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DOI:
10.1002/pmic.201300316

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Document Version
Publisher's PDF, also known as Version of record

Citation for published version (Harvard):
Edwards, R, Griffiths, P, Bunch, J & Cooper, HJ 2014, 'Compound heterozygotes and beta-thalassemia : top-down mass spectrometry for detection of hemoglobinopathies', Proteomics, vol. 14, no. 10, pp. 1232–1238. https://doi.org/10.1002/pmic.201300316

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RESEARCH ARTICLE

Compound heterozygotes and beta-thalassemia: Top-down mass spectrometry for detection of hemoglobinopathies

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We have shown previously that liquid extraction surface analysis of dried blood spots coupled to high resolution top-down MS may be applied for the diagnosis of hemoglobin (Hb) variants FS, FAS, FC, FAC, FAD in newborn samples. The objective of the current work was to determine whether the structural variant HbE, compound heterozygote variants FSC and FSD, and β-thalassemia were amenable to diagnosis by this approach. Anonymized residual neonatal dried blood spot samples, taken as part of the routine newborn screening program, were analyzed by liquid extraction surface analysis coupled to high resolution MS/MS. The samples had been previously screened and were known to be FAE, FSC, FSD, or β-thalassemia. Manual analysis of the mass spectra revealed that, in all cases, the variants may be confirmed. Direct surface sampling MS should be considered as an alternative to current screening techniques for the diagnosis of Hb variants.

Received: August 12, 2013
Revised: October 30, 2013
Accepted: December 10, 2013

Keywords:
Biomedicine / Direct surface sampling / Dried blood spots (DBS) / Hemoglobinopathies / LESA / Top-down

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1 Introduction

Annually, the UK newborn screening program screens ~700 000 neonates for five genetic disorders; phenylketonuria, congenital hypothyroidism, cystic fibrosis, medium chain acyl-CoA dehydrogenase deficiency, and sickle cell disease (SCD) [1]. SCD has the highest prevalence of all the disorders screened for by the National Health Service (NHS) in England with a rate of 1 in 2000 [2]. The term SCD refers to a set of disorders characterized by the inheritance of the structural variant HbS. The variant is formed as a consequence of a single substitution (GTG for GAG) on the sixth codon of the β-globin chain causing an amino acid substitution of a glutamic acid to a valine (Δm −29.9745 Da) at position six on the β-globin chain [3]. When the sickle variant is inherited in a homozygous state (HbSS) it is responsible for sickle cell anemia, however it is usually phenotypically silent when inherited in heterozygous form (HbAS).

SCD also includes compound heterozygotes disorders in which the HbS variant is coinherited with another hemoglobinopathy, e.g. HbC, HbD Punjab/Los Angeles or HbE [4]. Clinical significance of these compound heterozygote variants differ. The phenotype for the HbSC variant is variable: It is generally expressed as a milder form of sickle cell anemia requiring medical intervention and careful monitoring but in some cases it can be equally/more severe than HbSS [5]. HbSE syndrome is uncommon and the vast majority of patients experience no sickle-like symptoms. Rarely, symptoms may develop requiring medical assistance [6]. The

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Abbreviations: ceHPLC, cation exchange HPLC; **DBS**, dried blood spot; **Hb**, hemoglobin; **HbF**, fetal Hb; **LESA**, liquid extraction surface analysis; **NHS**, National Health Service (UK); **SCD**, sickle cell disease

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compound heterozygotes HbSD Punjab/Los Angeles and the rare HbSO-Arab phenotypes are severe and have a similar pathology to HbSS [7].

Thalassemias are hemoglobinopathies characterized by a reduction in the synthesis of (one of) the globin chains. β-thalassemia is caused by the reduction in the rate of synthesis of the β-globin chain leading to an excess accumulation of unbound α-globin chains. These unbound globin chains are released into erythroid precursors in the bone marrow causing their premature death and therefore unsuccessful erythropoiesis. β-thalassemia is classified into three subtypes. β-thalassemia major patients exhibit symptoms of severe anemia in the first year of life and require regular blood transfusions in order to survive. β-thalassemia intermedia patients experience milder anemia. Symptoms are usually apparent later in life (between ages 2 and 6 years). Individuals do not require transfusions to survive however growth and development is impaired. β-thalassemia minor patients are usually asymptomatic. In β-thalassemia major, normal Hb (HbA) is absent and fetal Hb (HbF) comprises the majority of Hb in the blood (92–95% total Hb). Less severe cases of β-thalassemia show HbF levels of 70–90% [8, 9] and some β-globin is present.

