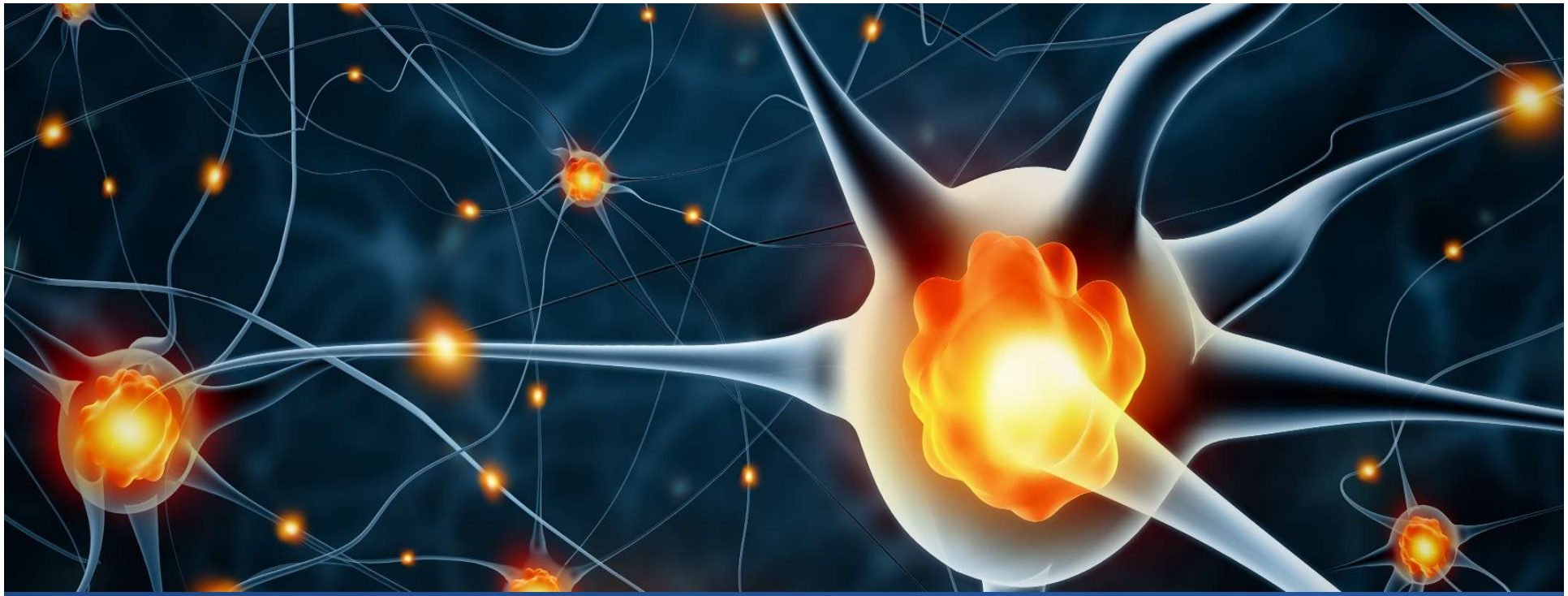


Development of IP-LC-TQMS methodology as biomarker read-out to quantify Tau phosphorylation around T217 in CSF clinical study samples from Alzheimer diseased patients

Sebastiaan Bijttebier, Clara Theunis, Farid Jahouh, Dina Rodrigues Martins, Marc Verhemeldonck, Karolien Grauwen, Lieve Dillen, Marc Mercken

13th EBF Open Symposium - November 2020

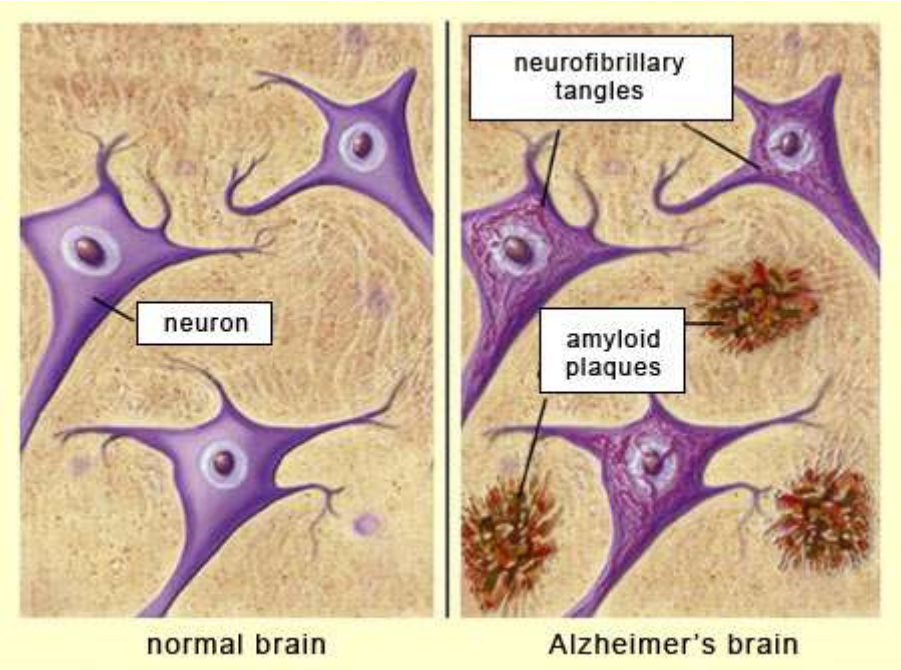
janssen | PHARMACEUTICAL COMPANIES OF Johnson & Johnson

