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High-Throughput Identification of Hemoglobinopathies & Thalassemias by SEC-HRAM-MS

Thomas Wiesinger[1], Thomas Mechtler[1], Markus Schwarz[1], Xiaolei Xie[2], David C. Kasper[1]

[1]ARCHIMED Life Science GmbH, Leberstraße 20, 1110 Vienna, [2]Thermo Fisher Scientific, 355 River Oaks Parkway, San Jose

[1] Introduction:

The diagnosis of hemoglobin disorders become more and more importance due to rising carrier-frequencies in several areas around the world. The challenging task is to develop a high-throughput screening, which is reliable, fast, robust and with perfect precision towards hemoglobinopathies or thalassemia positive patients.

These rare diseases mainly contain a mutation in one or both Hb-beta chains or a misbalanced ratio between α/β chains. Besides the identification of Hb S (sickle cell anemia, SCD) in homozygoteous form, Hb variants (e.g. Hb -C, -D, & -E) with co-inherited Hb S in a heterozyoteous form are the clinical most relevant types. Due to a single mutation in the beta chain, these variants differ only by 1 Dalton for the entire protein.

To tackle the main limitations for the identification of clinical relevant Hb mutations a "**double check strategy**" using dried blood spots was investigated.

[2] Method Development:

The presented HRAM-MS method consists of a "**double check**" strategy (intact hemoglobin chains plus trypsin-digested peptide sequences) to identify Hb positive patients. Therefore, dried blood spots (DBS) were treated with two different buffer solutions **[A]** & **[B]**.

Ⓐ Extraction of intact Hb chains

One 3.2mm DBS was extracted with 200 µL buffer ($MeOH:H_2O:AcOH$ 10:88:2) for 30 min in order to elute the intact Hb chains for the identification of thalassemias (α/β ratio) and Hb F or Hb S. Optionally, rare Hb variants with significant mass-shifts can also be identified.

Ⓑ Trypsine digest

For the identification of Hb variants with a mass difference of <1 Da in the beta chain, data dependent MS^n scans of Hb variant specific tryptic peptides are investigated (One additional DBS + 0.5 mg/mL porcine trypsin in 40 mmol/L ammonium bicarbonate, 9% acetonitrile).

