Development of Parallel Reaction Monitoring (PRM) Assays for the Validation of Biomarkers Associated to Alzheimer's Disease in CSF

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Introduction

Alzheimer's disease is an irreversible, progressive brain disorder and the most common cause of dementia among older adults. Damages in the brain are expected to starts a decade or more before cognitive problems become evident. The identification of biomarkers associated to early biological events in the onset and progression of Alzheimer's disease would be of tremendous importance in both prevention and treatment. Suspension bead arrays (SBA) has proven to be a convenient approach to profile hundreds of proteins in large cohorts of biological samples. The aim of this study was to develop and validate Parallel Reaction Monitoring (PRM) assays to perform an orthogonal verification of clinically rlevant protein profiles previously discoved by SBA[1]

[1] Julia Remnestål et al. Proteomics Clin Appl. 2016 Dec; 10(12): 1242–125

Material & Methods

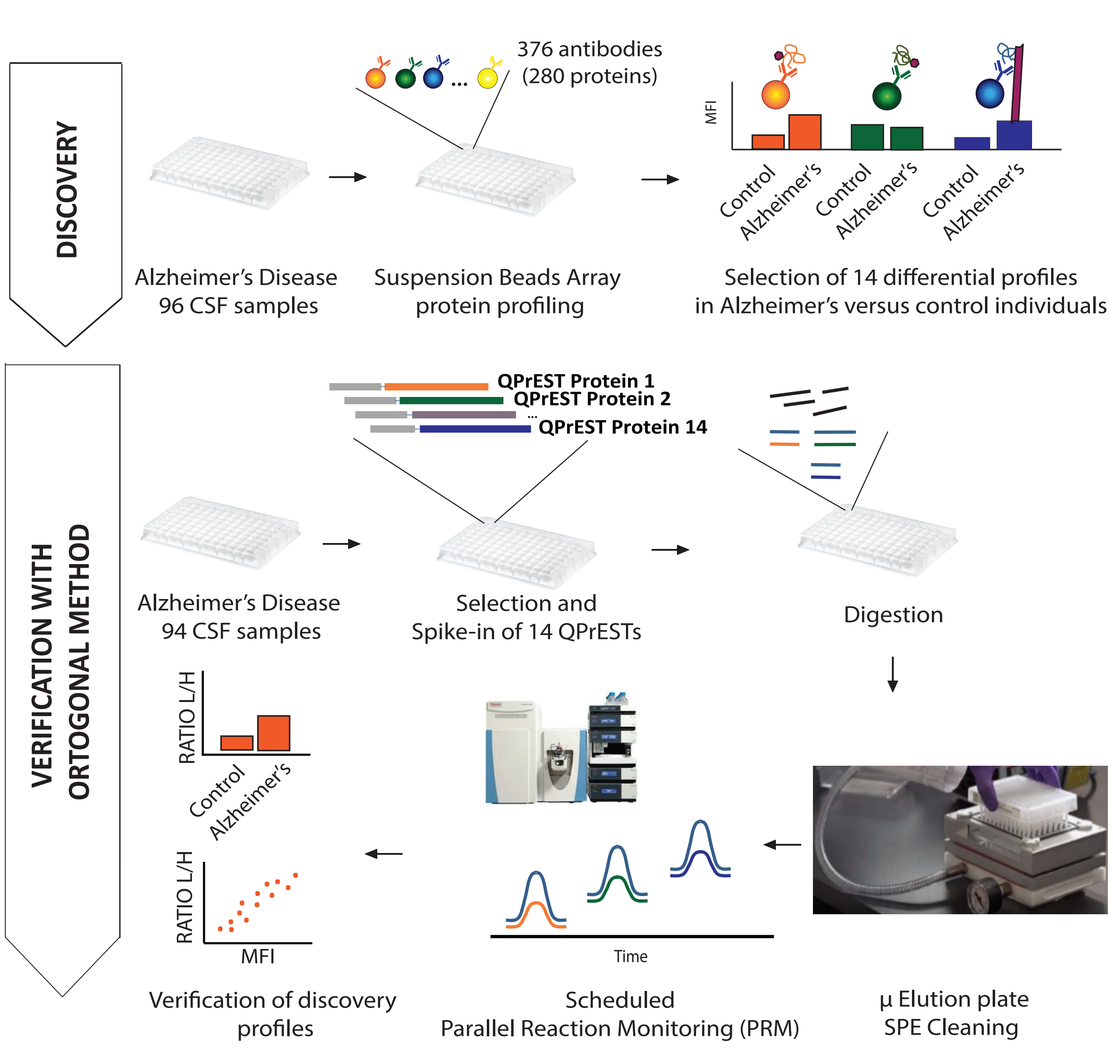

Figure 1. Graphical representation of the workflow

94 CSF samples were collected at Sahlgrenska Hospital in Gothenburg (Sweden) from patients with AD, preclinical AD (down A β42), prodromal AD (down Aβ42, up t-tau or p-tau), non-AD dementia, MCI (mild cognitive impairment) and age-matched healthy controls (Table 1). Protein profiling was performed with SBA as previously described