Biochemistry and Biophysics Reports 18 (2019) 100635

Contents lists available at ScienceDirect

Biochemistry and Biophysics Reports

journal homepage: www.elsevier.com/locate/bbrep

Relative proteome quantification of alpha, beta, gamma and delta globin chains in early eluting peaks of Bio-Rad variant II® CE-HPLC of hemoglobin from healthy and beta-thalassemia subjects in Malaysia

Uday Younis Hussein Abdullah[a,*], Hishamshah M. Ibrahim[b], Haitham Muhammed Jassim[c], Mohamad Zaki Salleh[d], Teh Lay Kek[d], Mohd Nur Fakhruzzaman bin Noorizhab[d], Bin Alwi Zilfalil[e], Prapin Wilairat[f], Suthat Fucharoen[g]

[a] Faculty of Medicine, Universiti Sultan Zainal Abidin, Medical Campus, 20400, Kuala Terengganu, Terengganu, Malaysia
[b] Paediatric Department, Hospital Kuala Lumpur (HKL), Jalan Pahang, 50586, Kuala Lumpur, Malaysia
[c] Emergency Department, Rockingham Peel Group, South Metropolitan Health Service, 33 Elanora Drive, Rockingham, 6967, PO Box 2033, West Australia, Australia
[d] Integrative Pharmacogenomics Institute (iPROMISE), Universiti Teknologi Mara (UiTM), Bandar Puncak Alam, 42300, Selangor, Malaysia
[e] School of Medical Science, Health Campus, Universiti Sains Malaysia (USM), 16150, Kubang Kerian, Kelantan, Malaysia
[f] National Doping Control Centre, Mahidol University, Rachathawee, Bangkok, 10400, Thailand
[g] Institute of Molecular Biosciences, Mahidol University, Salaya Campus, Nakornpathom, 73170, Thailand

ARTICLE INFO

Keywords:
Anaemia
Thalassemia
Mass spectrometry
Globin chain
Quantification
HPLC
Unknown peaks

ABSTRACT

This is the first report of QQQ-mass spectrometric identification and quantification of the Hb subunits, alpha, beta, delta and gamma globin peptides, derived from enzymatic-digestion of proteins in the early unknown peaks of the Bio-Rad cation-exchange chromatography of haemoglobin. The objectives were to assess the relationship of the quantity of the free alpha, beta, delta and gamma globin chains with the phenotypic diversity of beta-thalassaemias (β-thal). The results demonstrate that the pools of free globin chains in red blood cells were correlating with the severity of the disease in patients with different phenotypes of β-thal. The mechanism and the regulation of synthesis of free globin chains pool in a normal individual and in patients with different β-thal phenotypes could arise from several mechanisms which will require further investigation. The role of the free globin pool in patients with β-thal for development of novel therapeutic approaches based on these potential targets requires further investigation. Pertinent biomarkers improves the diagnosis of the β-thal, especially in low-income countries where they are most common and allows more effective therapeutic intervention leading to more successful therapeutic outcome.

1. Introduction

Employing multiple-reaction monitoring (MRM) analysis, a triple quadrupole mass spectrometer (QQQ-MS) can quantify compounds of a complex mixture with high sensitivity and specificity. In MRM analysis, the mass spectrometer first selects an ion corresponding to the compound of interest using the first quadrupole and then subsequently fragments the target compound (or the precursor ion) in the second collision cell to produce fragment ions which are specific to the precursor ion. The masses of the fragment ions are measured using the third quadruple. Quantification of the target compound employing MRM analysis can be done using either labeled or label-free approach. For labeled MRM analysis, an isotope-labeled standard compound is used and the peak area ratio between the labeled- and unlabeled compounds is employed for calculating the concentration of the compound in the sample. In contrast, a label-free MRM analysis does not employ an isotope-labeled compound. Instead, measurement of concentration is carried out using a calibration curve constructed using a known amount of the compound of interest.

The objectives of this study were to measure the alpha, beta, gamma and delta globin chains in the early-eluting fraction of unknown peaks of the Bio-Rad cation-exchange chromatography using Triple Quad Mass spectrometry of haemoglobin from normal subjects and patients with Beta-thalassemias (β-thal), and to assess the relationship of the amount of identified globins with the phenotypic diversity of β-thal.

* Corresponding author. Faculty of Medicine-Pathology department, University Sultan Zainal Abidin (UniSZA), Medical campus, Sultan Mahmud Street, 20400, Kuala, Terengganu, Malaysia.
E-mail address: udayyounis@unisza.edu.my (U.Y.H. Abdullah).

https://doi.org/10.1016/j.bbrep.2019.100635
Received 26 February 2019; Received in revised form 2 April 2019; Accepted 2 April 2019
Available online 28 April 2019

2. Methodology

Respondent's recruitment: In this cro-sectional study, the total recruited respondents were divided into four groups: 1) healthy blood donors with normal clinical and haematological profile, 2) newly diagnosed patients with β-thal trait, 3) newly diagnosed untreated patients with β-Thal major and Hb-E β-Thal, and 4) transfusion-dependent β-Thal major and Hb-E β-Thal. For the latter group blood samples were taken just before receiving allogeneic blood. Out of the total 210 recruited respondents, 52 individuals were healthy blood donors, 48 individuals with the β-Thal trait, 45 individuals having β-Thal major and 65 individuals with Hb-E β-Thal. The majority of patients with β-Thal major and Hb-E β-Thal were transfusion-dependent and were enrolled in the regular transfusion programme in the paediatric department of Hospital Kuala Lumpur-Kuala Lumpur (HKL-KL), Malaysia. Patients with chronic viral diseases, hyperthyroidism, ongoing inflammatory diseases and recently transfused (within last two months) non-transfusion dependent β-thal patients were excluded. Prior to the enrolment of each respondent, patient information and consent form was obtained. Ethical approval from National Medical Research Register (NMRR) (NMRR-13-1471-15105), Medical Research and Ethical Committee (MREC) ((6) dlm.KKM/NIHSEC/P14-462) and Jawatankuasa Etika Penyelidikan (Manusia) – Universiiti Sains Malaysia (USM) (JEPeM) (JEPeM Code: USM/JEPeM/15) and Universiti Sultan Zainal Abidin (UniSZA) (UniSZA/C/2/CRIM/431-2 (24)) were obtained at the initial stage of this study.

CE-HPLC: Haemoglobin analysis was carried out using the Bio-Rad cation-exchange chromatography system with the "β Thalassaemia Short" program for quantification of Hb variants for all blood samples [8–10]. The eluted fraction within the first minute of the chromatography program of each blood sample was manually collected in a sterile tube. This is the targeted fast-eluting unknown HPLC peaks. The eluate was collected from the time of injection (0 min) to 1.0 min for a total volume of 2.0 mL.

