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Potential of MALDI-TOF mass spectrometry to overcome the interference of hemoglobin variants on HbA_{1c} measurement

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Abstract

Objectives: Hemoglobin (Hb) variants remain an important cause of erroneous HbA_{1c} results. We present an approach to overcome the interference of Hb variants on HbA_{1c} measurements using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS).

Methods: Samples containing or not containing Hb variants were analyzed for HbA_{1c} using an MALDI-TOF MS system (QuanTOF) and a boronate affinity comparative method (Ultra^2). For QuanTOF, two sets of HbA_{1c} values were obtained through α- and β-chain glycation.

Results: A robust correlation between the glycation degrees of the α- and β-chains was found, and HbA_{1c} values derived from α- and β-chain glycation correlated well with the Ultra^2 results. Statistically significant differences ($p<0.01$) were found for all the Hb variants tested. When using the conventional β-chain glycation to determine HbA_{1c}, clinically significant differences were only found among samples containing β-chain variants detected by QuanTOF (i.e., Hb J-Bangkok, Hb G-Coushatta, and Hb G-Taipei). In contrast, based on α-chain glycation, no clinically significant differences were found for these three variants.

Conclusions: In addition to conventional β-chain glycation, α-chain glycation can be used to calculate HbA_{1c} values. The interference of Hb variants on HbA_{1c} quantification can be overcome by employing the glycation of the globin chain without a genetic variant to estimate HbA_{1c} values.

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Keywords: α-chain glycation; glycation degree; HbA_{1c}; hemoglobin variant; interference; matrix-assisted laser desorption/ionization time-of-flight mass spectrometry.

Introduction

Hemoglobin A_{1c} (HbA_{1c}) is characterized by the attachment of glucose to the N-terminal valine residue of the β-chain, which plays an essential role in the long-term monitoring of glycemic levels, the prediction of complications associated with diabetes mellitus, and diagnosing diabetes [1–3]. However, despite the considerable improvement in the assay performance of commercial automated HbA_{1c} analyzers, the accuracy of HbA_{1c} quantification is still adversely affected by the presence of Hb variants [4–6].

The interference caused by Hb variants depends on the method used and the type of Hb variant [7]. Various popular methods, such as cation-exchange high-performance liquid chromatography (HPLC), capillary electrophoresis (CE), boronate affinity HPLC, and immunoassays, have been developed and introduced to measure HbA_{1c} [8–10]; each method with different principles has specific advantages and potential pitfalls, as well as different capabilities for detecting and dealing with Hb variants. In regions such as southern China, ethnic diversity is associated with the existence of multiple Hb variants [11, 12], which further complicates this interference. For patients with an Hb variant trait but normal red blood cell turnover, an HbA_{1c} assay without Hb variant interference should be used [13]. Therefore, in case of such patients, it could be beneficial to eliminate or minimize such interference.

In addition to these widely used methods, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) has been proposed to determine HbA_{1c} values with β-chain glycation, which resulted in satisfactory agreement with diabetes control and complications trial (DCCT)-traceable methods [14–17]. However, some drawbacks, such as high-cost equipment, complex manual operation, and high requirements for personnel expertise, inhibit its wide utility in clinical laboratories. In addition, the effects of Hb variants on HbA_{1c}

measurements by MALDI-TOF MS remain unclear. Here, we evaluated the interference of Hb variants common in southern China on HbA_{1c} measurements by MALDI-TOF MS, and more importantly, we discuss the potential of MALDI-TOF MS to overcome the interference.

Materials and methods

Blood samples

Blood samples from a biobank at the Medicine Laboratory of Peking University Shenzhen Hospital were accessed following approval by the Ethics Committee of the same institution, and informed consent of all participants was obtained. In total, 614 samples, from patients with or without diabetes, were collected in EDTA tubes and tested. Samples without Hb variants (Hb AA, n=282) were used to determine the correlation between the α- and β-chain glycation degrees and to evaluate the HbA_{1c} values based on α- and β-chain glycation. The other samples (n=332) each harbored one of six heterozygous Hb variants common in southern China [11, 12]: Hb E (n=119), Hb J-Bangkok (n=44), Hb G-Coushatta (n=35), Hb G-Taipei (n=26), Hb Q-Thailand (n=72), and Hb G-Honolulu (n=36). These samples were used to evaluate the effects of Hb variants on HbA_{1c} measurements with MALDI-TOF MS. These Hb variants were originally identified by a CE-based HbA_{1c} procedure (Capillarys 3 TERA, Sebia) and confirmed by Sanger sequencing before the residual samples were placed in the biobank at −80 °C. The molecular characteristics of Hb variants are summarized in Table 1.