Neonatal screening for Hb variants allows for early detection, appropriate genetic counseling, and treatment to reduce deaths from sickle-related complications [10, 11]. In addition to HbS, the UK NHS newborn screening program requires that methods used in the screening process must also be able to reliably detect the structural variants; HbC, HbD Punjab/Los Angeles, HbE, and HbO-Arab as well as β-thalassemia [12]. Inheritance of the structural variants (without HbS) in either heterozygous or homozygous state is usually clinically benign.

The current screening protocol uses blood samples collected by midwives via heel prick onto standard NHS dried blood spot (DBS) cards from neonates between 5 and 8 days old. Small discs (approximately 3 mm in diameter) are punched out of the DBS and the sample is eluted for ~30 min [13]. The samples are either analyzed by cation exchange HPLC (ceHPLC) and/or IEF [14]. Both methods are presumptive and diagnosis of an Hb variant can only be achieved by second line testing using DNA analysis (sequencing) or amino acid analysis (MS) [15].

We have developed a method in which automated direct surface sampling of the DBS is coupled to top-down MS of the intact globin chains [16, 17]. The approach circumnavigates the need for sample preparation and confirms diagnosis in less than 3 min. In contrast to other MS-based approaches, there is no requirement for resolubilization of the DBS, use of tryspin digests [18–20], or HPLC-coupled runs [21]. In our approach, the DBS is mounted on a platform and a small volume (a few μL) of solvent is spotted onto the sample. After ~5 s (to allow dissolution of the sample), the solvent containing the sample is reaspirated and injected into the mass spectrometer via ESI. To diagnose the Hb variant, a precursor ion, corresponding to the intact globin chain, is selected for fragmentation (MS/MS) within the mass spectrometer. Fragmentation may be induced by collision induced dissociation (CID) or electron transfer dissociation (ETD). The result is a series of sequence fragments from which the presence and location of an amino acid substitution may be identified unambiguously. We have shown previously that the approach may be applied to the diagnosis of FS, FAS, FC, FAC, FAD in newborn samples [16]. In order to consider a suitable technique for use within the national screening program, the approach must also be capable of identifying structural variant HbE, compound heterozygotes, and β-thalassemia. Here, we show that our approach can meet those requirements.

2 Materials and methods

The research was approved by the University of Birmingham Science, Technology, Engineering and Mathematics Ethical Review Committee (ERN_11_0722B). Anonymized residual DBS from normal neonates (FA), heterozygous HbE (FAE), heterozygous (FAS) sickle trait, sickle cell HbC (FSC), sickle cell HbD (FSD) neonates, and β-thalassemia were supplied by Birmingham Children's Hospital in accordance with the Code of Practice for the Retention and Storage of Residual Spots [22]. Samples were collected by midwives 5–8 days after birth by heel prick onto standard NHS blood spot cards (ID Biological Systems, Greenville, South Carolina, USA). Following collection, samples were left to dry at room temperature, and stored in accordance with NHS newborn screening laboratories standard storage conditions. Prior to the current work, the samples were screened according to the standard NHS protocol by ceHPLC and IEF, and the Hb variant status was known.

2.1 Surface sampling

The DBS was placed onto the Advion Liquid Extraction Surface Analysis (LESA) universal plate adapter (Advion, Ithaca, NY). An image of the DBS was acquired using an Epson Perfection V300 photo scanner. The precise location of the DBS to be sampled was selected by use of the LESA Points software. The universal plate adapter was loaded into the Triversa Nanomate chip-based nano electrospray device (Advion, Ithaca, NY), coupled to Thermo Fisher Orbitrap Velos electron transfer dissociation mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). Surface sampling was as described in [16, 17]. Briefly, 7 μL of the electrospray/extraction solution (methanol (J.T. Baker, Denveter, The Netherlands) and water (J.T Baker; 48.5:48.5), with 3% formic acid (Sigma-Aldrich Company, Dorset, UK) was aspirated from the solvent well. Six microliters of the solution was dispensed onto the DBS forming a sample surface-to-tip liquid microjunction. The liquid microjunction was maintained for 5 s, before 5 μL was reaspirated and electrosprayed into the mass spectrometer.

