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Comparability of different methods of glycated hemoglobin measurement for samples of patients with variant and non-variant hemoglobin

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ABSTRACT

Background: Glycated hemoglobin (HbA_{1c}) is measured to monitor patients with diabetes. However, the measurement results can vary according to the analysis method and presence of variant hemoglobin. Thus, we compared HbA_{1c} results between liquid chromatography-tandem mass spectrometry (LC-MS/MS) as the reference method and matrix assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF MS).
Methods: $\%HbA_{1c}$ were measured using the two methods in 45 non-variant and 73 heterozygous variant samples. Precision was calculated; the results were compared using Passing-Bablok regression and the concordance correlation coefficient (CCC). The average bias between methods was compared with the lowest bias of 2.3% for biological variation.
Results: The precision of the two methods was $< 2\%$. The R^2 for the non-variant samples were 0.986 and the CCC was 0.99. Based on α- and β-chain, the variant samples were divided into four groups: α-chain, α-chain negligible, β-chain, and β-chain negligible variants. The R^2 between the two methods of the four groups were > 0.95; However, the average biases of α-chain and β-chain variants were above the minimum bias.
Conclusion: LC-MS/MS and MALDI-TOF MS had good comparability in the measurement of HbA_{1c} in non-variant samples, but the existence of variant hemoglobin caused discrepancies.

1. Introduction

Glycated hemoglobin (HbA_{1c}) is an important indicator for the long-term monitoring of blood glucose levels in patients with diabetes. HbA_{1c} is formed via an irreversible reaction in which glucose combines with the N-terminal valine residue of the hemoglobin β-chain through covalent bonds [1]. Since the metabolic cycle of red blood cells is approximately 120 days, measurement of HbA_{1c} can effectively help to monitor the average blood glucose level in patients with diabetes within 2–3 months. Variant hemoglobin is a condition characterized by an abnormal hemoglobin structure due to changes in the globin gene. Variant hemoglobin can affect the HbA_{1c} measurement results for some methods of measurement [2–4], thereby affecting the ability of clinicians to provide a timely diagnosis and treatment. Therefore, it is very important to ensure the accuracy of HbA_{1c} results and to identify cases of variant hemoglobin in the measurement process.

At present, the commonly used measurement methods for HbA_{1c} in clinical settings include ion-exchange chromatography, affinity chromatography, capillary electrophoresis, and immunoassay [5]. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) mainly obtains the peak area ratio of glycated and non-glycated hexapeptides according to the mass-to-charge ratios of ions, enabling calculation of the $\%HbA_{1c}$ in the sample. LC-MS/MS is recommended by the International Federation of Clinical Chemistry (IFCC) for its high sensitivity and good accuracy. With the continuous development of measurement technology, matrix assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF MS) has rapidly developed for its advantages of requiring only a small sample size and fast analysis, offering

Abbreviations: CCC, concordance correlation coefficient; HbA_{1c}, glycated hemoglobin; IFCC, International Federation of Clinical Chemistry; LC-MS/MS, liquid chromatography-tandem mass spectrometry; MALDI-TOF MS, matrix assisted laser desorption/ionization time of flight mass spectrometry; NGSP, National Glycohemoglobin Standardization Program.

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broad clinical application prospects [6].

Although it is known that the results of HbA_{1c} measurement differ among various methods owing to different measurement principles, measurement reagents, and instruments, there has been no study to directly compare the LC-MS/MS and MALDI-TOF MS measurement results of HbA_{1c} to date. Therefore, in this study, the performance of MALDI-TOF MS and the reference method by LC-MS/MS was compared in terms of $\%HbA_{1c}$, along with the ability of two methods to distinguish non-variant and heterozygous variant hemoglobin samples.