HbA_{1c} measurements

HbA_{1c} quantification was performed for all samples with a boronate affinity HPLC system (Ultra^2, Trinity Biotech) as a comparative method and an MALDI-TOF MS system (QuanTOF, Intelligene Biosystems). All operations were conducted according to the manufacturers' instructions. For QuanTOF, HbA_{1c} was tested following the approach described in our previous study [17]. Since QuanTOF can measure each chain and the corresponding glycated form, degrees of α- and β-chain glycation were calculated using the area (under each peak) ratio of glycated chains to total chains. Linear regression analysis was carried out to ascertain the correlation between the glycation degrees of α- and β-chains.

The standard curve for QuanTOF to quantify HbA_{1c} was determined directly based on the correlation between the target HbA_{1c} values and the glycation degrees of α- or β-chains. This curve was used to convert the MALDI-TOF MS measurements of α-or β-chain glycation in clinical samples into HbA_{1c} values. Thus, HbA_{1c} values calculated with α- and β-chain glycation were obtained.

Interference of Hb variants on MALDI-TOF MS

A total of 332 samples with four β-chain variants (Hb E, Hb J-Bangkok, Hb G-Coushatta, and Hb G-Taipei) and two α-chain variants (Hb Q-Thailand and Hb G-Honolulu) were analyzed to evaluate the interference of Hb variants on HbA_{1c} measurements by QuanTOF. A paired t-test was employed to ascertain whether the presence of Hb variants caused any statistically significant differences ($p<0.01$) relative to the comparative method (Ultra^2). Linear regression and Bland–Altman analyses were performed to evaluate the agreement of HbA_{1c} results obtained by Ultra^2 and QuanTOF. Deming regression was applied to estimate whether these Hb variants produced a clinically significant interference on HbA_{1c} results at clinical decision levels; clinically significant interference was designated as a difference exceeding ±7% of the evaluation levels at 6% (42 mmol/mol) or 9% (75 mmol/mol) HbA_{1c} [i.e., 0.42% at 6% (42 mmol/mol) HbA_{1c} and 0.63% at 9% (75 mmol/mol) HbA_{1c}] [18].

Data analysis

All data analyses and graphing were performed using GraphPad Prism 8.0 (GraphPad Software Inc., CA, USA).

Results

Correlation between glycation degrees of α- and β-chains

For Hb AA, a close correlation between the glycation degrees of α- and β-chains was observed over the glycation range measured. Linear regression analysis resulted in the following equations: α-chain = 0.6209β-chain + 0.5723, with a correlation coefficient (r^2) of 0.9669 ($p<0.0001$) (Figure 1A).

Table 1: Molecular characteristics of hemoglobin variants tested in this study.

Hb variant	n	Gene mutation (HGVS name)	Variant chain	Mass of variant chain, Da	Mass difference[a], Da	Detectable by QuanTOF
Hb E	119	*HBB*:c.79 G→A	β-chain	15867	~1	No
Hb J-Bangkok	44	*HBB*:c.170 G>A	β-chain	15926	58	Yes
Hb G-Coushatta	35	*HBB*:c.68 A>C	β-chain	15810	−58	Yes
Hb G-Taipei	26	*HBB*:c.68 A>G	β-chain	15796	−72	Yes
Hb Q-Thailand	72	*HBA1*:c.223G>C	α-chain	15148	21	Yes
Hb G-Honolulu	36	*HBA2*:c.91G>C	α-chain	15126	~1	No

[a]Mass difference between variant α- or β-chain and the corresponding normal α-chain (15127 Da) or β-chain (15868 Da).