by Remnestål et al. Twenthy proteins with interesting profiles were selected for further verification with PRM-MS. CSF samples were spiked with QPrEST internal standards and digested (Trypsin/Lys-C) overnight. Clean-up was performed with solid phase extraction (SPE) containing hydrophilic-lipophilic balance (HLB) sorbent in a µElution plate format (Waters). Liquid chromatography (LC) was used for peptide separation with a 35-min linear gradient on a 25 cm C18 analytical column (ThermoFisher). PRM analysis was performed on a Q-Exactive HF mass spectrometer (ThermoFisher) with a full scan event (mass range m/z 150-2000, resolution 60 000, AGC value 2e5 and maximum IT 55 ms) followed by up to 25 PRM events (resolution 30 000, AGC value 1e6, maximum IT 250 ms, precursor isolation window 2.0 Th). A minimum of three transitions was required for identification and peaks were manually inspected. Light-to-heavy ratio were exported from Skyline and analyzed using the statistical environment R.

Table 1. Sample demographics

Diagnosis	# Samples	Age Median (Range)	Gender F/M
Alzheimer Disease (AD)	43	81 (53-102)	28/15
Prodromal AD	2	90 (88-92)	2/0
Preclinical AD	14	85 (73-96)	10/4
Non-AD dementia	2	84 (82-86)	0/2
Non-AD mild cognitive impairment (MCI)	10	84.5 (56-93)	8/2
Healthy	23	79(44-91)	12/11
Total	94		

Results

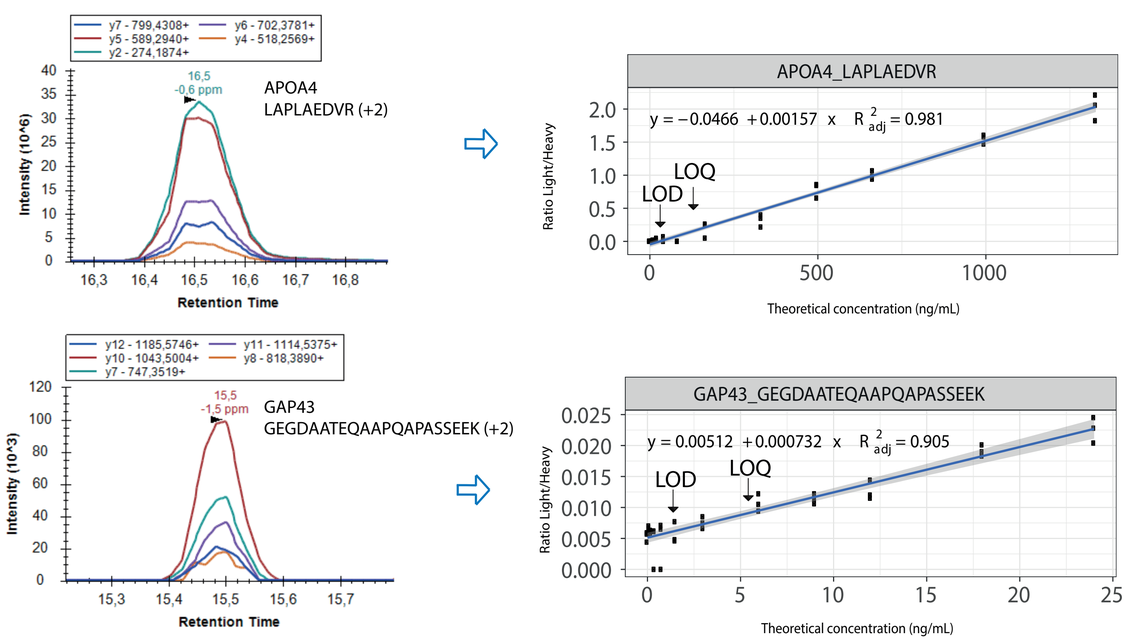

Figure 2. (Left) Chromatograms of PRM transitions.(Right)l Linear regression and 95% confidence band.