2. Materials and methods

2.1. Sample collection

2.1.1. Non-variant samples with different $\%HbA_{1c}$

Fresh whole blood was collected from 45 patients in Chaoyang Hospital, and HbA_{1c} was 4%-15% by HLC-723 G8 automatic glycated hemoglobin analyzer (Tosoh, Japan). 2 mL of blood was collected into EDTA-K2 anticoagulant tubes. Two portions of 200 μL each of whole blood were loaded into frozen tubes (one for MALDI-TOF MS measurement and the other for LC-MS/MS measurement) and immediately frozen at –80 °C. The remaining samples were pretreated for LC-MS/MS measurement with saline according to IFCC recommendations [7]. The pretreated samples and a set of 200 μL untreated samples were thawed and then a corresponding volume of enzyme (protease Glu-C, Sigma, St. Louis, MO, USA) and ammonium acetate solution (Sigma; pH = 4.2) were added. After incubation at 37 °C for 18–20 h, the samples were subpackaged into three parts and frozen at –80 °C until analysis. The samples were approved by the Ethics Review Committee of Beijing Chaoyang Hospital.

2.1.2. Heterozygous variant hemoglobin samples

Seventy-three heterozygous variant hemoglobin samples were identified during routine HbA_{1c} measurement. The variant status was measured in one portion of the samples by automatic capillary electrophoresis and confirmed by Sanger sequencing. Heterozygous variant hemoglobin samples were all trait samples, including Hb Q-Thailand, Hb G-Honolulu, Hb Ube-2, Hb New York, Hb J-Bangkok, Hb G-Coushatta and Hb E traits, as shown in Table 1. The remaining two portions were subjected to LC-MS/MS and MALDI-TOF MS, respectively, for HbA_{1c} measurement. In this experiment, sufficient protease Glu-C were added to ensure a complete reaction when the samples were digested. After enzymatic treatment, the samples were frozen at –80 °C until analysis. The samples were approved by the Ethics Review Committee of Beijing Chaoyang Hospital.

2.2. Measurement of HbA_{1c}

2.2.1. LC-MS/MS

After enzymolysis, the samples were restored to room temperature and centrifuged at 11964*g* for 5 min, and 5 μL of the supernatant was mixed with 500 μL of a 1:1 mixture of methanol (Sigma) and deionized water (Watsons, domestic). The multi-reaction monitoring mode of electrospray ionization was used for LC-MS/MS measurement with the LC-20AD liquid chromatograph (Shimadzu, Japan) and QTRAP 5500 mass spectrometer (AB Sciex, Framingham, MA, USA). Two ion pairs on the secondary mass spectrometer were selected to measure the non-glycated and glycated forms of the N-terminal hexapeptide of the hemoglobin β chain. The mass-to-charge ratio (m/z) of non-glycated forms was 348.2 → 237.2 and that of glycated forms was 429.2 → 245.2. The samples were loaded in the Shim-Pack XR-ODS C_{18} chromatographic column (3 mm × 50 mm, 2.2 μm, Shimadzu). The system was calibrated using the IFCC calibration products (2016-A, 2016-B, 2016-C, 2016-D, 2016-E, 2016-F).

The mobile phase consisted of 0.1% acetic acid aqueous solution and pure methanol. The gradient elution of mobile phase A and B was performed for 10 min. The proportions of mobile phase A and B were 95:5, 40:60, 0:100, and 95:5 in 0.01–0.5 min, 0.6–2 min, 2.1–4.5 min, and 4.6–10 min, respectively. The flow rate was 0.4 mL/min and the injection volume was 5 μL. The samples were measured twice. Three quality controls (Milanol IFCC quality control samples and RELA Sample 2020: 2020GHA, 2020GHB) were measured between each five samples to ensure the reliability of the data.

2.2.2. MALDI-TOF MS

Forty-five non-variant and 73 heterozygous variant samples were subjected to MALDI-TOF MS with the QuanGHb glycated hemoglobin quantitative mass spectrometry system (Rongzhi Biotechnology, Beijing, China), after qualifying the quality control with one high- and one low-level quality control samples. The sample (200 μL) was dissolved at room temperature, and 2 μL of sample and 18 μL mustard acid matrix solution (Rongzhi Biotechnology) were mixed after shaking. Two microliters of mixed sample were placed on the target plate (the first drop of sample was omitted), waiting for samples to film on the target. Each sample was measured three times, and the average value was taken for data analysis.

