

1. Introduction

Current routine clinical method for detecting and monitoring of Alzheimer's disease (AD) biomarkers are performed using cerebrospinal fluid (CSF). However, CSF sampling through lumbar puncture can be both tricky for the clinician and invasive for the patient.

Therefore, the quantification of plasma-based biomarkers such as phosphorylated Tau (p-Tau), Tau, APOE and A β 1-42 and alpha-synuclein would allow for a large-scale population screening and would facilitate patient monitoring through a non-invasive sampling procedure.

The collection of genetic and physiological data would enable easy and early diagnosis, would improve patient care and would support the development of new therapeutic drugs. Besides, LC-MRM method, in comparison with commercial immunoassays, allows multiplex analysis and detection of structural modifications such as post-translational modifications (PTMs).

This research project is part of a collaboration of Shimadzu Corporation with University Hospital of Montpellier (CHU).

Primary development was carried out with specific immunocapture targeting several phosphorylation sites on Tau protein in CSF before further implementation in plasma.

2. Objectives

The objective of this PhD is the development of a unique and innovative LC-MRM method using a LCMS-8060NX (Shimadzu) triple quadrupole to detect Alzheimer's blood biomarkers involved in diagnosis and prognosis : Tau, pTau, Apolipoprotein E, A β peptide and alpha-synuclein.

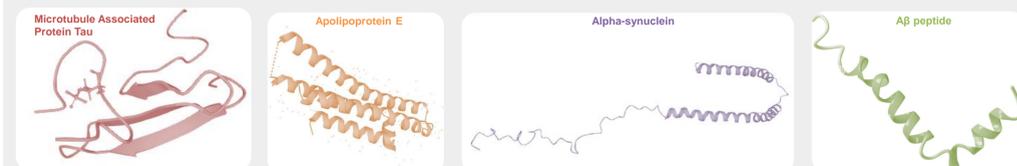


Figure 1 : Tau, APOE, alpha-synuclein and A β peptide 3D structures

The first part of this project was dedicated to the development of a workflow enabling the detection of Tau and pTau (T217,T205,T181) which are major biomarkers correlating with AD progression.

3. Materials and methods

3.1 – Materials and reagents

Pooled CSF and plasma were gathered from samples stored in the National Center of Biological Ressources (CRB) biobank and stored at -80°C.

Recombinant ¹⁵N-isotope-labelled 2N4R Tau was offered by Guy Lippens (CNRS-Université Lille 1, UMR8576, Villeneuve d'Ascq, France) and synthetic phosphorylated AQUA peptides (ThermoFisher) were used as internal standards.

Selective immunocapture was performed using pTau specific antibodies targeting phosphorylated threonines at sites 181,205,231 and 217 (abcam).

Before LC-MRM implementation, analysis were run on TIMS-TOF HT (Bruker) coupled with Evosep One Liquid chromatography instrument. (Evosep).



Figure 2 : Protein Tau and antibodies epitopes

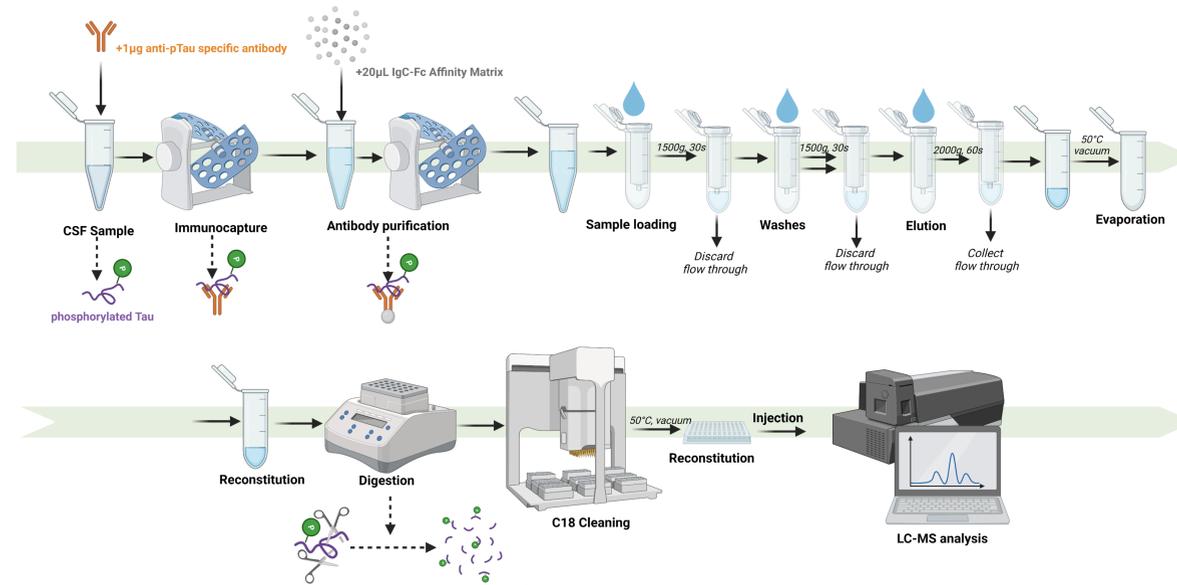


Figure 3 : Sample preparation workflow

3.2 - CSF Workflow :

Sample preparation

After thawing on ice, 250 μ L CSF was mixed with 250 μ L 1% NP-40 buffer (20mM Tris, 137mM NaCl, 2mM EDTA, 5mM Guanidine Hydrochloride, 1% NP-40, pH 7.4). 1 μ g antibody was added and incubated for 4 hours on a rotator. The complexed antibodies were then mixed with IgC-Fc affinity matrix and incubated 1 hour.