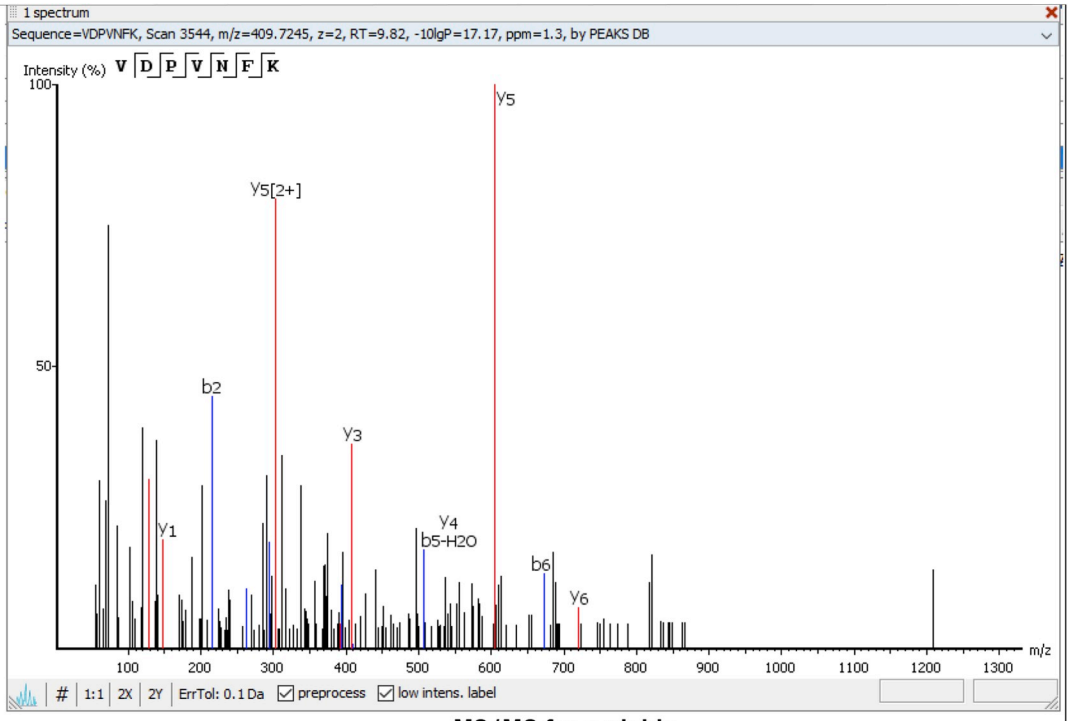
Tryptic digestion of proteins in HPLC-eluates: The 2 mL of the manually collected eluates during the first minute of the CE-HPLC analysis of blood samples were subjected for intensity-based label-free peptide quantitation of globin chain(s) by QQQ-MS. The total protein concentration of each sample was measured for all samples using the Nanodrop Spectrophotometer (Thermo Scientific NanoDropTM 2000/2000c). Then 100 µg of protein from each HPLC eluate was reconstituted and mixed with 100 µL of 6 M urea in 50 mM Tris-HCl, pH 8.0. Next a 5 µL of 200 mM dithiothreitol (DTT) in 50 mM ammonium bicarbonate, pH 8.0, was added into the mixture and incubated at room temperature for an hour. A 20 µL of 200 mM iodoacetamide (IAA) in 50 mM ammonium bicarbonate, pH 8.0, was added into the mixture, followed by incubation at room temperature in dark. The excess IAA was chelated by the addition of 20 µL of 50 mM DTT in 50 mM ammonium bicarbonate, pH 8.0, and incubated in dark for another one hour at room temperature. A 775 µL of 50 mM ammonium bicarbonate was added into the mixture to reduce the concentration of urea to 0.6 M prior to the addition of trypsin. A 2 µg MS grade trypsin (Thermo, USA) was added into the mixture giving the final ratio of 1:50 (w/w, trypsin: protein). The mixture was vortexed gently and incubated at 37 °C for at least 18 h. Finally, 2 µL of neat formic acid was added to the digested protein mixture to stop the trypsin activity. Following the proteolytic digestion, the samples were placed in a receiver tube and stored at −20 °C until use. Prior for injection into the LC/MS-MS system, all tryptic digests were desalted using Pierce C18 Spin Column (Thermo Scientific, USA, product number 89873) following equilibration in 50% acetonitrile (ACN) in 0.1% formic acid.

Pierce C18 Spin Column containing 8 mg of C18 is a ready-to-use centrifuge column of porous C18 reverse phase resin with excellent binding and recovery characteristics for peptide sample preparation for mass spectrometry. the new spin column was used for the processing of each sample. After tapping the C18 spin column to settle resin and removal of the top and bottom cap, 200 µL of activation solution and 50% Acetonitrile (ACN), were added to rinse walls of the C18 spin tube and to wet resin. Centrifugation at 1500 × *g* for one minute and discard of the flow-through was repeated. Equilibration solution prepared by adding 7.5 µL of 0.5% fomic acid to 75 µL of ACN in 1417.5 double-distilled (DD) water; 400 µL per sample was added followed by centrifugation at 1500 × *g* for one minute and then discard flow-through and this step was repeated. the sample was loaded on top of resin bed by preparing sample buffer (2% fomic acid with 20% ACN in 780 µL of DD water). Three parts sample (500 µL) was mixed with 1 part sample buffer (167 µL). C18 spin tube was placed into a receiver tube and centrifuged at 1500 × *g* for 1 min and this step was repeated to ensure complete binding and recover flow-through. After placing C18 spin tube into a receiver tube, 200 µL wash solution was prepared by adding 0.5% fomic acid in double distilled water then centrifuge at 1500 × *g* for 1 min and discard the flow-through. After repeating the last step, C18 spin tube was placed in a new receiver tube and 20 µL of elution buffer was added to the top of the resin bed followed by centrifugation at 1500 × *g* for one minute and repeated addition of 10 µL of elution buffer was done. Finally, the sample was gently dried in a vacuum evaporator for 40 min and the sample was suspended in 10 µL of water with 0.1% formic acid.

Preparation of peptide standards and method development for quantitation of enzymatically-derived globin chain peptides: Four synthetic peptides representing four globin chains (Fig. 1) were purchased from Sigma Aldrich, USA. Each of the peptide was reconstituted with 0.1% formic acid to produce a stock solution of peptide standard with the concentration of 10.2 ng/µL. For method development, a solution containing all four peptides at a concentration of 1 ng/µL was prepared. Then, the peptide sequences of each peptide standard were entered into the Skyline software (MacCoss Lab Sofware, USA). The Skyline software produces a list of transitions that are derived from the respective peptides following collision-induced-dissociation (CID) at given collision energy. The software also gives the MS acquisition method used to obtain theoretical transitions. The Agilent 6400 Series AJS ESI-LC-MS-QQQ (Santa Clara, CA, USA) was set according to the acquisition method generated by the Skyline software followed by direct injection of 1 µL of the standard peptide solution in the MS-QQQ system. The resulting transitions detected by the QQQ system were assessed in terms of abundance and reproducibility in order to select the quantitation and qualifier ions.

LC/MS-QQQ Setup: The Agilent 1200 infinity UHPLC system coupled with an Agilent 6460 triple-quadruple mass spectrometer (Santa Clara, CA, USA) was used in this experiment. The instrument was controlled by Agilent Mass Hunter Workstation Data Acquisition (B.07.00). Chromatographic separation was performed at 40° C using a ZORBAX Eclipse Plus C18 column (2.1 × 100 mm, 1.8-µm) (Agilent, USA) with 0.1% formic acid and acetonitrile containing 0.1% formic acid as the mobile phases. The gradient elution program was 3–97% acetonitrile in 9 min with equilibration at 3% acetonitrile for 3 min. The injection volume of the sample was 8 µl and the flow rate was fixed at 0.4 mL/min. The analytes were determined in positive ionization mode at 3500 V and quantified by multiple-reaction-monitoring (MRM) mode. The ESI was at an optimum gas temperature of 150° C, flow at 13 l/min, and nebulizer at 30 psi.

