[Application Note]

Human Cerebrospinal Fluid HRM Profiling using N-Glycocapture Enrichment

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Introduction

Cerebrospinal fluid (CSF) is a clear colorless bodily fluid found in the brain and spine. CSF is produced in the choroid plexus of the brain. Its functions are amongst others the basic mechanical and immunological protection of the brain inside the skull. CSF can be tested for the diagnosis of a variety of neurological diseases like subarachnoid hemorrhage, central nervous system infections (such as meningitis), multiple sclerosis or Alzheimer's disease. CSF offers several advantages as a biological fluid for biomarker research. One of these advantages is that CSF is in direct continuity with the brain and therefore more directly reflects chemical changes occurring there.

The protein content of CSF is about 0.2 to 0.8 mg/ml. The protein concentration distribution is large, identified proteins cover a dynamic range of 8-10 orders of magnitude (serum albumin 130-350mg/L to 14-3-3 protein 4-6pg/l) (Hühmer et al.). Therefore, direct analysis of CSF proteins by mass spectrometric means is limited to the maximal injectable amount of peptides (up to 5ug) and the dynamic range of the mass spectrometer (up to 6 orders of magnitude). This results in robust detection of only a small fraction of the CSF proteome. Recent approaches to increase the coverage of proteins by mass spectrometric means include extensive fractionation of large amounts of CSF including depletion of the 14 most abundant proteins, strong cation exchange chromatography of peptides and reversed phase separation. This resulted in the identification of up to 2'630 proteins (Schutzer et al.). The application of such fractionations required large amounts of pooled CSF, long sample preparation protocol and days of mass spectrometric measurement.

To mitigate the limitation imposed by asymmetric protein concentration distribution and increase reproducibility of detection, a protocol to selectively enrich the N-glyco subproteome was developed (Ossola et al.). This protocol was optimized for the validation of biomarkers in plasma and enables measurement of proteins in the low ng/ml concentration range. We evaluated the performance of the N-Glycocapture protocol to maximize the depth analysis and reduce the required amount of CSF and mass spectrometric analysis time. Secreted proteins are glycosylated during their maturation and secretion. The N-Glycocapture protocol enables a selective enrichment of plasma proteins thereby increasing the dynamic range and localization of glycosylation sites. Using this approach, we can follow the changes in the CSF proteome that occur with aging.

Methods and materials

Materials Cerebrospinal fluid was purchased from Sera Laboratories, five samples of males ranging from 23 to 76 years of 1ml. Zeba™ Spin Desalting Column, 7k MWCO, 0.5ml and Sodium meta-periodate were purchased from Thermo Scientific. Affi-prep Hz Hydrazide resin was purchased from Bio-Rad.

Sample preparation N-glycocapture was performed as described by Ossola et al. The following alterations were performed. The CSF sample concentrations was determined using the BCA assay. 700 ug of CSF was prepared according to Biognosys Sample preparation kit. Then the sample was dried on a speed vac and resuspended in 6ul 1M sodium acetate and 6ul 5M sodium chloride plus 88ul H2O. 19ul of 50 mM sodium meta-periodate was added to the sample. The sample was incubated at RT in darkness for 60min. Subsequently, the samples were processed as described by Ossola et al. The peptide concentration of the final sample was determined using microBCA. The Biognosys' HRM Calibration Kit was added according to the manufacturers instructions.

Liquid chromatography and mass spectrometry. LC solvents were A: 1% acetonitrile in water with 0.1% formic acid; B: 3% water in acetonitrile with 0.1% formic acid. The LC gradient was 5-35% solvent B in 120 minutes followed by 35-100% B in 2 minutes and 100% B for 8 minutes (total gradient length was 130 minutes). For DDA/shot gun analyses on the Thermo Scientific Q Exactive™ a Top10 method was used. The DIA/HRM method used is described in Bruderer et al.

Data analysis. The LC-MS/MS (shotgun) mass spectrometric data was analyzed using the MaxQuant Software (maxquant.org). As an additional variable modification to the default, "Asn to Asp" was added. A canonical human Uniprot FASTA sequences database was used for the search. The spectral library was generated using the Spectral library generation functionality of the Spectronaut™ software. DIA mass spectrometric data was analyzed using Spectronaut™ software from Biognosys. Statistical testing was performed using the post analysis processing functionality of Spectronaut™. The candidates were selected using a cut of of 1.5 fold and a q-value filtering of 0.01. The gene ontology analyses were performed using the DAVID Bioinformatics Resources 6.7 and PANTHER Classification System 9.0.

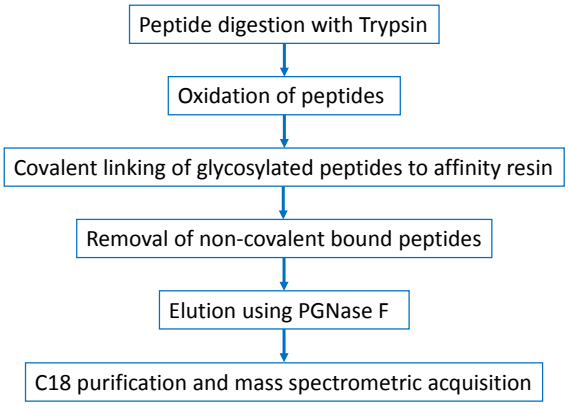

Figure 1: N-Glycocapture of CSF. The N-Glycocapture protocol was developed for the sensitive and reproducible detection of the N-glyco subproteome of plasma (4). Thee protocol was adopted to satisfy the characteristics of CSF. As starting material 1ml of individual healthy male donors was used. The resulting peptide amount was about 7ug corresponding to about a 100 fold enrichment.