2.3. Precision

The precision of LC-MS/MS and MALDI-TOF MS were evaluated according to the low-, medium-, and high-level quality controls. Each level was measured three times for five batches. The standard deviation and coefficient of variation (CV) of each level of quality controls were calculated.

Table 1
Heterozygous variant hemoglobin types and groups.

Heterozygous variant hemoglobin	HGVS name	Amino acid change	Electrophoresis position [a]	N[b]	Identified by MALDI-TOF MS	Group
Hb Q-Thailand	HBA1: c.223G > C	Asp > His	Z12	13	Yes	α-chain variant
Hb G-Honolulu	HBA1: c.91G > C	Glu > Gln	Z(D)	7	No	α-chain negligible variant
Hb Ube-2	HBA2: c.205A > G	Asn > Asp	Z12	6	No	α-chain negligible variant
Hb New York	HBB: c.341 T > A	Val > Glu	Z11	10	Yes	β-chain variant
Hb J-Bangkok	HBB: c.170G > A	Gly > Asp	Z12	12	Yes	β-chain variant
Hb G-Coushatta	HBB: c.68A > C	Glu > Ala	Z(D)	11	Yes	β-chain variant
Hb E	HBB: c.79G > A	Glu > Lys	Z(E)	14	No	β-chain negligible variant

[a] Electrophoresis position: peak area in the capillary electrophoresis region.
[b] N: number.

2.4. Data analysis

LC-MS/MS converts the peak area ratio of the glycated and non-glycated hexapeptide of the calibration product into the corresponding %HbA_{1c} to construct a standard curve. According to the peak area ratio of the standard curve and the sample to be measured, the %HbA_{1c} of the sample to be measured was obtained. The measurement result was harmonized in National Glycohemoglobin Standardization Program (NGSP) units (NGSP %) using the following formula:

$$\mathrm{NGSP}\,(\%) = 0.0915 \times \mathrm{IFCC}\,(\mathrm{mmol/mol}) + 2.15$$

2.5. Statistical analysis

The results were statistically analyzed using Excel, MedCalc, and SPSS 17.0 packages. Passing-Bablok regression and the concordance correlation coefficient (CCC) were used to compare the correlation and consistency between the two methods, interpreted as follows: CCC > 0.99, excellent consistency; 0.99–0.95, good consistency; 0.94–0.90, general consistency; <0.90, poor consistency [8]. Differences between quality standards derived from biological variation data and the R^2 value from the regression were used to evaluate the results of heterozygous variant hemoglobin HbA_{1c}. The intra-individual biological variation of HbA_{1c} derived from the Westguard website (https://www.westgard.com/biodatabase1.htm) was 1.9% and inter-individual biological variation was 5.7%, and the lowest bias was calculated at ≤ 2.3%. An R^2 value > 0.95 was considered to indicate a good correlation between methods.

3. Results

3.1. Hemoglobin results using LC-MS/MS with and without pretreatment

Before the experiment, we used different pretreatments to measure HbA_{1c} in 30 samples with different known %HbA_{1c}. There was no significant difference in HbA_{1c} measurement results between samples incubated in saline and untreated samples (8.25% ± 3.00 and 8.20% ± 2.96, respectively; $t = 1.699$, $P = 0.1$).

3.2. Characterization of heterozygous variant hemoglobin samples

The whole blood samples after enzymatic hydrolysis were directly measured in this study. According to the gene sequencing results, we divided variant hemoglobin into α- and β-chain variants. We defined samples with small variations in hemoglobin that could not be identified by MALDI-TOF MS as negligible variations. Therefore, samples were divided into four groups: α-chain variant (13 cases), α-chain negligible variant (13 cases), β-chain variant (33 cases), and β-chain negligible variant (14 cases), as shown in Table 1.

