Can we validate an assay for which we have not identified the analyte ?

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Summary

Topics

- The challenge faced
- Comparable bioanalytical challenges in PK/PD domain
- Current approach
- Future needs
- Conclusions

The challenge

In summary

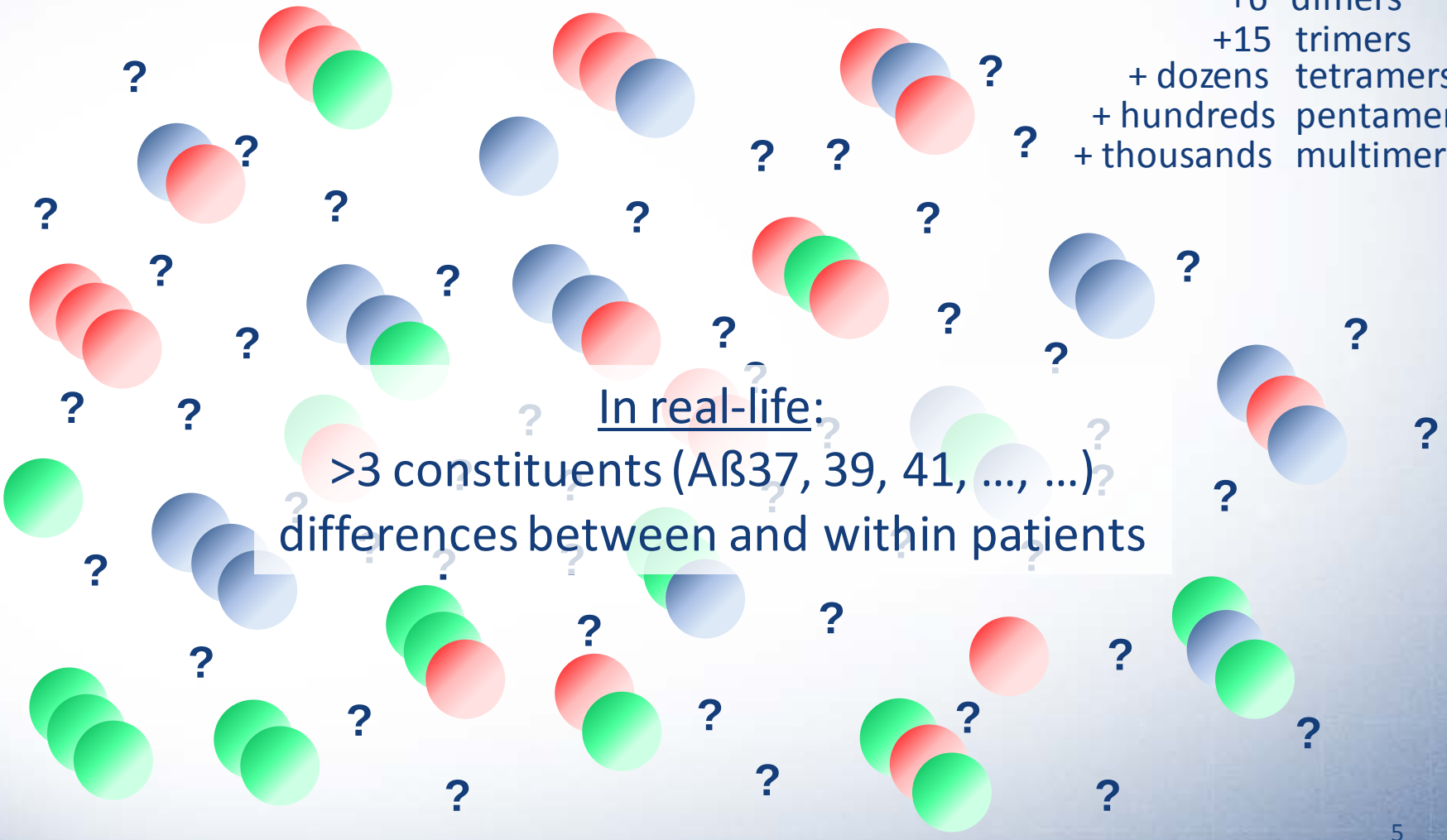
Amyloid-ß (Aß, Abeta) aggregates in Alzheimer's

- Aß peptide monomers aggregate to form plaques
- Plaques cause neurodegeneration in brain and hence Alzheimer's Disease
- Currently monomer bioanalysis used as biomarkers
- As a PD marker, a goal should become to
  - measure effect of therapy on plaque formation, degradation
  - In stead of monitoring monomer Aß, ideally monitor all different aggregates (or selection, signatures, pattern)

A complex mixture

Assuming only 3 different constituents (e.g. Aß38, 40, 42)

3 monomers
+6 dimers
+15 trimers
+ dozens tetramers
+ hundreds pentamers
+ thousands multimers



In real-life:
>3 constituents (Aß37, 39, 41, …, …)
differences between and within patients

A complex mixture

How can we monitor such a complex mixture and evaluate therapies ?