Alzheimer

Lack of knowledge of the mechanisms involved in disease pathogenesis

Aggregation of **amyloid β** and **phosphorylated Tau** in brain

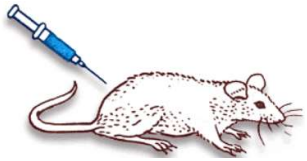
→ Linked to **neurodegeneration**



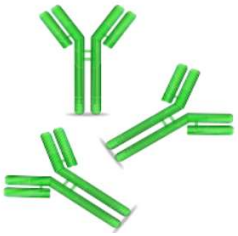
→ **Project aim**: develop Ab specific for pTau to prevent aggregation

Hanger et al., 2009, Trends in Molecular Medicine 15, 112-119
Lee et al., 2019, Future Medicinal Chemistry 11, 1845-1848

Anti-pTau Ab



Immunizing mice with Tau ePHF (paired helical filaments extracted from AD patients)

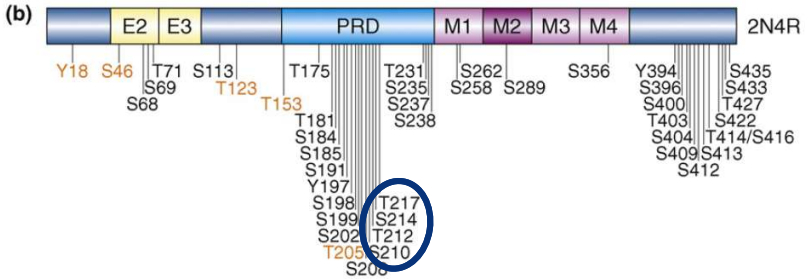


Screening

Ab selection

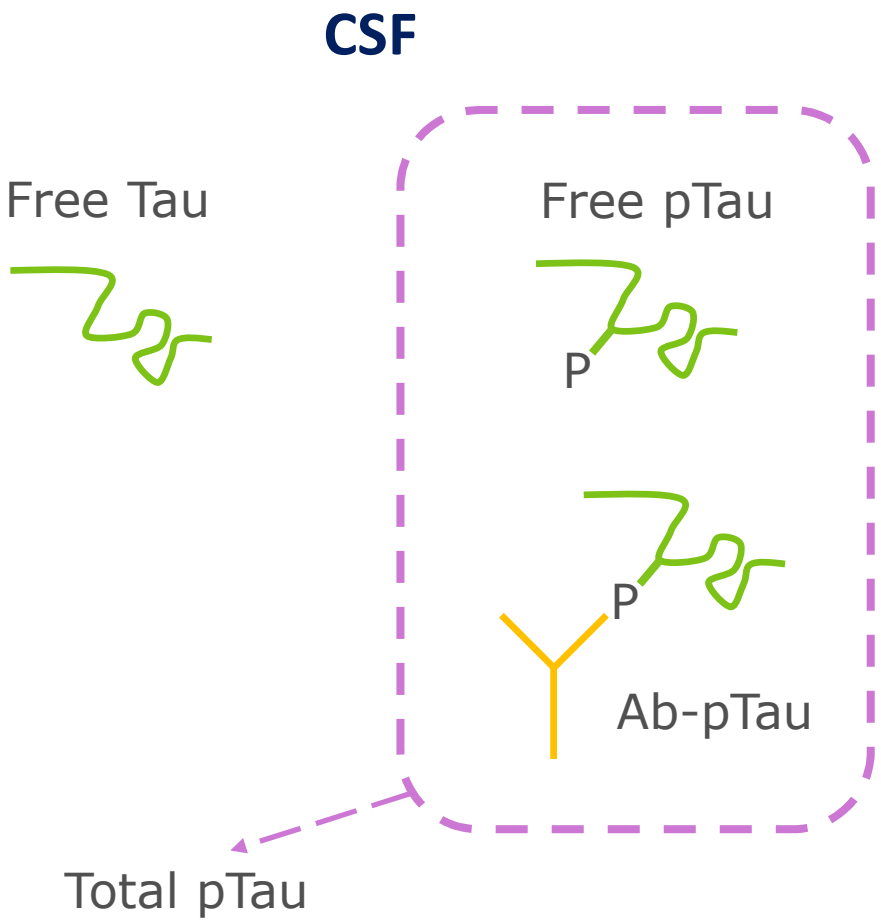
- High affinity for AD tau PHF
- Strong efficacy in *in vitro* and *in vivo* experiments of AD tau seeding
- Selectivity for phosphorylated Tau

