Workflow development for the characterization of neurofilament light chain (NfL) by LC-MS/MS for clinical applications

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✓ Introduction

- Neurofilament light chain is a 68kDa protein biomarker used in clinics to assess axonal degradation, as it is known to be increased in patients with neurological disease, in cerebrospinal fluid (CSF) and in blood(1, 2, 3).
- Most of today's rapid detection methods are based on immunoassays, which could lead to cross-reactivity(1, 4).
- Understanding which epitope is detected by commercial antibodies and how this impacts detection efficiency could lead to a better understanding of this protein metabolism and its involvement in associated diseases, owning that degradation mechanism in situ is poorly understood. The use of different epitopes would enable us to better characterize the fragments found in biological fluids(1, 3).

✓ Objectives of the study

- **Develop a characterization method for NfL in a model using digested standards and CSF, with or without immunocapture, using mass spectrometry.**
- We first set out to optimize a sample preparation method that would deliver the best possible analytical performance.
- Secondly, the complexity of the model matrix was increased to approximate a biological CSF sample.

✓ Material

Reagent list : Anti-68kDa NF-L antibodies purchased from Abcam°, Recombinant Human 68kDa NF-L protein from Abcam°, Trypsin Protease MS Grade from ThermoFisher Scientific°, Bovine Serum Albumin from Sigma Aldrich°, MS-grade water and acetonitrile, Formic acid from Fisher Chemical°.

✓ Methods

Operating mode :

1) We assessed the best concentration ratio between NfL and trypsin for simulation models using standard without immunocapture. A total of 18 samples were analyzed. 2 solvents were selected for the dilution of the NfL standard (STD): a 90:10 v/v H_2O/ACN mixture and a 90:10 v/v H_2O/ACN mixture spiked with 10mg/mL BSA. Final STD concentrations were 10µg/mL, 1µg/mL and 0.1µg/mL respectively. Trypsin aliquots were first diluted in NH_4HCO_3 and then mixed with diluted NfL STDs to obtain 3 ratios of 10:1, 1:1 and 1:10 for each medium and concentration.

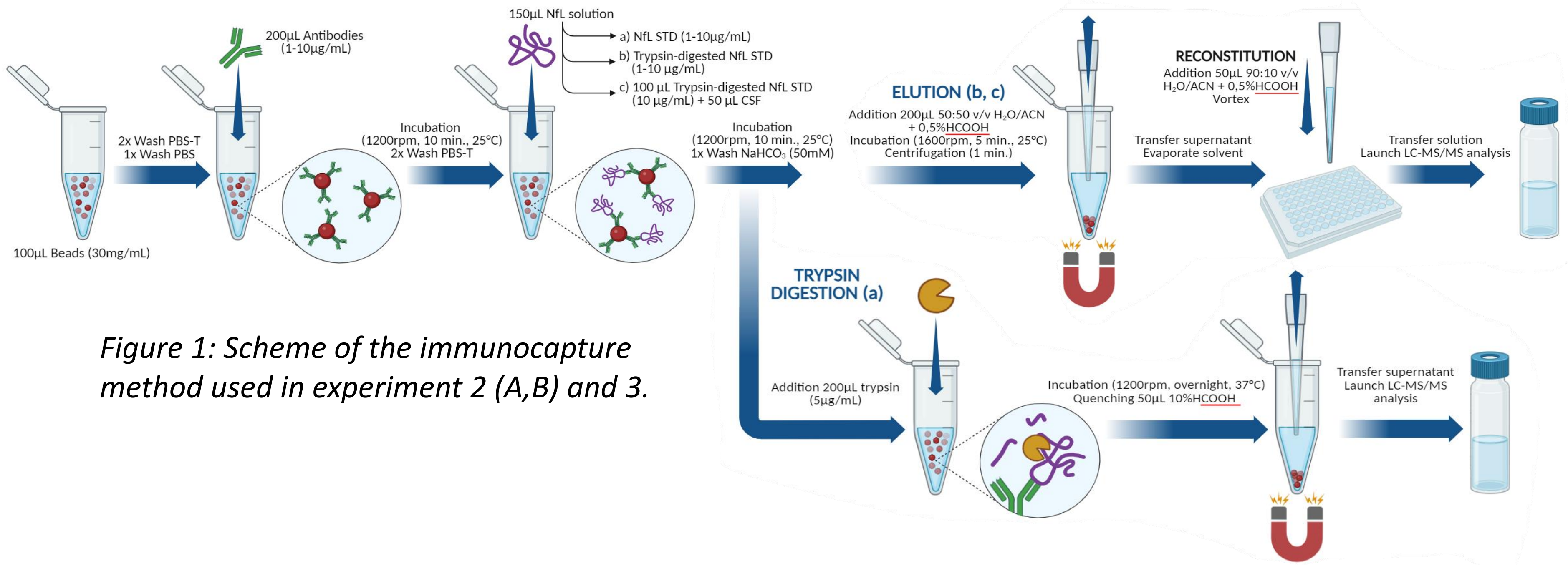

Figure 1: Scheme of the immunocapture method used in experiment 2 (A,B) and 3.

