSC
- – x = y

Fig. 28: Results from the Tina-quant® HbA1c Gen.2 assay on the COBAS INTEGRA® 800 analyzer in the presence of rare hemoglobin variants

Menarini HA-8180V

All investigated rare variants except for Hb Indonesia and Hb Hopkins-2 gave an abnormal chromatogram and no HbA1c result

Tosoh G8

All investigated rare, variants except for Hb Indonesia and Hb Hopkins-2, gave an abnormal chromatogram and no HbA1c result

Variant type	HbA1c (mmol/mol)	n
HbAA	0 – 100	50
Hb Setif	38 – 58	2
Hb Okayama	74	1
Hb Philadelphia (HbH)	41	1
Hb Zurich	20	1
HbEE	60	1
HbSE	36	1
HbO Indonesia	39	1
HbSD	21	1
Hb Coushatta (C4+C9)	No indications possible	2
Hb Queens	No indications possible	1
Hb Hopkins-2	43	1
HbCC	51	1
HbSC	21	1

Table 3: Rare hemoglobin variants used on all systems
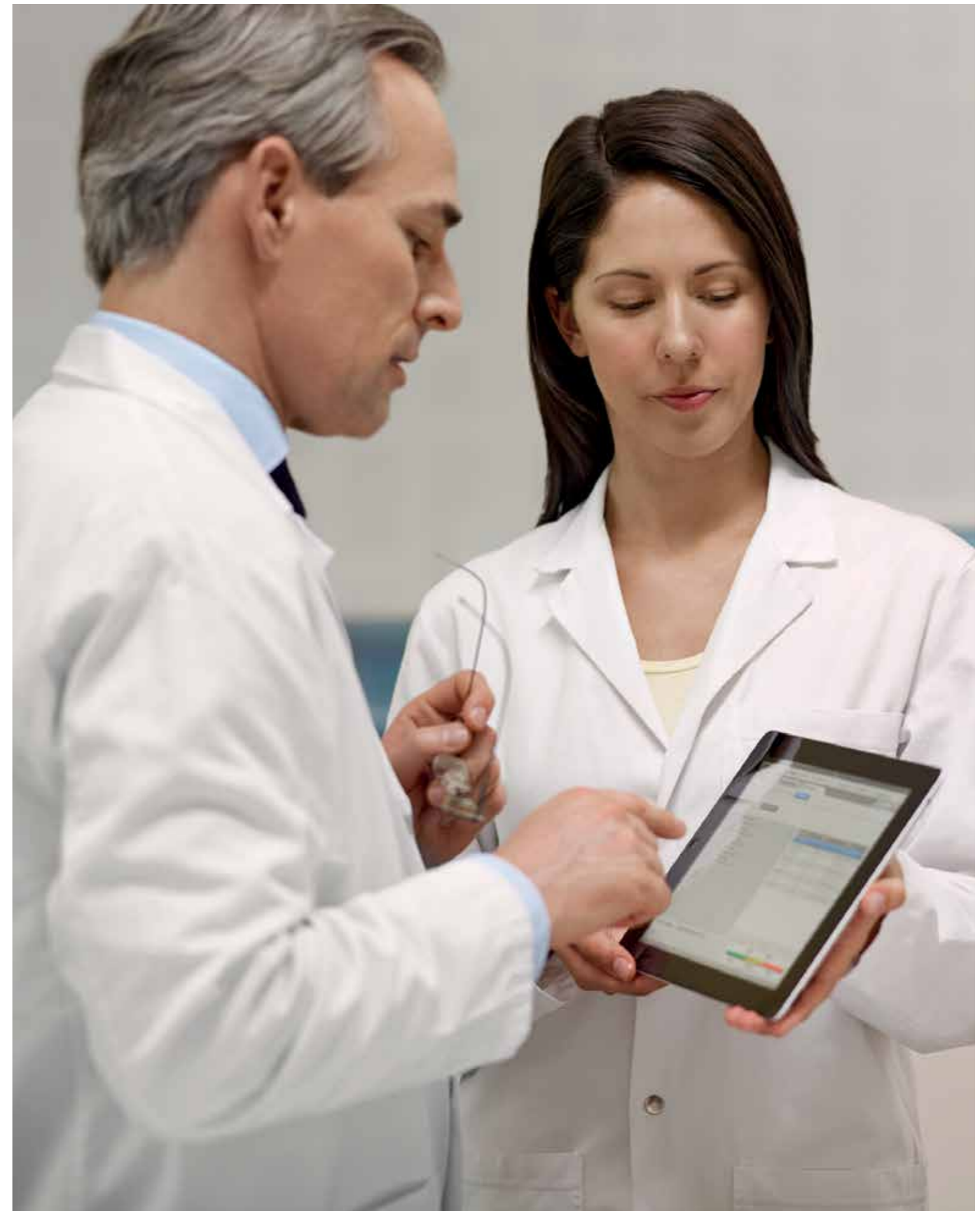
Part 2: Literature collection of variant interference from CE-HPLC

The inherited hemoglobin disorders are the commonest diseases attributable to single defective genes. Approximately 7 percent of the world's population is a carrier.[6] For combination of diabetes with variant carrier is not rare in clinical practise, people have the endless debating on which method is superior in HbA1c measurement with the least interference from variants. However, there is no simple answer there.