Humanized form: "Ab X"

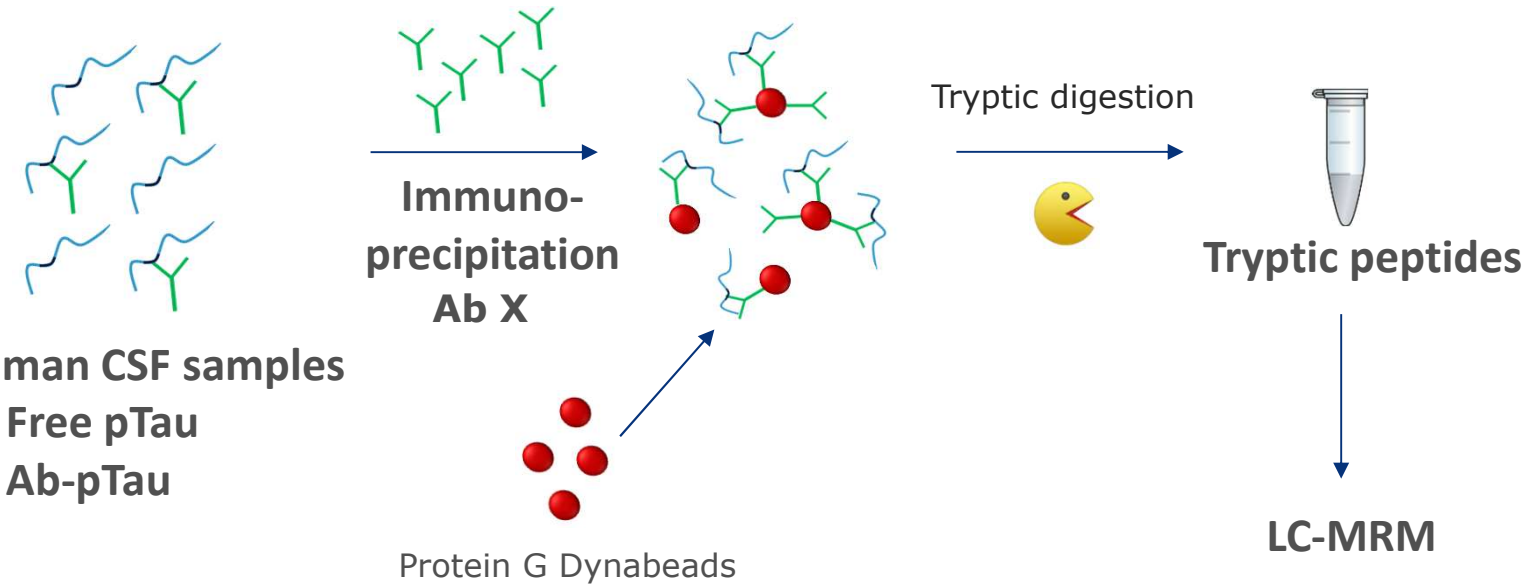


Van Kolen et al., in preparation

Humanized Ab X dosed in clinic



IP-LC-MS/MS approach



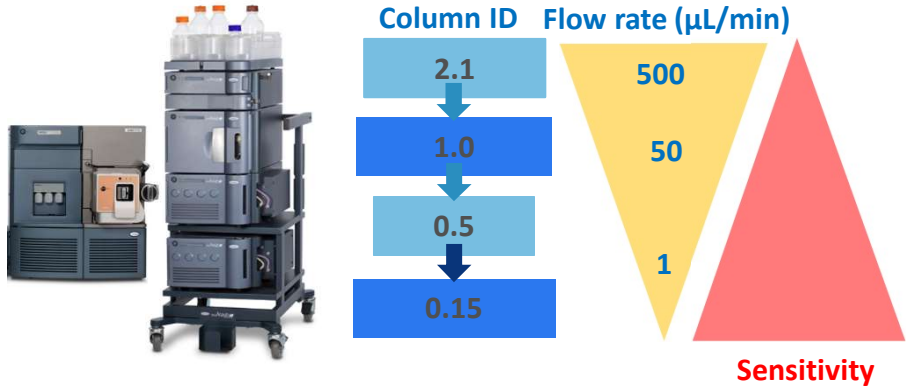
LC-MSMS systems

AB Sciex 6500

Advantages:
- Most sensitive system in development BA
- High throughput
- Compatible to a wide pH range