Calibration curve procedure and quantitation of globin chains peptides by QQQ-MS: To establish the calibration curves for human α-globin 1, α-globin 2, β-globin, γ-globin and δ-globin chain peptides, 100 µL of blank 0.1% formic acid and 100 µL of each of the globin chain peptide solutions at concentrations of 0.937, 0.469, 0.234, 0.117, 0.058, 0.029, 0.0146, 0.0073, 0.00365, 0.001875, 0.0009375 and 0.00046875 ng/100 µL, respectively, were prepared. The calibration curve was plotted using the target peptide response versus peptide concentrations for each globin chain. Control standards, consisting of synthetically produced peptides (as listed in Fig. 1), were prepared to achieve reliable and accurate quantitative results. The within-day

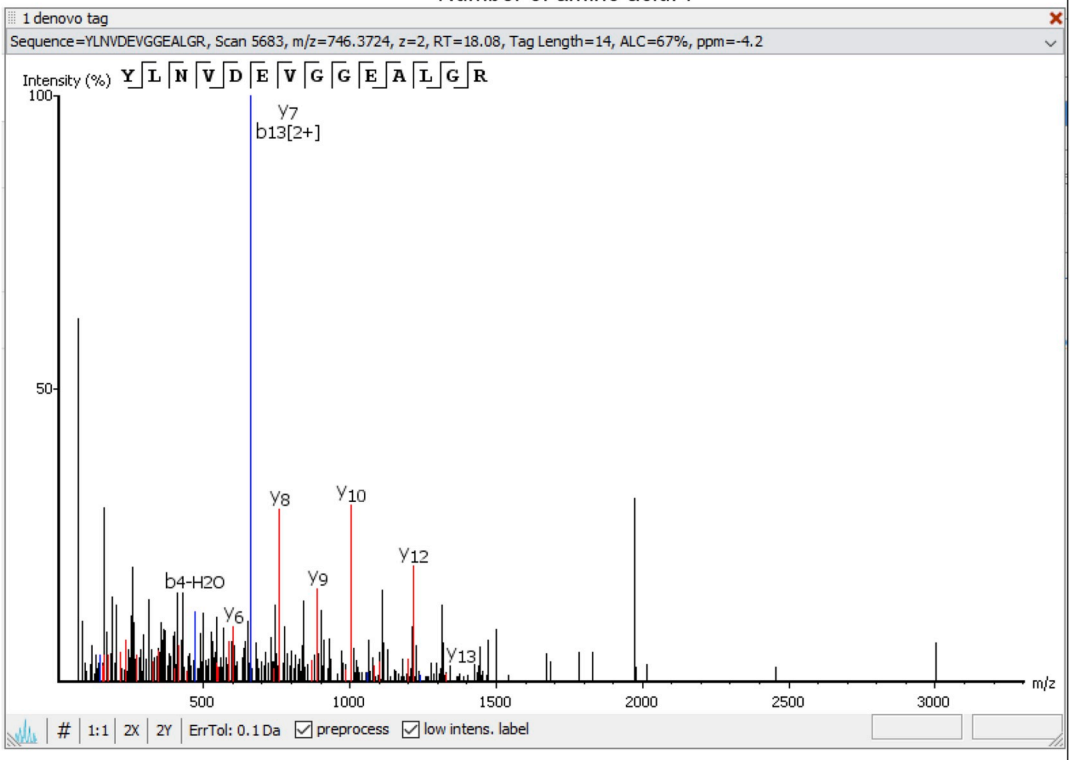


MS/MS for α-globin

Peptide sequence: VDPVNFK

Molecular formula: $C_{38}H_{59}N_9O_{11}$

Number of amino acid: 7



MS/MS for β-globin

Peptide sequence: VNVDEVGGEALGR

Molecular formula: $C_{75}H_{120}N_{20}O_{23}$

Number of amino acid: 13

(caption on next page)

Fig. 1. Mass spectrum (MS/ MS) of each peptide in the MRM analysis. The eluted fractions of every sample were collected in two screw-cap sterile plastic tubes. The two ml volume of the early-eluted fraction during the first 60 s was collected in one tube and the remaining second-eluted fraction was collected in a separate second tube starting strictly after elapse of the first 60 sec. till the end of the cycle time of each sample. All four globin types were consistently detected in the eluted fractions in both tubes. The second eluted fraction was considered as the positive control for the globin as the eluting Hb-A, Hb-A2 and Hb-F contain the four globin chains (α-, β-, δ and γ-globin). Molecules that elute before 60 s are not designated as Hb variant by the detector of the HPLC instrument. Moreover, only tetramers that are composed of one globin chain type such, as Hb-Bart's (γ4) or Hb-H (β4), are known to have rapidly-eluting peaks within the first 60 s of the HPLC analysis. Accordingly, the α, β, δ, or γ globin chain that were detected in the eluates collected within the first 60-s of the HPLC analysis of the Hb are considered representative of the α, β, δ, or γ globin chain whether in the form of monomers, dimers or tetramers. The findings of the same peptide fragments in the early eluted-fraction collected during the first 60 s corresponding to respective globin chains in the second-eluted fraction after 60 s support our hypothesis for the presence of globin chains in the content of the early-eluting peaks of the CE-HPLC. For each globin chain type, only the most consistent peptide detected in all samples was selected. Unique synthetic peptides were used for globin chain identification from the database search.

variability of the assay was determined by repeated analysis of quality control samples at concentrations ranging from 0.00046875 to 0.937 ng/100 μl on the same day. The day-to-day variability of the assay was determined by repeated analysis of quality control samples at concentrations ranging from 0.00046875 to 0.937 ng/100 μl on three different days.

The experimental data were evaluated using statistical software (IBM Corp. Released in 2013. IBM SPSS Statistics for Windows, Version 22.0. Armonk, NY: IBM Corp). One-way ANOVA was used for comparing the means of the parametric data and Mann-Whitney test and Spearman's correlation were used for the analysis of the non-parametric data of the studied groups of individuals. The *p*-value of less than 0.05 was considered significant.

3. Results

HPLC analysis: Each globin chain is readily distinguished through ion-exchange chromatography by having a distinct charge at pH 7.0: values of 4.3, 1.0, 2.6 and 0.6 for α-, β-, δ and γ-globin chain respectively. This supports our observation that even in blood samples from healthy individuals, peaks at the retention time of less than 60 s occur. Even though these early eluting peaks were observed at low intensity, the differences between the patient's groups make them potential biomarkers for thalassaemias.

A criterion has been proposed by various certifying authorities such as the World Anti-Doping Agency (WADA) to ensure unambiguous identification of the compound of interest. Under the guideline, at least two precursor-product ion transitions of the peptide are required (Fig. 2). A label-free MRM analysis was employed to quantitate the amount of free globulin chains which were present in the early eluting peaks (RT $<$ 1.0 min) from CE-HPLC of healthy blood donors and patients with β-thalassemias.

Quantitation Approach: in addition to confirming the presence of globin-chains in the early eluting peaks, the current study also used intensity-based label-free peptide quantitation by QQQ-MS for the first time. Label-free quantitation is possible due to the highly specific and sensitive nature of the MRM analysis where a QQQ-MS system is capable of selectively quantify compounds within a complex mixture. The method of quantitation is based on comparing the response of the targeted analyte in the sample to the calibration curve established using a known concentration of the same analyte.

Four calibration curves were constructed for the quantitation of alpha, beta, delta and gamma globin chains using the respective synthetic diagnostic peptides as shown in Fig. 1. Each calibration curves were constructed using 24 points with the $R^2 > 0.99$. The MRM chromatogram of the selected transitions and their corresponding retention times were used for identifying and quantifying the target compounds. All the results are expressed in terms of femtogram (fg). At the start of the quantitation method development, MRM analysis was done on each of the diagnostic peptides to determine their fragmentation profiles following collision-induced-dissociation (CID). The MRM resulted in 5, 9, 8 and 6 stable product-ions produced from alpha, beta, delta and gamma peptides respectively. To identify the target peptides in a sample, two product-ions from the peptide must be detected in the chromatogram. In addition, one product-ion for each peptide is assigned as the quantifier ion which is used to establish the calibration curve. The list of product-ions for each diagnostic peptide is shown in Fig. 2.

Preparation of standard solutions of the synthetic peptides was done by serial dilution of a stock solution of 10.2 μg/μl (dilution factor: 1:2). Linear calibration equation for α-globin chain peptide is $y = 77657.1111x + 0.3504$, $R^2 = 0.9997$, for β-globin chain peptide $y = 343.9862x + 0.3409$, $R^2 = 0.9986$, for γ-globin chain peptide $y = 15018.3710x\text{-}36.0384$, $R^2 = 0.9919$, and for δ-globin chain peptide $y = 12790.3914x\text{-}155.1080$, $R^2 = 0.9950$, respectively Fig. 2.

