Physikalisch-Technische Bundesanstalt
Braunschweig and Berlin
National Metrology Institute

Role of metals and metal containing biomolecules in neurodegenerative diseases such as Alzheimer's disease

(15HLT02 ReMiND)

C. Swart, C. Frank, G. Köllensperger, E. del Castillo

EURAMET

Al
Fe
Zn
Cu
Mg

Why metrology and traceability?

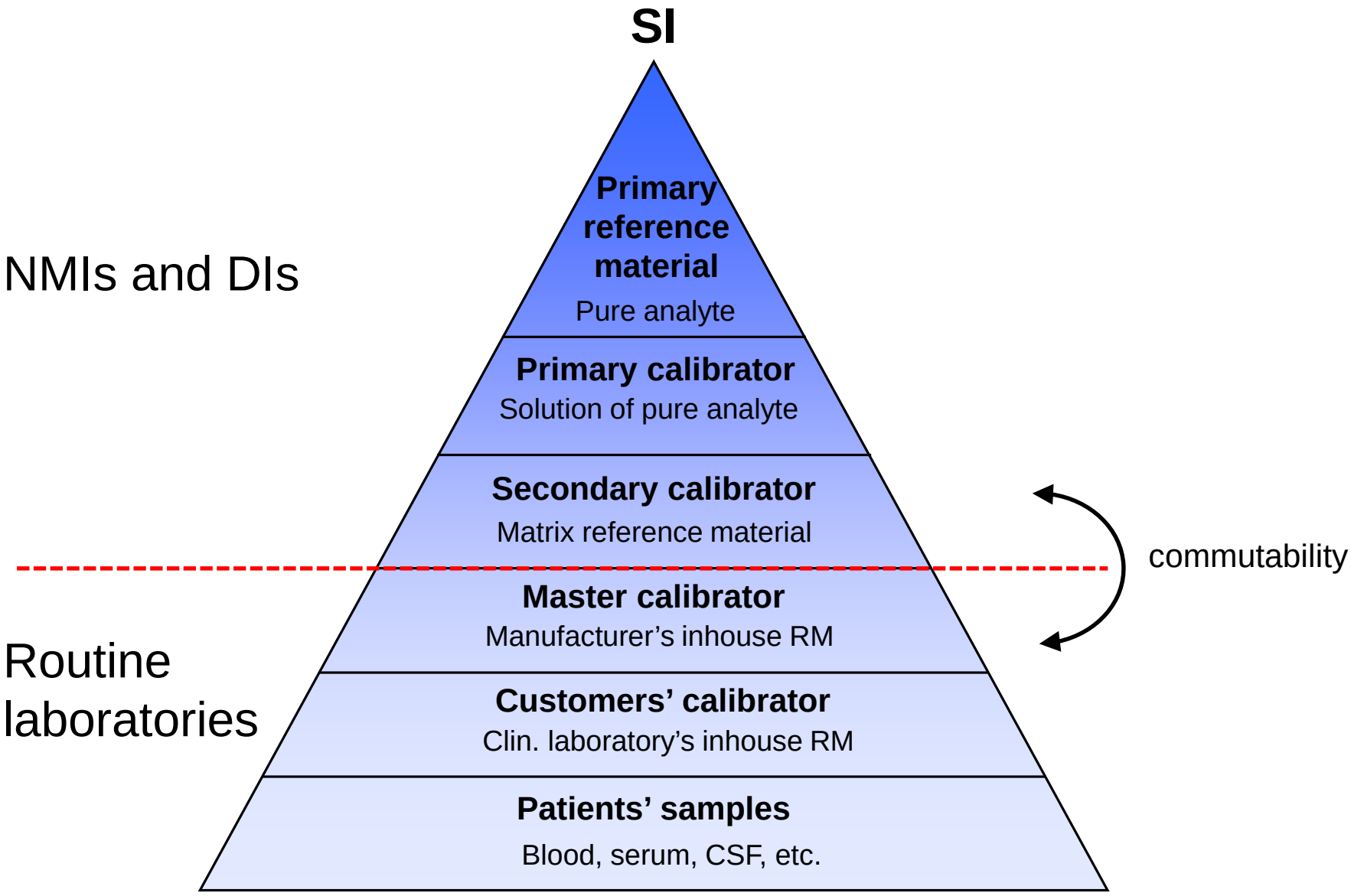
Metrological traceability

Property of a measurement result whereby the result can be related to a reference through a documented **unbroken chain of calibrations**, each contributing to the measurement uncertainty

Advantages of results traceable to the SI

- Comparability over a longer period of time
- Comparability even between laboratories and different measurement procedures
- Enable establishing universal cut-off values
- Results not depending on one calibrator

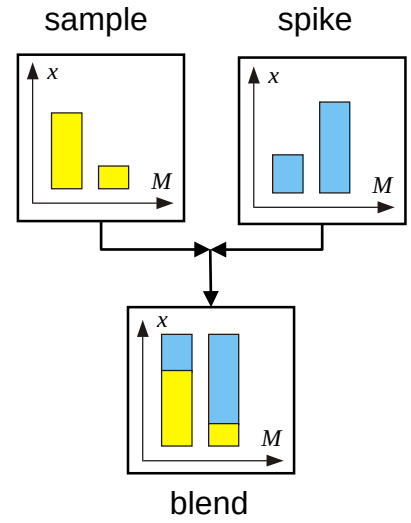
Traceability in laboratory medicine



Requirements and challenges

Reference measurement procedures

- Only for a few peptides and proteins available yet
- Exact definition of the analyte
- Mainly immune based methods ⇒ rather harmonisation then standardisation



and/or

Certified reference materials

- Only very few commercially available
- Clear definition of the important quantity (total content, specific modifications,…)

⇒ Alzheimer‘s Association Global Biomarker Standardization Consortium (GBSC) for QC; IFCC working group CSF for standardisation of β amyoid$_{1-42}$ for development of RMP and CRM (J. Kuhlmann et al., Clin. Chim. Acta 2017, 467, 27-33)

Motivation

β amyloid 1-42

Physiological function unknown, formation of plaques in the brain during aging, increased in Alzheimer patients

τ-protein

Stabilisation of microtubules in neurons, formation of tangles in the brain during aging, increased in Alzheimer patients

		N	Median	Ref	normal	borderline	pathological
Aß1-42	Kit 1	39	**517**	med. **500**	**18**	**11**	**10**
Aß1-42	Kit 2	8	**330**	**651**	**0**	**0**	**8**
tot Tau	Kit 1	42	442	med. 450	**21**	**6**	**12**
tot Tau	Kit 2	8	501	466	**4**	**2**	**2**
P Tau	Kit 1	40	33		**40**	**0**	**0**

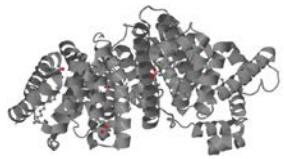
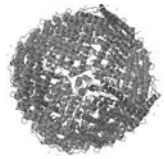
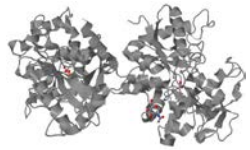
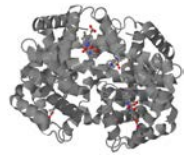
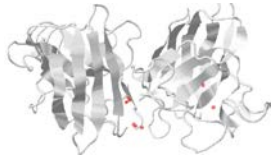
All concentrations given in ng/L

"The unacceptable large variation of the laboratory own cut-off values leads to false negative and false positive diagnostic interpretations. This questions the practical relevance of dementia marker analysis. "
(Reiber et al., J Alzheimers Dis Parkinsonism 2014, 4(3))