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2.2 MS

The sample was introduced at flow rate of ~80 nL/min, with a gas pressure of 0.5 psi, tip voltage of 1.75 kV, and capillary temperature of 250°C. MS data were collected in full-scan mode (m/z 600–2000) and SIM mode (m/z 1055–1090) at a resolution of 100 000 at m/z 400 in the orbitrap. Each scan comprises 30 coadded microscans. Mass spectra shown comprise approximately five scans. Automatic gain control target was 1×10^6 with a maximum fill time of 2 s. CID was performed in the ion trap (isolation width of 10 Th) and the fragment ions detected in the orbitrap with a resolution of 100 000 at m/z 400. CID experiments were performed with helium gas at a normalized collision energy of 30%. Each CID scan comprises 30 coadded microscans. MS/MS spectra shown (m/z 290–2000) comprise five coadded scans. Data were analyzed using Xcalibur 2.10 software (Thermo Fisher Scientific) where the Xtract program was used to calculate monoisotopic masses (44% fit factor, 25% remainder). All spectra were manually analyzed.

3 Results

3.1 HbE

The HbE variant occurs at position 26 on the β-globin chain where a glutamic acid is substituted for a lysine, resulting in a mass shift of Δm –0.9476 Da [23]. Figure 1a shows a comparison of SIM-mode mass spectra from a normal (FA) and FAE neonatal sample. The spectra show peaks corresponding to the isotopic distributions of multiply charged $[M+14H]^{14+}$ ions of the α-chain (MW_{calc} 15117.8924), $[M+15H]^{15+}$ ions of the β-chain (MW_{calc} 15858.2570), and $[M+15H]^{15+}$ ions of the γ-chains (from HbF; γ_A MW_{calc} 15986.2626 and γ_G MW_{calc} 16000.2782). The resolving power of the orbitrap mass spectrometer used in the work is capable of differentiating between isotopic species containing ^{13}C and therefore it is possible to determine the monoisotopic mass experimentally by use of deconvolution software such as Xtract. (Mass spectrometers with low resolving powers are only capable of determining average masses.) Nevertheless, it is not possible to unambiguously determine the presence of the HbE variant because the small mass shift (Δm −0.9476 Da) of the variant means that isotope peaks from the intact variant globin overlap with those from the intact β-globin. Using a broad isolation window (10 Th), it is possible to select both precursor ion types and subject them to simultaneous fragmentation. This step can be achieved without a priori knowledge of the presence of the variant and so could be easily incorporated into a screening protocol. The resulting fragmentation allowed unambiguous diagnosis of the phenotype. A peak corresponding to normal b_{24}^{3+} fragment ions was observed (m/z_{meas} 852.1195) verifying that the Hb variant is not present before position 25 on the globin chain. Figure 1b shows the regions of the MS/MS spectra containing the b_{32}^{4+} fragment