3.3. Precision

The precision results of the two methods are summarized in Table 2. After conversion to the harmonized NGSP unit (%HbA_{1c}), the total CV for low-, medium-, and high-level quality controls were the highest for MALDI-TOF MS. However, the CVs of the two methods were ≤ 2%, which met the requirements of IFCC precision.

3.4. Comparison and bias evaluation of the two methods for HbA_{1c} measurement in non-variant hemoglobin samples

The R^2 value, CCC, and average biases of the comparison of HbA_{1c} results of non-variant samples measured by LC-MS/MS and MALDI-TOF MS are shown in Table 3. The R^2 values were 0.986 and the CCC was 0.99, demonstrating good correlation and consistency between the two methods. The Passing-Bablok regression analysis and relative bias distribution between the two methods are shown in Fig. 1. The average biases between MALDI-TOF MS and LC-MS/MS was below the minimum bias requirement of biological variation of 2.3% (Table 3).

Table 2
Precision results of HbA_{1c} measurement by LC-MS/MS and MALDI-TOF MS.

	LC-MS			MALDI-TOF MS		
	Low level	Medium level	High level	Low level	Medium level	High level
Intra-batch SD	0.07	0.35	0.51	0.06	0.07	0.08
Intra-batch CV	0.24%	0.91%	0.71%	1.24%	1.27%	0.88%
Inter-batch SD	0.25	0.07	0.86	0.05	0.07	0.09
Inter-batch CV	0.81%	0.19%	0.79%	1.15%	1.25%	0.92%
Total SD	0.26	0.35	0.76	0.07	0.07	0.12
Total CV	0.84%	0.93%	1.06%	1.54%	1.21%	1.27%
Average value (%)	5.07	5.58	8.63	4.56	5.64	9.60

Table 3
Comparison of HbA_{1c} in non-variant hemoglobin samples measured by different methods.

Statistic	MALDI-TOF MS vs.LC-MS/MS	
	%Glycated α globin	%Glycated β globin
R^2	0.956	0.986
Consistent correlation coefficient	0.98	0.99
Average bias (%)	1.5	–1.6

3.5. Comparison and bias evaluation of the two methods for HbA_{1c} measurement of heterozygous variant hemoglobin samples

The Passing-Bablok regression equations, R^2 values, CCCs, and average biases for the comparison of HbA_{1c} results of heterozygous variant hemoglobin samples measured by LC-MS/MS and MALDI-TOF MS are shown in Tables 4. The R^2 values between the results of MALDI-TOF MS and LC-MS/MS in the four variant groups was in the range of 0.954–0.988 (β-chain variant assigned according to glycated α-globin), indicating a good correlation. The average biases of glycated hemoglobin in the four groups ranged from –3.1% to 0.1%, and the average biases of the α-chain variant and β-chain variant groups were higher than that of the biological variation standard of 2.3% (Table 4).

Fig. 2 shows the HbA_{1c} spectra of heterozygous variant hemoglobin samples from LC-MS/MS and MALDI-TOF MS. Compared with non-variant hemoglobin (Fig. 2B), the presence of an abnormal peak, which was near the normal β peak in MALDI-TOF MS, was indicated to be a β-chain variation (Fig. 2C).

We previously compared the LC-MS/MS and capillary electrophoresis (CE) methods with respect to the measurement of heterozygous variant hemoglobin, and the results are shown in Supplement Table 1. The average biases of glycated hemoglobin in the four groups ranged from 1.0% to 3.5%, and average biases of the α-chain negligible variant and β-chain negligible variant groups were higher than the biological variation standard of 2.3%.