Table 2. Method validation

Gene	Peptide	Calibration curve					Precision			Accuracy		Carry-over	
		Adj R^2	LOD (ng/mL)	LOQ (ng/mL)	Ratio at LOD	Ratio at LOQ	Intra-day mean CV% (n=3)	Inter-day mean CV% (n=4)**	Inter-day CV% (n=3)***	Mean ± SD (µg/mL)	Closeness (%)	Light (%)	Heavy (%)
ALDOC	YTPEEIAMATVTALR	-	-	-	-	-	5.52	31.1	30.8	0.88 ± 0.24	1.80	0.00	0.00
	ALQASALNAWR	0.964	12.2	37.0	0.014	0.048	13.2	30.1	30.2	0.69 ± 0.20	4.01	0.00	0.00
	DNAGAATEEFIK	-	-	-	-	-	3.13	29.7	29.7	0.54 ± 0.14	0.84	0.00	0.00
APOA4	LAPLAEDVR	0.981	42.3	128	0.020	0.154	7.42	27.6	27.6	1.51 ± 0.37	6.84	0.00	0.00
	ALVQQMEQLR	0.957	67.1	203	0.023	0.214	10.5	32.3	31.7	2.30 ± 0.66	11.3	0.00	0.00
	VNSFFSTFK	0.975	48.6	147	0.063	0.220	13.1	23.4	21.5	2.20 ± 0.48	10.3	0.00	0.00
CCK	AHLGALLAR	-	-	-	-	-	38.5	47.5	32.2	0.03 ± 0.02	11.2	0.00	0.00
CKB	FCTGLTQIETLFK	-	-	-	-	-	19.3	34.3	35.6	0.01 ± 0.003	18.2	0.00	2.47
	LGFSEVELVQMVVDGVK	0.926	0.305	0.924	0.0003	0.002	12.6	14.6	12.8	0.01 ± 0.001	2.84	0.00	2.23
	GTGGVDTAAVGGVFDVSNADR	-	-	-	-	-	-	-	-	-	-	-	-
CTSD	LLDIACWIHHK	0.931	174	529	0.125	0.607	24.9	30.4	21.2	15.4 ± 4.75	16.6	0.00	0.00
	FDGILGMAYPR	0.973	148	450	0.191	0.543	10.5	15.9	14.5	8.79 ± 1.42	16.3	0.21	0.09
	ISVNNVLPVFDNLMQQK	0.960	137	414	0.294	0.665	4.81	14.8	14.7	8.57 ± 1.14	14.3	0.00	0.00
	LVDQNIFSFYLSR	0.986	80.2	243	0.070	0.260	5.85	12.9	12.6	7.53 ± 0.91	6.24	1.09	0.06
GAP43	GEGDAATEQAAPQAPASSEEK	0.905	1.79	5.41	0.006	0.009	11.9	31.2	32.8	0.02 ± 0.01	1.06	0.00	0.00
FGA	PNNPDWGTFEEVSGNVSPGTR	0.931	156	472	0.008	0.018	11.4	24.1	23.7	5.92 ± 1.4	3.12	0.00	0.00
ITIH1	EVAFDLEIPK	0.980	6.49	19.7	0.009	0.014	3.76	29.0	29.0	0.52 ± 0.13	9.25	0.42	0.26