Limit of detection and quantitation (LOD and LOQ) is highly dependent on the nature of the analyte, its fragmentation and the sample matrix. Generally, it is difficult to detect analytes at less than 0.1 μmol/L using direct injection methods and also problematic to perform quantitative determinations using absolute MS responses Fig. 2.

The quantities of the globin chains were significantly different, depending on the clinical phenotypes of B-Thal (Table 1). The delta globin levels were significantly higher at *p*-value 0.014 and 0.021 in patients with moderate and severe scoring criteria respectively than in patients with mild scoring criteria while moderate and severe scoring criteria showed no significant difference (*p*-value 0.192) respectively. The alpha, beta and gamma globin values showed no significant difference among three scoring criteria (Table 2).

A significant fair direct positive correlation between Hb-F and the Delta together with the Gamma globin levels in patients with Beta thalassaemia major ($r = 0.320$ and 0.410 respectively) at *p*-value ($p = 0.036$ and 0.006 respectively) (Table 3) found.

A significant good direct positive correlation between the alpha globin and the beta, gamma as well as delta globin levels in patients with Hb-E beta thalassaemia ($n = 65$) as well as those with Beta thalassaemia major ($n = 45$) ($r = 0.685$, 0.639, 0.743 and 0.684, 0.721, 0.636 respectively) at *p*-value = 0.001 (Table 4) found.

Moreover, a significant fair direct positive correlation between Hb-F and Hb-A2 levels in patients with Hb-E beta thalassaemia ($n = 65$) ($r = 0.492$) at *p*-value = 0.001 (Table 5) found.

4. Discussion

Quantitative impairment of beta globin synthesis is an inherent defect of the individual red blood cell in patients with β-thal resulting in accumulation of excess, free other globins in all stages of red blood cells maturation [11,12]. The disease severity correlates with the degree of imbalance between the alpha and non-alpha globin chains and the resulting amount of the free globin pool in the erythrocytes rather than the underproduction of Hb [13–15].

This is the first report of QQQ mass spectrometric quantification of globin peptides (globin digest) identified in the fast-eluting unknown HPLC peaks. Acylated or altered Hb-F is the only Hb type that was reported to be present in the fast-eluted fraction within the first minute during Hb analysis by CE-HPLC for patients with B-Thal [16]. However, we found that the quantities of the globin chains were significantly

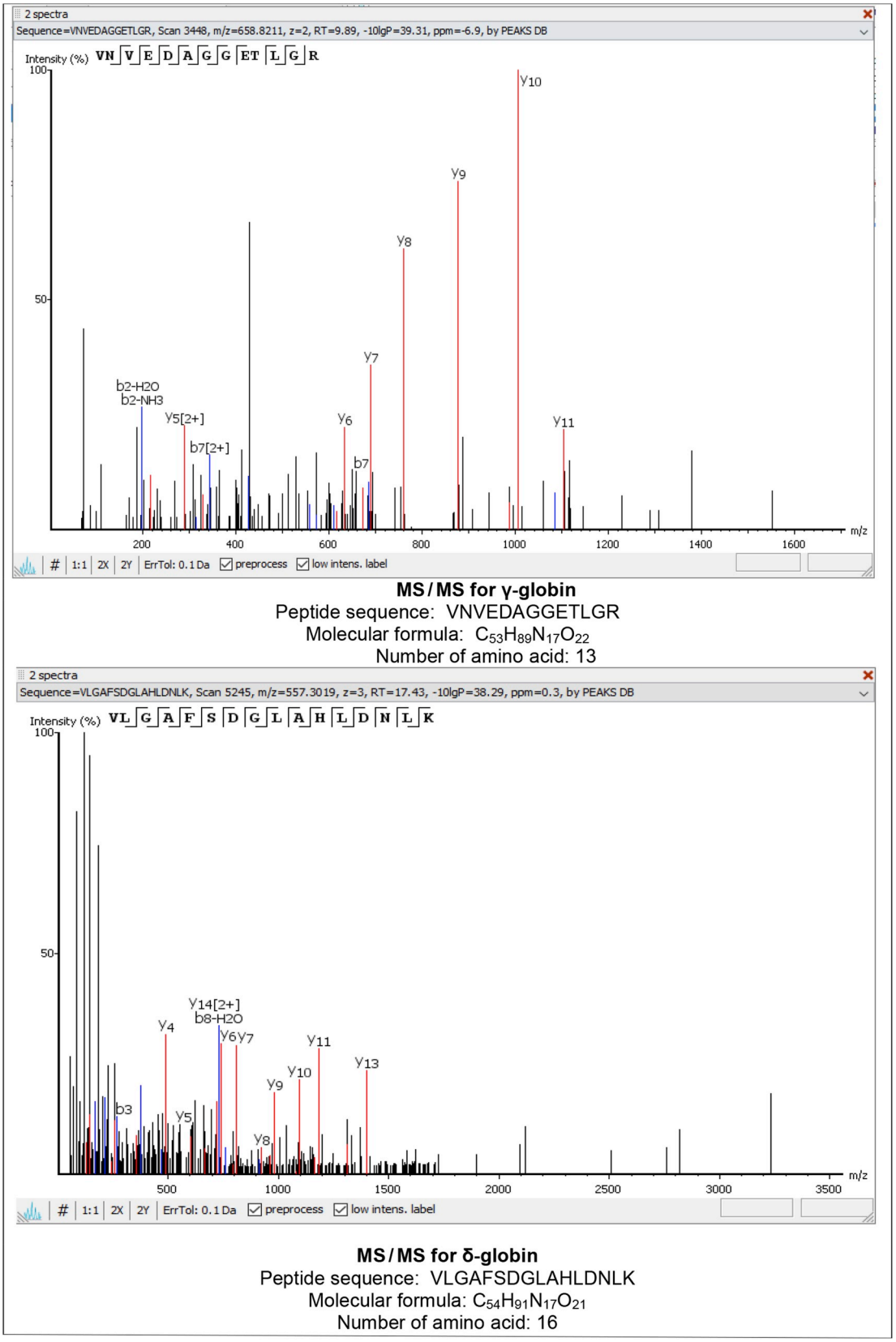


Fig. 1. (*continued*)

different, depending on the clinical phenotypes of B-Thal (Table 1), as well as within the group of patients with Hb-E B-Thal that have different categories of disease severity (Table 2). These findings support the proposal that these globin chains were not a result of the tryptic digestion of eluted intact Hb tetramer molecules. Moreover, acylated or altered Hb-F, with the assigned retention time of 0.5 min, was described only in the CE-HPLC analysis of blood samples from neonates [16]. The assumption of altered Hb-F might indicate that it is altered from