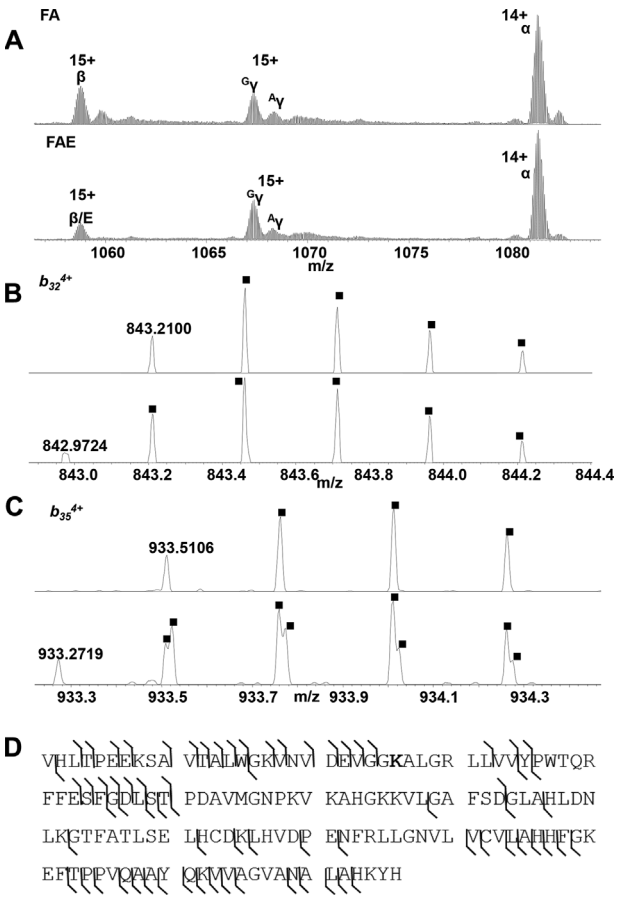

Figure 1. (A) SIM mass spectra of FA (top) and FAE (bottom). Expanded m/z region showing peaks corresponding to the isotopic distributions of (B) b_{32}^{4+} fragments observed following CID of β-chain ions (m/z_{calc} 843.2096; top) and variant HbE (m/z_{calc} 842.9727) (bottom), and (C) b_{35}^{4+} fragments following CID of β-chain ions (m/z_{calc} 933.5096; top) and HbE variant ions (m/z_{calc} 933.2727; bottom). (D) CID sequence coverage of heterozygous FAE.

ion from the FA and FAE samples. The peak at m/z 842.9724 in the FAE MS/MS spectrum (and absent in the FA spectrum) has a mass shift of −0.9476 Da when compared with the fragment from the FA sample, i.e. substitution of glutamic acid for lysine. The only glutamic acid residue between positions 24 and 32 on the β-globin chain is located at position 26 therefore the HbE variant can be confirmed. The presence of b_{34}^{4+}, b_{35}^{3+}, and b_{35}^{4+} fragment ions with mass shifts of −0.9476 Da, and of y_{111}^{10+}, y_{111}^{11+}, y_{111}^{12+}, y_{112}^{10+}, and y_{112}^{12+} fragment ions at the expected masses all confirm the presence of the HbE variant. The region of the mass spectrum containing the b_{35}^{4+} fragment ions is shown in Fig. 1c. Full details of the observed fragments are given in Supporting Information Table 1. It is also possible to determine from this fragment that the variant is present in the heterozygous state. Two sets of overlapping isotope peaks can be clearly distinguished (although not baseline resolved). In England,

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FAE carrier rate has been reported as 1 in 639 neonates [24]. CID resulted in a total sequence coverage of 43%, i.e. fragments resulting from cleavage of 63/145 individual peptide bonds were observed. The sequence coverage, i.e. the experimentally observed fragment ions, is shown in Fig. 1d, but it should be noted that, as the sample is heterozygous and both normal and variant globin chains were selected for fragmentation, the sequence coverage has contributions from both. Thirteen of the fragments are unique to HbE, whereas the remainder are common to both.

3.2 Compound heterozygotes

3.2.1 HbSC

HbSC is a compound heterozygote composed of two normal α-chains and two variant β-chains. One abnormal chain is caused by a substitution of a Glu→Lys at position 6 (HbC) on the β-chain, resulting in a mass shift of –0.9476 Da, and the other abnormal chain is caused by the HbS variant, a substitution also at position 6 on the β-chain, Glu→Val (Δm + 29.9745 Da). Figure 2a shows SIM-mode mass spectra of heterozygous sickle (FAS) and compound heterozygous FSC samples. As we have described previously, it is possible to determine the presence of the sickle variant and its nature (homo- or heterozygous) in this mode [16]. Nevertheless, there exists the possibility of misdiagnosis of compound heterozygotes—the mass spectra in Fig. 2a top (FAS) and bottom (FSC) appear identical. That is significant because whereas FAS is generally benign, the clinical severity of FSC is similar to that of homozygous sickle. FSC has a prevalence rate of 1 in 7174 in England [24]. For both the FAS and FSC sample, ions centered at m/z 1056 were isolated and subjected to CID. The isolation width was 10 Th meaning that both HbS and β-chain ions (in the case of FAS) or both HbS and HbC ions (in the case of FSC) were selected for fragmentation. The protein sequence coverage (i.e. the experimentally observed fragment ions) obtained for the HbC variant chain is 24% and is shown in Fig. 2b. Full details of the fragment ion assignments are given in Supporting Information Table 2. Figure 2c shows the expanded m/z region containing the $b_{47}{}^{4+}$ fragments of the FAS and FSC variants. The peaks at m/z 1300.4444 and 1307.6914 in Fig. 2c, bottom, confirm the presence of both HbS and HbC, i.e. the sample is a compound heterozygote FSC. Figure 2d and e shows further examples that confirm the presence of the HbC variant.

