Analysis of hemoglobin variants by top-down mass spectrometry

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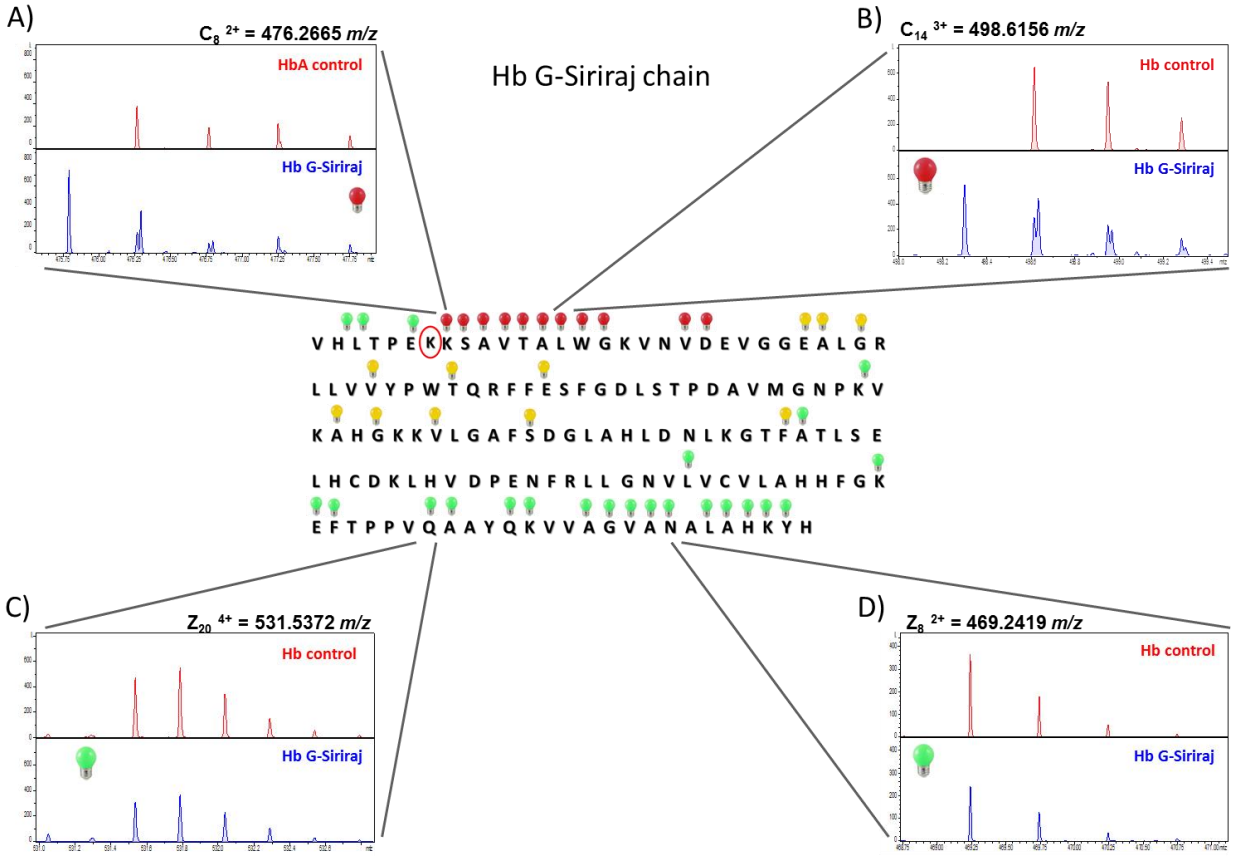
Hemoglobin (Hb) is a tetrameric blood protein that transports oxygen to organs and tissues. The main Hb form in adults (HbA) is composed of two α and two β chains. A healthy person also carries two minor forms: HbA_2 and HbF composed of two α and two δ or γ chains, respectively. Hemoglobin disorders named hemoglobinopathies are divided in two categories. First, qualitative disorders where a Hb variant is present, typically due to a single point mutation or a gene deletion. To date, 1206 Hb variants have been described. Second, quantitative disorders, named Thalassemias, where the decreased synthesis of one or several globin chains result in an unbalance between Hb chains. The biological diagnosis of Hb disorders relies on the combination of hematological tests, protein analysis techniques and molecular biology assays. The protein analysis method provides putative identifications of Hb variants, which must be confirmed by gene sequencing. The process is relatively fast and efficient for the identification of the most common Hb variants, such as HbS, HbC or HbE. The identification of less common mutations requires laboratory expertise. Protein screening techniques allow in the majority of cases to know if the patient carries a mutation on the α or the β chain to select correct primers for the polymerase chain reaction for gene sequencing. However, due to the lack of specificity of the protein analysis methods, identification of rare Hb variants can become a hard task to

accomplish, especially when the variants co-elute with non-mutated chains or in cases of fusion proteins.

In this work, we present a top-down mass spectrometry (MS) method using electron transfer dissociation (ETD) to identify and characterize mutated Hb chains. Reference product ions, named diagnostic ions, distributed all along Hb β chain were selected based on their abundance, resolution and reproducibility. To simplify and speed-up top-down ETD data interpretation, the diagnostic ion list was associated with a color code strategy allowing to quickly and specifically localize a mutation in the Hb β chain sequence. If a diagnostic ion was present as in the control sample, the color is green. If the diagnostic ion was not present in the sample or if there was a shift of the monoisotopic peak, the color was red. The red color thus signifies that a sequence variation is detected compare to the normal globin chain. If the signal is not well resolved or if there is an overlapping signal from a particular sample, the color is yellow (Figure 1).