	AAISGENAGLVR	0.984*	2.53	7.66	0.008	0.011	20.3	31.3	30.2	0.34 ± 0.11	11.2	0.00	0.00
LRG1	DLLLPQPDLR	0.982	20.0	60.7	0.006	0.018	4.25	19.5	19.4	1.05 ± 0.18	5.33	0.26	0.17
	VAAGAFQGLR	0.933	32.8	99.3	0.016	0.037	11.5	33.2	33.5	1.90 ± 0.60	12.6	0.00	0.00
NBEA	IHTTSDGMSSISER	-	-	-	-	-	-	-	-	-	-	-	-
	GLEYAEMTATTLETESSSSK	-	-	-	-	-	-	-	-	-	-	-	-
NEFM	EIEAEIQALR	-	-	-	-	-	-	-	-	-	-	-	-
	VQSLQDEVAFLR	-	-	-	-	-	22.5	23.4	19.7	-	-	0.00	0.61
PLG	WELCDIPR	0.963	228	692	0.150	0.320	4.22	41.4	41.5	10.8 ± 3.87	6.01	0.11	0.00
	CTTPPPSSGPTYQCLK	-	-	-	-	-	3.23	35.7	35.6	6.02 ± 1.83	3.16	0.00	0.00
PSAP	LGPGMADICK	0.877	74.1	225	0.064	0.139	9.62	29.1	28.5	1.16 ± 0.30	16.5	0.00	0.00
	EICALVGFCDEVK	0.889	9.92	30.1	0.004	0.017	-	-	-	-	-	-	-
	NVIPALELVEPIK	0.964	3.30	9.99	0.007	0.010	6.39	15.2	14.3	0.15 ± 0.02	4.08	0.48	0.97
	SDVYCEVCEFLVK	0.959	8.82	26.7	0.008	0.018	8.20	26.4	26.1	0.27 ± 0.06	11.1	2.00	1.67
	EILDAFDK	0.926	54.2	164	0.031	0.089	8.98	26.9	25.7	0.97 ± 0.23	18.9	0.00	0.00
SERPINA1	LSITGTYDLK	0.986	798	2417	0.128	0.475	11.0	25.4	24.3	81.5 ± 19.0	16.5	0.09	0.11
	SVLGQLGITK	0.958	1364	4132	0.319	0.902	5.32	28.5	28.2	66.1 ± 16.3	15.9	0.04	0.07
	VFSNGADLSGVTEEAPLK	0.982	992	3005	0.143	0.569	11.0	27.7	26.7	74.1 ± 18.3	28.2	0.68	0.80
SERPINA3	AVLDVFEEGTEASAATAVK	0.979	264	800	0.097	0.235	9.79	19.2	16.6	25.3 ± 4.32	5.51	0.46	0.46
	ITLLSALVETR	0.976	267	810	0.078	0.225	10.2	25.6	24.7	24.4 ± 5.62	4.47	0.14	0.26
SPARCL1	QEEDNTQSDDILEESDQPTQVSK	0.833	5.85	17.7	0.007	0.010	11.4	16.7	13.2	0.09 ± 0.01	10.6	0.00	0.37
	TGLEAISNHK	0.948	31.9	96.6	0.009	0.027	17.3	42.7	41.9	0.38 ± 0.15	33.4	0.00	0.00
VCAM1	GIQVELYSFPR	-	-	-	-	-	10.3	35.6	35.0	0.57 ± 0.18	3.50	0.00	0.00

