Development of a blood-based multiplex quantitative LC-MS method for neurodegenerative diseases patient stratification : towards an innovative diagnostic tool

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1. Introduction and objectives

Current neurodegenerative biomarkers measurements for diagnosis purposes are performed using CSF samples which requires invasive procedures for the patient.

Besides, AD and other associated dementias are very complex pathologies that can have intricate symptoms and pathological deleterious effects. Therefore, the developed quantitative multiplex method includes 22 blood neuroinflammatory, brain function-linked, genetic and general state biomarkers for disease state evaluation and monitoring, patient stratification and differential diagnosis purposes.

2. Materials and methods

2.1 – Targeted proteins

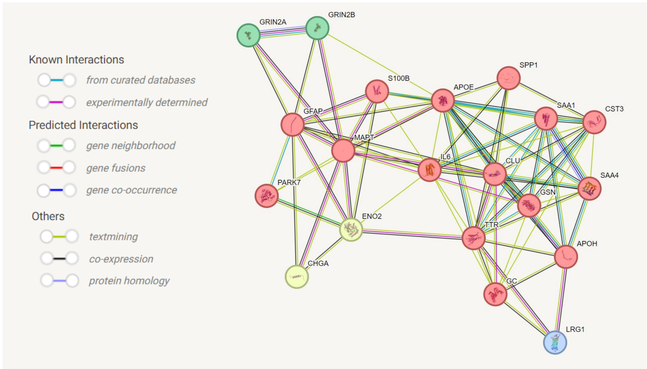

Figure 1 : Protein links and clusters (acquired with stringDB)

2.2 – Materials and reagents

Pooled plasma and individual plasma samples were gathered from samples stored in the National Center of Biological Ressources (CRB) biobank and stored at -80ºC. Recombinant cleavable wing-^{13}C-^{15}N-heavy-labelled peptides for the 22 protein targets were purchased from jpt peptides and used as internal standards

2.3 – Sample preparation worfklow :

Plasma sample preparation (Figure 2)

Sample preparation was performed on 96-well protein LoBind plates. After thawing on ice, 10µL EDTA plasma sample was reduced with 20µL a 300mM Tris, 9M Urea and 100mM DTT Buffer and further alkylated with 20µL of 100mM IAA. Sample was diluted with 225µL 100mM Tris Buffer. Enzymatic digestion was initiated with 15µg trypsin/LysC (Promega). Proteolysis proceeded overnight at 37ºC with 450rpm constant shaking. Digestion was stopped with 20% formic acid.

Peptide clean-up was automatically processed using BRAVO-Assay MAP (Agilent) on RPS cartridges and eluted with 125µL 70% ACN + 0,1% formic acid in LCMS grade H_2O.

The concentrated eluates were lyophilized to dryness and reconstituted with 60µL phase A. Samples were then kept frozen until LC-MS/MS analysis.

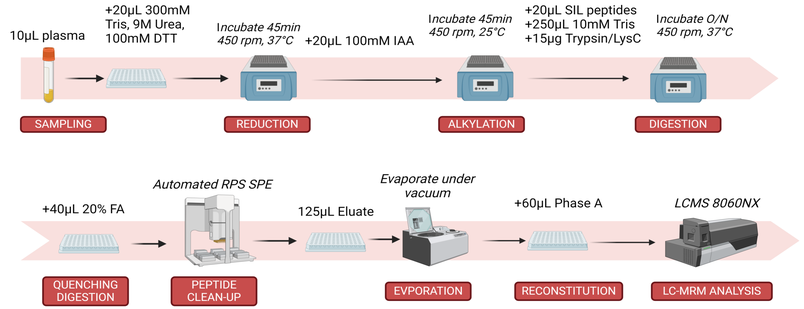

Figure 2: Automated sample preparation automated workflow

LC/MS analysis

Samples were analysed using liquid chromatography tandem mass spectrometry using Nexera Inert LC (Shimadzu) coupled to LC-MS 8060NX Triple Quadrupole (Shimadzu). 40µL of the reconstituted extract was injected on a Shimadzu Shim-pack Scepter Claris C18-300 (3µm;150x2.1mm). Separation was achieved with a 45min binary gradient. The mobile phases consisted of (A) 0,5% Acetic Acid and (B) ACN + 0,5% Acetic Acid. The instrument was operated in LC-MRM mode using ESI positive ionisation. .

Data Processing

Data were acquired in LabSolutions 5.120 (Shimadzu). Raw data processing was performed using Skyline software 22.2.0 version.

3. Digestion optimization

3.1 – Enzyme quantity

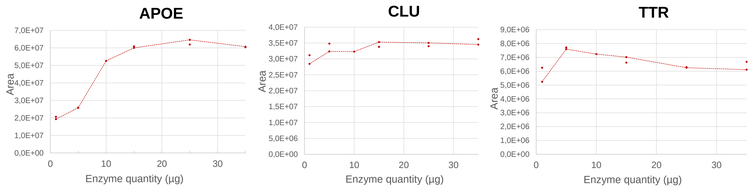

Figure 3: Area measured with increasing enzyme quantity

3.2 – Digestion duration

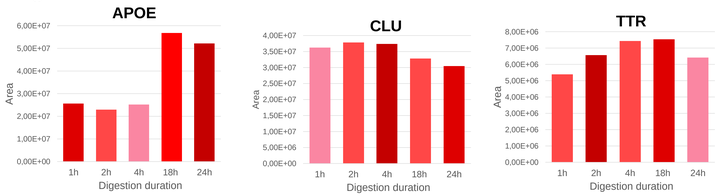

Figure 4: Digestion kinetics

4. Analytical validation

Blank matrix regarding all analytes was not available therefore SIL cleavable peptides were used as analytes and peptides derived from the endogenous proteins were used as internal standard.