Potential markers for Alzheimer‘s disease

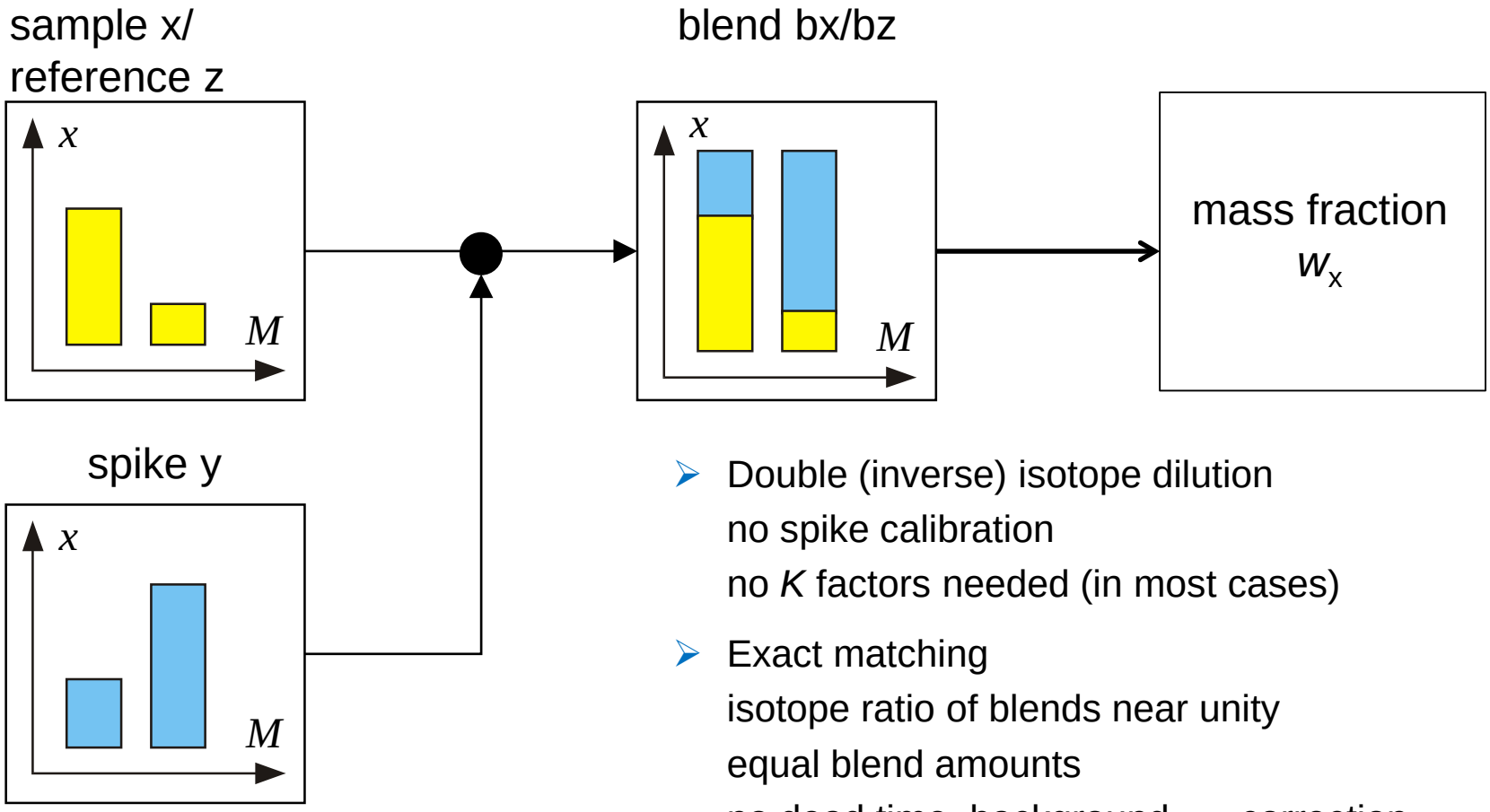
SHUTTLES FOR METALS:

OXIDATIVE STRESS PREVENTION

	Human serum albumin (HSA)	Regulation of colloidal osmotic pressure of blood, unspecific binding of metals (including toxic ones)
	Ferritin (FER)	Fe storage protein, incorporation also of other metals (including toxic ones) with similar properties
	Transferrin (TRF)	Fe transport protein, Fe is suspected to be involved in plaque formation, free Fe causes oxidative stress
	Haemoglobin (HGB)	O_2 transport protein, used for control of blood contamination in CSF
	Cu, Zn-superoxide dismutase (SOD1)	Oxidative stress preventation, acute phase protein, indication for inflammation processes
	Ceruloplasmin (CER)	Cu storage protein, ferroxidase, Cu is suspected to be involved in plaque formation