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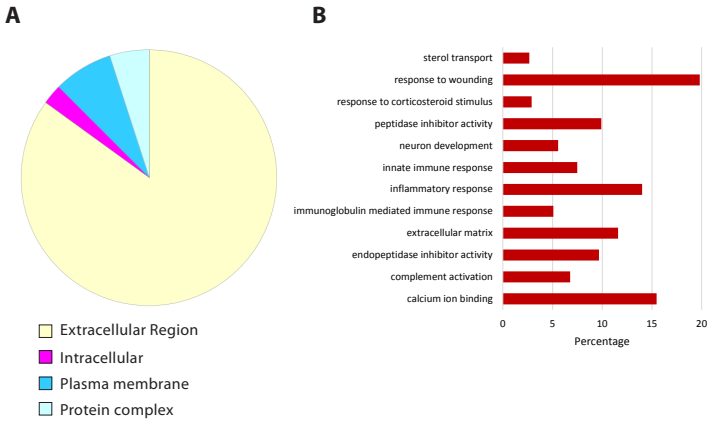
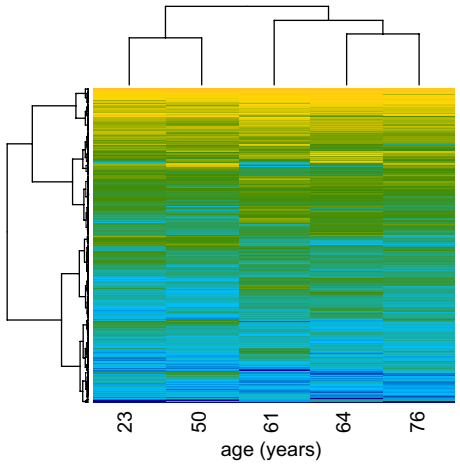


Figure 2: Spectral library generation The CSF samples prepared by N-glycocapture were subjected to shotgun proteomic analysis for protein inventory generation. The average enrichment for N-glycosylated peptides was 66%. A combined spectral library of the CSF was generated using the spectral library generation functionality of Spectronaut™. This resulted in a spectral library of 511 proteins (including direct digest 855). A comparison with the literature absolute concentration values of detected proteins shows that protein were detected down to the low ng/ml range. GO annotation demonstrated, that the majority of proteins identified stem from the extracellular region and the membrane fraction (**A**). This is in agreement with the published results of the N-glycocapture in plasma (Ossola et al.) **B** Enriched biological pathways (GO) were selected and visualized with important biological functions in CSF.

Figure 3: Age dependent differences of the CSF proteome dominate the biological variance between individuals To profile the CSF samples of the individual male donors of different ages, we performed block randomized acquisition of the five samples in HRM-DIA mode. The samples were acquired in technical triplicates. The data was analyzed, the measurements were normalized and an unsupervised clustering was performed. Interestingly, the samples clustered according to the age of the donors. This shows that the biological variation between the individuals is lower than the age dependent changes in the samples analyzed.



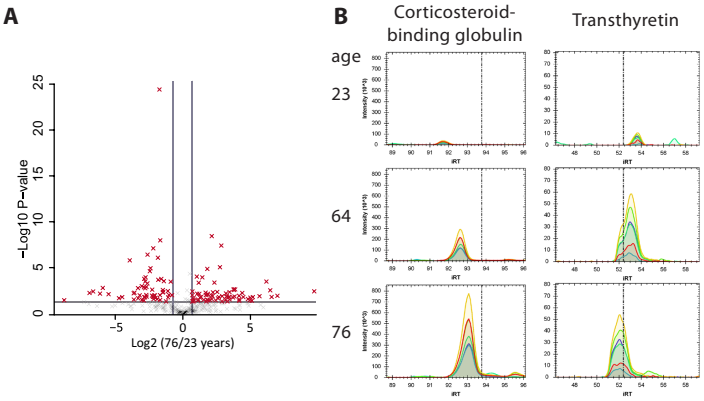


Figure 4: Statistical analysis fo significant differences between young and old A statistical comparison of the CSF samples was performed using a t-test analysis. All samples were compared against the samples of the youngest donor (23 years old). **A** A volcano plot visualization of the comparison of the samples from the 76 and the 23 year old donors. Multiple proteins are significantly differentially expressed. The lines in the plot show 50% regulation and a p-value of 0.01 **B** Extracted ion current of two candidates are visualized with increased expression with age (left column Corticosteroid binding globuli (CBG), GSPAINVAVHVFR with charge state 3 and right column Transthyretin, AQLLQGLGFNLTER with charge state 2).

Conclusions

The patented and published N-glycocapture protocol was successfully adapted to CSF. A combined spectral library was generated that covered 855 proteins. The HRM-DIA analysis showed that the biological variation amoung individuals was smaller than the variation upon aging in this study. HRM-DIA comparative study resulted in the discovery of protein of differential expression between old and young donors. 8 candidates increased consistently with age. The two presented candidates are of importance in the protection of the brain from neurodegenerative diseases upon aging. The protein transthyretin was tested as a biomarker for Alzheimer's disease (Merched et al.). The level of free cortisol in higher age is correlated with lower brain performance, therefore binding of free cholesterol by elevated CBG levels might serve as a protective mechanism (Comijs et al.). The spectral library protein list and the candidate list can be obtained from Biognosys.

The conclusion of this study is, that N-Glycocapture in conjunction with HRM-DIA is well suited to study CSF and the discovery of potential biomarkers for neuropathology.

References

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