Analytical performances were evaluated according to international guidelines recommendations and reported in the table below (Figure 5). Out of the 22, 16 endogenous proteins were quantifiable.

4.1 – Linearity, repeatability and matrix effect

Analyte	Linearity		Within run repeatability (n=6)						Between run repeatability (n=18)						Matrix Effect
			QC Low		QC Med		QC High		QC Low		QC Med		QC High		
	Range (pmol/µL)	Regression coeff.	Acc.	Pre.	Acc.	Pre.	Acc.	Pre.	Acc.	Pre.	Acc.	Pre.	Acc.	Pre.	
CHGA	**0,04-1**	**0,948-0,983**	**83%**	**2%**	**88%**	**4%**	**93%**	**4%**	**78%**	**11%**	**83%**	**16%**	**88%**	**17%**	**-36%**
IL6	0,0025-0,05	0,948-0,963	60%	26%	75%	15%	88%	15%	66%	28%	73%	18%	81%	20%	-44%
ENO2	0,05-0,5	0,852-0,941	65%	6%	77%	8%	96%	6%	65%	18%	76%	7%	88%	20%	-97%
SPP1	**0,0025-0,05**	**0,986-0,975**	**75%**	**10%**	**86%**	**6%**	**96%**	**5%**	**59%**	**27%**	**77%**	**21%**	**87%**	**21%**	**-42%**
MAPT	0,005-0,01	0,911-0,989	92%	4%	91%	6%	88%	5%	87%	14%	92%	9%	92%	10%	-37%
CLU	**0,05-2**	**0,990-0,998**	**90%**	**9%**	**94%**	**1%**	**97%**	**4%**	**85%**	**6%**	**94%**	**3%**	**96%**	**3%**	**-40%**
GFAP	**0,0025-0,05**	**0,981-0,934**	**53%**	**10%**	**81%**	**8%**	**78%**	**1%**	**50%**	**10%**	**76%**	**14%**	**80%**	**5%**	**-32%**
SAA4	**0,0625-2**	**0,933-0,996**	**87%**	**5%**	**93%**	**4%**	**96%**	**3%**	**75%**	**37%**	**85%**	**18%**	**84%**	**13%**	**-27%**
GRIN2A	0,00625-0,1	0,981-0,906	86%	16%	89%	14%	93%	8%	69%	10%	81%	13%	89%	7%	-80%
LRG1	**0,0625-2**	**0,987-0,997**	**78%**	**2%**	**86%**	**3%**	**87%**	**5%**	**72%**	**2%**	**82%**	**4%**	**87%**	**5%**	**-8%**
GC	**0,025-2**	**0,996-0,997**	**93%**	**4%**	**96%**	**4%**	**96%**	**4%**	**86%**	**8%**	**95%**	**6%**	**95%**	**6%**	**-66%**
GSN	**0,03125-1**	**0,987-0,995**	**76%**	**2%**	**88%**	**3%**	**89%**	**4%**	**82%**	**6%**	**90%**	**7%**	**90%**	**10%**	**-44%**
PARK7	**0,0005-0,01**	**0,974-0,994**	**81%**	**18%**	**88%**	**15%**	**88%**	**11%**	**81%**	**13%**	**86%**	**14%**	**87%**	**15%**	**-57%**
SAA1	**0,025-1**	**0,999-0,998**	**98%**	**2%**	**97%**	**2%**	**97%**	**3%**	**96%**	**3%**	**97%**	**4%**	**97%**	**4%**	**-83%**
CST3	**0,025-0,5**	**0,933-0,995**	**89%**	**4%**	**98%**	**2%**	**97%**	**4%**	**89%**	**11%**	**98%**	**6%**	**93%**	**7%**	**-57%**
APOE	**0,03125-1**	**0,986-0,994**	**78%**	**4%**	**90%**	**3%**	**96%**	**4%**	**83%**	**5%**	**94%**	**7%**	**94%**	**6%**	**-60%**
APOH	**0,025-1**	**0,989-0,996**	**88%**	**3%**	**94%**	**5%**	**92%**	**5%**	**88%**	**4%**	**95%**	**5%**	**95%**	**4%**	**-84%**
GRIN2B	0,0005-0,01	0,943-0,979	88%	11%	94%	7%	90%	12%	86%	13%	95%	6%	88%	12%	-63%
APOE e3/e4	**0,0005-0,01**	**0,988-0,989**	**93%**	**6%**	**91%**	**9%**	**97%**	**4%**	**92%**	**5%**	**93%**	**6%**	**97%**	**4%**	**-68%**
APOE e4	**0,0005-0,01**	**0,937-0,963**	**63%**	**42%**	**80%**	**24%**	**87%**	**11%**	**63%**	**39%**	**80%**	**24%**	**82%**	**11%**	**-54%**

Figure 5 : Validation results table for absolute quantification

Acc. : Accuracy; Pre. : Precision; Bold : endogenous peptide detected

5. Conclusions and Perspectives

Further analytical validation would be performed to fully assess method performances (stabilities, intra-laboratory reproducibility). Then, large patient cohorts would be analyzed to evaluate and clinically validate this innovative tool and its capacity to differentiate diseases, severity and co-pathologies. Such tool would bring dementia diagnosis within the landscape of personalized medicine fostering better patient care..