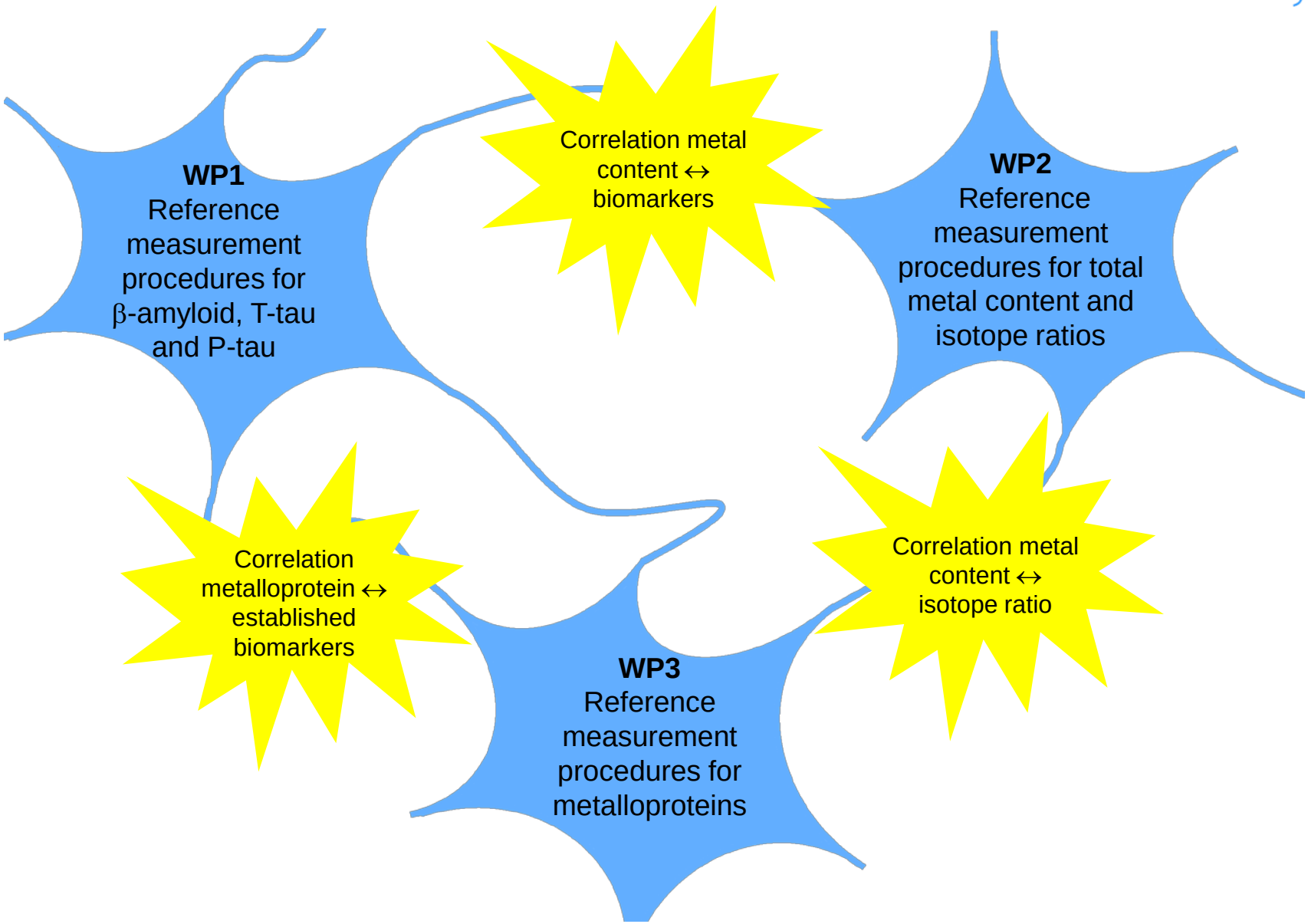
Reference measurement procedure

Isotope Dilution (ID)



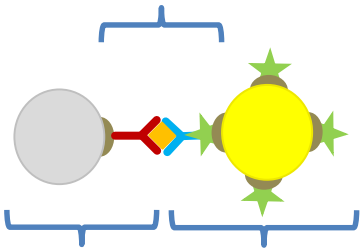
- Double (inverse) isotope dilution
  no spike calibration
  no *K* factors needed (in most cases)
- Exact matching
  isotope ratio of blends near unity
  equal blend amounts
  no dead time, background, … correction
  both blends share the same “fate”

EMPIR project 15HLT02 ReMiND



SERS based immuno assay for quantification of T-tau

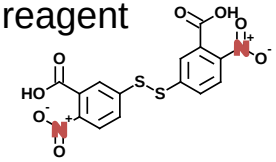
specific interactions



separation

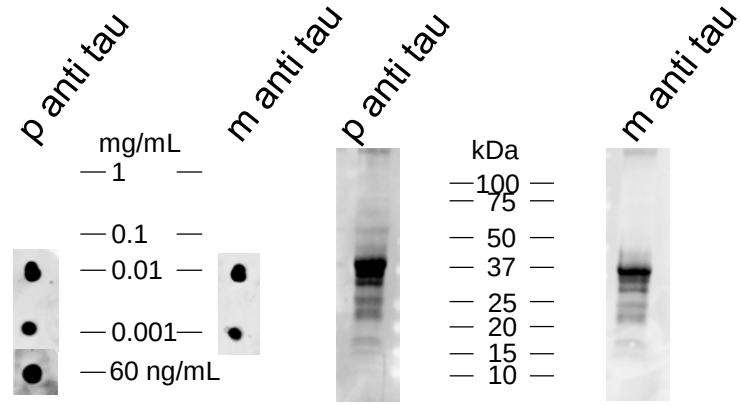
detection, quantification

DTNB:
Ellman‘s reagent

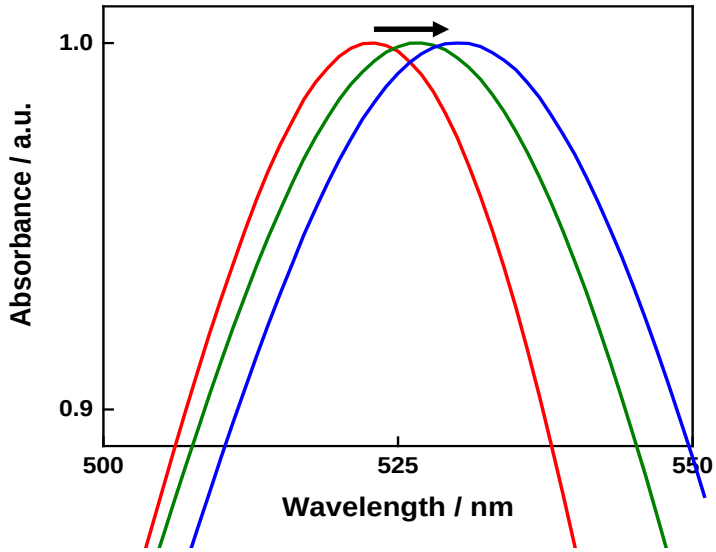


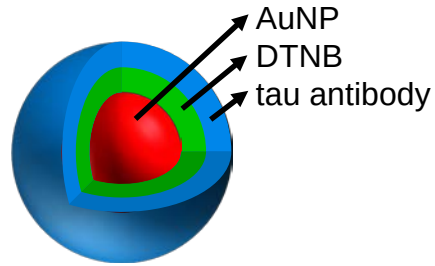
Dot Blot (tau protein)

Western Blot (pig brain extract)

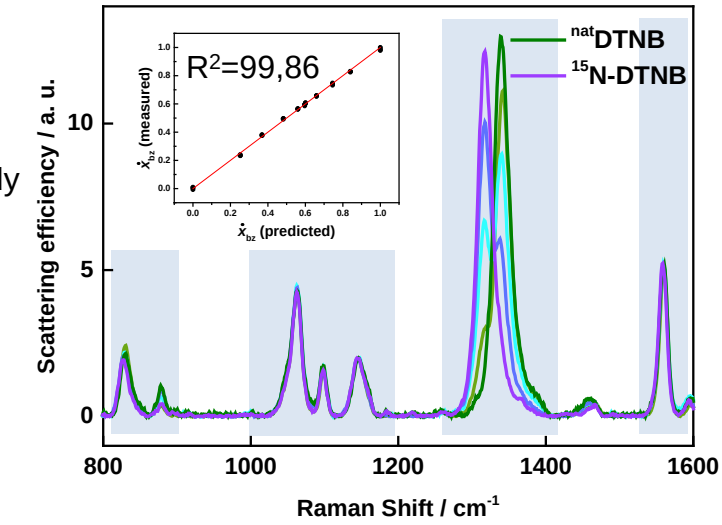


UV/Vis



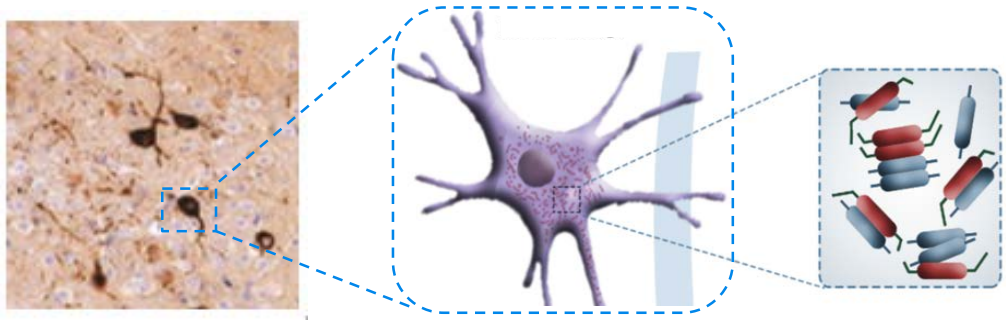


Raman



Determination of T-tau and P-tau

Workflow

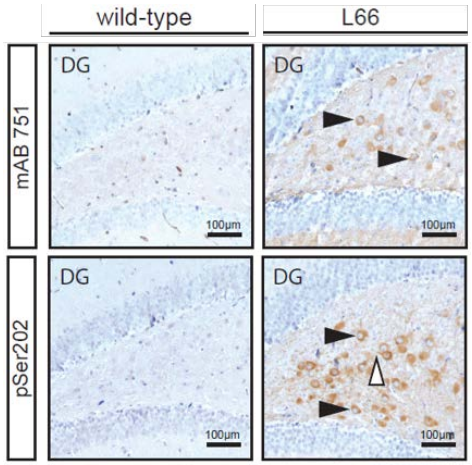


Brain from tau transgenic mice
Alzheimer‘s disease model

Neuron

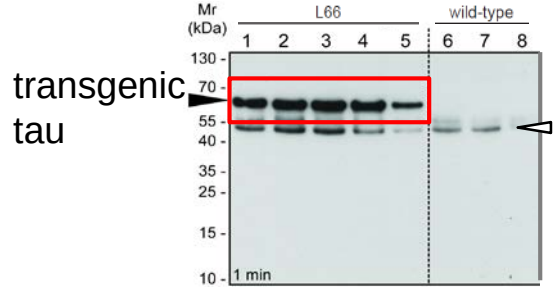
Tau protein

Preliminary tests:



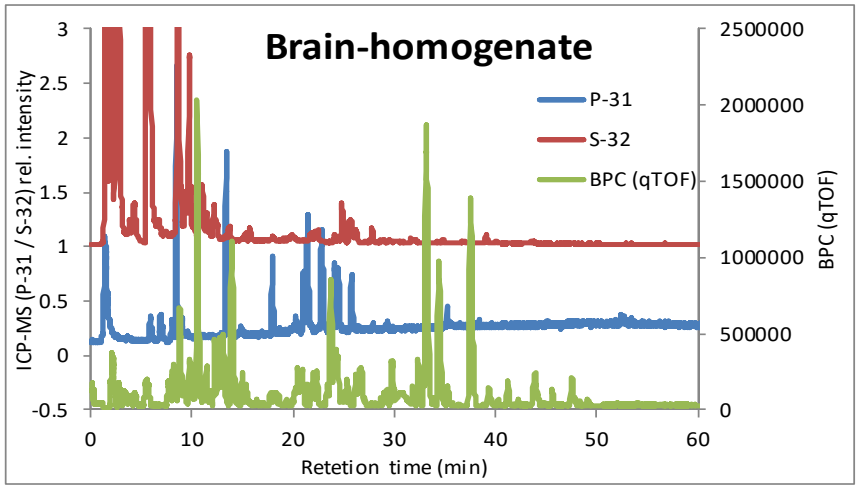
Immunohistopathological staining of tau and phospho-tau in mouse brain
DG: Dentate Gyrus

- Biochemical evaluation of antibodies
- Testing of different protein extraction and immunopurification methods



Immuno-blotting of tau
in mouse brain lysate

Determination of T-tau and P-tau

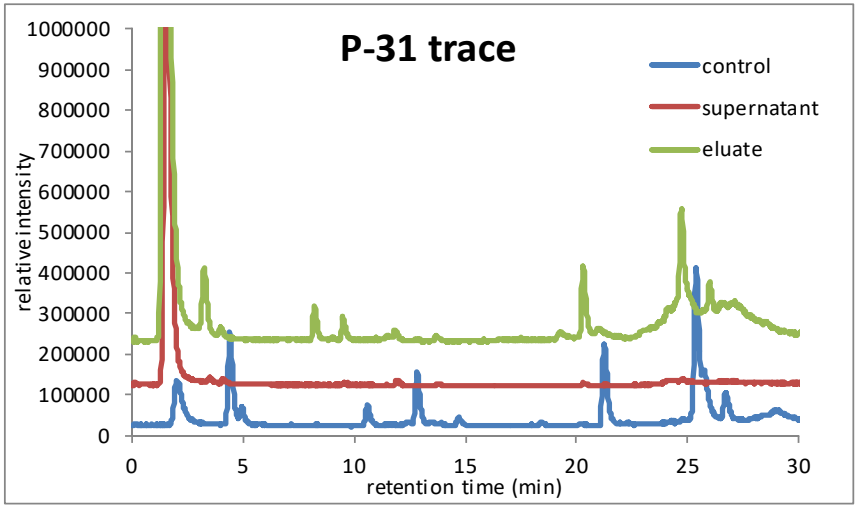


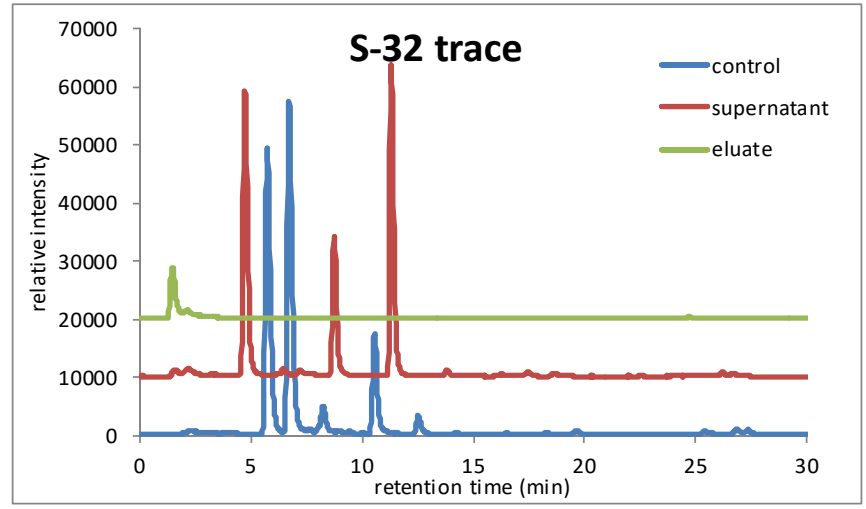
To many peptides in brain homogenate to allow identification of tau derived peptides

P-enrichement via

Al-silicate spin column (75 % recovery, S-depletion 10 %)

GdO-nanoparticles (60-80 % recovery, S-depletion 20 %)