Disadvantages:
- Works at classical UHPLC flow rates

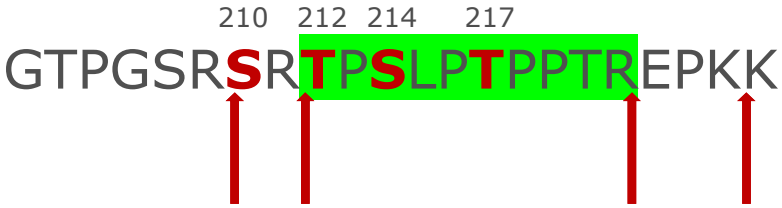
Waters TQS multi-D LC system



Advantages:
- High samples loading capacity
- Elution at a low flow rate (best sensitivity of the system)

Disadvantages:
- Not possible to work at high pH
- Complexity of the system (novelty)
- Longer run time

Tryptic fragments of extended peptides



	Missed cleavages (mc)
TPSLPTPPTR	0
SRTPSLPTPPTR	1
TPSLPTPPTR**EPK**	1
SRTPSLPTPPTR**EPK**	2

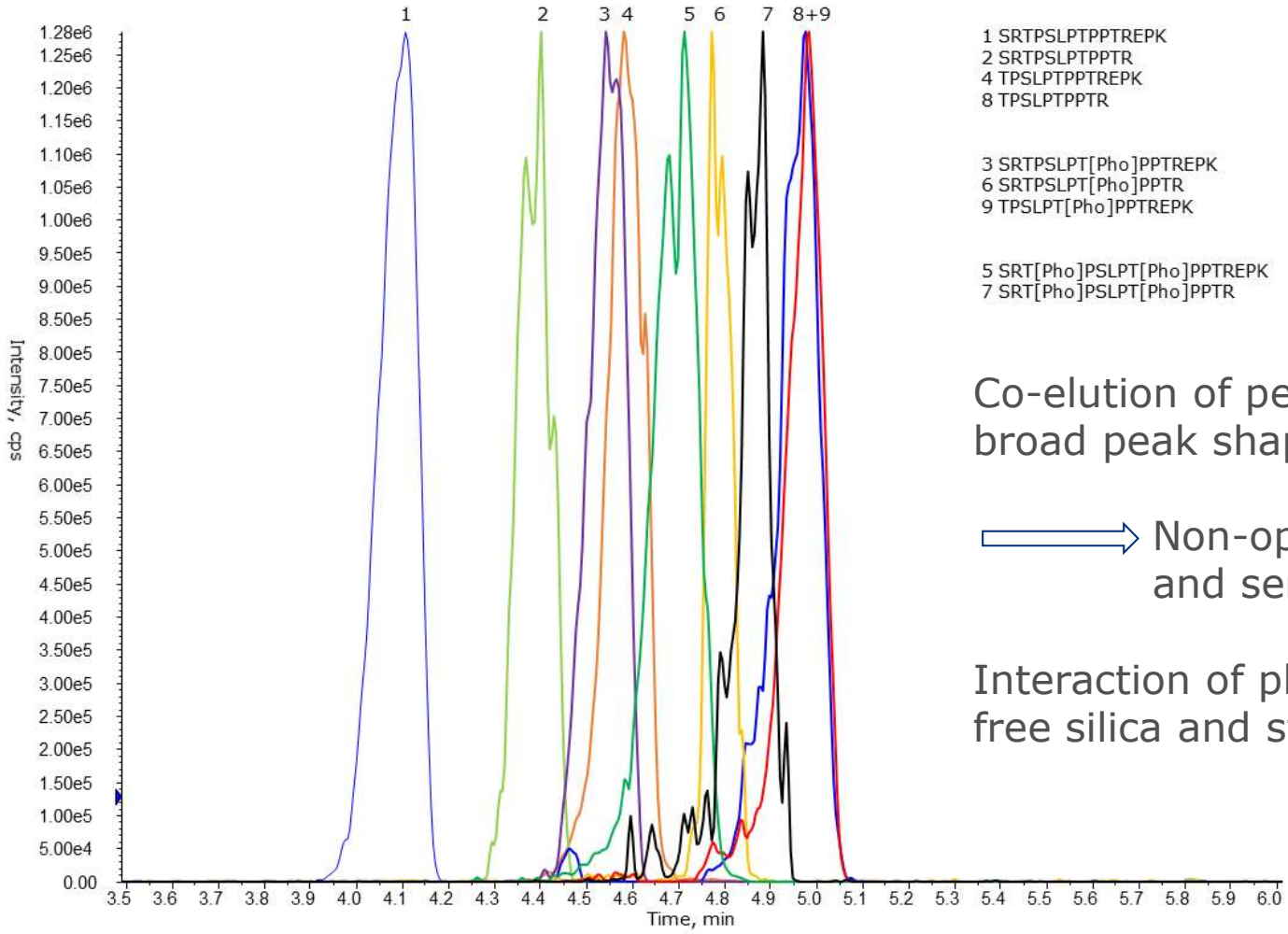
relative abundance relative to the most abundant ion per peptide:

	-	p217	P214/p217	p212/p217	p212/p214	P212/p214/p217	p210/p214/p217
TPSLPTPPTR	100	100	100	2.5	2.3	-	75*
SRTPSLPTPPTR	-	-	53	100	100	100	100
TPSLPTPPTREPK	12	18	32	-	-	-	26*
SRTPSLPTPPTREPK	-	-	19	50	15	64	52

*loss of phospho-moiety

Major influence of phosphorylation on trypsinization – site dependent

Chromatography at pH 3



Co-elution of peptides of interest, broad peak shapes

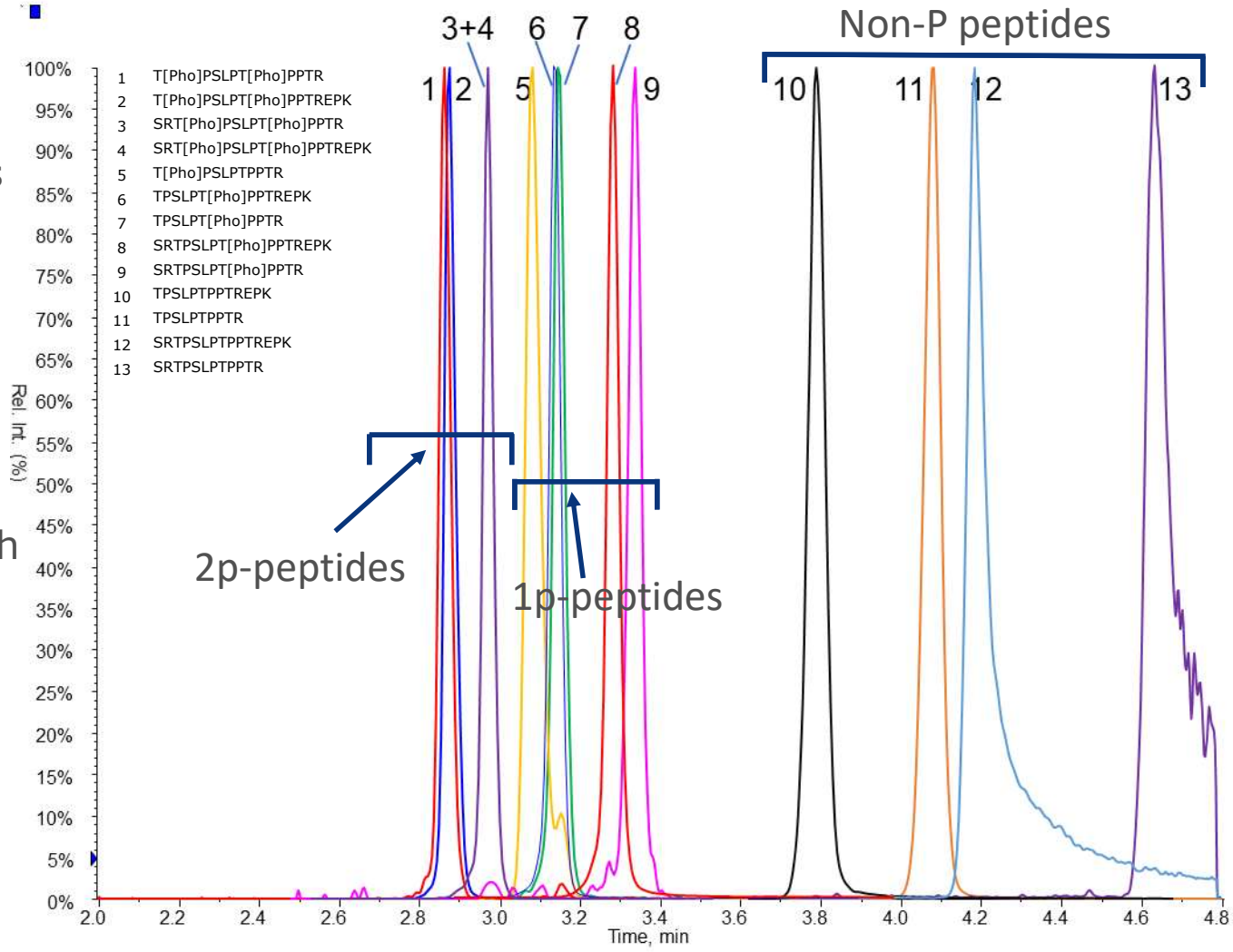
⟹ Non-optimal selectivity and sensitivity

Interaction of phospho-peptides with free silica and stainless steel at low pH

Chromatography at pH 11

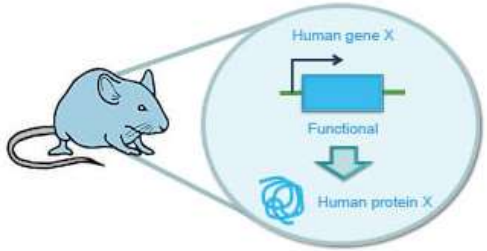
- Higher selectivity
- Narrow peak widths
- Higher sensitivity

pH 11 not possible with multi-DLC due to silica tubing!