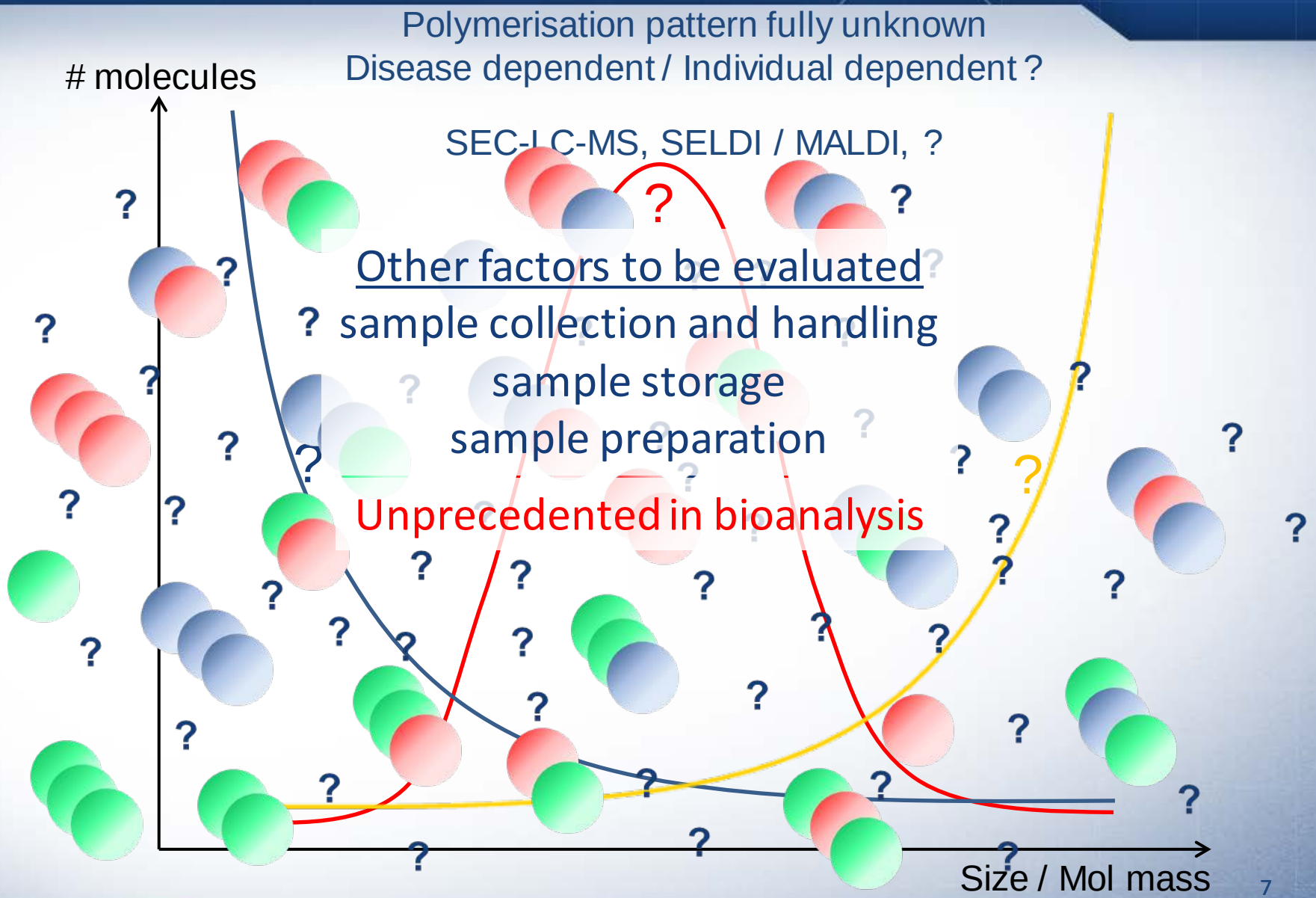
Monitoring all will proof impossible

Pattern recognition ?

Select and monitor signature/model aggregates ?

Overcome differences between and within patients ?

Intact aggregates bioanalysis



Other complex analyte challenges for PK, efficacy and safety analysis

Expl #1 - Copaxone

Glatiramer acetate (formerly: copolymer-1)

- Complex ***heterogeneous*** mixture of acetate salts of synthetic polypeptides, containing
  - 4 naturally occurring AAs:

L-glutamic acid	Avg mole fraction=	0.141
L-alanine		0.427
L-tyrosine		0.095
L-lysine		0.338

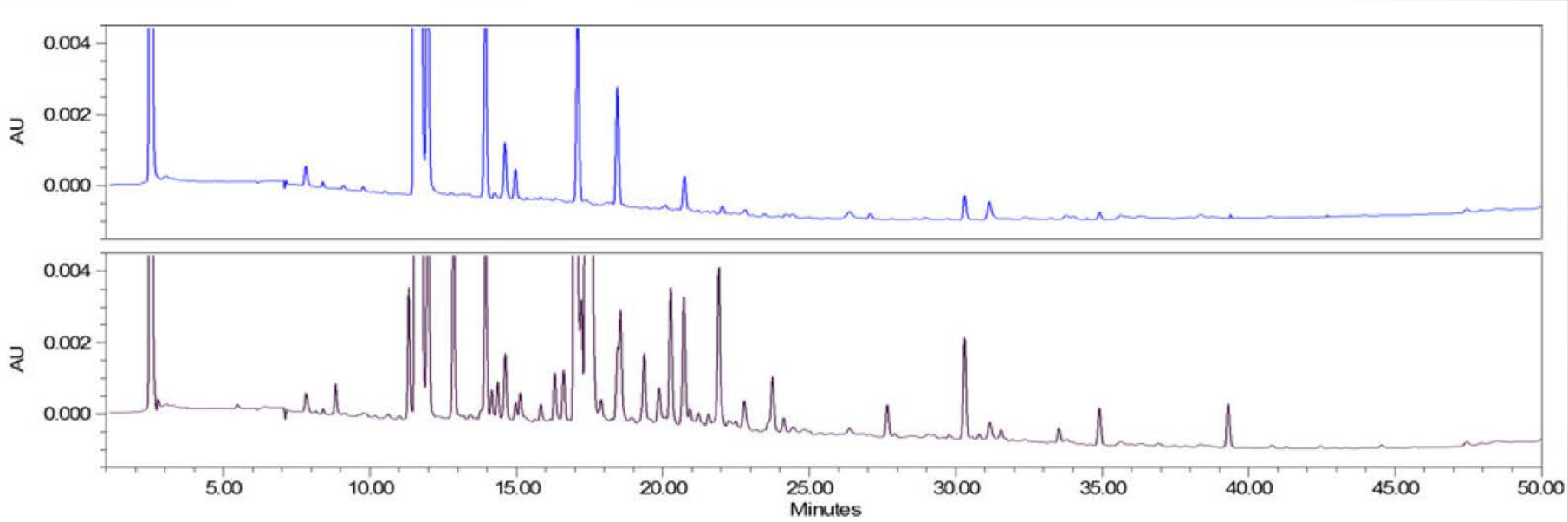
$$(Glu, Ala, Lys, Tyr)_x \bullet xCH_3COOH$$