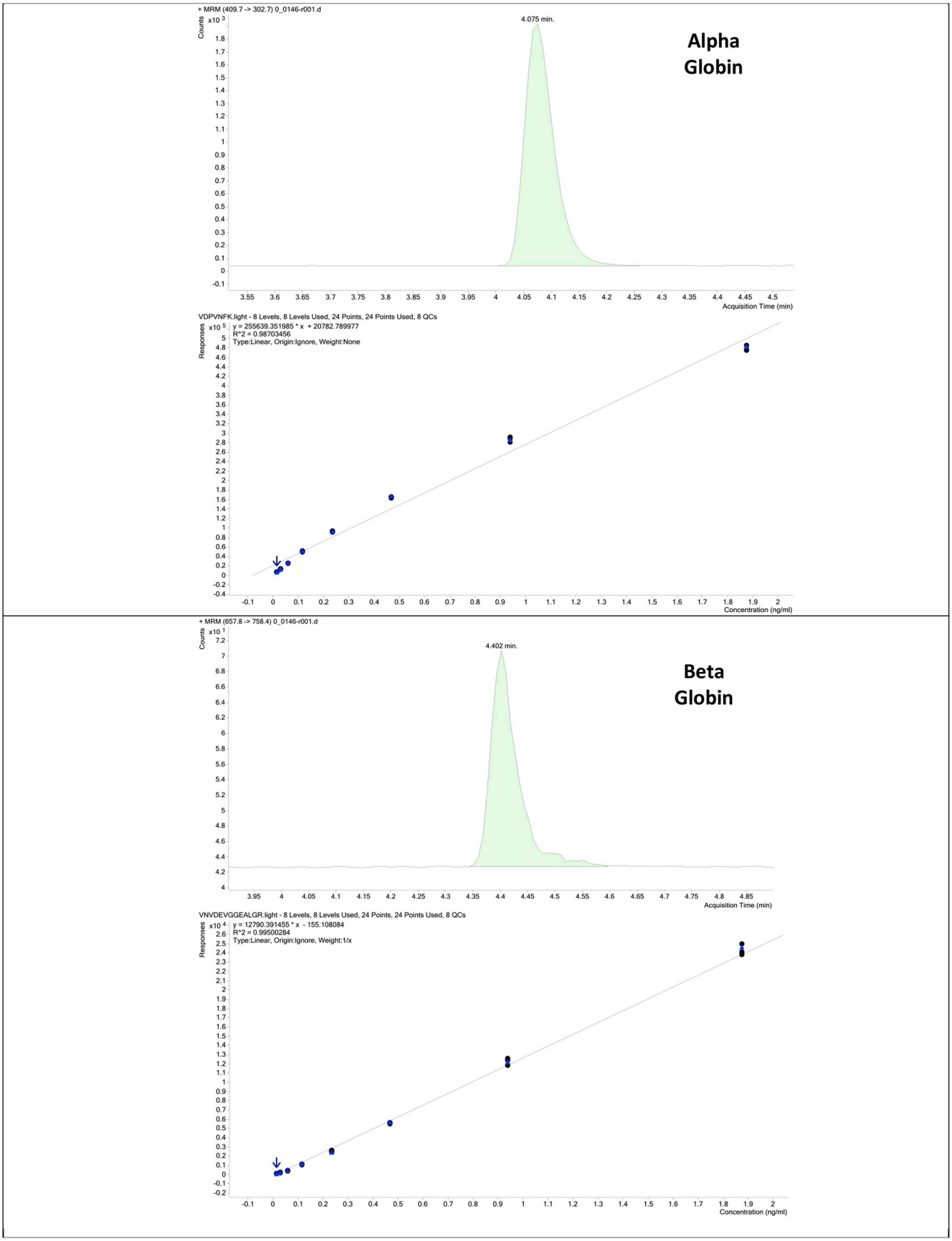


Fig. 2. QQQ-MS Chromatography results of the calibration curves of the analysed peptides and the limit of detection and quantitation (LOD and LOQ).

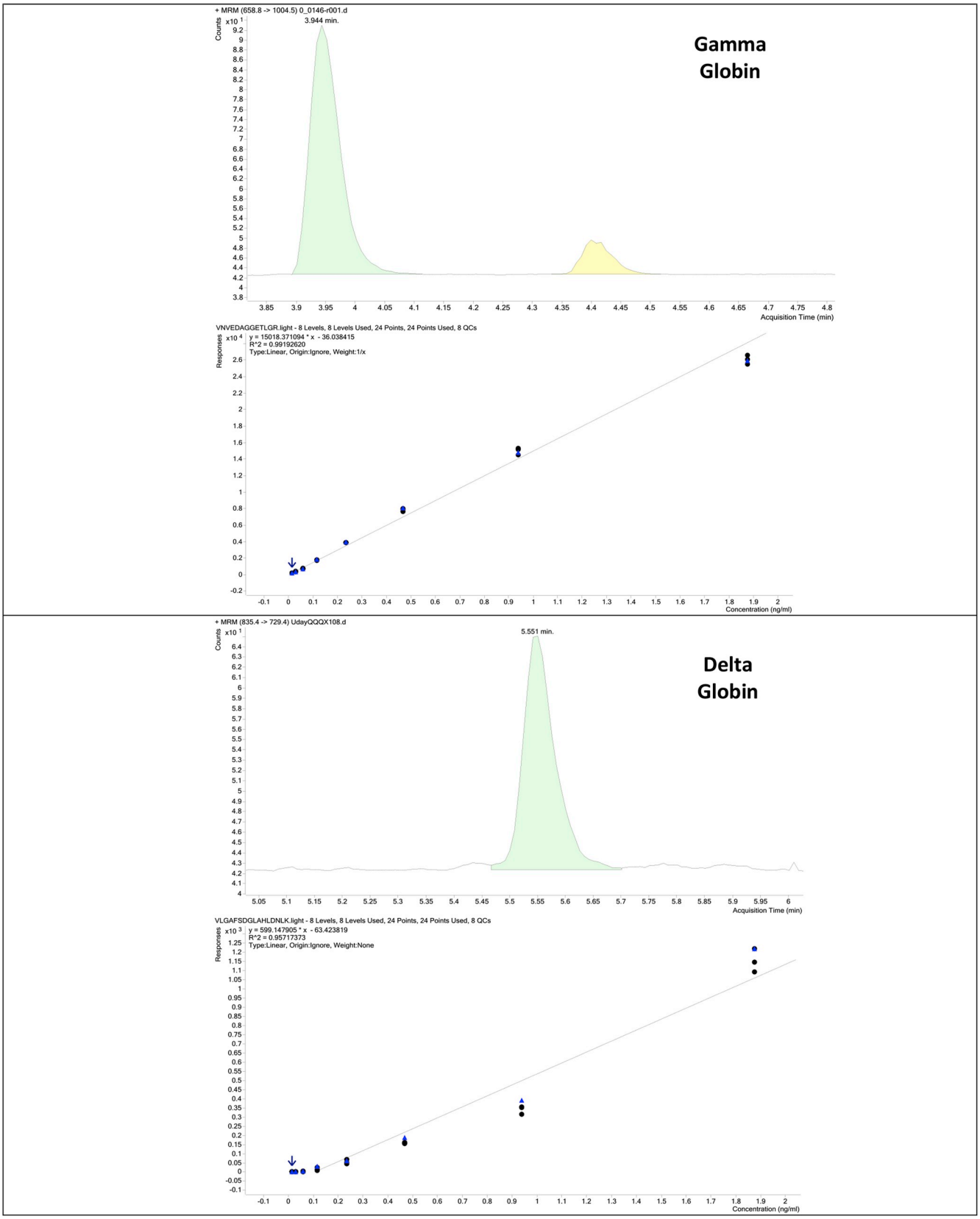
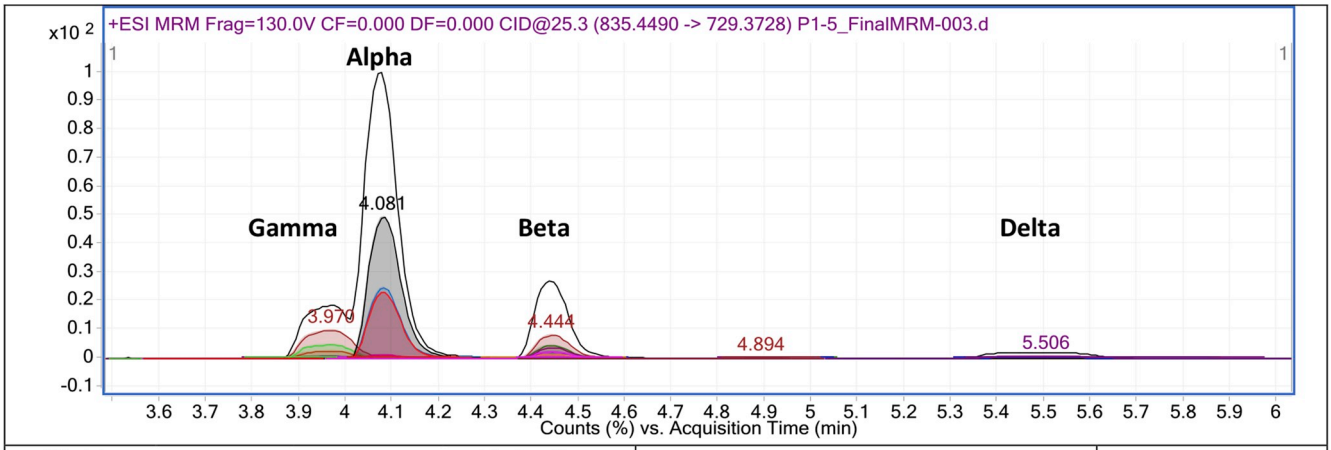