Optimization of immunoprecipitation

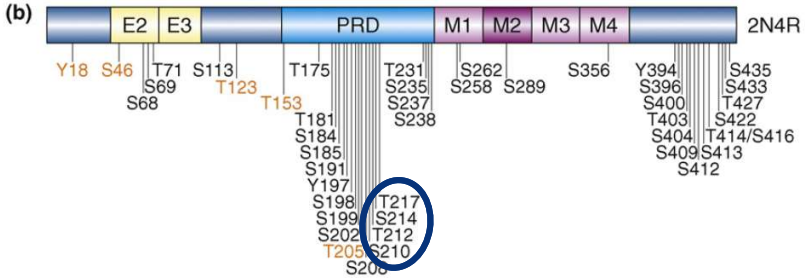
First optimization experiments were carried out on **brain homogenates of transgenic mouse expressing human Tau**, diluted in aCSF:



- Higher (p)Tau concentrations
- Availability for method optimization

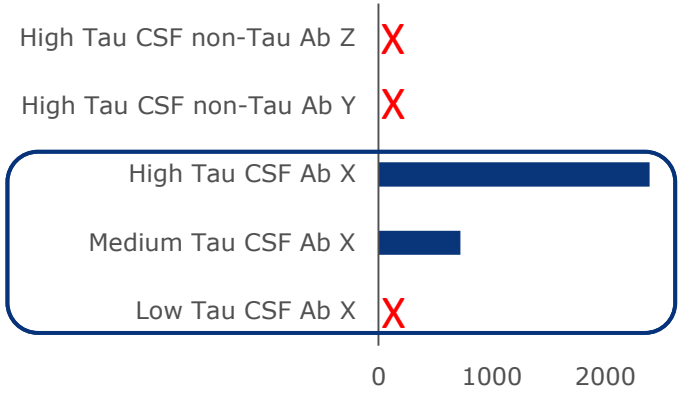
Sample preparation optimization → **Focus on pT212 and/or pT217**

- Amount of DTT
- Ab concentration
- Amount of beads
- On-bead digestion or elution



IP experiments: human CSF

Di-phospho peptide (2 miscl.): peak areas



Detection of **di-phospho peptide** in CSF not expected:

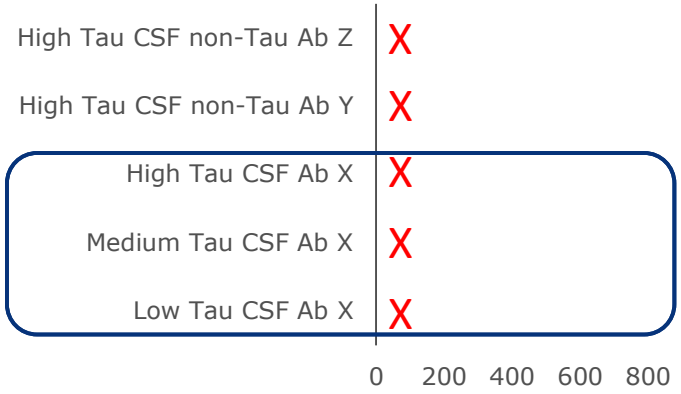
- Not reported in literature
- Ab highest affinity for Tau with pT212/pT217

→ FOCUS on "2 p 2 m" peptide

- Reference standard + SIL ordered
- High noise levels and ionisation suppression