Both extracts (**[A]** & **[B]**) were pooled in a 1:1 ratio (50 µL) and loaded into the autosampler for direct injection onto an size exclusion (SEC) column (flow rate: 1.0 mL/min; 18% acetonitrile) for introduction into the Q Exactive Focus mass spectrometer.
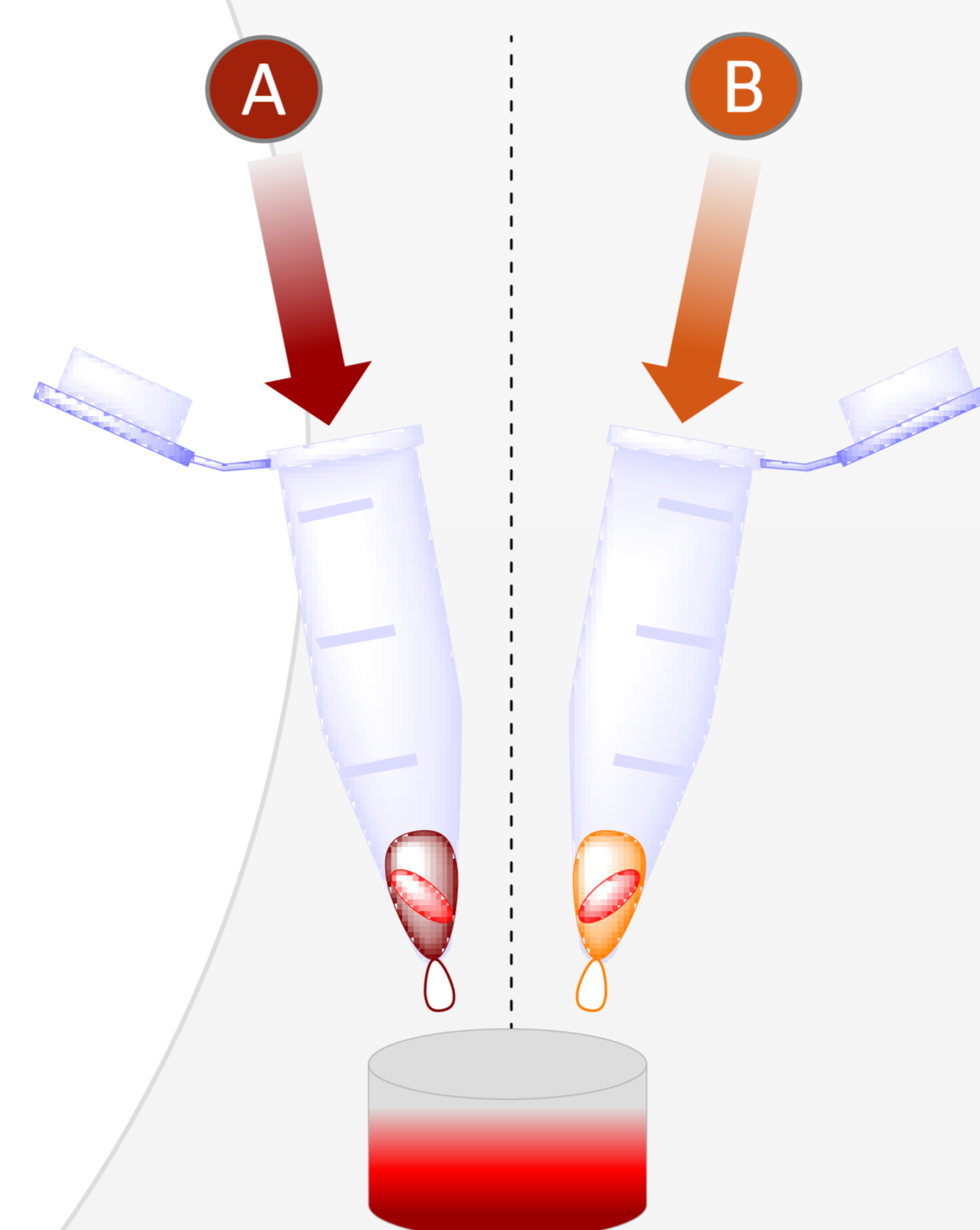
SEC-HRAM-MS

[3] Results & Discussion:

Within the presented "**double check strategy**", a robust Hb screening tool (>1000 injections of crude Hb extracts, without loss of instrument performance) was developed for the identification of control material as well as Hb negative samples.

Intact hemoglobin was analyzed by monitoring higher charged ion-species (z=15), which are separated from the related tryptic peptides by using size exclusion chromatography (**Figure 1**). These proteinase treated samples and the analysis of the resulting peptides were conducted by data dependent MS^n scans. Automated data processing was performed by using Tracefinder (Thermo Fisher Scientific).