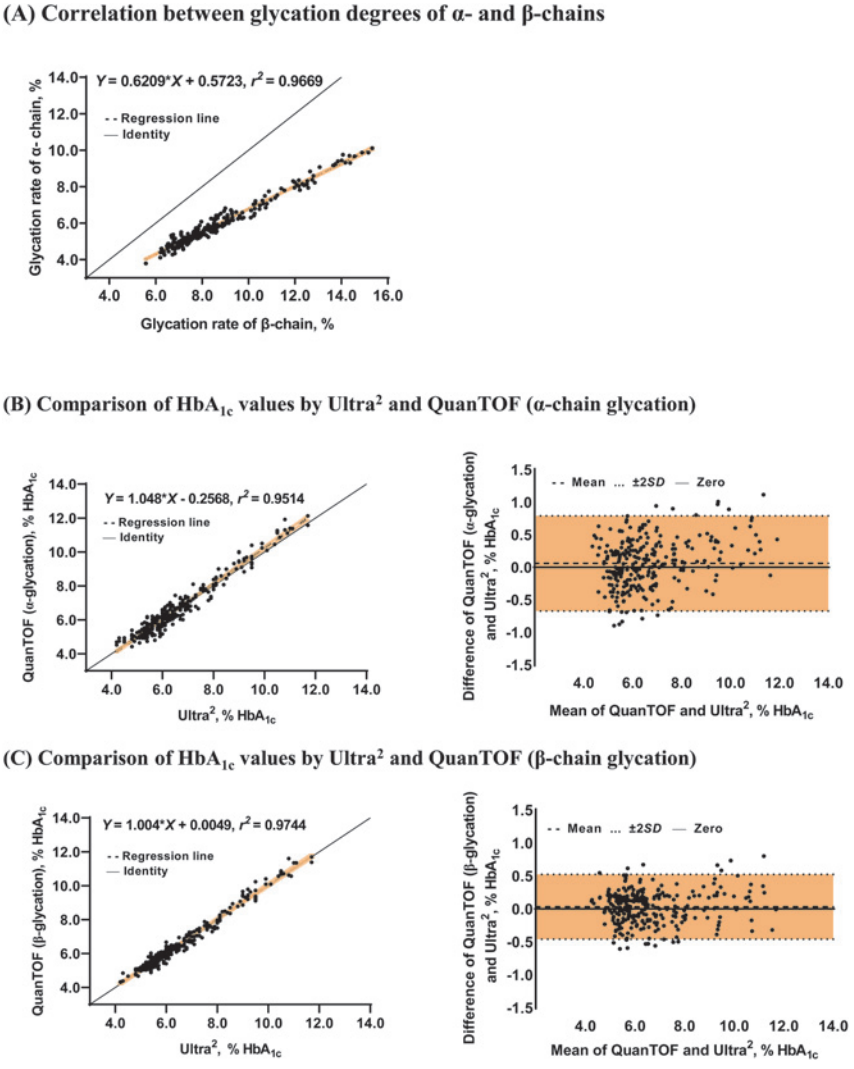

Figure 1: Samples without hemoglobin variants. (A) Correlation between glycation degrees of α- and β-chains. (B) Comparison of HbA_{1c} values determined by Ultra² and QuanTOF (α-chain glycation). (C) Comparison of HbA_{1c} values determined by Ultra² and QuanTOF (β-chain glycation). Left, linear regression. Right, Bland-Altman difference. Orange shaded region, 95% confidence interval.

HbA_{1c} results based on α- and β-chain glycation

For samples without Hb variants, two sets of HbA_{1c} values obtained with α- and β-chain glycation correlated well with the Ultra² results. Line equations were Y = 1.048X – 0.2568 and Y = 1.004X + 0.0049, with a correlation r^2 of 0.9514 and 0.9744 for QuanTOF (α-chain glycation) versus Ultra² and QuanTOF (β-chain glycation) versus Ultra², respectively (Figure 1B, C).

Interference of Hb variants on MALDI-TOF MS

Three β-chain variants (Hb J-Bangkok, Hb G-Coushatta, and Hb G-Taipei) and one α-chain variant (Hb Q-Thailand) were detected by QuanTOF, and mass differences between the normal and variant chains were consistent with the theoretically calculated values (Supplementary Figure 1). In contrast, QuanTOF detected no sign of Hb E and Hb G-Honolulu owing to minor mass differences (Supplementary Figure 2). Statistically significant differences ($p<0.01$) were found in HbA_{1c} results calculated by α- and β-chain glycation for all Hb variants tested when compared with Ultra² results (Table 2).

Bland–Altman analysis of samples with detected β-chain variants (Hb J-Bangkok, Hb G-Coushatta, and Hb G-Taipei) revealed significant positive bias for the HbA_{1c} results determined by conventional β-chain glycation (Figure 2). Deming regression further confirmed the presence of clinically significant interference due to these variants (Table 2). The results for other samples with Hb E, Hb Q-Thailand, and Hb G-Honolulu instead showed good

Table 2: Comparison of HbA_{1c} results determined by QuanTOF and Ultra².[a]