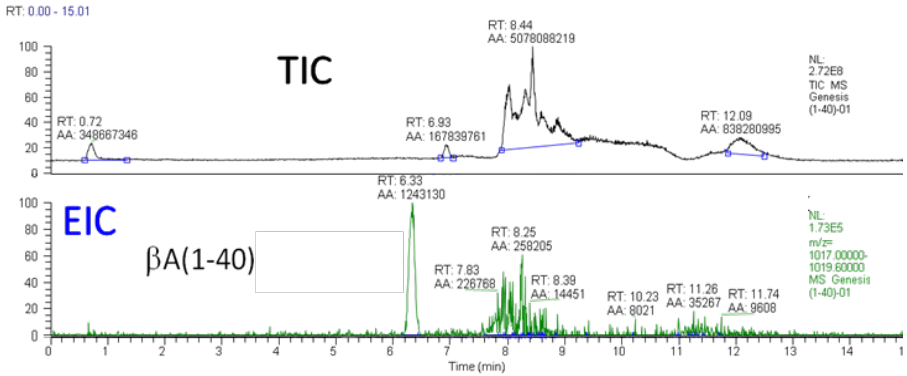


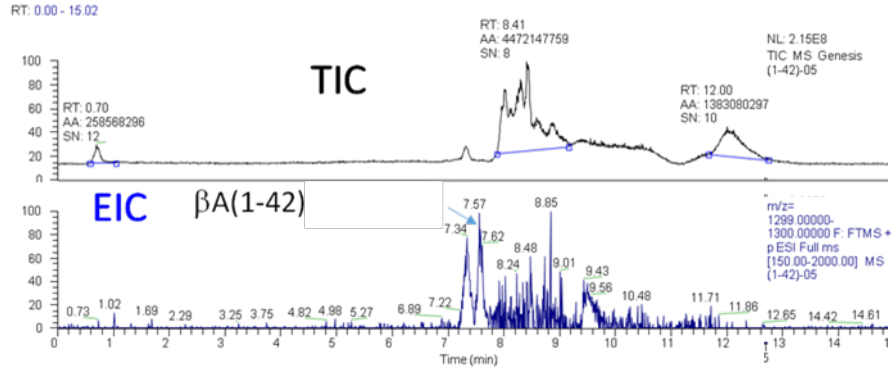


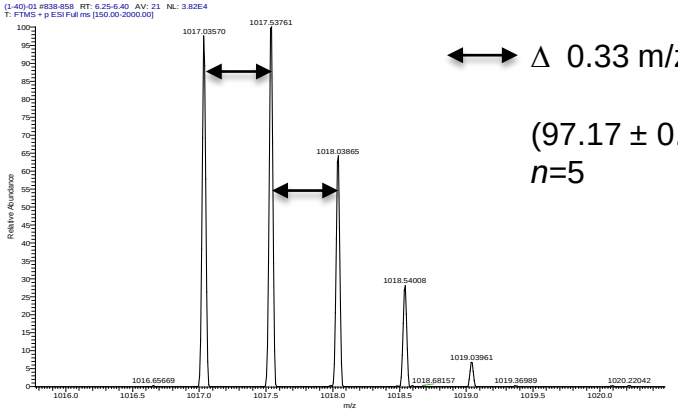
Quantification of β-amyloid using amino acid analysis (AAA)

β-Amyloid (1-40)
DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVV

β-Amyloid (1-42)
DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVVIA

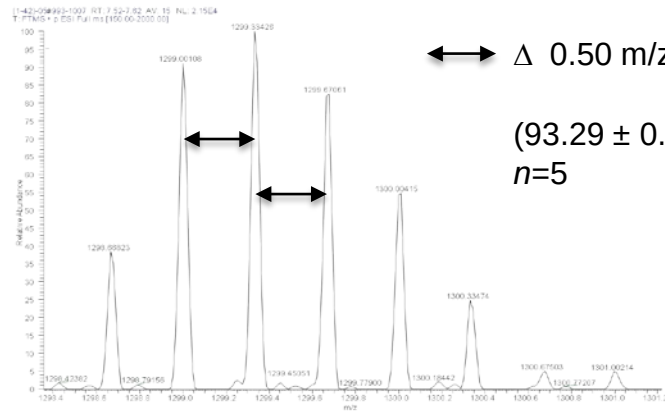






↔ Δ 0.33 m/z / +3

(97.17 ± 0.04) %
n=5



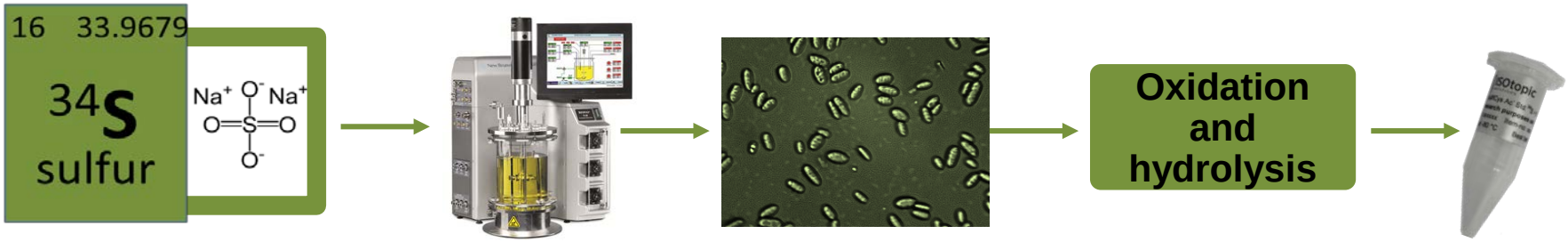
↔ Δ 0.50 m/z / +2

(93.29 ± 0.11) %
n=5

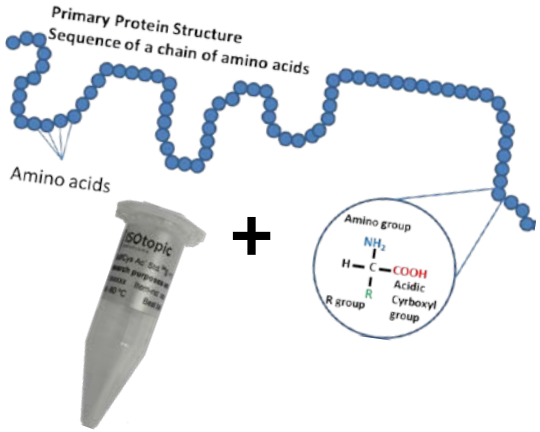
Quantification of β-amyloid using AAA

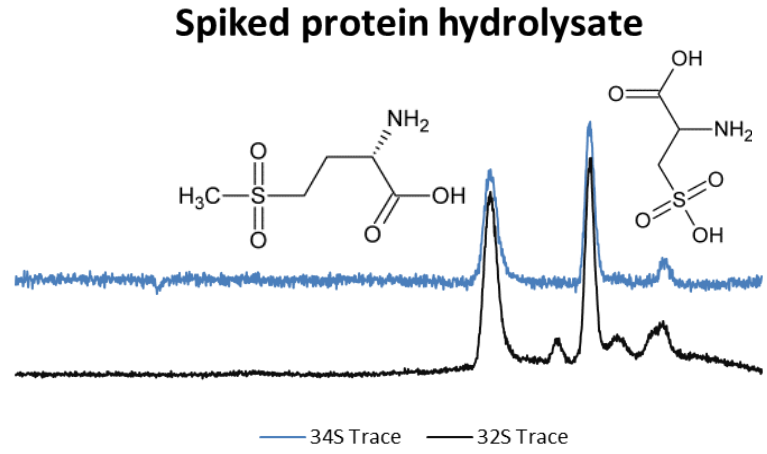
Sodium sulfate to label biomass to obtain biomass highly enriched in ^{34}S

> 95% ^{34}S Met sulf and Cys Ac$^-$
For absolute protein quantification via ICP-MS

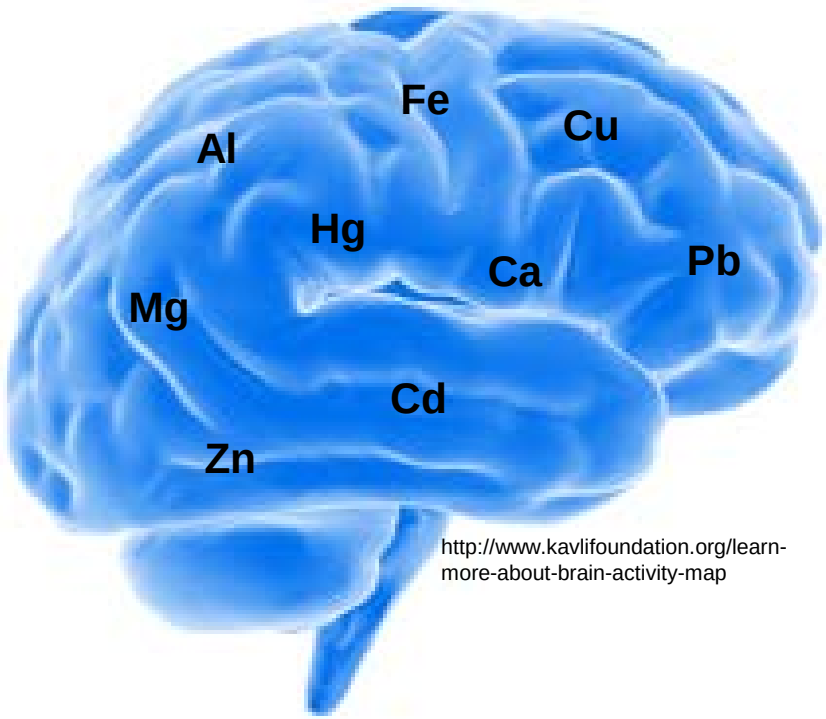


Oxidation and spiking → Hydrolysis → LC-ICP-MS





Total metal content and isotope ratios



http://www.kavlifoundation.org/learn-more-about-brain-activity-map

- Metals suspected to facilitate aggregation of β-amyloid
- Leasions in the brain caused by free and/or toxic metal ions
- Lack of essential elements can lead to brain malfunction