$$(C_5H_9NO_4 \bullet C_3H_7NO_2 \bullet C_6H_{14}N_2O_2 \bullet C_9H_{11}NO_3)_x \bullet xC_2H_4O_2$$

  - Average MW: 5000-9000 Da, >1000 amino acid sequences
  - Batch-to-batch consistency, safety and efficacy 'ensured' by:
    - well-controlled manufacturing processes
    - rigorous QC procedures specifically for GA analysis

Expl #1 - Copaxone

Approved originator and approved generic API comparison



Schellekens et al, The AAPS J, 2013

Fig. 2. Comparative peptide mapping of originator product Copaxone (*blue*, upper trace) and a follow-on glatiramoid (*black*, lower trace) showing clear indication of differences in the primary structure of the drug amino acid sequences (22)

Expl #1 - Copaxone

For further background reading:
Schellekens et al, Reg. Tox. Pharmacol. 59 (2011) 176–183

Current approach for clinical evaluation of safety, efficacy

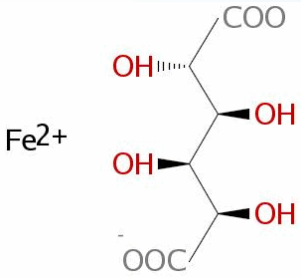
- Glatiramoid products complexity is much higher than most biological products
  - Minor manufacturing differences may produce altered sequences, which display different efficacy and safety profiles
  - impossible to isolate and identify active sequences in GA
  - not possible to predict glatiramoid toxicity from structural characterization and in vitro studies
  - Multi-epitopal nature thought of being the source of its widespread biological and clinical activity
  - Active sequences, epitopes within the GA mixture responsible for efficacy and safety unknown.

How can we relate efficacy & safety to exposure if we have no concrete PK ?

PD is not an alternative due to the mixture.

Expl #2 – Iron Sucrose

Iron-oxyhydroxide carbohydrate drugs



- Colloidal IV iron preparations
  - colloids of spheroidal iron-carbohydrate nanoparticles.
    - at particle core there is an iron-oxyhydroxide gel
    - core is surrounded by a shell of carbohydrate to stabilizes the iron-oxyhydroxide - slows the release of bioactive iron
      - maintains colloidal suspension

	Particle		Core		Shell Carbohydrate
	Diameter (nm)	Shape	Diameter (nm)	Shape	
Ferric gluconate	3 ± 1	Spheroid	2 ± 1	Spheroid	Bound gluconate, loosely associated sucrose
Iron sucrose	7 ± 4	Spheroid	3 ± 2	Spheroid	Bound sucrose
Iron dextran	30 ± 10	Spheroid	20–35 × 6	Ellipsoid	Bound dextran polysaccharide

Expl #2 – Iron Sucrose

Iron-oxyhydroxide carbohydrate drugs

- Treatment of iron deficiency anemia
- Clearance rapid to very slow, depending on the molecular weight (rate ↓; small → large)
- Pharmacological activity highly affected by
  - physico-chemical properties (incl. size etc.).
  - manufacturing conditions determine the variety of different iron–sucrose complexes produced, e.g.
    - differences in the structure and MW distribution
    - different stabilities of iron-oxyhydroxide core and iron–sucrose complex

Expl #2 – Iron Sucrose

BA/BE by PK or PD

- Standard approaches are not appropriate when assessing PK of iron supplements, due to
  - the ubiquity of endogenous iron and its active compartment
  - the complexity of the iron metabolism.
  - primary iron site of action is erythrocyte, without drug-receptor interaction taking place
  - Notably, the process of erythrocyte formation of new erythrocytes, takes 3–4 weeks. Accordingly, serum iron concentration and area under the curve (AUC) are clinically irrelevant for assessing iron utilization.