4. Discussion

HbA_{1c} is a complex of hemoglobin and glucose in the human body, forming unstable glycated hemoglobin (also known as HbA_{1c} precursor), and slowly forming stable HbA_{1c} [9]. The diversity of HbA_{1c} measurement methods, different sources of measurement reagents, and differences in operation among operators will lead to large differences in

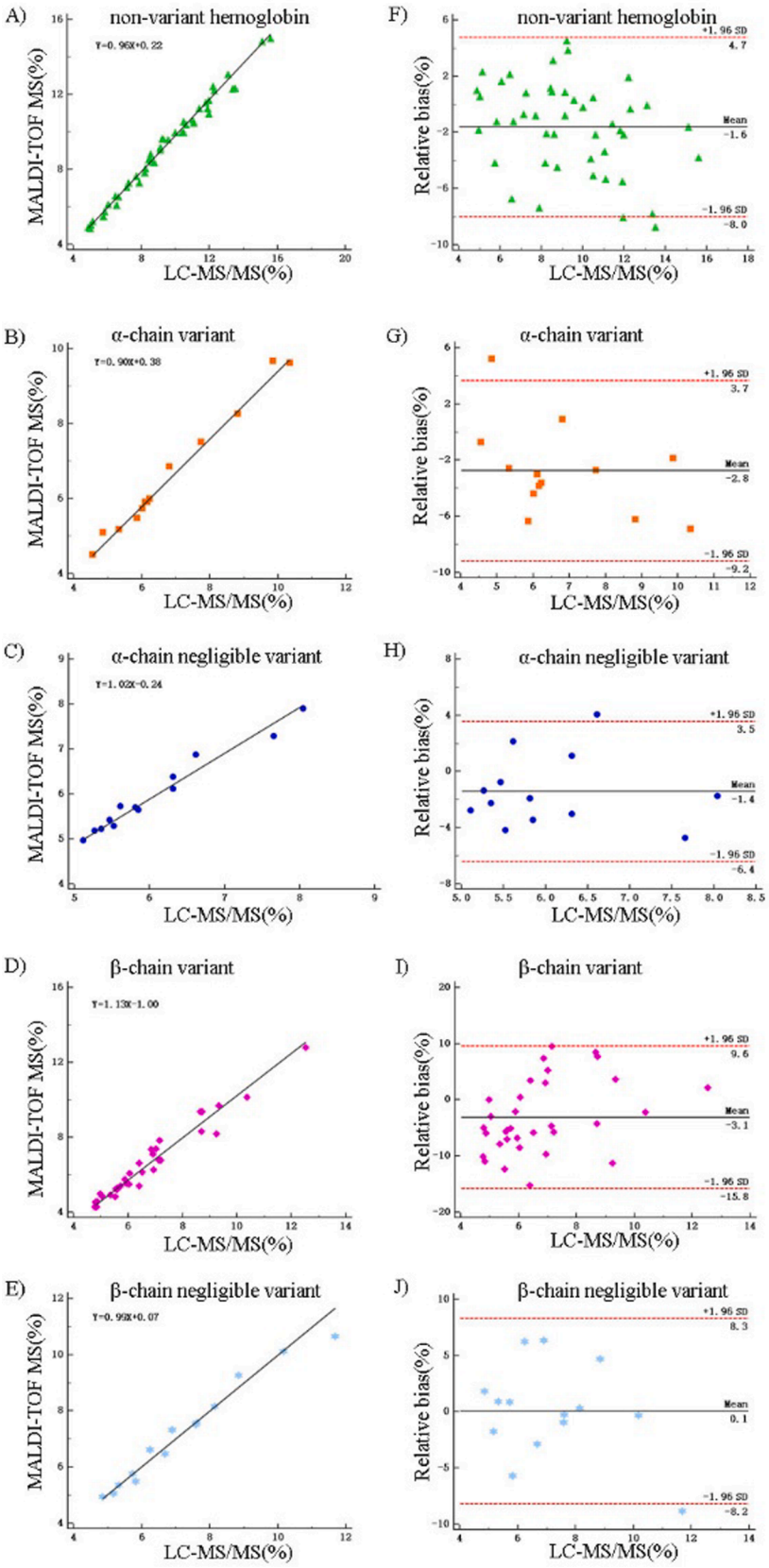

Fig. 1. Method comparison with Passing-Bablok regression analysis and relative bias distribution. (A–E) Passing-Bablok regression lines between LC-MS/MS and MALDI-TOF MS non-variant hemoglobin, α-chain variant, α-chain negligible variant, β-chain variant, and β-chain negligible variant respectively. The Passing-Bablok regression of the results. (F–J) Relative bias distributions of LC-MS/MS and MALDI-TOF MS non-variant hemoglobin, α-chain variant, α-chain negligible variant, β-chain variant, and β-chain negligible variant respectively. The black solid line represents the mean, and the red dashed line shows the ± 1.96 standard deviation.