	β-Glycation				α-Glycation			
	p	6% (42 mmol/mol) HbA_{1c}	9% (75 mmol/mol) HbA_{1c}	Mean biases	p	6% (42 mmol/mol) HbA_{1c}	9% (75 mmol/mol) HbA_{1c}	Mean biases
Hb AA	0.0306	0.03	0.08	0.03	0.0069[b]	0.02	0.25	0.06
Hb E	<0.0001[b]	0.16	0.3	0.16	0.0015[b]	−0.09	−0.15	−0.08
Hb J-Bangkok	<0.0001[b]	1.54[c]	1.61[c]	1.54	<0.0001[b]	−0.24	−0.34	−0.24
Hb Coushatta	<0.0001[b]	4.49[c]	3.47[c]	4.60	<0.0001[b]	−0.22	−0.33	−0.21
Hb G-Taipei	<0.0001[b]	1.39[c]	1.6[c]	1.35	0.0018[b]	−0.05	0.14	−0.09
Hb Q-Thailand	<0.0001[b]	−0.16	−0.27	−0.16	<0.0001[b]	1.18[c]	1.03[c]	1.19
Hb G-Honolulu	0.0001[b]	0.12	0.16	0.12	<0.0007[b]	0.09	0.05	0.12

[a]Using Ultra² as the comparative method, p-values, mean biases, and the biases at clinical decision levels were determined for each variant. All biases were calculated using HbA_{1c} values expressed in NGSP units (%).
[b]Statistically significant difference (p<0.01).
[c]Clinically significant difference [>0.42% at 6% (42 mmol/mol) HbA_{1c} or >0.63% at 9% (75 mmol/mol) HbA_{1c}].

agreement with those obtained by Ultra² (Figure 3; Table 2; Supplementary Figure 2). Importantly, for these three β-chain variants, HbA_{1c} values determined by α-chain glycation showed no clinically significant differences between MALDI-TOF MS and Ultra². That is, the interference could be fully eliminated by the use of α-chain glycation to estimate HbA_{1c} values. As shown in Table 2 and Figure 2, the mean biases and correlation coefficients were substantially improved for Hb J-Bangkok, Hb G-Coushatta, and Hb G-Taipei. Similarly, clinically significant positive interference caused by Hb Q-Thailand could be overcome by using β-chain glycation to estimate HbA_{1c} values, which changed the correlation r^2 values from 0.7743 to 0.9614 and mean biases from 1.29 to −0.16 (Table 2, Figure 3).

Discussion

In normal adults, Hb consists of a major component, Hb A ($\alpha_2\beta_2$), and two minor components, Hb F ($\alpha_2\gamma_2$) and HbA_2 ($\alpha_2\delta_2$). The heterogeneity of human Hb arises mainly from post-translational modifications, principally glycation, such as HbA_{1c} [19]. Moreover, both the β- and α-chains can be glycated on the N-terminal valine or any of the lysine residues in the primary sequence [20, 21]. Glycation produces a mass increase of 162 Da derived from the elimination of water (18 Da) from a glucose adduct (180 Da), which makes it feasible to distinguish the glycated forms of globin chains by MALDI-TOF MS [14–17]. Unlike traditional methods for HbA_{1c} analysis (i.e., cation-exchange HPLC, CE, boronate affinity HPLC, and immunoassay) that distinguish only between symmetric Hb tetramers, MALDI-TOF MS analysis reveals free globin chains of symmetric Hb because the tetramer was denatured before its injection into the MALDI-TOF MS system, which makes it possible to calculate the glycation degree of each chain.

In this study, the addition of only one glucose was seen at each chain, with no peak of two glucose molecule adducts being detected, following a mass increase of 324 Da at any HbA_{1c} value, consistent with previous studies [14–17]. As speculated by Roberts et al. [14], the percentages of two or more glycations are so low that we have not been able to reliably detect them till date. Although glycation modifications are distributed differently between the two chains [20, 21], our results revealed the stable relationship between the glycation degrees of the α- and β-chains; the degree of α-chain glycation was approximately 0.69-fold that of the β-chain, which is consistent with the formerly reported ratio of 0.66 [14]. In addition, both α- and β-chain glycation were found to be proportional to HbA_{1c} concentrations, again confirming earlier studies [14–17].

A boronate affinity HPLC method is often used as a comparative method of choice in most studies regarding the impact of Hb variants, as it is generally thought to be unaffected by most Hb variants because total glycated Hb is measured regardless of the Hb species [18]. However, this method cannot detect the presence of Hb variants and is not always applicable as a comparative method, especially under conditions in which the amino acids at key sites of glycation change due to gene mutations. Under these circumstances, the glycation degrees of Hb variants will be inconsistent with that of Hb A and the efficacy of this method will be significantly influenced by these Hb variants [22]. Despite this limitation in this