Expl #2 – Iron Sucrose

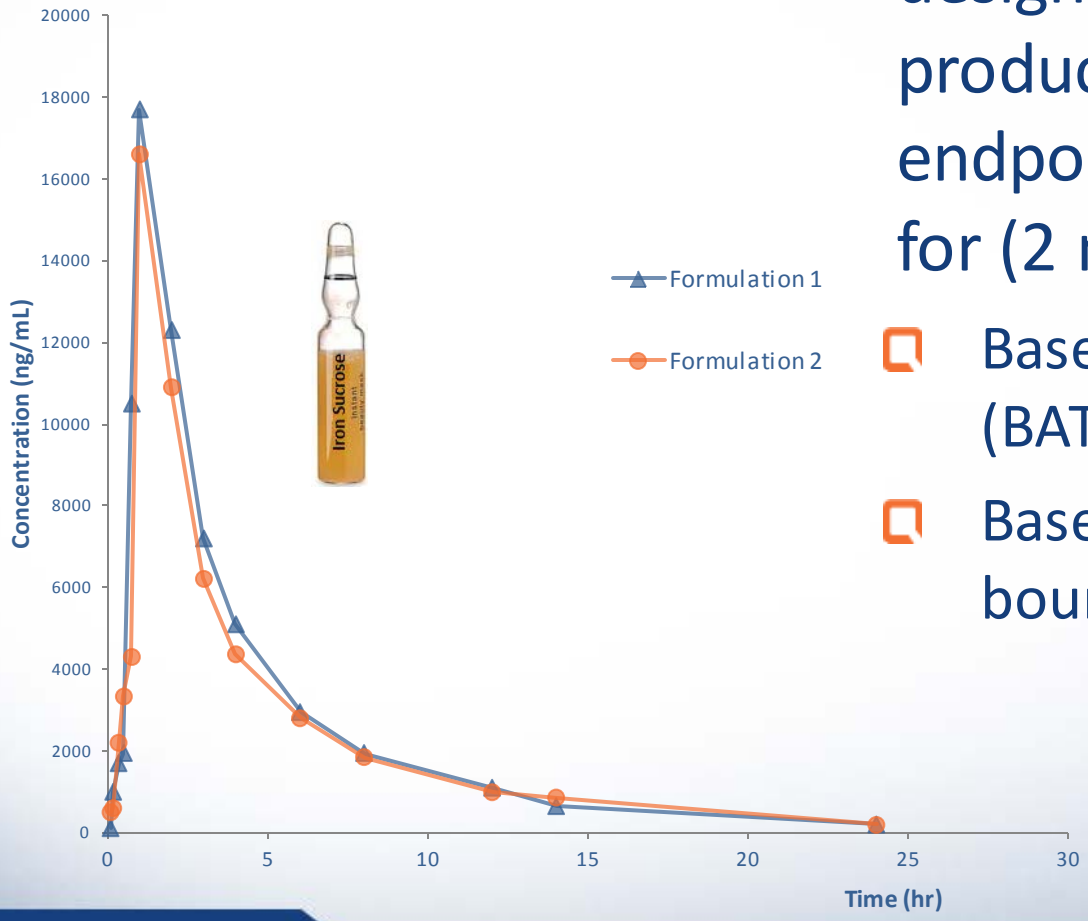
Safety issues for iron complexes

- oxidative stress as a result of
  - redox cycling → formation of highly reactive OH-radicals → radical-interaction with DNA, proteins, lipids, etc.
  - depending mainly on iron complex compositions, and interaction between $Fe(OH)_3$ core and carbohydrates
  - if not stable, weakly bound Fe saturates Tf and leads to generation of redox-active non-Tf bound Fe (NTBI)
  - AE incidence (IV dosing) correlates with NTBI

Conclusion: subtle structural modifications may affect stability and reactivity of macromolecular iron–sucrose complexes with safety implications as a result. This was also observed for Pharmacopeia-based release tested formulations.

Expl #2 – Iron Sucrose

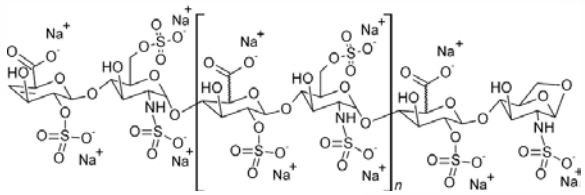
Current standard study



- (EMA, FDA guidance) designed to assess BE of 2 products through PK endpoints of AUC and Cmax for (2 min IV injection):
  - Baseline-adjusted total iron (BATI), and
  - Baseline-adjusted Transferrin-bound iron (BATBI)

Complex bioanalysis

Other examples



- LMWH (e.g. enoxaparin)
- Immunogenicity, we validate and measure one response for a probably complex mixture (pt, stage, gender, .., .., dependent ?)
- Metabolomics profiles, biomarker patterns thought of being descriptive and predictive
- …??

All are unique in their own background, and not comparable to Aß aggregates

Current approach for Amyloid-ß aggregates biomarker testing

Current bioanalysis of Abeta

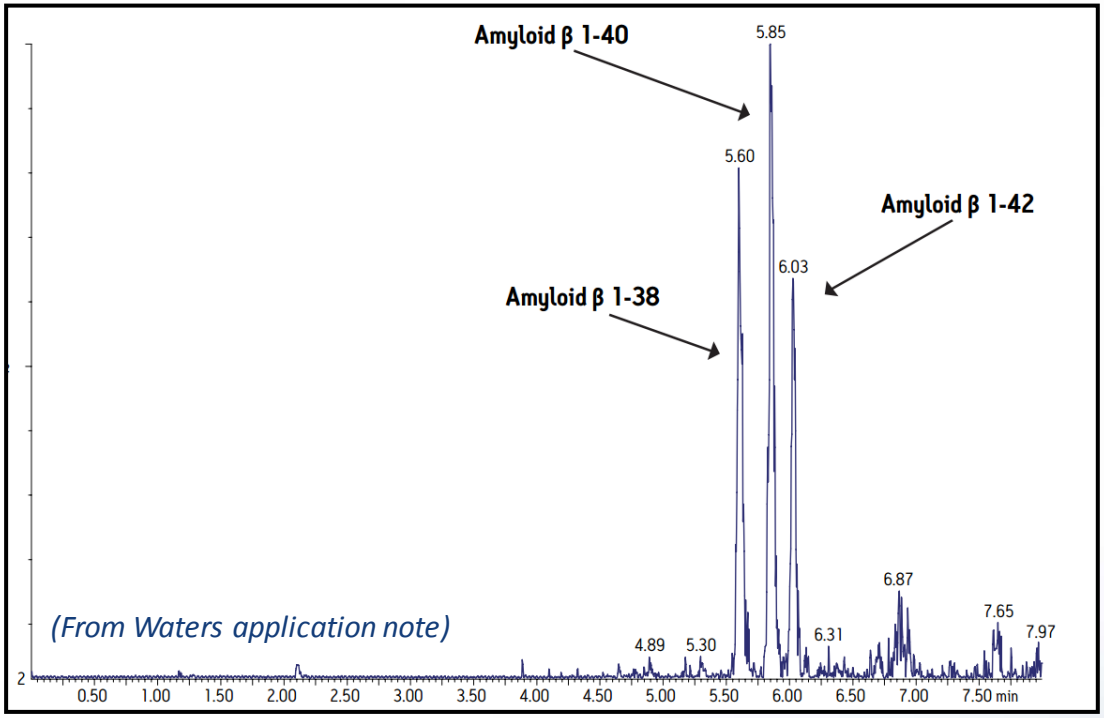
Diversity of in-house available *validated* assays: wide variety of analytes, species, matrices, kit providers, ranges