Table 4
Comparison of HbA_{1c} measurement of heterozygous variant hemoglobin samples (N = 73) by LC-MS/MS and MALDI-TOF MS.[a]

Statistic	Variant Group							
	α-chain variant (n = 13)		α-chain negligible variant (n = 13)		β-chain variant (n = 33)		β-chain negligible variant (n = 14)	
	%Glycated α globin	%Glycated β globin	%Glycated α globin	%Glycated β globin	%Glycated α globin	%Glycated β globin	%Glycated α globin	%Glycated β globin
Passing-Bablok linear regression	Y = 1.02X + 0.91	Y = 0.90X + 0.38	Y = 1.06X – 0.42	Y = 1.02X– 0.24	Y = 1.13X– 1.00	Y = 1.29X + 0.43	Y = 0.99X + 0.07	Y = 1.06X + 0.02
R^2	0.969	0.988	0.945	0.967	0.954	0.141	0.969	0.989
Consistent correlation coefficient	0.82	0.98	0.96	0.98	0.97	0.18	0.98	0.97
Average bias (%)	17.8	–2.8 [b]	–2.0	–1.4	–3.1 [b]	53.8	0.1	6.4

[a] Passing-Bablok regression analysis was carried out with LC-MS/MS as the reference method to calculate the average bias between the methods for measuring the heterozygous variant hemoglobin samples.
[b] Clinically significant difference (average bias > minimum bias of biological variation of 2.3%).

HbA_{1c} measurement results. Having a reference method for methodological comparison is very important for the standardization of HbA_{1c} measurement results. In this study, the LC-MS/MS reference method recommended by the IFCC was used as a comparison method to evaluate the correlation and consistency of MALDI-TOF MS and LC-MS/MS in HbA_{1c} measurement, and to investigate the comparability of the two methods in the measurement of heterozygous variant hemoglobin samples.

The IFCC recommendations [7] indicate that whole blood samples should be pretreated before HbA_{1c} measurement by LC-MS/MS (37 °C saline incubation for 4 h) to eliminate any unstable HbA_{1c}. Svendsen et al. [10] and Tornqvist et al. [11] confirmed that the HbA_{1c} precursor interfered with the measurement of HbA_{1c} by chromatography. However, in practice, the results of LC-MS/MS measurement will also be affected by several factors, including operation procedures and others. Therefore, in this study, whole blood samples were both pretreated with saline and left unpretreated for comparison, with no significant difference found, which was inconsistent with the results reported in the literature. The reason for this discrepancy may be that the chromatographic column used in this study (Shim-pack XR-ODS C_{18} column; 3 mm × 50 mm, 2.2 μm, Shimadzu, Japan) was different from that used by Svendsen et al. [10] (Bio-Rex 70 column; 0.9 × 7 cm, Bio-Rad, Hercules, CA, USA). The Bio-Rex 70 and Shim-pack XR-ODS C_{18} chromatographic columns perform separation according to the different affinities of each component and the stationary phase in the sample to be measured by reverse chromatography. The main difference between the two columns is that the stationary phase of the former is resin with low column efficiency and a poor separation effect on HbA_{1c} and its precursors, whereas the stationary phase of the latter is silica gel, with a better separation effect, thereby effectively reducing the interference of HbA_{1c} precursor on the measurement results.

LC-MS/MS calculates the $\%HbA_{1c}$ of the sample by measuring the peak area ratio of glycation and non-glycation hexapeptide segments. MALDI-TOF MS mainly measures the free globin chain, which can be identified according to the mass difference of 162 Da between glycated and non-glycated β-globin [6]. MALDI-TOF MS has the advantages of rapid measurement, a simple operation, and only requiring a small sample volume. Among the measurement results of 45 clinical samples measured, we found that the precision of the two methods met the requirements of the IFCC, and the difference between the measurement results of the two methods was small. Two methods showed good correlation and consistency.