Fig. 2. (*continued*)

tetramers to homopolymers composed of free or unbound gamma globin chains. Identification of all types of Hb subunits in the retention time before one minute suggest that altered Hb- A, Hb-F and Hb-A2 is the nature of these fast-eluting peaks. It may also indicate that the nature of these globin chains is aggregates of homopolymers (macro-globin molecules) consisting of identical free globin of α, β, δ, or γ chains that are representative of total Hb, Hb-A, Hb-A2, or Hb-F types, respectively. Relevancy of thalassaemias to the protein-aggregation



Globin chain	LOD	LOQ	Retention time (min.)	Product ion*		Precursor-ion
Gamma	5.474^{-13}	3.105^{-11}	3.97	**1004.464329***	345.22448	658.82
				760.394792	345.182477	
				609.291473	214.118617	
Alpha	1.790^{-13}	5.968^{-13}	4.08	604.345323		409.72
				507.292559	**302.676299***	
				360.189771	215.102633	
Beta	4.525^{-12}	3.015^{-10}	4.44	1329.679741	923.530892	835.44
				1182.611327	**810.446828***	
				1095.579299	**785.914829***	
				980.552356	739.409714	
					729.372797	
Delta	2.354^{-10}	3.34^{-07}	5.51	1002.485064	602.32565	657.83
				887.458121	**545.304186***	
				758.415528*	**416.261593***	
				659.347114	232.140415	

Fig. 2. (*continued*)

Table 1
Comparison of the total alpha, beta, gamma and delta globin levels (fg) in healthy blood donors and their levels in respondents with B-Thal trait, Hb-E B Thal and B-Thal major.

Groups		Total free Alpha globin (VDPVNFK)	Total free Beta globin (VNVDEVGGEALGR)	Total free Gamma globin (VNVEDAGGETLGR)	Total free Delta globin (VLGAFSDGLAHLDNLK)
Healthy blood donors (n = 52)	Mean	646.0**	114.2*	29.9	285.8
	SD	2664.7	225.9	6.0	132.6
B-Thal Trait (n = 48)	Mean	12.3	171.9	24.9	126.8
	SD	18.5	872.7	1.3	5.2
Hb-E B Thal (n = 65)	Mean	146.2	325.8	117.2*	363.2*
	SD	154.7	1456.7	203.3	707.9
B-Thal Major (n = 45)	Mean	394.1	238.9	410.6*	374.3*
	SD	1542.1	571.6	1777.5	321.3

fg = femtogram.
*One-way ANOVA: indicate differences between the groups.
(*) $p \leq 0.05$ represents statistical significance of >95%.
(**) $p \leq 0.01$ represents statistical significance of >99%.

disorders will require a review of the role of free globin chains in the pathology of the disease. There is a positive correlation of the level of Hb-F with the gamma (representing Hb-F) and delta (representing Hb-A2) globin levels (r = 0.320 and 0.410, p = 0.036 and 0.006, respectively (Table 3). The finding of a positive correlation between the alpha globin and the beta, gamma as well as delta globin levels in patients with Hb-E B-Thal and those with B-Thal major (r = 0.685, 0.639, 0.743 and 0.684, 0.721, 0.636 respectively) at p value 0.001 (Table 4) and the absence of such correlation in healthy blood donors and in patients with B-Thal trait groups confirm the presence of the these globin chains in the eluted fraction during the first minute of the Hb analysis by CE-HPLC. These free globins seem to attributes to the higher amplitude of the unknown HPLC peaks found in Hb analysis in respondents with Hb-E B-Thal and those with B-Thal major that are associated with elevated

Table 2
Comparison between the total alpha, beta, gamma and delta globin levels (fg) in patients with Hb-E B-Thal classified according to the scoring criteria [9].

Hb-E B-Thal		Total Alpha globin (VDPVNFK)	Total Beta globin (VNVDEVGGEALGR)	Total Gamma globin (VNVEDAGGETLGR)	Total Delta globin (VLGAFSDGLAHLDNLK)
Mild (n = 4)	Mean	19.7	56.4	34.1	124.8
	SE	7.2	10.1	8.3	1.9
Moderate (n = 57)	Mean	153.3	373.2	113.7	349.8*
	SE	21.2	31.5	26.6	95.7
Severe (n = 4)	Mean	66.9	53.3	232.9	714.3*
	SE	34.2	26.7	151.9	389.5

fg = femtogram.
*One-way ANOVA: indicate differences between the groups.
(*) $p \leq 0.01$ represents statistical significance of >99%.

Table 3
Correlation between the Hb-F (%) and the delta and gamma globin levels (fg) in respondents with Beta thalassaemia major (n = 45).

Total globin levels in β-thal major	Hb-F level	
	r	*p*-value
Delta globin	0.320	0.036*
Gamma globin	0.410	0.006**

fg = femtogram.
* Spearman's at < 0.05 level (2-tailed) represents the statistical significance of >95%.
** Spearman's at 0.01 level (2-tailed) represents the statistical significance of >99%.

Table 4
Correlation among the total alpha globin and the beta, gamma and delta globin levels (fg) in respondents with Hb-E beta thalassaemia (n = 65) and those with Beta thalassaemia major (n = 45).

Total globin levels	Alpha globin level	
	β-thal major	Hb-E β-thal
	r	r
Beta globin	0.685	0.684
Gamma globin	0.639	0.721
Delta globin	0.743	0.636
p-value*	0.001	0.001

fg = femtogram.
* Spearman's at 0.01 level (2-tailed) represents the statistical significance of >99%.

Table 5
Correlation between Hb-F and Hb-A2 levels (%) in respondents with Hb-E beta thalassaemia (n = 65).

	Hb-F level	
	r	*p*-value*
Hb-A2 level	0.492	0.001

* Spearman's at 0.01 level (2-tailed) represents the statistical significance of >99%.

Hb-F and Hb-A2 levels (Fig. 3). Moreover, the Hb-E variant is known to cause weak union between α- and β-globin that leads to fast elution of these globin chains within the first minute as a free globin that causes higher amplitudes of the unknown HPLC peaks particularly in patients with Hb-E B-Thal type [8,17–19].

There are 4 alpha globin genes and 2 beta globin genes to produce a normal level of Hb A in a normal subject. The finding of significantly larger quantity of total alpha-globin in samples from healthy blood donors (Table 1) might be attributed to the higher total Hb level in healthy subjects than the patients group as the amount of α globin chain represents the total amount of Hb, or to the spectral characteristic of the alpha globin for the measurement at 540 nm wavelength. Moreover, the alpha globin chains that are bound to its chaperone AHSP (alpha haemoglobin stabilizing protein) which in turn control the "excess" amount of alpha globin was also digested during the sample preparation before analysis by the QQQ-MS. The significantly higher total alpha, gamma and delta globin levels found in Hb-E B-Thal and B-Thal major groups compared to their levels in B-Thal trait group (Table 1) indicate that the level of the imbalance in the globin production in Hb-E B-Thal and B-Thal major is significantly more severe compared with its counterpart in patients with B-Thal trait. Delta globin chain levels were found to be significantly higher (p = 0.001) in patients with moderate and severe category compared to the mild category of the scoring criteria [20] and this is attributed to the known characteristic of the occurrence of markedly elevated Hb-A2 levels in patients with Hb-E B-Thal (Table 2).