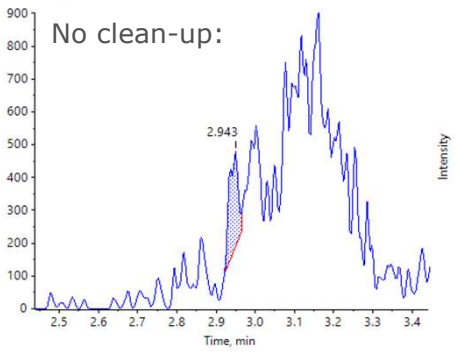
→ Optimization of LC-MSMS methods

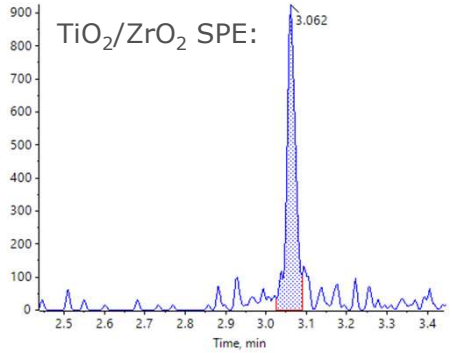
Mono-p peptide: peak areas



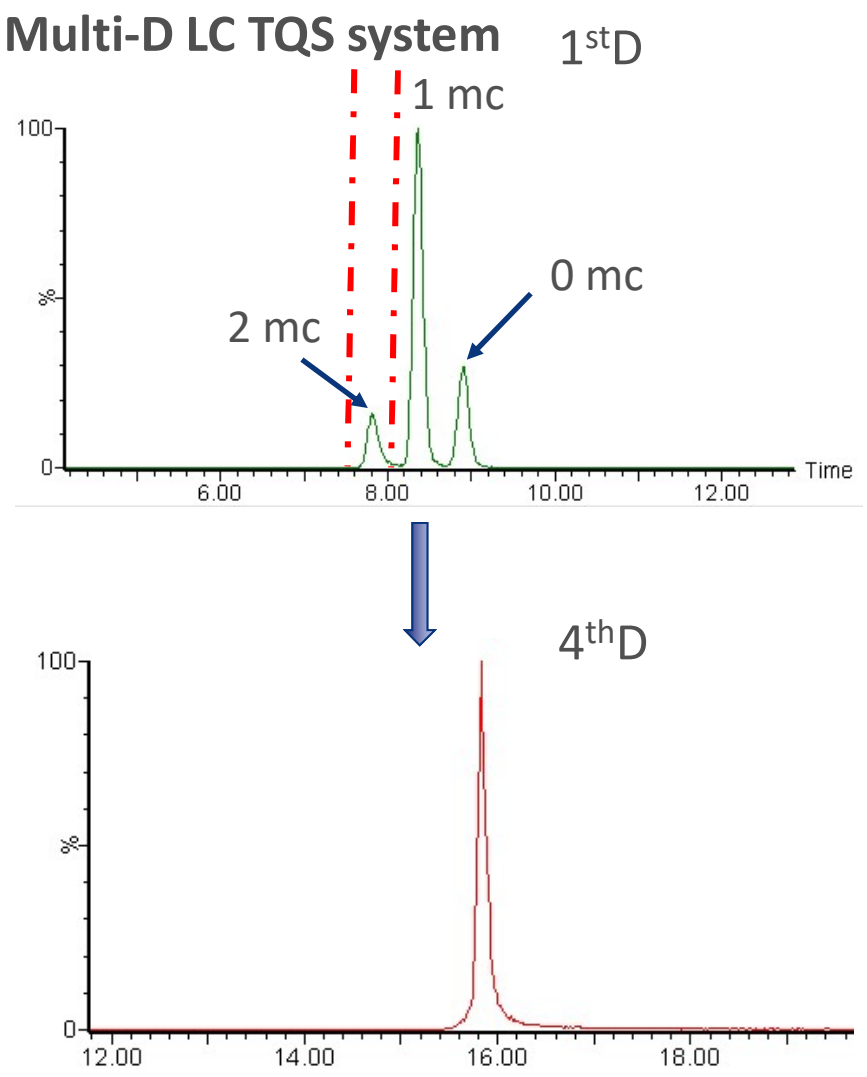
Mono-phospho peptides not detected!