As Dr. Randie said, "Following manufacturer instructions and training for interpretation of ionexchange HPLC chromatograms and CE electropherograms, careful examination usually, but not always, prevented reporting of inaccurate results."[7]

Some incorrect result is reported in silence, as mentioned by Prof. Song, "Laboratories should be aware that the fast mode HPLC methods could not detect relatively common Hb variants in the Korean population, which is likely to lead to inaccurate results, especially incorrectly low results. Clinical management of patients that depend solely on these results may lead to inappropriate treatment."[8] Moreover, even as the most classic method in Hb Variants identification, the study from Mayo Clinic concludes, "It (CE-HPLC) should not be used as a stand-alone method for definitive identification of Hb variants."[9]

Hence, a few paper collected since 2000 to remind audiences the possibility which misreporting happens due to the interference of variants in CE-HPLC as the stand-alone method for HbA1c measurement in diabetes management. In the CAP survey in 2018, as calculated there were more than 50 % participants reported as immunoassay users.[11]

Paper	Link	Paper	Link
Hb variants in Korea: effect on HbA1c using five routine methods		Effects of hemoglobin (Hb) E and HbD traits on measurements of glycated Hb (HbA1c) by 23 methods	
Effects of Hemoglobin C, D, E and S traits on Measurements of Hemoglobin A1c by Twelve Methods		Effects of 7 hemoglobin variants on the measurement of glycohemoglobin by 14 analytical methods	
Effects of 49 Different Rare Hb Variants on HbA1c Measurement in Eight Methods		Silent hemoglobin variants and determination of HbA(1c) with the high-resolution program of the HPLC HA-8160 analyzer	
A Large Cohort of Hemoglobin Variants in Thailand: Molecular Epidemiological Study and Diagnostic Consideration		Effects of hemoglobin C and S traits on glycohemoglobin measurements by eleven methods	
Effects of Hemoglobin Variants on Hemoglobin A1c Values Measured Using a HPLC Method		Effects of hemoglobin C and S traits on seven glycohemoglobin methods	
Interference of hemoglobin (Hb) N-Baltimore on measurement of HbA1c using the HA-8160 HPLC method		Effects of Hemoglobin Variants and Chemically Modified Derivatives on Assays for Glycohemoglobin	
Effects of hemoglobin C, D, E, and S traits on measurements of HbA1c by six methods		Unexpected Hemoglobin A1c Results	
A comprehensive analysis of hemoglobin variants by high-performance liquid chromatography (HPLC)			

There is no "perfect" method for measurement of HbA1c, we need a close knowledge exchange between clinical chemists and health care providers.[10]

References

1   *NGSP. HbA1c methods: Effects of hemoglobin variants (HbC, HbS, HbE and HbD traits) and elevated fetal hemoglobin (HbF). Updated July 2013. Available at: http://www.ngsp.org/interf.asp (accessed October 2013).*

2   *HbVar. A database of human hemoglobin variants and thalassemias. Available at: http://globin.cse.psu.edu/globin/ hbvar/ (accessed October 2013).*

3   *Consensus Committee. (2007). Consensus statement on the worldwide standardization of the hemoglobin A1C measurement: the American Diabetes Association, European Association for the Study of Diabetes, International Federation of Clinical Chemistry and Laboratory Medicine, and the International Diabetes Federation.* **Diabetes Care 30,** *2399-2400.*

4   *Rohlfing, C.L., Connolly, S.M., England, J.D., Hanson, S.E., Moellering, C.M., Bachelder, J.R., Little, R.R. (2008). The effect of elevated fetal hemoglobin on hemoglobin A1c results: five common hemoglobin A1c methods compared with the IFCC reference method.* **Am J Clin Pathol 129,** *811-814.*

5   *Little, R.R., Rohlfing, C.L., Hanson, S.E., Schmidt, R.L., Lin, C.N., Madsen, R.W., Roberts, W.L. (2012). The effect of increased fetal hemoglobin on 7 common Hb A1c assay methods.* **Clin Chem 58,** *945-947.*

6   *Report of the Fifth WHO Working Group on the Feasibility Study on Hereditary Disease Community Control Programmes (Hereditary Anaemias) Cagliari, Sardinia. WHO/HDP/WG/HA/89.2. Geneva: WHO.*

7   *Randie, R., Little, et al. (2015).* **Effects of 49 Different Rare Hb Variants on HbA1c Measurement in Eight Methods 9(4), 849-856** *.*

8   *Junghan, S. et al. (2017).* **Hb variants in Korea: effect on HbA1c using five routine methods 55(8),** *1234-1242.*

9   *Hoyer, J.D. et al. (2012). Department of Laboratory Medicine and Pathology, Mayo Clinic. A comprehensive analysis of hemoglobin variants by high-performance liquid chromatography (HPLC).* **Int J Lab Hematol 34**(6), *594-604.*

10 *Lenters-Westra, E. (2013). Haemoglobin A1c: Historical overview and current concepts. Diabetes research and clinical practice 99, 75-84*

11 *Ngsp.org.*