The findings depicted in Tables 1 and 2 seems to suggest the usefulness of the measurement of these free globin in the 1) stratification of the B-Thal subtypes, 2) enhancing the established scoring criteria for the Hb-E B-Thal [20], 3) optimising the diagnostic criteria for the B-Thal subtypes as the differentiation between transfusion-dependent B-Thal major and Hb-E B-Thal types, as well as within the Hb-E B-Thal group, is currently not easily feasible mainly in the regions where over 90% of patients with these disorders live in low-to middle-income countries [21].

The significant positive correlation between Hb-F and Hb-A2 levels in patients with Hb-E B Thal (r = 0.492) at p-value 0.001 (Table 5) might attribute to the unique characteristic of Hb-E B Thal in having higher Hb-A2 levels due to the elution of the Hb-E at the same time as that of the Hb-A2 [8]. In the chromatograms shown in Fig. 3, there is at the retention time of 1.0 min a leading edge of a relatively large peak eluting after 1.0 min for samples from thalassemic subjects. This is the peak of Hb-F. However, the significant difference in the gamma globin chain levels among the studied groups does not show that the measured gamma chain may be overestimated.

Accordingly, we propose that the more severe imbalance of globin production might contribute to the higher production of the different levels of free globin in the different beta thalassaemia phenotypes. It seems that the globin production imbalance is more complicated than the currently proposed mechanism of imbalanced globin production in β-thal. Excess alpha globin chains stimulate beta globin chain synthesis and release from the ribosomes [14]. Red cell precursors containing significant amounts of excessive alpha or beta globin chains would be preferentially destroyed. There is evidence that the cells containing the most Hb-B4 have the shortest half-life in patients with Hb-H disease [15]. With the relevance of free globin in red cells to haemoglobin biosynthesis and metabolism, it seems that the free globin represents 1)

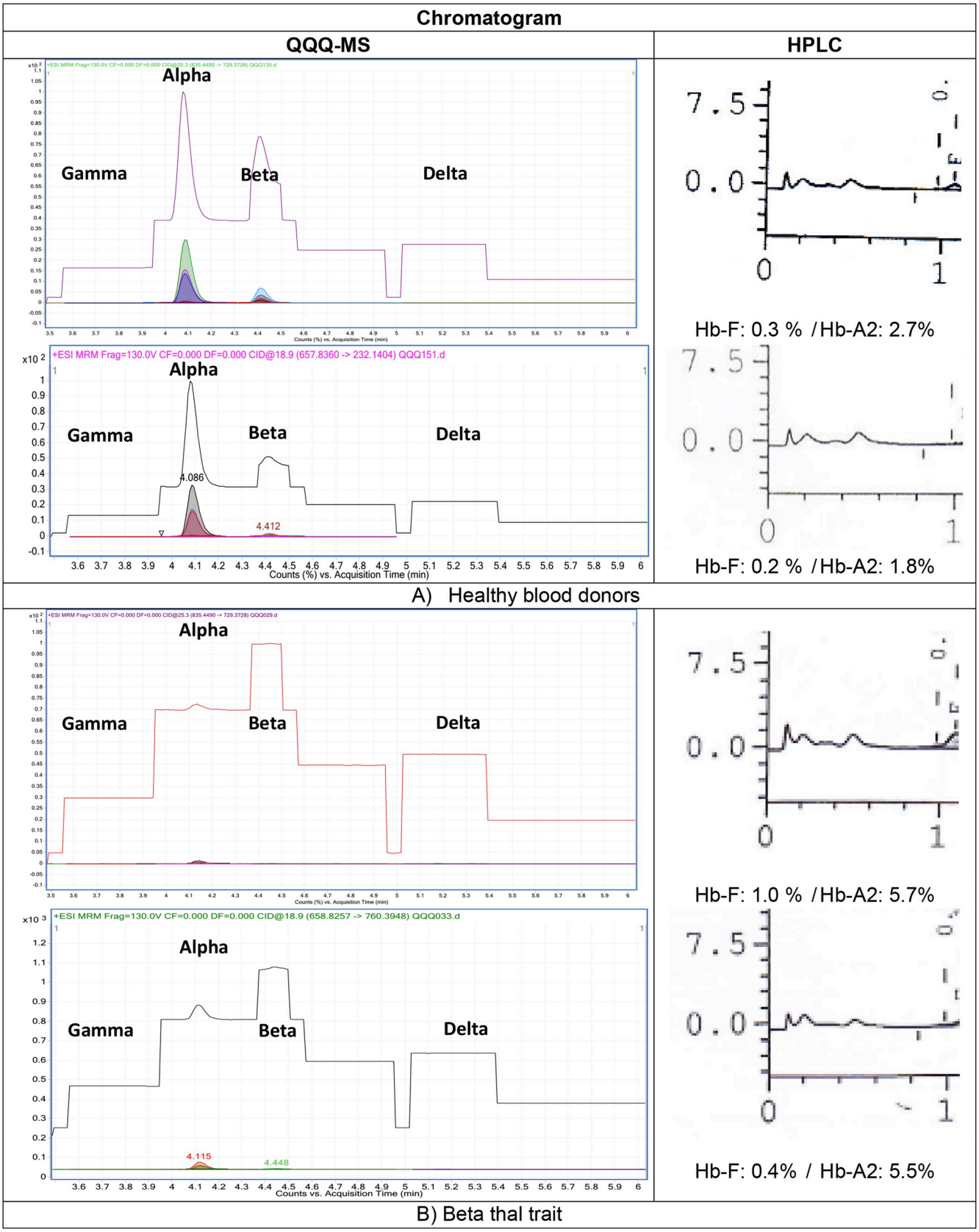


Fig. 3. Chromatograms of the fast-eluting unknown peaks during the first minute of HPLC analysis of blood from patients with different types of β-thal and from healthy blood donors.