Spin Column (ThermoFisher) was equilibrated twice with 300 μ L LCMS grade H₂O, centrifuged 30s at 1500g and the flow through was discarded. The sample was loaded on the column, incubated 1 hour while shaking at 750rpm and then centrifuged 30s at 1500g. Flow through was discarded. Washes were performed with 400 μ L 1% NP-40 buffer and 1X PBS. Captured proteins were then eluted with 80/20 (v/v ; 100mM formic acid in LCMS grade H₂O/ACN) and centrifuged 1min at 2000g to collect flow through.

Before enzymatic proteolysis, the sample was dried under vacuum and reconstituted with 60 μ L 12,5/87,5 (v/v ; ACN/ 50mM Ammonium Bicarbonate in LCMS grade H₂O).

Enzymatic digestion was initiated with trypsin/LysC (Promega) at 1/50 (w/w) enzyme to substrate ratio. Proteolysis proceeded overnight at 37°C with 450rpm constant shaking. Digestion was stopped with 1 μ L formic acid.

Peptide clean-up was automatically processed using BRAVO-Assay MAP (Agilent) on C18 cartridges and eluted with 20 μ L 40% ACN + 0,1% formic acid in LCMS grade H₂O. The concentrated eluates were lyophilized to dryness and reconstituted with 20 μ L phase A. Samples were then kept frozen at -20°C until LC-MS/MS analysis.

LC/MS analysis

The 20 μ L peptide sample was loaded onto Evotip . Separation was achieved using performance Evosep EV1137 (15cm x 150 μ m, 1.5 μ m) column with a 44min binary gradient (30SPD method). The mobile phases consisted of (A) 0,1% Formic Acid and (B) ACN + 0,1% Formic Acid. The mass spectrometer was operated in DDA Parallel Accumulation-Serial Fragmentation (PASEF).

Data Processing

Data were acquired in Compass Hystar 6.0 software (Bruker). Raw data processing was performed using Skyline software 22.2.0 version.

4. Preliminary results on Tau

This method, using three different antibodies enabled to detect several peptides located from the N-terminal to the proline rich region of Tau protein. No C-terminal peptides were identified.

Anti pTau-231 and anti pTau-181 led to detection of unphosphorylated, phosphorylated and di-phosphorylated peptides while anti-pTau-205 led to the detection of none.

Peptide sequence	Charge state	m/z	Capture with anti pTau-231 antibody	Capture with anti pTau-181 antibody	Capture with anti pTau-205 antibody
QEFEVMEDHAGTYGLGDR [6;23]	3	685,3022	Detected	Not detected	Not detected
DQGGYTMHQDQEGDQGLK [25;44]	3	722,6395	Detected	Detected	Not detected
ESPLQPTEDGSEEPGSETSDAK [45;67]	2	1196,0195	Detected	Detected	Not detected
TPPAPKpTPSSGEPK [175;190]	2	834,4057	Detected	Detected	Not detected
SGYSSPGSPGTPGSR [195;209]	2	697,3208	Detected	Detected	Not detected
TPSLPTPPTTR [212;221]	2	533,7982	Detected	Detected	Not detected
SRpTPSLPpTPPTR [210;221]	2	735,3311	Detected	Detected	Not detected
VAVVRpTPPK [226;234]	2	523,7915	Detected	Detected	Not detected
VAVVRpTPPKSPSSAK [226;240]	3	226,240	Detected	Detected	Not detected
LQTAPVMPDLK [243,254]	2	655,3629	Detected	Detected	Not detected

Figure 4 :Detected peptides according to the antibody used

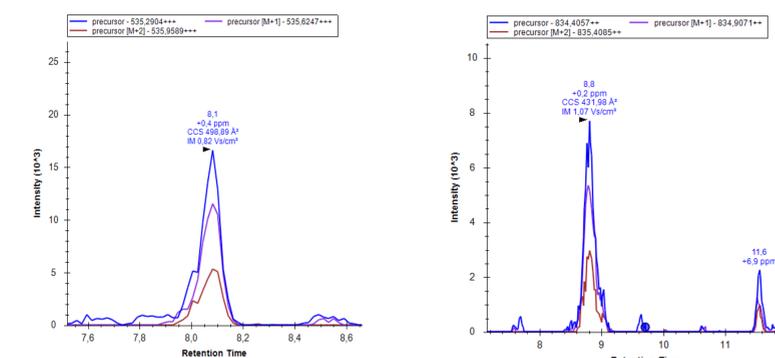


Figure 5 and 6 : VAVVRpTPPKSPSSAK chromatogram with anti pTau-231 antibody (left) and TPPAPKpTPSSGEPK chromatogram with anti pTau-181 antibody (right)

5. Conclusions and Perspectives

The preliminary development of this innovative method allowed the detection of phosphorylated peptides corresponding to specific phosphorylation sites correlated with Alzheimer's disease progression as previously reported. Among which, pTau217, pTau231 and pTau181.

Further optimization of the sample preparation would be performed in order to increase the performances of the method and combine with other biomarkers for multiplexing. This would be carried on in order to use the method with plasma samples and implement the latter on triple quadrupole systems.

Disclaimer : The products and applications in this presentation are intended for Research Use Only (RUO). Not for use in diagnostic procedures.