→ Presence confirmed with additional off-line TiO_2/ZrO_2 SPE clean-up





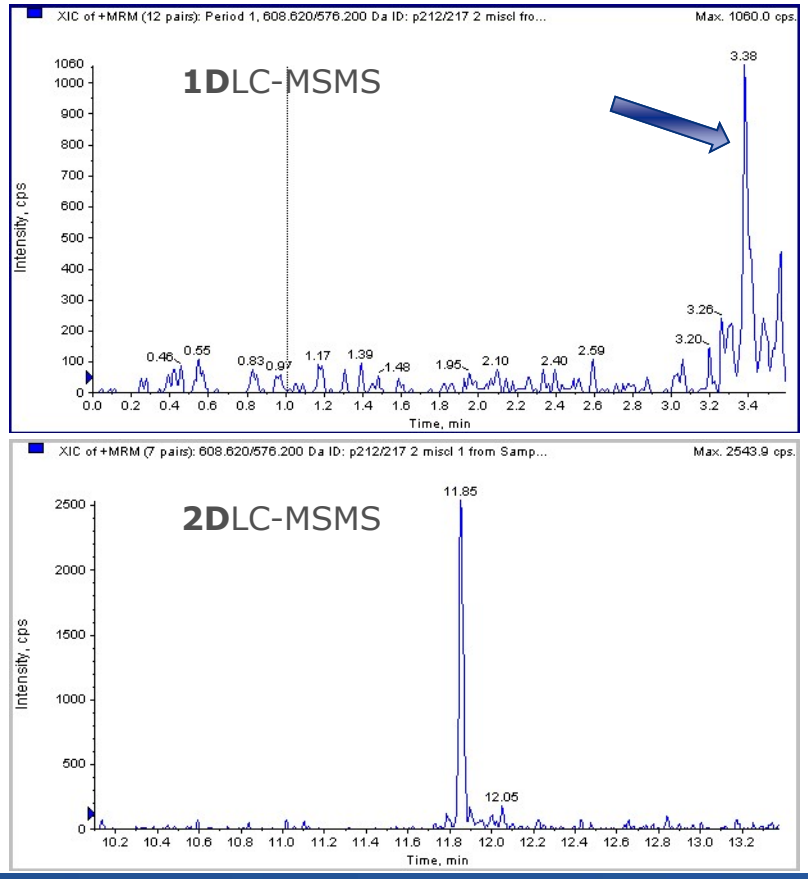
Focus on "2 p 2 m peptide" – multi-dimensional LC



Removal of matrix interferences with **2DLC-6500**:

Retention mechanism: metal oxide chromatography

Reduction of noise levels and increase in peak height



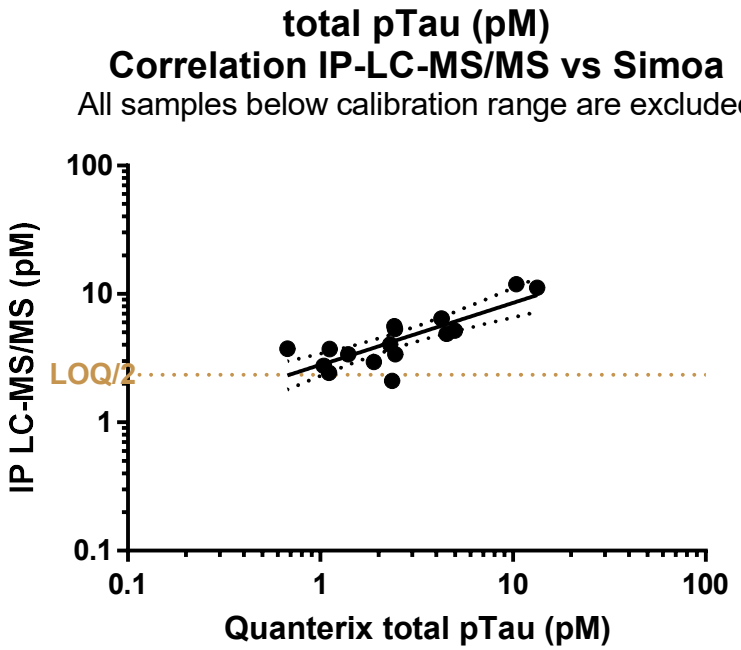
Quality control criteria

Method validation not possible in the traditional way due to scarcity of human CSF and endogenous presence of pTau.

Quality control criteria:

- Use of **stable isotope internal standard** (SIL)
- **Calibration curve:** method blanks (IP-ed artificial CSF) spiked at 0.5, 1, 2, 4, 6, 10 pM. Linear regression, $1/X^2$. Acceptance criteria: $|\%RE| \leq 20\%$ (LLOQ ≤ 25%), at least 4 standards accepted.
- **Blank:** method blank, no SIL
- **Zero:** method blank with SIL
- **Carry over**
- **QC samples:**
    - **IP depletion samples** were analysed with **SIMOA** to confirm all pTau is captured during IP
    - **Human CSF samples** from CSF pools with **low, medium and high (p)Tau** concentrations. Evaluation criterium: concentration 2 p 2 m peptide: low Tau CSF < medium Tau CSF < high Tau CSF
    - **Repeatability:** 3 replicates of 2-fold diluted medium Tau CSF. Acceptance criterium: at least 2 out of 3 should be ≤ 20% of the mean value.
    - **QC for batch acceptance:** method blanks spiked at 4 pM and 8 pM, analysis before and after samples. Acceptance criteria: $|\%RE| \leq 20\%$, at least 2 accepted, 1 of each set and 1 of each concentration.

Results clinical samples IP-LC-MSMS



Total pTau measured with **IP LC-MS/MS correlates with Simoa** measurement

Acknowledgments

Neurosciences	Discovery Bioanalysis	Development Bioanalysis
Clara Theunis	Farid Jahouh	Marc Verhemeldonck
Dina Rodrigues Martins	Tine Loomans	Luc Diels
Karolien Grauwen		Lieve Dillen
Marc Mercken		