3.2.2 HbSD

HbSD is another compound heterozygote where two variant chains are inherited: one HbS chain (substitution also at position 6 of the β-chain, Glu→Val (+29.974)) and one HbD chain (Glu→Gln substitution at position 121 of the β-chain, Δm −0.9840 Da). The SIM-mode mass spectra of FAS and FSD samples are shown in Fig. 3a look identical. This is because the normal β-globin chain differs in mass from the variant D Punjab/Los Angeles chain by only 0.9840 Da. The presence of the sickle variant in both samples is clear in SIM mode. CID fragmentation is required to differentiate between clinically benign FAS and the severe medical disorder FSD. The protein sequence coverage (i.e. the experimentally observed fragment ions) obtained for the HbD variant chain is 15% and is shown in Fig. 3b. Full details of the fragment ion assignments are given in Supporting Information Table 3. FSD is unambiguously diagnosed as the fragment ion $y_{23}{}^{4+}$ (m/z_{meas} 608.8415) is normal but the next fragment observed, $y_{32}{}^{4+}$, is present at m/z_{meas} 872.4143 corresponding to a variant with a mass shift of −0.9840 Da (Glu→Gln), Fig. 3c.

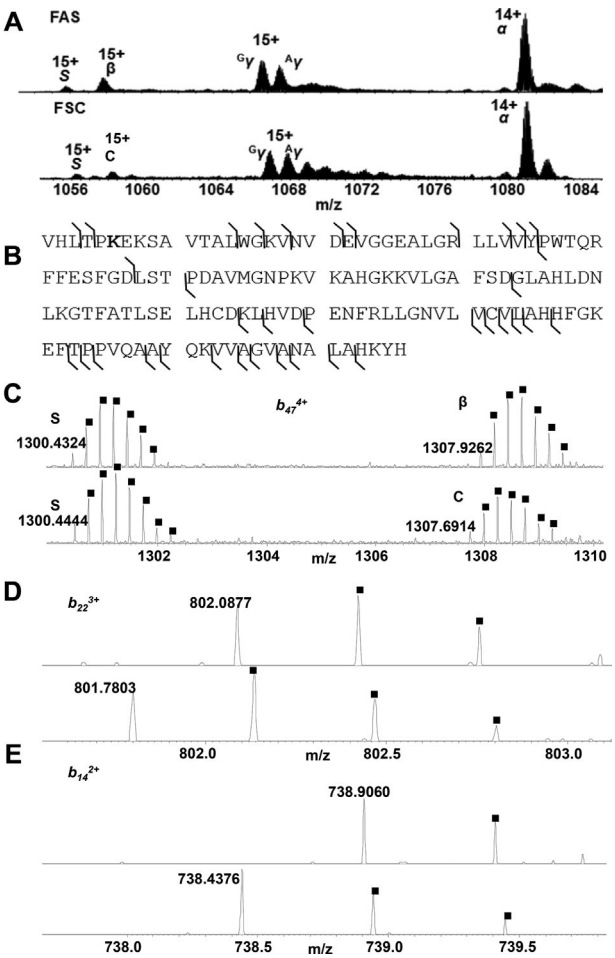

Figure 2. (A) SIM mass spectra of heterozygous FAS (top) and compound heterozygous FSC (bottom). (B) CID sequence coverage of heterozygous FSC (variant HbC ions only). (C) Expanded m/z region showing the $b_{47}{}^{4+}$ fragments observed following CID of the sickle ions (m/z_{calc} 1300.4330), β-chain ions (m/z_{calc} 1307.9266), and HbC variant ions (m/z_{calc} 1307.6897). (D) Expanded m/z region showing $b_{22}{}^{3+}$ fragments for the β-chain ions (m/z_{calc} 802.0884; top) and HbC variant ions (m/z_{calc} 801.7726; bottom). (E) Expanded m/z region showing $b_{14}{}^{2+}$ fragments for the β-chain ions (m/z_{calc} 738.9065; top) and HbC variant ions (m/z_{calc} 738.4327; bottom).