2) We then carried out immunoprecipitations using Dynabeads Protein G kit, adapting their data sheet, with on-beads digestion, followed by digestion prior to capture(4). A) Digestion was performed on 4 duplicates containing 100µL of beads (30mg/mL), 200µL of anti-NfL antibody (1-10µg/mL), 150µL of NfL (1-10µg/mL). 200µL of trypsin (5µg/mL) was added to all vials. B) Capture on digested STD samples was performed in parallel using 100µL of beads (30mg/mL), 200µL of anti-NfL antibody (1-10µg/mL) and 150µL of A-B-D-E NfL-trypsin ratios from experiment 1 *(Table 2).*
3) Finally, we performed capture on digested samples of NfL STD mixed with patient CSF. A single capture condition containing 100µL beads (30mg/mL) coated with 200µL of anti-NfL antibody (1µg/mL) and 100µL of condition E digested NfL mixed with 50µL of pure CSF was carried out in triplicates.

Analytical settings :

- Column : Waters nanoEase M/Z Peptide BEH C18 Column, 130Å, 1.7 µm, 300 µm X 150 mm, temperature set at 40°C
- Gradient mode:
  - Flow: 10µl/min
  - Injection volume: 5µl
  - Mobile phases: A: H_2O, 0.1% HCOOH ; B: ACN, 0.1% HCOOH

Time (min)	0.00	2.00	45.00	52.00	52.00	60.0
Solvent B (%)	0.0	0.0	90.0	90.0	5.0	5.0

Table 2: Gradient elution details.

- Detection performed on a NanoACQUITY UPLC system coupled with a SYNAPT XS instrument operating in positive ion mode.
- All data were acquired using Waters UDMSe (DIA using ion mobility) acquisition mode and processed using Progenesis QI for proteomics and PEAKS DB.

✓ Protein to trypsin ratio optimization

- The best performances were obtained with 10% ACN and 1% BSA spike matrix as well as high NfL standard concentration up to 10 µg/ml. Several trypsin ratios showed good results, with the most peptides found for 10:1 and 1:1 ratios of trypsin to NfL STDs.
- We continued with condition “D” composed of a BSA spiked 90:10 v/v H2O/ACN mixture, 10µg/mL NfL STD and 10:1 tryspin to NfL ratio. This condition can be further used to check instrument performances.

	Solvent	[NfL] (µg/mL)	Trypsin* ratio	Amount of peptides detected
A	H_2O/ACN	10	x10	5
B	H_2O/ACN	10	X1	NA
C	H_2O/ACN	10	X0.1	1
D	H_2O/ACN +BSA	10	X10	15
E	H_2O/ACN +BSA	10	X1	14
F	H_2O/ACN +BSA	10	X0.1	11
G	H_2O/ACN	1	X10	1
H	H_2O/ACN	1	X1	NA
I	H_2O/ACN	1	X0.1	1
J	H_2O/ACN +BSA	1	X10	5
K	H_2O/ACN +BSA	1	X1	1
L	H_2O/ACN +BSA	1	X0.1	1
M	H_2O/ACN	0.1	X10	NA
N	H_2O/ACN	0.1	X1	1
O	H_2O/ACN	0.1	X0.1	1
P	H_2O/ACN +BSA	0.1	X10	3
Q	H_2O/ACN +BSA	0.1	X1	3
R	H_2O/ACN +BSA	0.1	X0.1	3
*All trypsinized samples were incubated at 37°C and 1200 rpm, for approx. 19 hours.				

Table 2: Number of peptides identified from protein sequence database in each sample by the number of missed cleavages.

✓ Immunocapture on standard samples optimization

- All diluted standard samples were analyzed simultaneously. Regarding on-beads digestion, good results were obtained with antibodies concentrated at 10µg/mL, and 10µg/mL or 1µg/mL NFL STD.
- One sample from upstream digestion with immunocapture, containing anti-NFL antibodies at 1 µg/mL and digested NfL E derived from experiment 1, stood out from the other conditions.

1	MSSFSYEPYY	STSYKRRYVE	TPRVHISSVR	SGYSTARSAY	SSYSAPVSSS	LSVRRSYSSS	SGSLMPSLEN	LDLSQVAAIS
81	NDLKSIRTQE	KAQLQDLNDR	FASFIERVHE	LEQQNKVLEA	ELLVLRQKHS	EPSRFRALYE	QEIRDLRLAA	EDATNEKQAL
161	QGEREGLEET	LRNLQARYEE	EVLSREDAEG	RLMEARKGAD	EAALARAELE	KRIDSLMDEI	SFLKKVHEEE	IAELQAQIQY
241	AQISVEMDVT	KPDLSAALKD	IRAQYEKLAA	KNMQNAEEWF	KSRFTVLTES	AAKNTDAVRA	AKDEVSESRR	LLKAKTLEIE
321	ACRGMNEALE	KQLQELEDKQ	NADISAMQDT	INKLENELRT	TKSEMARYLK	EYQDLLNVKM	ALDIEIAAYR	KLLEGEETRL
401	SFTSVGSITS	GYSQSSQVFG	RSAYGGLQTS	SYLMSTRSFP	SYYTSHVQEE	QIEVEETIEA	AKAEEAKDEP	PSEGEAEEEE
481	KDKEEAEEEE	AAEEEEAAKE	ESEEAKEEEE	GGEGEEGEET	KEAEEEEKKV	EGAGEEQAAK	KKD	

Figure 2: Labeling of identified peptides (16) on the amino acid sequence of NfL.