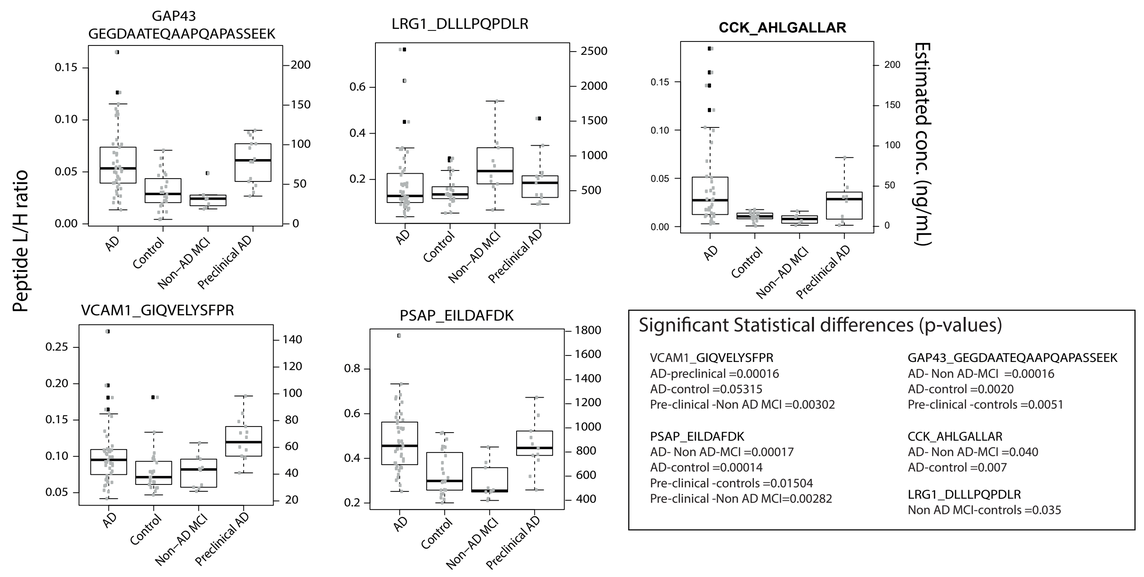

Figure 3. Box-and-whisker plot visualization of protein profiles.Pairwise comparison between group levels was performed by Wilcoxon Rank um Test with Bonferroni correction.

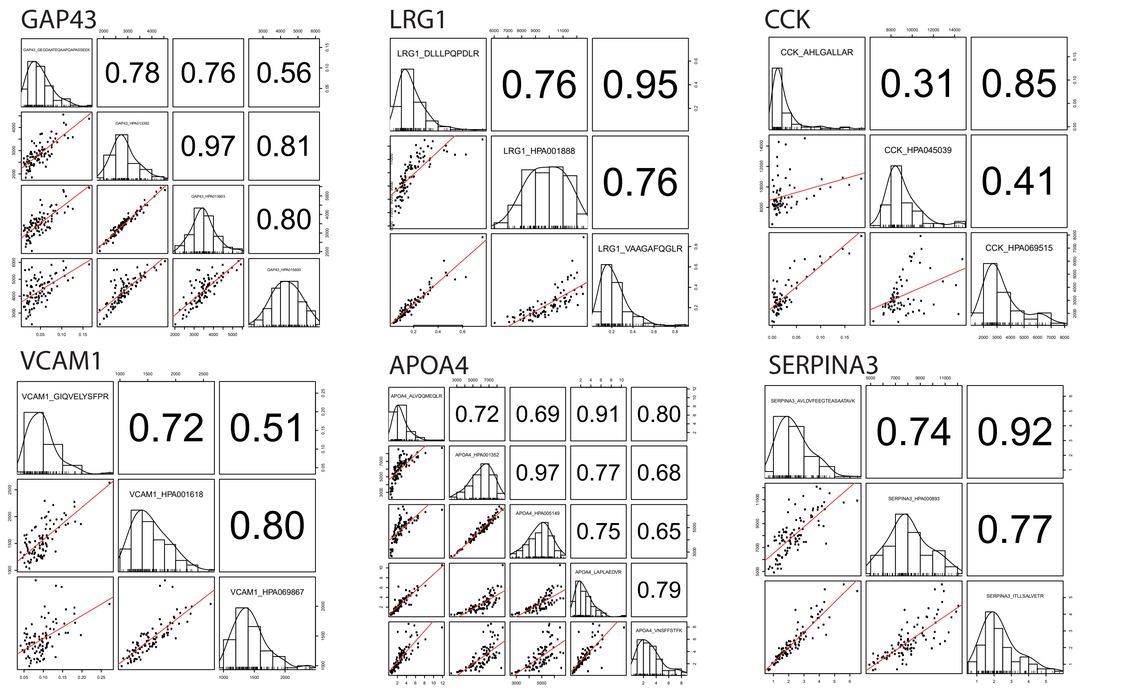

Figure 4. Six examples of scatterplots with value of correlation (Pearson's coefficient) of protein levels measured by suspension bead arrays (MFI) and PRM (L/H ratio).

Conclusions

- PRM assays for the quantification of 13 brain enriched proteins in CSF were developed and validated.
- Quantification by PRM confirmed the profiles observed by SBA for 7 proteins: GAP43, SERPINA3, LRG, APOA4 NEFM, VCAM and CCK
- This study strongly suggest GAP43,VCAM and CCK as CSF molecular markers of early onset of Alzheimer's disease
- PRM data suggest also clinically interesting profiles for PSAP even if the correlation with SBA data was low
- Peptide quantification in digested crude CSF show a sensitivity in the range of high ng/mLto mg/mL. Therefore,further method development is required to obtain an accurate quantificaton of low abundant proteins such as CCK, eventually including immuno-enrichment.