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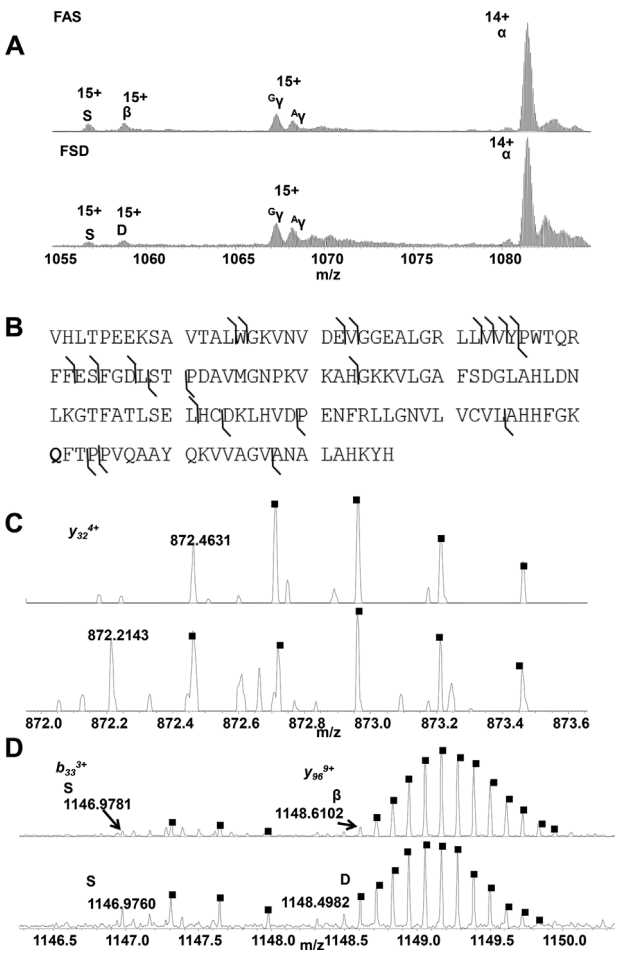

Figure 3. (A) SIM mass spectra of normal neonate Hb heterozygous and homozygous FAS (top) and FSD (bottom). (B) CID sequence coverage of heterozygous FSD (variant HbD ions only). (C) Expanded *m/z* region showing the y_{32}^{4+} fragments observed following CID of the β-chain ions (m/z_{calc} 1307.9266; top) in the FAS sample and HbD variant ions (m/z_{calc} 872.2140; bottom) in the FSD sample. (D) Expanded *m/z* region showing both the b_{33}^{3+} fragments observed following CID of the HbS variant ions (m/z_{calc} 1146.9751) and the y_{96}^{9+} fragment ions of the β-chain ions (m/z_{calc} 1148.6083) and HbD variant ions (m/z_{calc} 1148.4982).

The only glutamic acid residue present between y_{23} and y_{32} is at position 121. This variant is therefore confirmed as HbD Punjab/Los Angeles (β121 Glu→Gln Δm −0.9840 Da), and the individual is diagnosed as compound heterozygote HbSD.

3.2.3 β-Thalassemia

Five different β-thalassemia DBS samples were investigated in this study. The variant DBS underwent standard hospital screening (ceHPLC) and the samples were diagnosed as being β-thalassemia major, i.e. no β-chain is present. All the samples were then analyzed by use of the LESA protocol. In full MS mode, no peaks corresponding to β-globin chains were observed in any of the five β-thalassemia samples. Figure 4 shows a representative SIM-mode mass spectrum (*m/z* range 1055–1090) for a β-thalassemia variant. The spectrum was deconvoluted by use of the Xtract software to determine the monoisotopic masses of the measured ions. The measured MW of the α-chain from the spectrum shown in Fig. 4 was 15 117.8346 Da (Δm −3.8 ppm), γ_G-chain 15 986.1963 Da (Δm −4.2 ppm), and for the γ_A is 16 000.2017 (Δm −4.8 ppm). The β-globin chain was absent. Supporting Information Fig. 1 shows SIM-mode mass spectrum (*m/z* range 1055–1090) of all five β-thalassemia samples. All samples were analyzed from full term neonates so the absence of β -globin cannot be explained by neonatal prematurity (production of β-chain not yet switched on). The absence of the β-globin chain in the samples must therefore be explained by the presence of β-thalassemia major. Clinically symptomatic β-thalassemia has an incidence of about 1 in 10 000 within the European Union [8].