<u>Figure 1:</u> Hb G-Siriraj sequence and diagnostic ion color code results. Hb G-Siriraj sequence is presented with the color code. The mutation at position 7 (Glu>Lys) is pointed out with a red

circle. The mass spectra of four diagnostic ions are highlighted. In the upper part, the diagnostic ion signal for Hb A β chain. In the lower part, the diagnostic ion signal for the analyzed sample. A) A double peak is present instead of the monoisotopic peak and the monoisotopic peak is clearly shifted compared to Hb A mass spectrum for diagnostic ion c_8^{2+}. Thus, a red label represents this diagnostic ion. B) A double peak is present instead of the monoisotopic peak and the monoisotopic peak is clearly shifted compared to HbA mass spectrum for the diagnostic ion c_{14}^{3+}. Thus, a red label represents this diagnostic ion. C) The diagnostic ion z_{20}^{4+} yielded signal as the control sample. It is represented with green label. D) The diagnostic ion z_8^{2+} yielded signal as the control sample. It is represented with a green label. The color code showed that almost all lights are red or yellow from position 7 to 78 on the β chain. The C-terminal part of the protein sequence corresponds to the normal β chain. Indeed, all lights are green, from position 78 to 146. These data indicate that the N-terminal part of the sequence is affected by a mutation between positions 4 and 7.

This process allowed successfully characterizing several uncommon Hb variants including variants with only 1 Da mass shift, such as Hb G-Siriraj, and also an $^{A}\gamma$-β fusion protein named Hb Kenya. The method brings more precise information at the proteoform level compared to protein analysis methods currently used for hemoglobin disorder diagnosis. The data analysis strategy allows to a person without MS knowledge to interpret top-down ETD MS data. This process complements our previously descried automated MS methods for the identification of common Hb variants[1] and quantification of Hb chains[2] (Figure 2). The platform will allow a more precise and efficient protein analysis in Hb diagnosis process. A validation study is ongoing for the identification of Hb variants and quantification of HbA_2 in clinical samples. Diagnostic ion lists for HbA α, δ and γ chains will be determined. Automation of diagnostic ions and color code strategy will also be done.

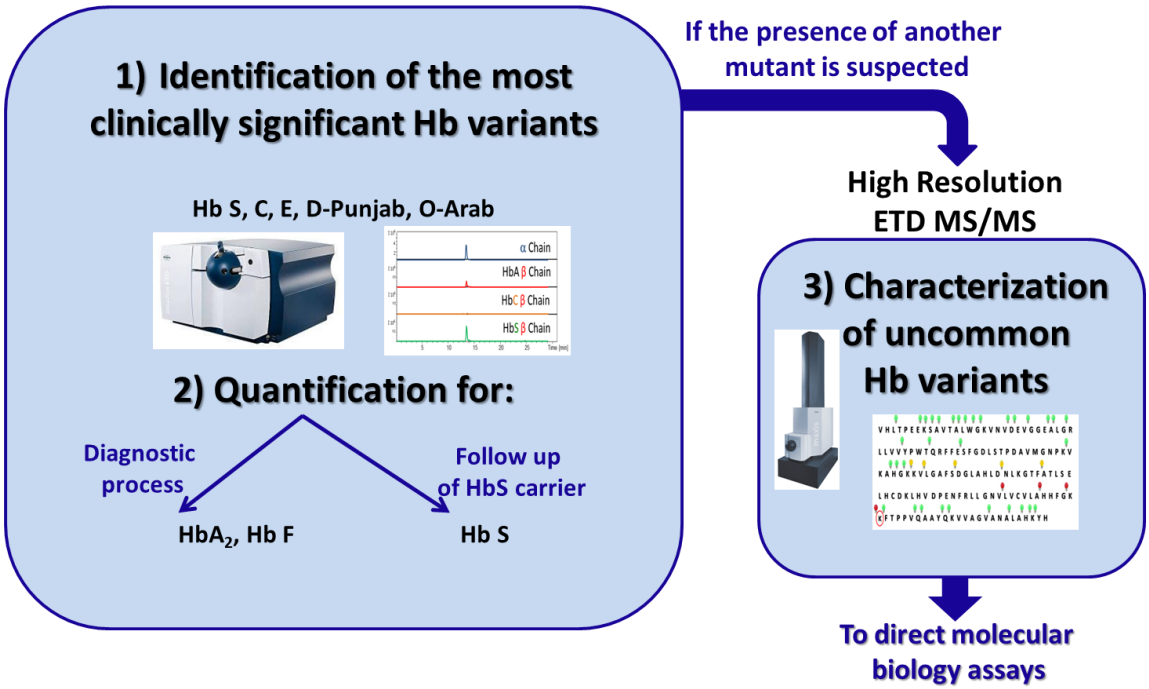


Figure 2: Proposed MS platform for protein analysis step in Hb diagnostic process. On the left box, identification and quantification method performed in an ion trap mass spectrometer. 1) Identification of the most clinically significant Hb variants by top-down SRM ETD. 2) Precise quantification of Hb chains for the diagnostic process and the follow up of HbS carriers. On the right box, top-down ETD method in a high resolution mass spectrometer. 3) Characterization of uncommon Hb variants by diagnostic product ion from top-down ETD strategy allowing to precisely direct molecular biology confirmation.

1. Coelho Graça, D. et al. (2012). "Electron Transfer Dissociation Mass Spectrometry of Hemoglobin on Clinical Samples." Journal of The American Society for Mass Spectrometry **23**(10): 1750-1756.
2. Acosta-Martin, A. E. et al. (2013). "Quantitative Mass Spectrometry Analysis of Intact Hemoglobin A2 by Precursor Ion Isolation and Detection." Analytical Chemistry **85**(16): 7971-7975.