Legend : **X** → *digestion prior to capture*
X → *on-beads digestion*

✓ Last results on standard spiked in CSF samples

- We could not obtain conclusive data for this test. We tried to find the malfunction source by repeating the same modus operandi a second time with system-check samples from experiments 1 and 2. However, the results were once again unconclusive.
- We currently suspect a technical issue related to the separation and/or detection equipment.

✓ Work in progress

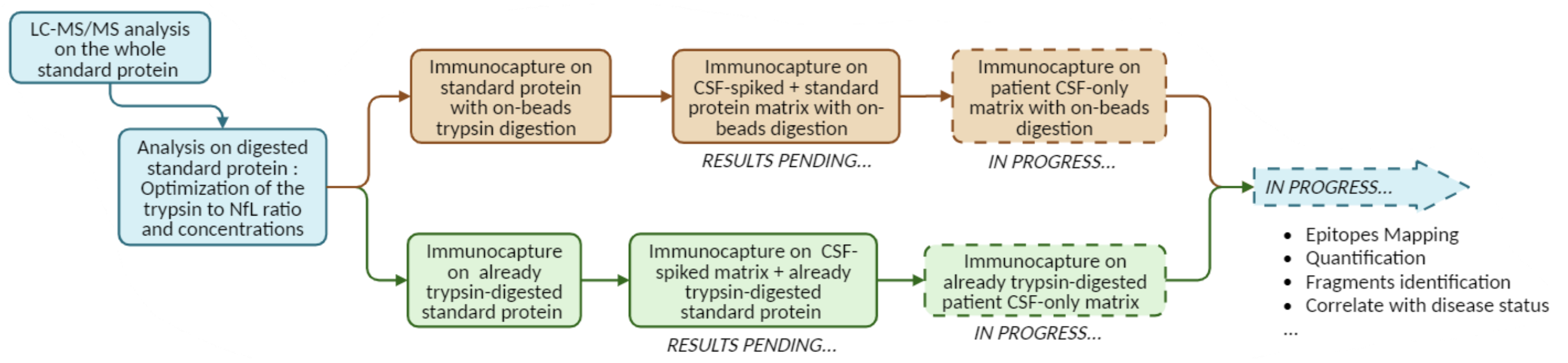

Figure 3: Workflow development diagram.

- Transpose and test the effectiveness of the method on CSF samples from healthy subjects or patients.
- Other statistical analysis tool will be considered.
- Evaluation of sample preparation: simplify washing steps, question the influence of trypsin digestion.
- Working with different antibodies to obtain paratope mapping(1), or even without immunocapture.
- Comparison between plasma and CSF samples.
- Develop a (semi-)quantitative method.

References

(1) Budelier, M. M.; He, Y.; Barthélemy, N. R.; Jiang, H.; Li, Y.; Park, E.; Henson, R. L.; Schindler, S. E.; Holtzman, D. M.; Bateman, R. J. A map of neurofilament light chain species in brain and cerebrospinal fluid and alterations in Alzheimer’s disease. *Brain Communications* **2022**, *4* (2). https://doi.org/10.1093/braincomms/fcac045.

(2) Thomas, I.; Dickens, A. M.; Posti, J. P.; Mohammadian, M.; Ledig, C.; Takala, R.; Hyötyläinen, T.; Tenovuo, O.; Orešič, M. Integrative Analysis of Circulating Metabolite Profiles and Magnetic Resonance Imaging Metrics in Patients with Traumatic Brain Injury. *International Journal of Molecular Sciences* **2020**, *21* (4), 1395. https://doi.org/10.3390/ijms21041395.

(3) Delaby, C.; Bousiges, O.; Bouvier, D.; Fillée, C.; Fourier, A.; Mondésert, E.; Nezry, N.; Omar, S.; Quadrio, I.; Rucheton, B.; Schraen-Maschke, S.; Van Pesch, V.; Vicca, S.; Lehmann, S.; Bedel, A. Neurofilaments contribution in clinic: state of the art. *Frontiers in Aging Neuroscience* **2022**, *14*. https://doi.org/10.3389/fnagi.2022.1034684.

(4) Massonnet, P.; Grifnée, E.; Farré-Segura, J.; Demeuse, J.; Huyghebaert, L.; Dubrowski, T.; Dufour, P.; Schoumacher, M.; Peeters, S.; Goff, C. L.; Cavalier, É. Concise review on the combined use of immunocapture, mass spectrometry and liquid chromatography for clinical applications. *Clinical Chemistry and Laboratory Medicine* **2023**, *61* (10), 1700–1707. https://doi.org/10.1515/cclm-2023-0253.