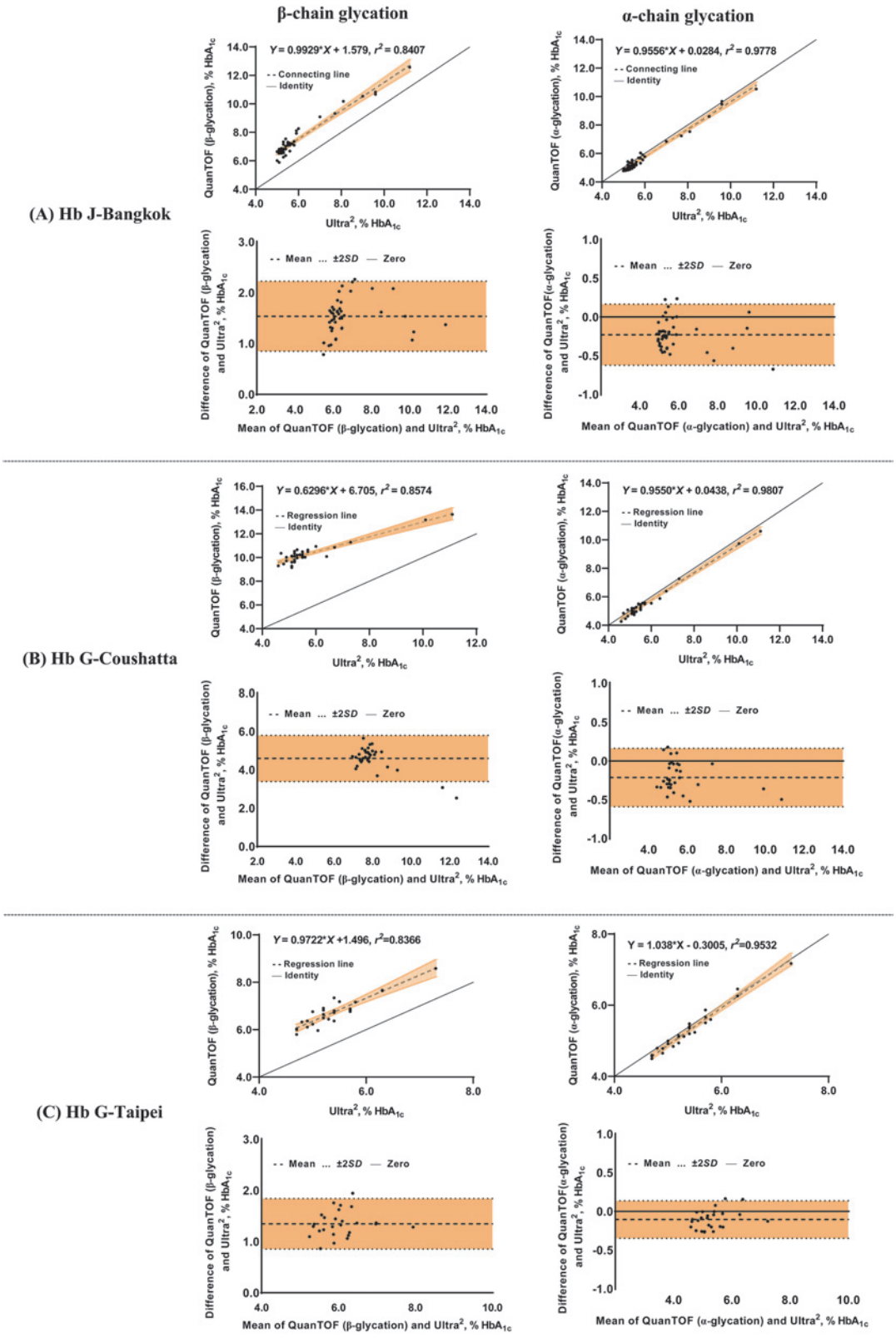

Figure 2: Comparison of HbA_{1c} values determined by Ultra² and QuanTOF for samples with detected β-chain variants by QuanTOF. (A) Hb J-Bangkok. (B) Hb G-Coushatta. (C) Hb G-Taipei. Left, β-glycation. Right, α-glycation. Top of each, linear regression. Bottom of each, Bland-Altman difference. Orange shaded region, 95% confidence interval.