4 Discussion

The UK NHS newborn screening program requires that the following Hb variants must be detected: HbSS, HbS/β-thalassemia, HbSC, HbSD, HbSE, HbSO-Arab, and HbS/HPFH (hereditary persistence of HbF). Direct surface sampling of DBS coupled to high resolution top-down MS allows for the rapid and unequivocal diagnosis of Hb variants. We have shown previously that our approach is suitable for screening for variants HbS (homo- and heterozygous), HbC (homo- and heterozygous), and HbD (heterozygous). Here, we show that the approach is also suitable for the structural variant HbE. This result is perhaps not surprising as the HbE variant is the result of a Glu→Lys substitution, as is the HbC variant. More importantly, we have shown that the approach is suitable for the diagnosis of compound heterozygotes (HbSC and HbSD) and that this diagnosis requires a single MS/MS analysis. That is, both the HbS and other variant are selected and fragmented in a single event. Given the identical mass shift of HbC and HbE, and the fact that (normal) heterozygous HbE can be determined, it is reasonable to predict that the compound heterozygous HbSE can be diagnosed by this approach. The HbO-Arab variant is rare [7] and no samples were available for analysis during this study. (Variants FSE, FSO-Arab, and other sickle compound heterozygotes including FSD have a combined prevalence of rate of 1 in 24 871 [24]). HbO-Arab occurs at position 121 on the β-chain, i.e. at the same position as the HbD Punjab/Los Angeles variant, and is the result of a substitution of glutamic acid for lysine, i.e. an identical mass shift to variants HbC and HbE. We have clearly shown that HbC, HbD, and HbE can all be diagnosed by our direct surface sampling top-down MS approach, and therefore fully anticipate being able to diagnose the Hb O-Arab variant as well.

We have also shown that the method is capable of confirming β-thalassemia major, a clinically significant

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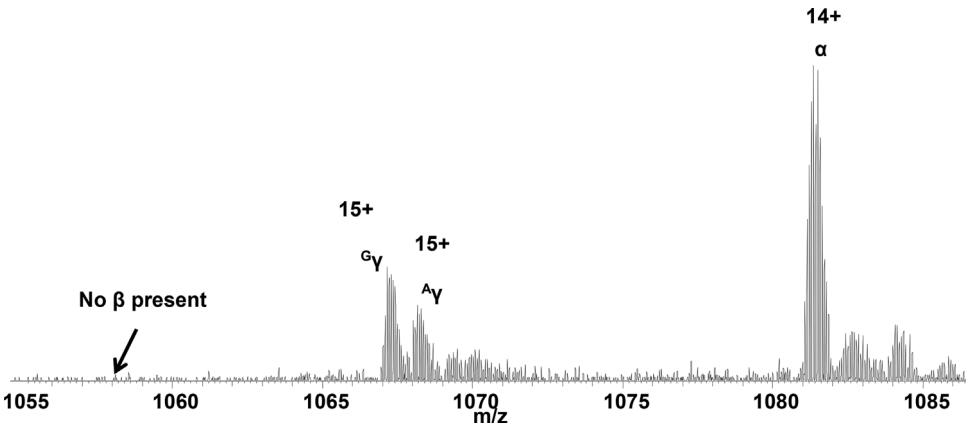

Figure 4. SIM-mode mass spectrum of a neonatal β-thalassemia sample.

hemoglobinopathy, which should be detected in newborn screening. Nevertheless, caution is advised: the absence of a mass spectral feature cannot automatically be interpreted as evidence of absence of the analyte. For β-thalassemia major screening, the method would be suitable either as first line testing with subsequent diagnosis via DNA analysis, or as a second line test. It is also worth noting, that in neonatal samples, the same results would be expected for HbSS, HbS/β-thalassemia, and HbS/HPFH, i.e. peaks corresponding to HbS, α-chains and γ-chains and an absence of peaks corresponding to β-chain.

Results from this study show the method can be realistically considered as an alternative to the current protocol. The use of MS as a first line screening method would speed up definitive diagnosis of clinically significant Hb variants, which can currently take several weeks. The direct surface sampling aspect of this method is advantageous in the clinical setting as it offers minimal sample handling and does not require any sample preparation. The process has the potential to become fully automated with the development of a device that could automatically load and remove hundreds of DBS cards into and out of the Triversa Nanomate ESI source for the surface sampling. The method could also be easily transferred for use in antenatal hemoglobinopathy screening.

R.L.E. was in receipt of an EPSRC-funded studentship. The Advion Triversa Nanomate and Thermo Fisher Orbitrap mass spectrometer used in this research were funded through the Birmingham Science City Translational Medicine: Experimental Medicine Network of Excellence Project, with support from Advantage West Midlands (AWM).

The authors have declared no conflict of interest.

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