Platform	Assay	Human Matrix	MRD	Assay Range	Accuracy (%RE)	Precision (%CV)	antibodies	Notes
ECL (MSD)	MSD's original format Abeta 3-Plex (1-38, 1-40, 1-42)	CSF	2X	1-38: 37 - 3,000 pg/mL 1-40: 41 - 10,000 pg/mL 1-42: 37 - 3,000 pg/mL	-2.0 to 19.2% 1.7 to 2.1% -0.5 to -0.1%	≤ 12.2% ≤ 5.0% ≤ 7.9%	1-38-, 1-40-, 1-42-specific capture abs, sulfo-tagged 6E10 as detect	
	MSD's new format Abeta 3-Plex (1-38, 1-40, 1-42)	CSF	4X	1-38: 9.77 - 10,000 pg/mL 1-40: 14.6 - 15,000 pg/mL 1-42: 1.42 - 1,450 pg/mL	-1.1 to 1.8% -1.5 to 1.1% 11.3 to 11.9%	≤ 13.9% ≤ 15.5% ≤ 18.1%	1-38-, 1-40-, 1-42-specific capture abs, sulfo-tagged 6E10 as detect	
	Total Abeta (x-42)	K_2EDTA plasma	2X	50 - 8,000 pg/mL	-13.6 to 14.0%	≤ 13.4%	biotinylated 4G8 (Covance Research) as capture, sulfo-tagged 6E10	developed at QPS using a biotinylated 4G8 capture antibody, MSD plates and MSD's sulfo-tagged 6E10 detect antibody
	MSD's Total Abeta (x-42)	CSF	2X	200 - 10,000 pg/mL	-1.5 to 10.4%	≤ 16.7%	4G8 as capture, 6E10 as detect	
	MSD's Abeta 1-40	extracted plasma	2X	10 - 10,000 pg/mL	6.6 - 8.2%	≤ 6.6%	6E10 as capture, detect specific to Abeta 1-40	
	Abeta 42 (MSD's validated kit)	CSF	8X	0.189 - 775 pg/mL	-15.8 to 0.4%	≤ 4.3%	Abeta 1-42-specific capture, sulfo-tagged 6E10 as detect	QPS participated in MSD's multi-site validation and is a qualified lab for this assay.
	Total Tau (MSD's validated kit)	CSF	4X	4.4 - 3,227 pg/mL	-2.9 to 21.6%	≤ 6.1%	Not disclosed	QPS participated in MSD's multi-site validation and is a qualified lab for this assay.
Luminex (xMap)	Innogenetic's AlzBio3: totalTau, Abeta 1-42, phosphoTau	CSF	2X	tTau: 12 - 1,384 pg/mL 1-42: 28.5 - 1,721 pg/mL pTau: 7 - 264 pg/mL	-8.3 to -0.4% -19.6 to 5.1% -19.1 to 0.5%	≤ 16.5% ≤ 18.8% ≤ 24.6%	4D7A3 for 1-42 capture, 3D6 for 1-42 detect	
	Innogenetic's Abeta Forms: 1-40, 1-42	K_2EDTA plasma	3X	1-40: 6 - 422 pg/mL 1-42: 5 - 499 pg/mL	-16.5 to -0.6% -14.3 to 4.7%	≤ 6.6% ≤ 5.5%	2G3 for 1-40 capture, 21F12 for 1-42 capture, 3D6 for detect for both	
ELISA	Wako Chemical's Abeta 1-40 ELISA	K_2EDTA plasma	2X	1.00 - 100 pmol/L	-15.2 to 28.8 %	< 13.4 %	BAN50 anti-human Abeta 1-16 capture, detect not disclosed	
	Wako Chemical's Abeta 1-42 ELISA	K_2EDTA plasma	4X	0.50 - 20.0 pmol/L	-13.9 to 22.6%	< 17.2 %	BAN50 anti-human Abeta 1-16 capture, detect not disclosed	