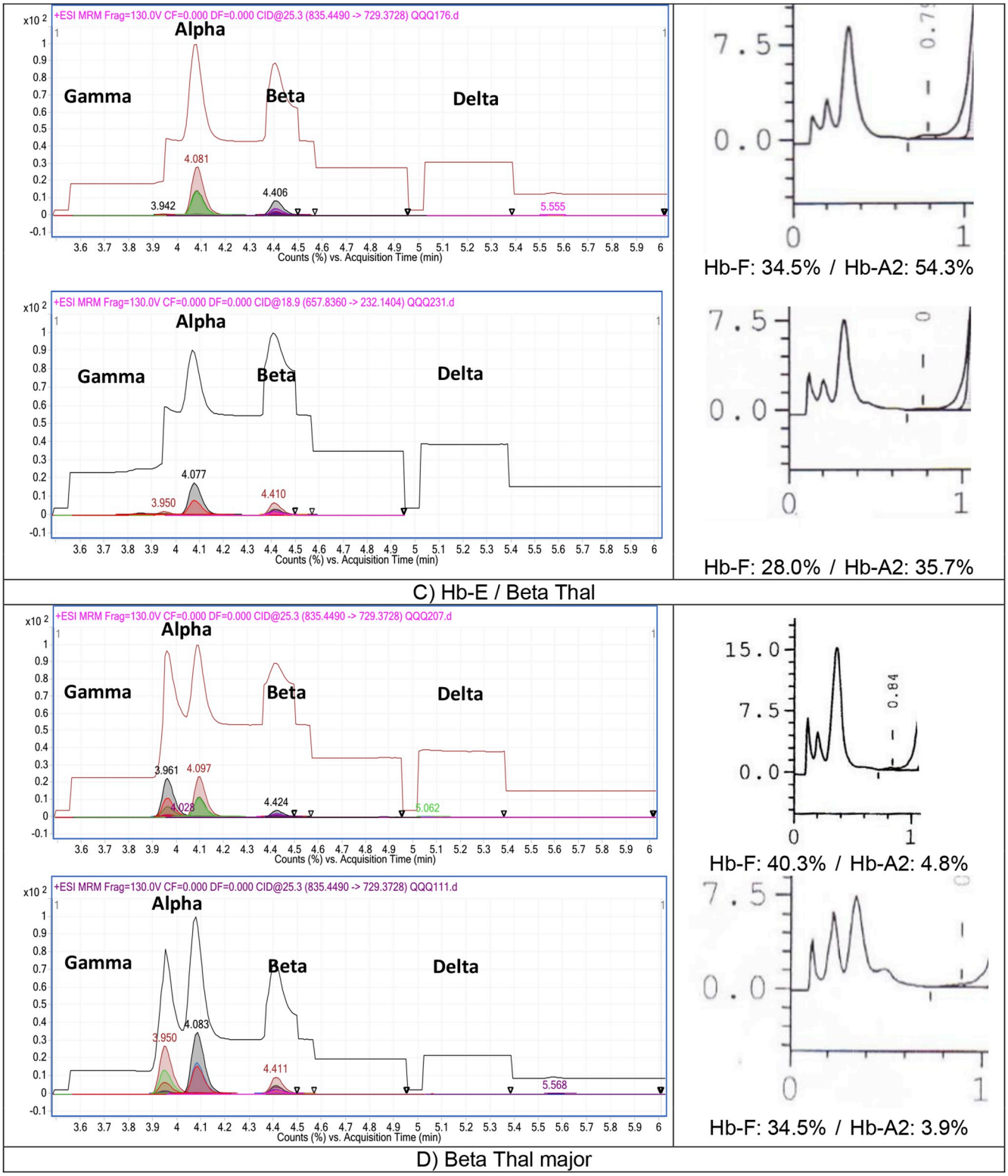


Fig. 3. (*continued*)

the result of intracellular Hb catabolism or instability, 2) aggregation of globin within the red cell as globin synthesis exceeds the haem synthesis in the late erythroblast cells, and/or 3) an intermediate in Hb synthesis, [22,23].

The lack of prediction of the severity of the disease and prognoses in β-Thal via quantification of Hb-F & Hb-A2 necessitate the search for relevant biomarkers of diagnostic and prognostic usefulness. Therefore, development of cost-effective test with diagnostic and prognostic clinical usefulness provides early therapeutic intervention and improve diagnosis efficiency at an early stage of the disease will contribute towards decreases the financial burden of patients with B-Thal on the human blood resources.

5. Conclusions and recommendations

This research reveals the existence of globin chains in the fast-eluting unknown HPLC peaks in normal human red blood cells and the presence of larger pools of globin chains that are directly correlated with the severity of the disease in patients with β-thal. The regulation and conjunction of several mechanisms in the synthesis of larger free globin chains pool in patients with different β-thal phenotypes is a hypothesis requiring further investigation. Early prognosis using pertinent biomarker with diagnostic and prognostic utility allows more effective and early therapeutic intervention with more successful therapeutic outcome. The current therapeutic options are the correction of globin chain imbalance, addressing ineffective erythropoiesis, and improving how the body handles iron [3].

The limitations of the study are the recruitment of patients with newly diagnosed untreated B-Thal major and technical limitations in the management of the time elapsed from the blood sample collection to the time of the analysis (the storage period) that might affect the integrity of the sample and subsequently, the detectable quantity of the free globin.

Conflicts of interest

The authors state that they have no conflict of interest.

Acknowledgment

This research project was supported by research grants: UniSZA/C/2/CRlM/431-2 (24) (FRGS, University Sultan Zainal Abidin (UniSZA)). Authors' sincere gratitude and thanks to the head (Dr Raudhawati Osman) and the MLT staff (Mrs Norafzam Binti Muhamad) in the haematology unit of the pathology department at the HKL-KL for their invaluable contribution in the data collection and samples processing and storage. Our gratitude extends to Dr Mohd Salleh Roffie from the Integrative Pharmacogenomics Institute (iPROMISE), Universiti Teknologi Mara (UiTM) for the technical support in the QQQ MS experiment. We are grateful to all the respondents who participated in this study.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrep.2019.100635.

References

[3] A.T. Taher, D.J. Weatherall, M.D. Cappellini, Thalassaemia. Lancet 391 (2018) 155–167.
[8] L.C.P. Gupta, C.H. Kumar, L.C.S. Kumar, B.M. Jaiprakash, Exchange high performance Liquid chromatography for diagnosis of Haemoglobinopathies, MJAFI (Med. J. Armed Forces India) 65 (2009) 33–37.
[9] G. Chalevelakis, J.B. Clegg, D.J. Weatherall, Imbalanced globin chain synthesis in heterozygous f3-thalassemic bone marrow, J. Clin. Investig. 72 (1975) 3853–3857.
[10] U.Y.H. Abdullah, A.G.F. Al-Attraqchia, Reasons of unknown HPLC peaks during hemoglobin analysis for patients with beta thalassemias, J Hematol Thrombo Dis 2 (5) (2014).
[11] P. Lahiry, S.A. Al-Attar, R.A. Hegele, Understanding beta-thalassemia with hocus on the Indian Subcontinent and the Middle East, Open Hematol. J. 2 (2008) 5–13.
[12] U.Y.H. Abdullah, A.G.F. Al-Attraqchia, H.M. Ibrahim, Z.B. Alwi, A.A. Baig, I. Othman, et al., Free alpha globin: a Promising biomarker for β-thalassemia, J. Mol. Biomark. Diagn. 5 (5) (2014).
[13] K.H. Winterhalter, J.D. Hywood, E.R. Huehns, C.A. Finch, The free globin in human erythrocytes. I, Br. J. Haematol. 16 (1969) 523.
[14] F.M. Gill, E. Schwartz, Free a-globin pool in human bone marrow, J of Clin Invest 52 (1973) 3057–3063.
[15] A. Bank, J.V. O'donnell, Hemoglobin synthesis in i3-thalassemia: the sroperties of the free a – chains, J Clin Invest 47 (1968) 860–866.
[16] BJ B, B W. Variant Haemoglobins: A Guide to Interpretation 2010.
[17] A.E. Acosta-Martin, D.C. Graça, P. Antinori, L. Clerici, R. Hartmer, M. Meyer, et al., Quantitative mass spectrometry analysis of intact hemoglobin A2 by Precursor ion Isolation and detection, Anal. Chem. 85 (2013) 7971–7975.
[18] A. Joutovsky, J. Hadzi-Nesic, M.A. Nardi, HPLC retention time as a diagnostic Tool for hemoglobin variants and Hemoglobinopathies: a study of 60 000 samples in a clinical diagnostic iaboratory, Clin. Chem. 50 (2004) 1736–1747.
[19] A. Premawardhena, C.A. Fisher, N.F. Olivieri, Sd Silva, M. Arambepola, W. Perera, et al., Hemoglobin E beta thalassaemia in Sri Lanka, Lancet 366 (2005) 1467–1470.
[20] O. Sripichai, W. Makarasara, T. Munkongdee, C. Kumkhaek, I. Nuchprayoon, A. Chuansumrit, et al., A scoring system for the classification of b-thalassemia/Hb E disease severity, Am. J. Hematol. 83 (2008) 482–484.
[21] D. Chiabrando, S. Mercurio, E. Tolosano, Heme and erythropoiesis: more than a structural role, Haematologica 99 (2014) 973–983.
[22] J.-A. Ribeil, J.-B. Arlet, M. Dussiot, I.C. Moura, G. Courtois, O. Hermine, Ineffective Erythropoiesis in Beta-Thalassemia vol. 2013, Hindawi Publishing Corporation,The Scientific World Journal, 2013, pp. 1–11.
[23] S. Mettananda, R.J. Gibbons, D.R. Higgs, a-Globin as a molecular target in the treatment of b-thalassemia, Blood 125 (2015) 3694–3701.