First results for CSF (Randox L2)

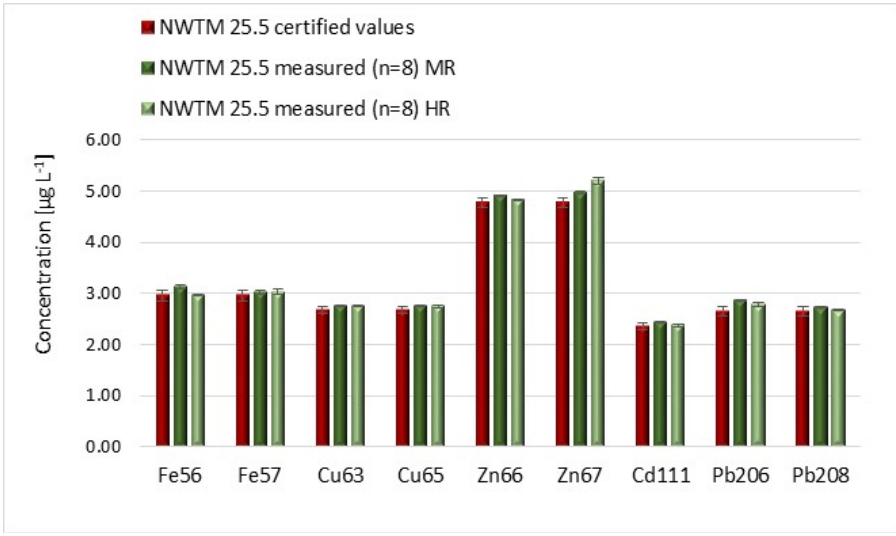
Element	Conc. / ng/g
Cu	1.3 ± 0.04
Zn	30.4 ± 0.3

Determined with double IDMS after acidic microwave digestion and detection with ICP-MS

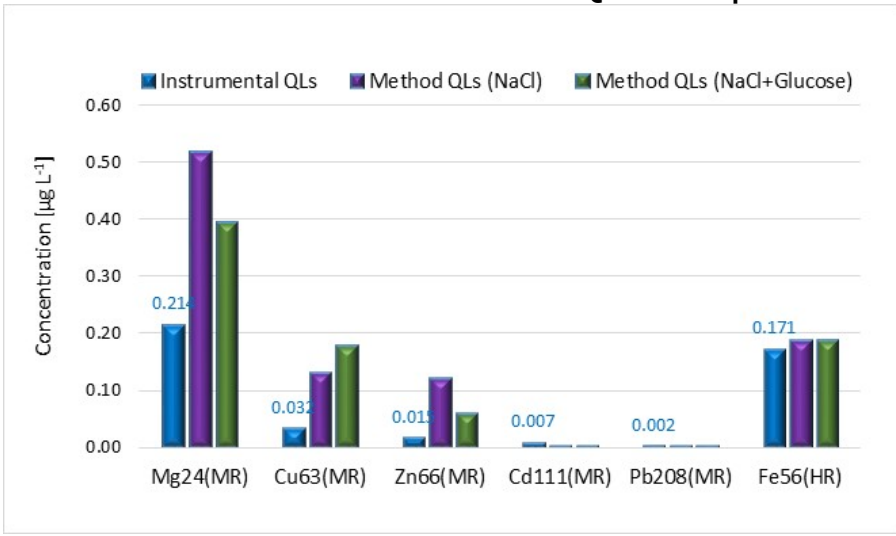
Changes in biological pathways already early on in the course of disease can lead to changes in isotope ratios of elements involved

⇒ **Sensitive markers as early-warning system**

Total metal content



Water reference material/ QC sample



Instrumental/procedural limits of quantification

Sector field ICP-MS

Advantage:

High resolution of interferences

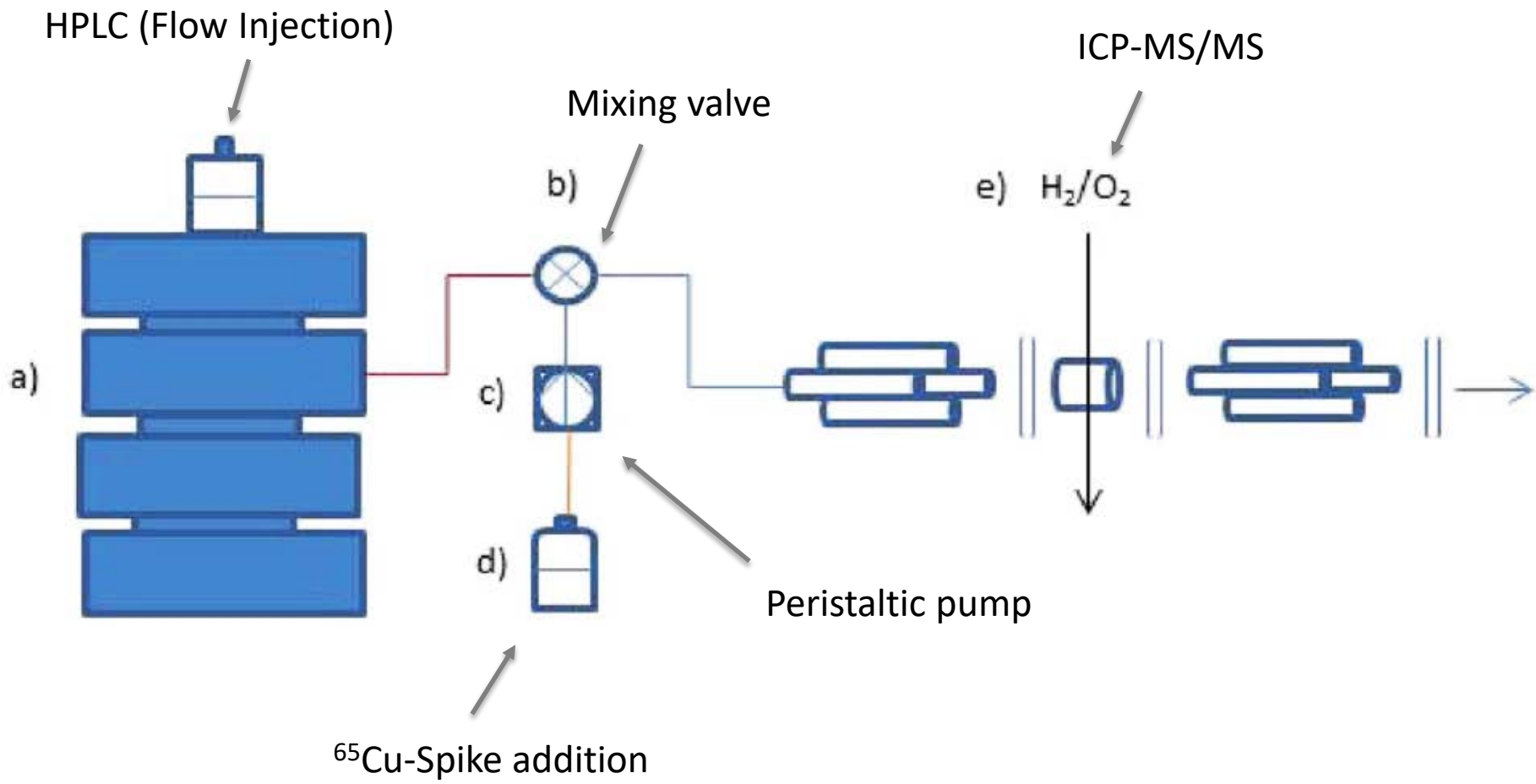
High sensitivity at low resolution

Disadvantage:

Large sample volumes required

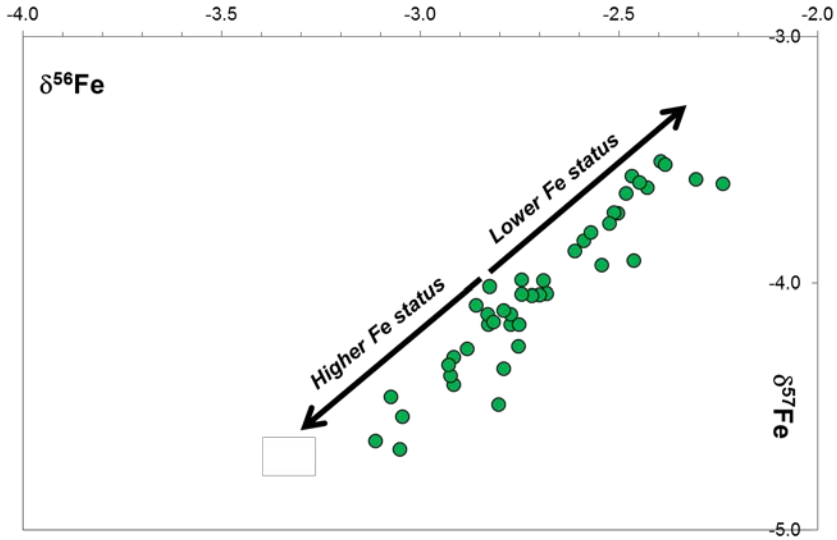
⇒ Not available for CSF samples

Total metal content in CSF



Metal isotope ratios

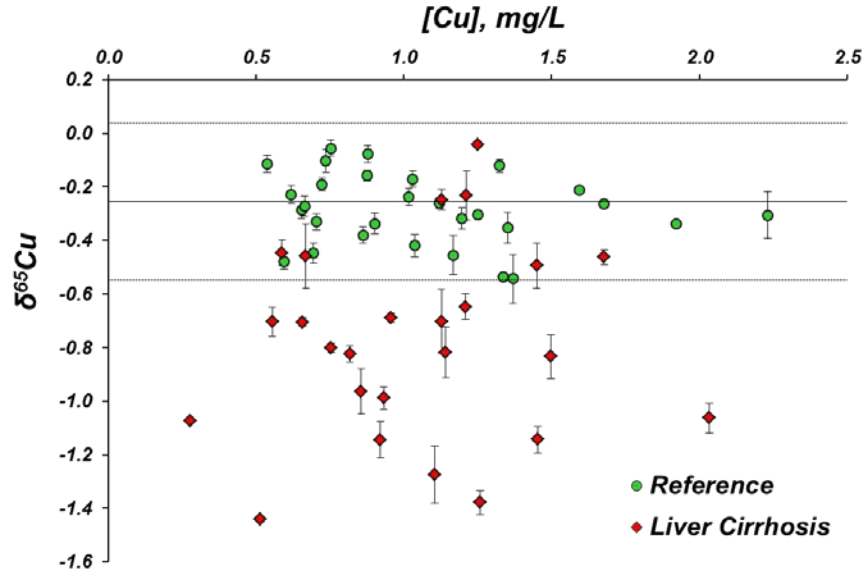
Whole blood Fe isotope ratios reflect iron status



- Individuals with anemia of chronic disease show abberant results
- Individuals with heochromatosis show abberant results

Van Heghe et al, Metallomics, 5, 1503-1509, 2013

Serum Cu isotope ratio indicates liver problems



- Link between $^{65}Cu/^{63}Cu$ & severity of condition
- Successful $^{65}Cu/^{63}Cu$ follow-up after liver transplantation

Costas-Rodriguez et al, Metallomics, 7,491-498, 2015

Traceable quantification of metalloproteins

Key Analytical Challenges

Analytical challenges → **Metrological approach**

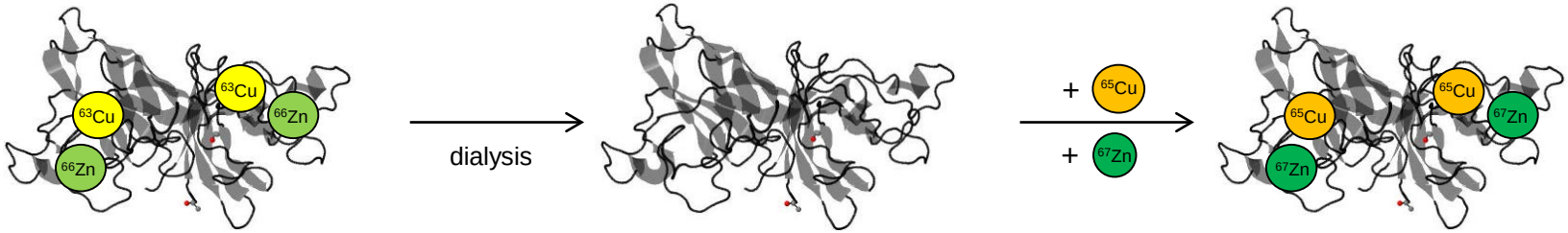
- Low concentrations of metals/ biomolecules in serum, CSF and brain tissues
- Very small samples (low µL)
- Complex matrices
- Potential species transformation?
- Unavailable well characterised calibrants/spikes

- Miniaturised high resolution separation techniques (µl/nl flow rates and sample injection) and minimised interaction of the biomolecules with surfaces
- Interference-reducing ICP-MS (e.g. QQQICP-MS)
- Strategies for the production and characterisation of calibrants/spikes
- Isotope dilution calibration