ECL = Electrochemiluminescence

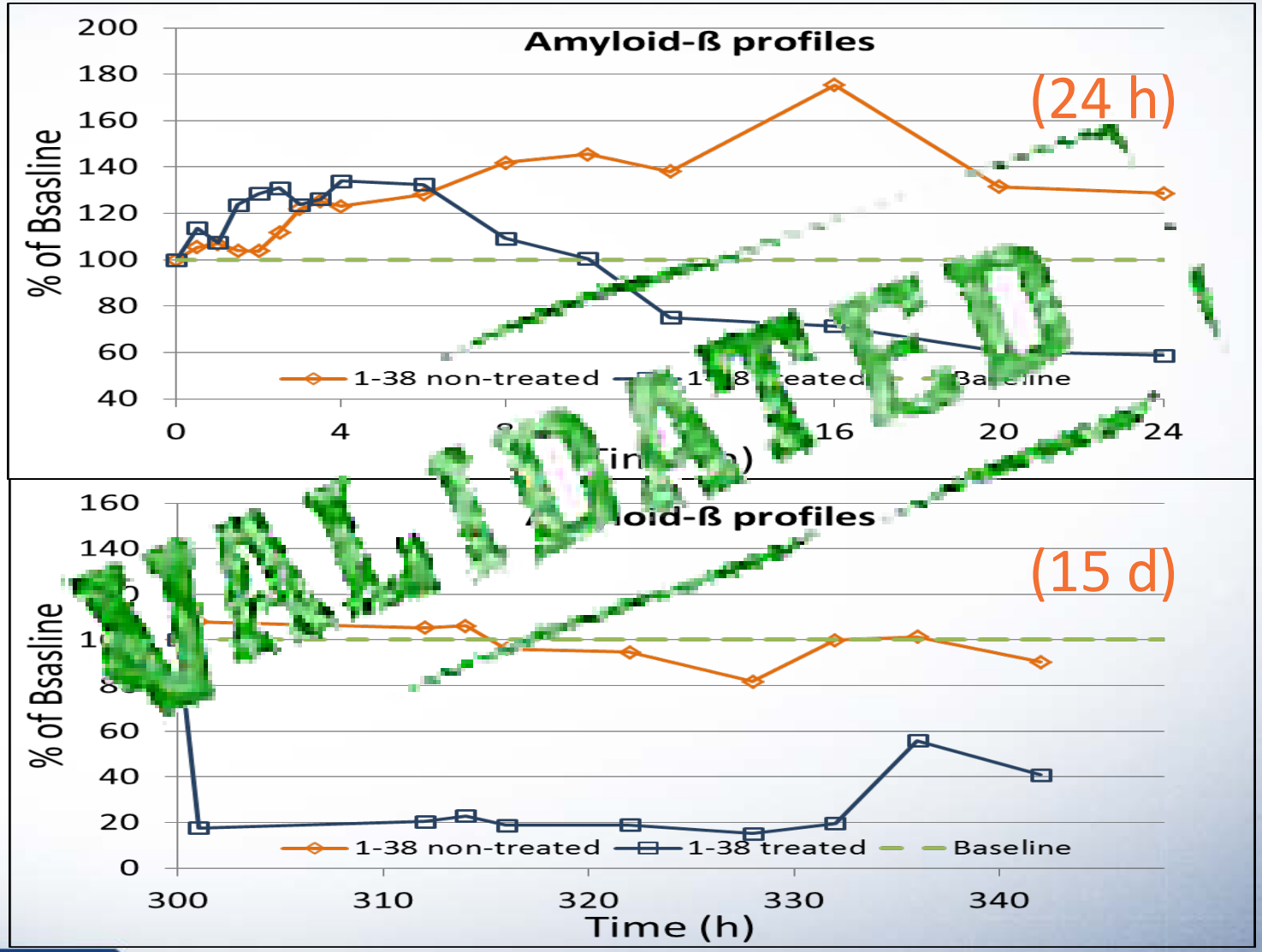
Current bioanalysis of Abeta

LBA assays standard, LCMS approaches underway



Bioanalysis of Abeta

Clinical studies in patients, cCSF sampling (24 h)