The presence of variant hemoglobin affects the accuracy of HbA_{1c} measurement results [2–3]. In this study, 73 heterozygous variant hemoglobin samples were measured by LC-MS/MS and MALDI-TOF MS, and the heterozygous variant types were confirmed by gene sequencing. The variant was divided into α-chain variant, α-chain negligible variant, β-chain variant, and β-chain negligible variant groups. LC-MS/MS is mainly used to measure the six peptides formed after enzymatic hydrolysis. If the variant occurs in the first six amino acids at the end of the β-chain, it may affect the measurement results of LC-MS/MS. In this study, seven heterozygous hemoglobin variants were not in the first six amino acids at the end of the β-chain. Therefore, in theory, the heterozygous hemoglobin variants did not interfere with the measurement of LC-MS/MS, suggesting its suitability as a reference method for methodological comparison. However, since MALDI-TOF MS measures the glycated free globin chain, it is more difficult to distinguish the globins with a small mass difference, although multiple variant hemoglobins can still be measured [12]. It has been reported that the α-chain subunit is similar to the β-chain subunit and can be glycated at either the N-terminal or another terminal amino acid [5]. Previous studies have found a good correlation between glycated α-globin and glycated β-globin [13–14]. Therefore, when the β-globin chain changes, MALDI-TOF MS can be used to calculate the $\%HbA_{1c}$ according to the α-globin chain to avoid interference of the measurement results. The results of this study showed that the average biases of MALDI-TOF MS and LC-MS/MS in measuring α-chain and β-chain variants were higher than the minimum 2.3% biological variation bias. In addition, the β-chain variant group showed substantial interference in the MALDI-TOF MS results (glycated β-globin results), resulting in higher values. The HbA_{1c} results calculated according to %glycated α-globin were more consistent with the LC-MS/MS results, which was in line with previous research [13–14]. In a previous analysis, we also compared 73 heterozygous variant hemoglobin samples between LC-MS/MS and CE, which showed that the average biases of the α-chain negligible variant and β-chain negligible variant groups were higher than the standard of 2.3%. Thus, a variety of methods should be used to evaluate the measurement of variant hemoglobin to confirm the reliability of results obtained.

In summary, LC-MS/MS and MALDI-TOF MS methods had good comparability for the measurement of HbA_{1c} in non-variant samples, but there were differences in the measurement results of some heterozygous variant hemoglobin samples. The main limitation of this study was the relatively low sample size and variant types of variant hemoglobin samples, and not all variant types were covered. Therefore, these results should be further verified. Different methods have advantages and disadvantages, especially when there is variant hemoglobin, and there are varying degrees of interference to the measurement system. Laboratory personnel should fully understand the principle of the measurement methods used and identify them with a spectrum, so as to correctly interpret the measurement results and provide appropriate guidance for clinical practice.

CRediT authorship contribution statement

Yichuan Song: Resources, Data curation, Investigation, Methodology, Writing – original draft. **Anping Xu:** Resources, Funding acquisition, Validation, Writing – review & editing, Supervision. **Mo Wang:** Data curation, Investigation, Methodology, Supervision. **Ling Ji:**