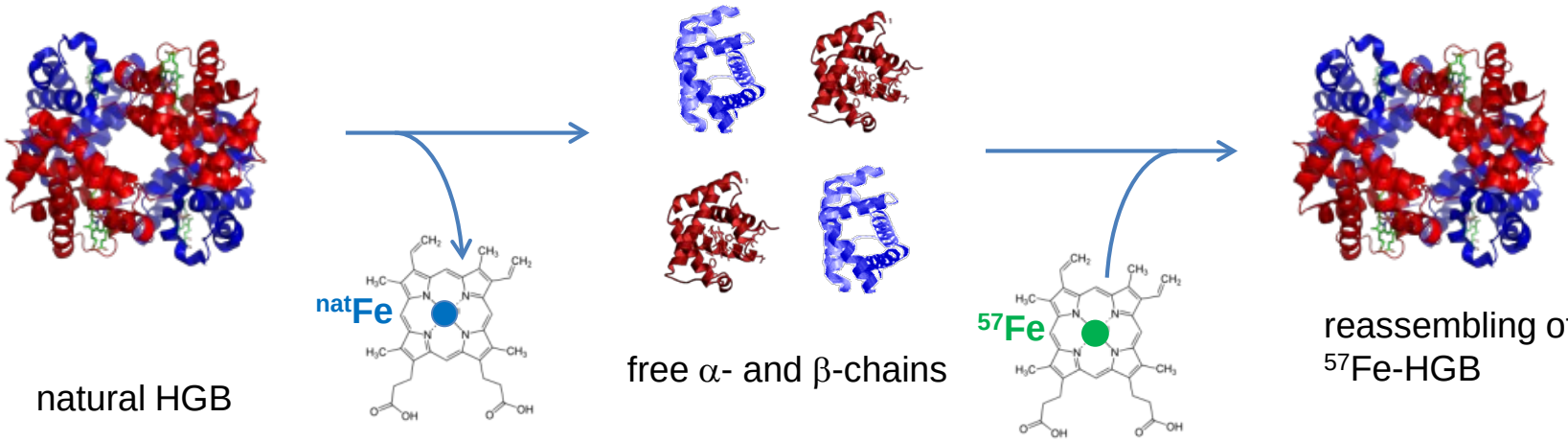
Traceable quantification of metalloproteins

Species specific IDMS

- Exchange of natural metal ions in proteins: SOD1, CER, ALB, TRF, FER



- Exchange of metal containing prosthetic groups



natural HGB

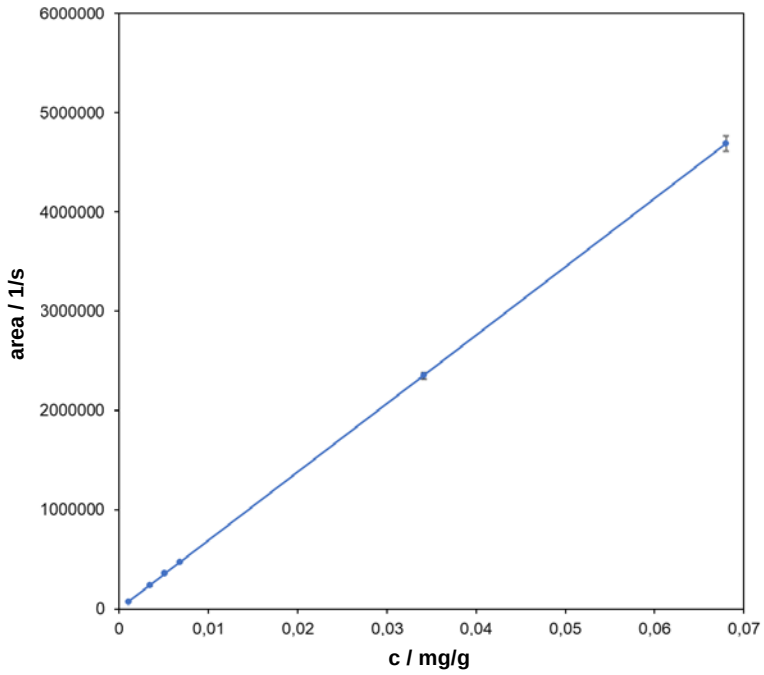
free α- and β-chains

reassembling of ^{57}Fe-HGB

- Recombinant production of isotopically enriched proteins and peptides: FER, P-peptides

Quantification of metalloproteins in CSF

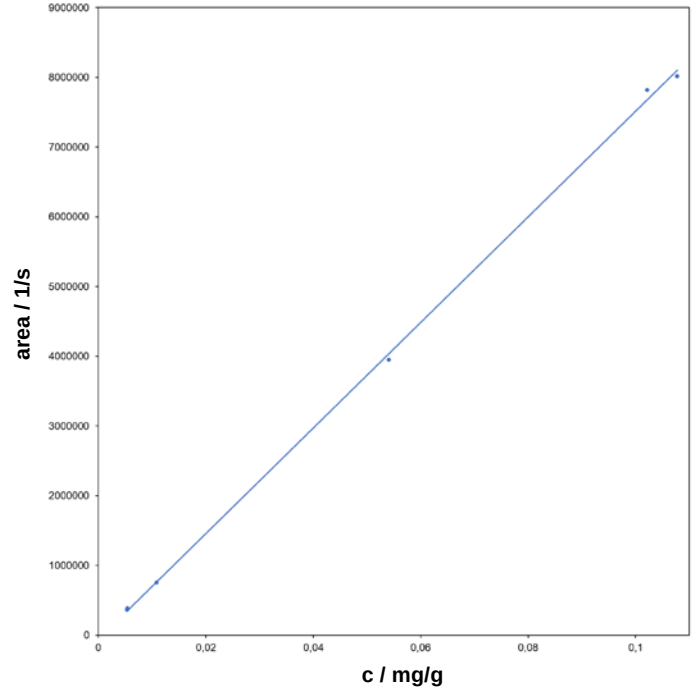
SOD1



LOD: 0.053 µg/g

LOQ: 0.158 µg/g

HBA_0



LOD: 0.733 µg/g

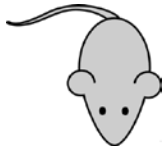
LOQ: 2.20 µg/g

Planned acitivities

MODEL SAMPLES

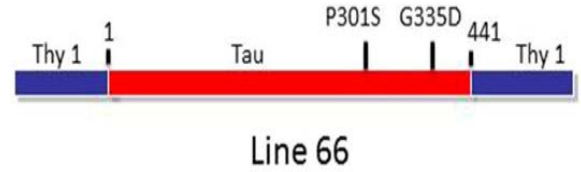
- Serum
- Artificial CSF
- Brain homogenates

REAL SAMPLES



- Mice samples
- Human samples

- **Tau-transgenic** mice samples (Charité)
  - L66 transgenic mice overexpress longest human tau isoform (441 AA)
  - Htau40 including 2 aggregation promoting mutations, P301S & G335D



Line 66

Melis, V., et al. *Cellular and molecular life sciences* 72.11 (2015): 2199-2222.

- **β-amyloid overexpressing** mice samples (University of Aberdeen)

Impact

- Stakeholder workshop at kick-off meeting 2016
- Workshop at Metallomics 2017 with invited speakers
- Presentations (oral / poster) at conferences a.o.:
  Metallomics 2017
  Emerging Analytical Professionals Conference 2017
  14th International Conference on Nanosciences and Nanotechnologies
- Presentation at Euramet TC-MC meeting

Consortium

Coordination, lead WP1
Determination of proteins using ICP-MS and Raman

Lead WP2
Multielemental and isotopic analysis

Lead WP 3
Quantification of metal containing biomolecules

Lead WP4 (Impact)
Quantification of potential biomarkers, provision of animal models

Multielemental and isotopic analysis

Quantification of metal and provision of animal models

Quantification of metals and metalloproteins

Isotopic analysis in metalloproteins

Collaborators

- INSTAND e.V.
- Sigma-Aldrich
- McGill University
- NIST
- Biobank of the Hertie Institute for Brain research
- NIS
- NIM China

15HLT02 ReMiND has received funding from the EMPIR programme co-financed by the Participating States and from the European Union's Horizon 2020 research and innovation programme.

Physikalisch-Technische Bundesanstalt
Braunschweig and Berlin
Bundesallee 100
38116 Braunschweig

Claudia Swart
Telefon: 0531 592-3150
E-Mail: claudia.swart@ptb.de
www.ptb.de