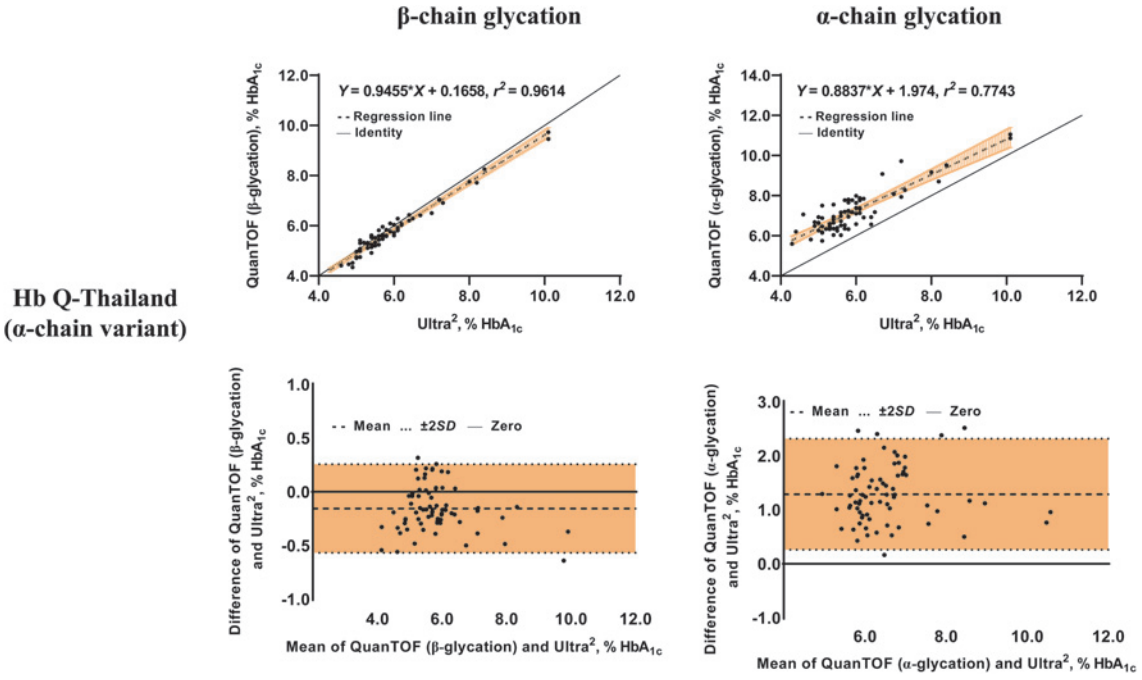

Figure 3: Comparison of HbA_{1c} values determined by Ultra² and QuanTOF for samples with Hb Q-Thailand.
Left, β-glycation. Right, α-glycation. Top of each, linear regression. Bottom of each, Bland-Altman difference. Orange shaded region, 95% confidence interval.

study, Ultra² is theoretically suitable for use as a comparative method, because important sites of glycation have not changed for all Hb variants involved [20–22], which was supported by our results.

MALDI-TOF MS has been the tool of choice to characterize Hb variants as they can be distinguished by their mass differences [23]. This allows the α- or β-chain variants with significantly different masses from each other, such as Hb J-Bangkok, Hb G-Coushatta, Hb G-Taipei, and Hb Q-Thailand tested in this study, to be detected separately. In contrast, for Hb E and Hb G-Honolulu, exhibiting mass differences of <1 Da, their separation requires instruments with higher resolution.

Although statistically significant differences were found for all the Hb variants tested, irrespective of β- or α-chain glycation, clinically significant differences were assumed to be a more effective indicator to determine whether there was interference [18]. Our results indicated that HbA_{1c} results obtained with β-chain glycation were prone to interference due to the variant β-chains, which presented specific variant peaks on the mass spectrum due to the sufficient mass differences from normal β-chains. Instead, the other Hb variants, including β-chain variants undetected by QuanTOF and all α-chain variants, had no obvious influence on the technique. We speculate that the reason for this is that the mass peaks of the glycated forms of variants are merged with those of normal chains, and the measured percentages of glycated forms remain basically unchanged.

In addition to quantifying HbA_{1c} based on conventional β-chain glycation, our data showed good agreement between HbA_{1c} values calculated with α-chain glycation and Ultra² results, thus supporting the use of α-chain glycation to estimate the levels of HbA_{1c}. The most important finding is that the interference on the measurement of HbA_{1c} caused by the presence of β-chain variants can be fully overcome by using α-chain glycation. Similarly, clinically significant interference generated by α-chain glycation can be eliminated by using the β-chain glycation. The obvious reason is that variant β-chains cannot affect both the α-chain and its glycated form, owing to sufficient mass differences between the two, and vice versa. Moreover, independent HbA_{1c} results calculated on the basis of α-chain glycation can be used to validate those based on β-chain glycation, and marked discordance between the two may indicate the presence of Hb variants.

In conclusion, our results confirmed the stable relationship between glycation degrees of α- and β-chains and substantiated the use of α-chain glycation as an additional strategy to estimate HbA_{1c} values by MALDI-TOF MS.