Moreover, porcine Hb was implemented to the method, which is acting as control for the tryptic digest and as a internal standard to continuously monitor the analytical performance of the instrument.
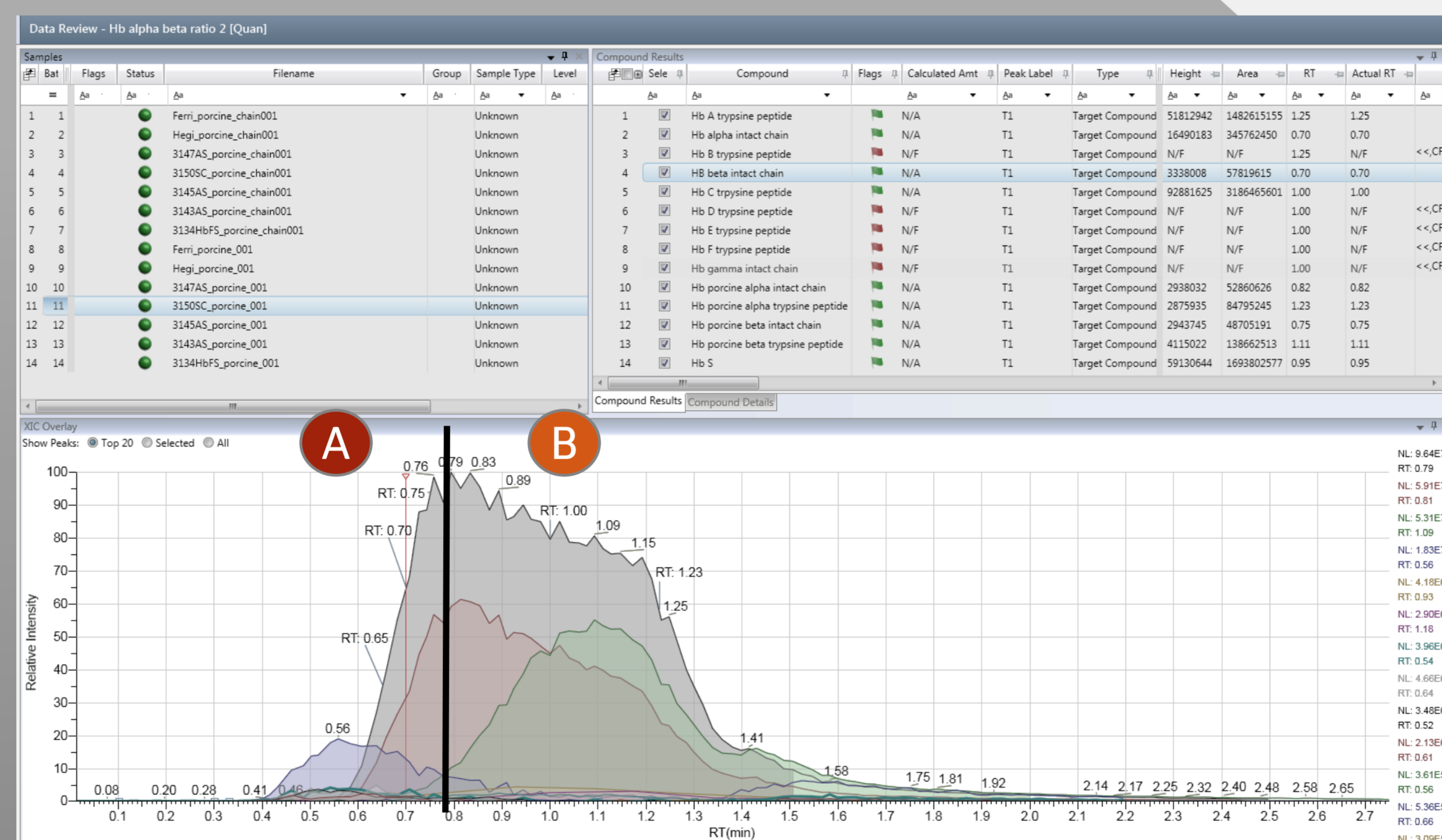

Figure 1 Illustration of an "artificial" hemoglobin positive sample (Hb S/C) applied to the established "confirmation strategy" (A) intact proteins are eluting between 0.4-0.8 min; (B) tryptic peptides (0.6-1.6 min).

As illustrated in **Figure 1**, the intact Hb beta chain is confirmed by the FS experiment, but with a closer look to the tryptic peptide sequence, a mutation in the beta chain, namely Hb C, was identified (Compound heterozygotic Hb S/C).

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[4] Conclusion:

A high throughput assay for the identification of selected Hb variants and thalassemias in dried blood spots by applying a fast dual-channel size exclusion liquid chromatography analysis, coupled with a high resolution mass spectrometer (SEC-LC-HRAM-MS) has been developed. Suitability and accuracy of the method was confirmed by "artificial" SCD patients in homo- or compound heterozygotic form.

References:
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