Bioanalysis of Aß aggregates

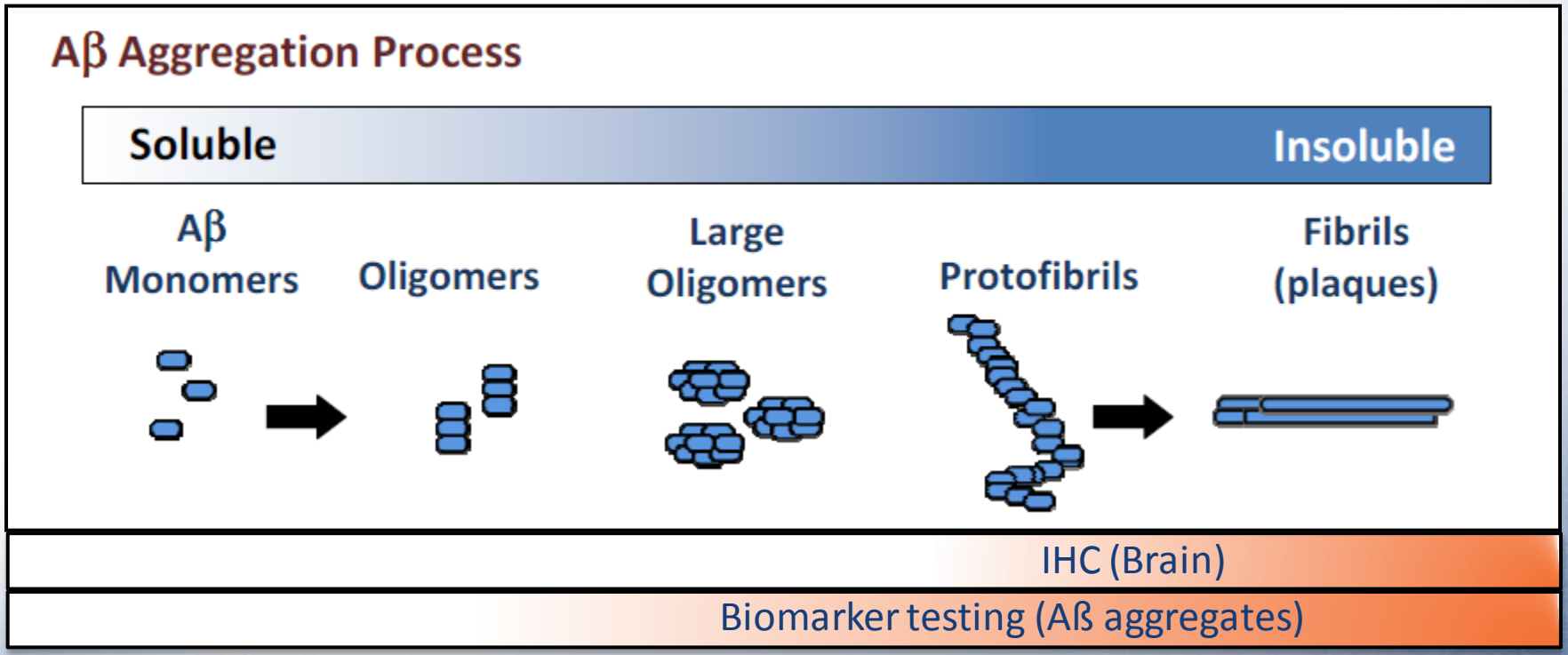
Objective

- as aggregates and profibrils cause AD, and not Aß monomers, Aß data may not be the correct markers
- replace ‘surrogate’ with a better assay for actual situation in brain, CSF
- apply a quantitative and sensitive biomarker assay that detects aggregated Aß in brain, plasma, CSF
- early detection in preclinical R&D will limit required resources, allowing for faster screening of lead candidates

Bioanalysis of Aß aggregates

Detection sensitivity of aggregated Aß (vs monomers)

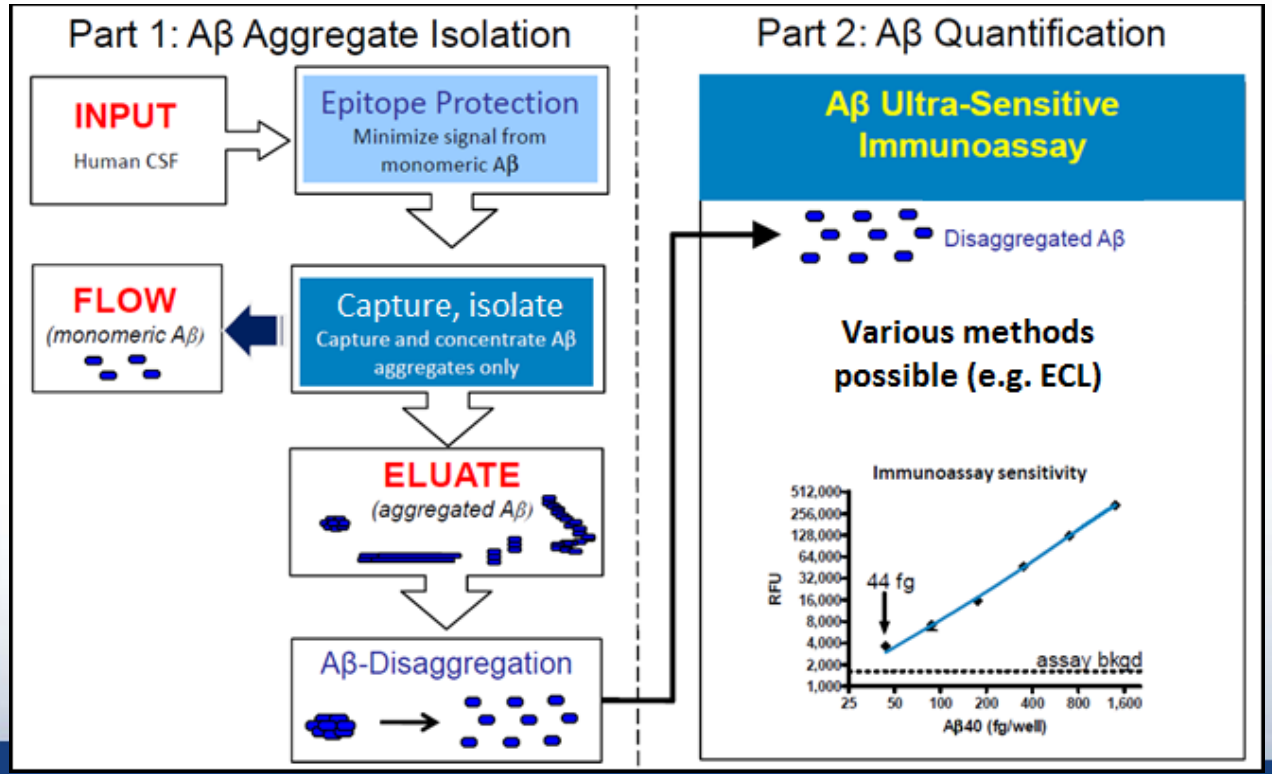
- Formation of insoluble plaques is the final step in Aß aggregation
- Goal: detect Aß aggregation before plaques are visible with IHC