Furthermore, we report that QuanTOF measurements through α- or β-chain glycation are affected by the corresponding detected variant α- or β-chain. In particular, this interference could be overcome by using alternative α- or β-chain glycation to calculate HbA_{1c} values.

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Author contributions: All authors have accepted responsibility for the entire content of this manuscript and approved its submission.

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Informed consent: Informed consent was obtained from all individuals included in this study.

Ethical approval: The study was approved by the Ethics Committee of the Peking University Shenzhen Hospital.

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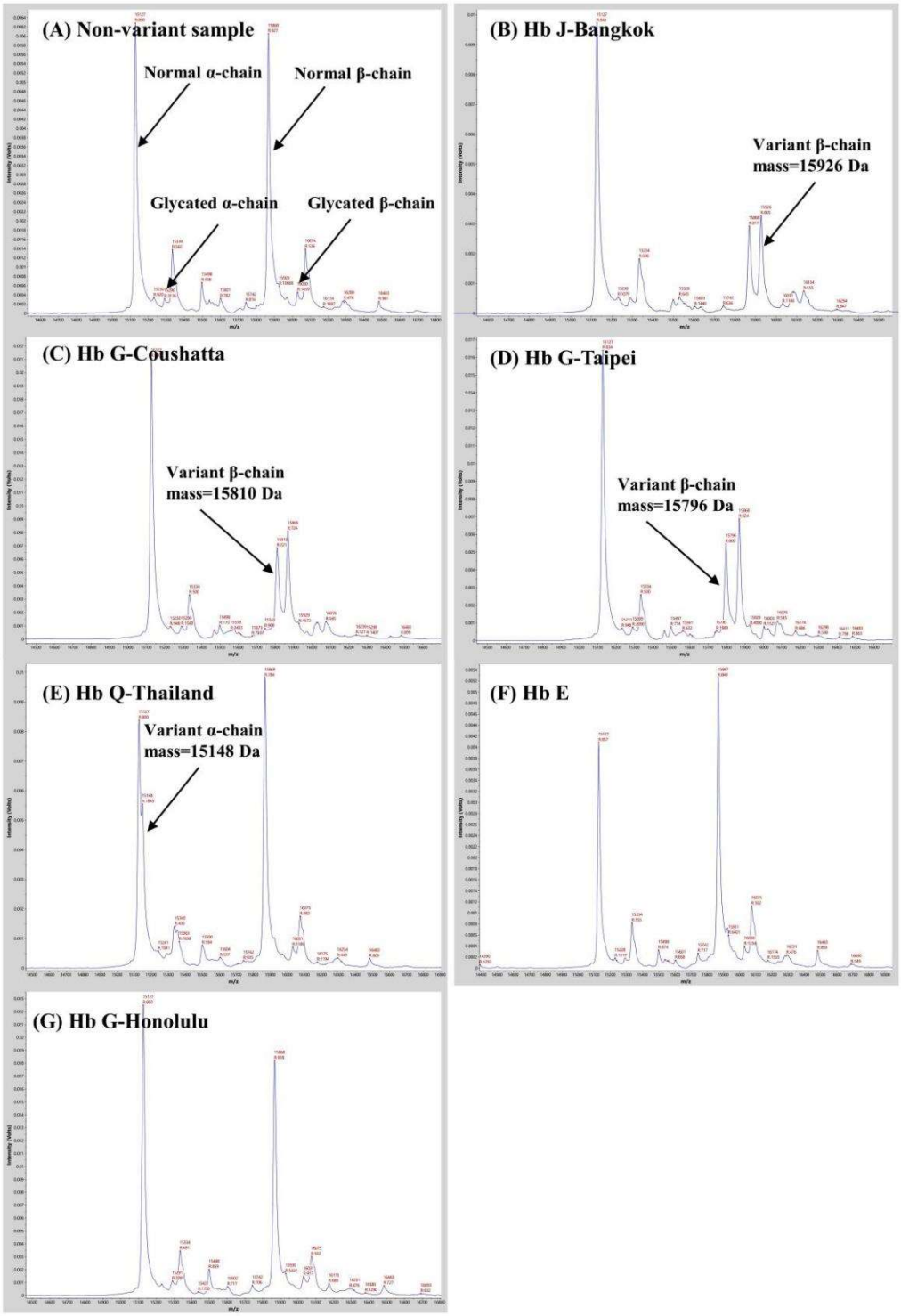