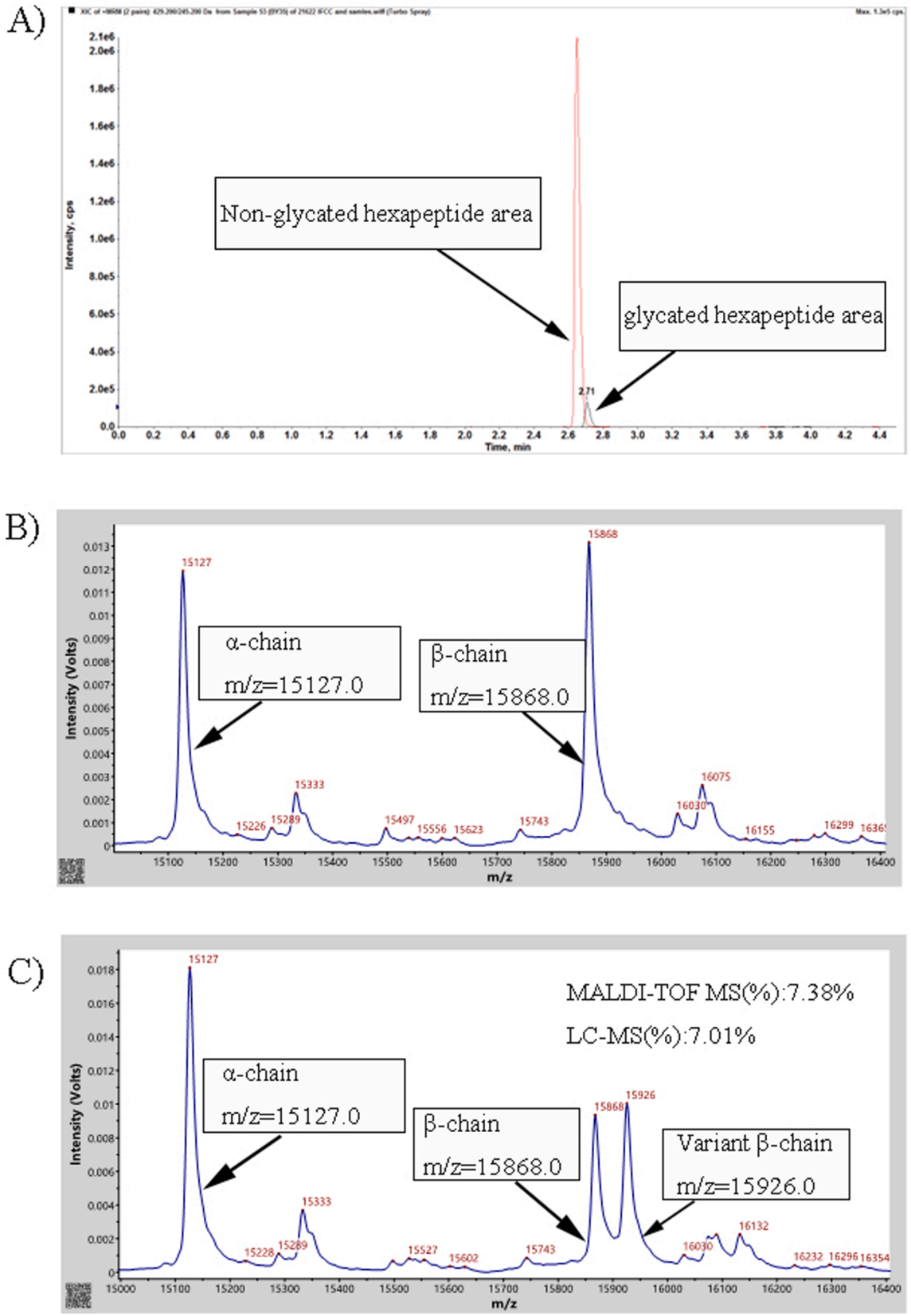

Fig. 2. (A) LC-MS/MS spectra and (B, C) MALDI-TOF MS spectra of HbA_{1c} measurement results of non-variant hemoglobin and heterozygous variant hemoglobin samples, respectively.

Conceptualization, Resources, Visualization. **Qingtao Wang:** Conceptualization, Visualization, Project administration, Supervision. **Jie Shi:** Resources, Writing – review & editing. **Rui Zhao:** Resources, Writing – review & editing. **Wenxaun Fu:** Resources, Writing – review & editing. **Rui Zhang:** Conceptualization, Data curation, Funding acquisition, Investigation, Methodology, Project administration, Supervision, Visualization, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cca.2022.06.024.

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