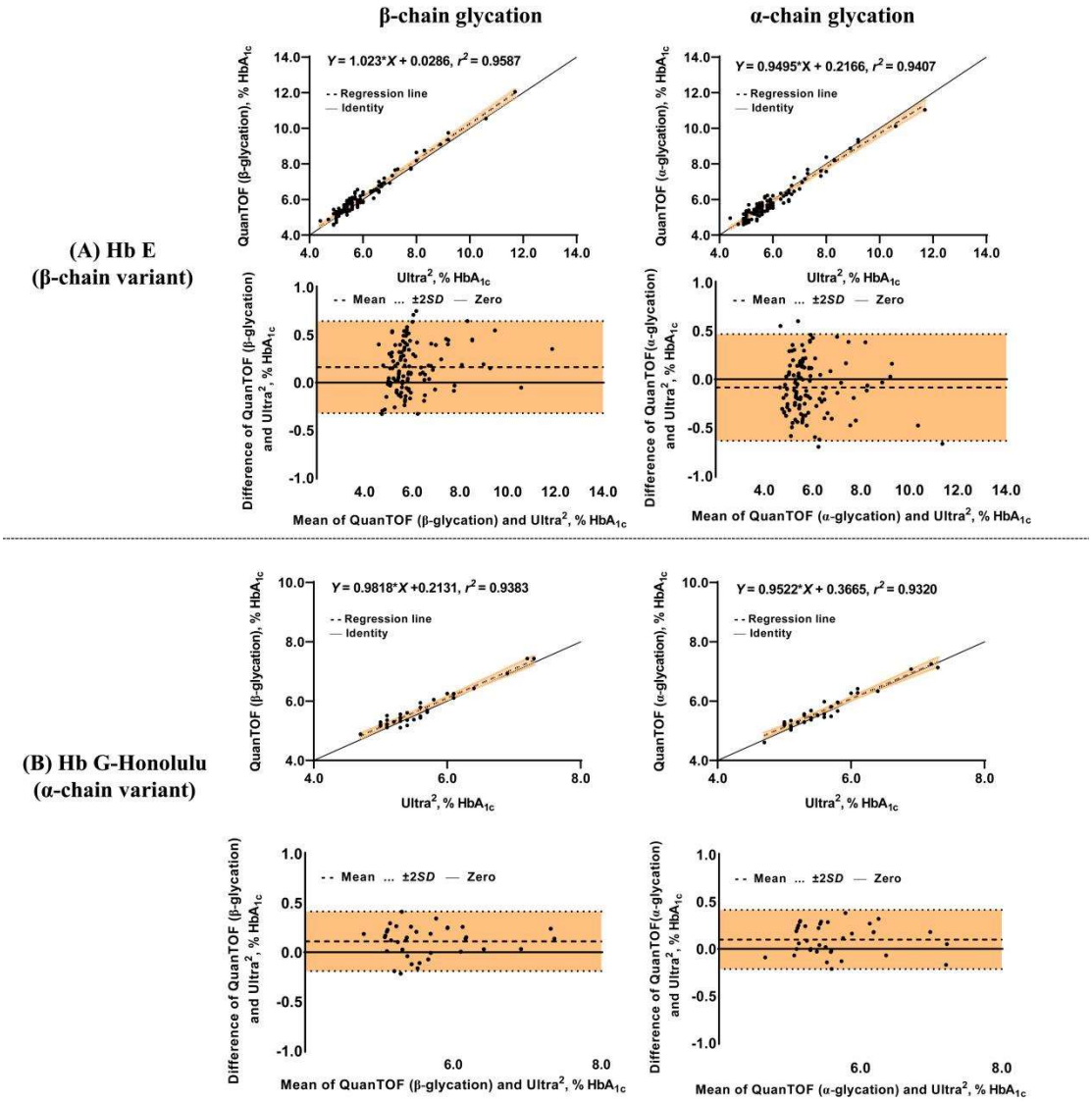


Supplementary Figure 1. Mass spectra results for samples with and without hemoglobin variants. (A) Normal α-chain and β-chain. (B) Hb E. (C) Hb J-Bangkok. (D) Hb G-Coushatta. (E) Hb G-Taipei. (F) Hb Q-Thailand. (G) Hb G-Honolulu. Arrows indicate the variant chains detected by QuanTOF.



Supplementary Figure 2. Comparison of HbA_{1c} values determined by Ultra^2 and QuanTOF for samples with undetected variants by QuanTOF. (A) Hb E. (B) Hb G-Honolulu. HbA_{1c} values obtained with both β- and α-glycation were compared with Ultra^2 results. Left, β-glycation. Right, α-glycation. Top of each, linear regression. Bottom of each, Bland-Altman difference. Orange shaded region, 95% confidence interval.