Bioanalysis of Aß aggregates

Current in-house standard

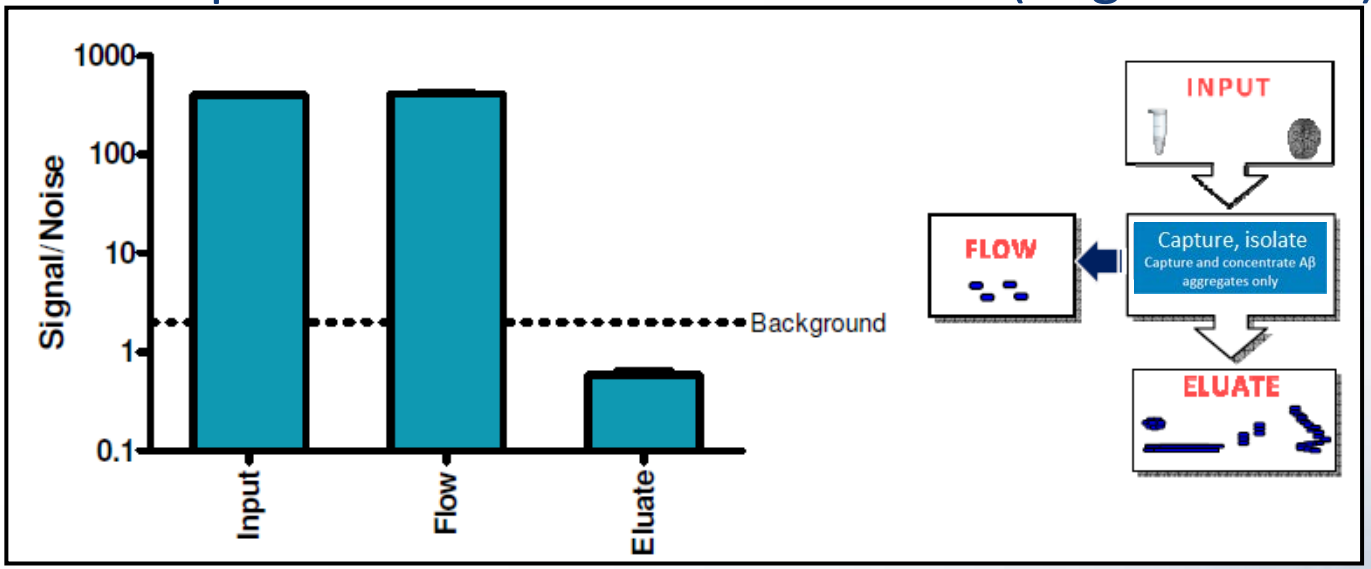
- Monomer analysis after aggregate separation and disaggregation
- Monomer assays fully validated, additional validation for aggregate capture and isolation



Specifics for Aß aggregates

Additional validation

- Validation of Aß aggregates capture, isolation
  - buffer spiked with monomeric Aß1-40 (bkgd= buffer)



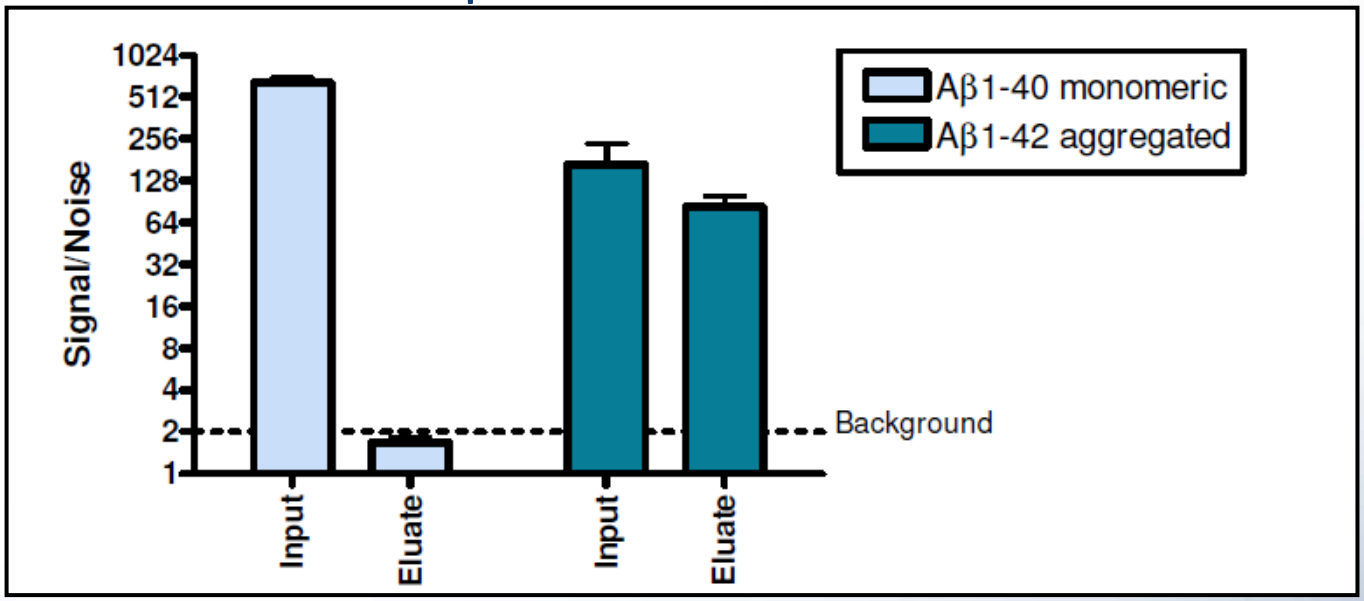
Eluate does not contain monomeric Aß1-40

→ monomers not captured

Specifics for Aß aggregates

Additional validation

- Validation of Aß aggregates capture, isolation
  - culture medium spiked with monomeric Aß1-40 or Aß1-42 aggr.

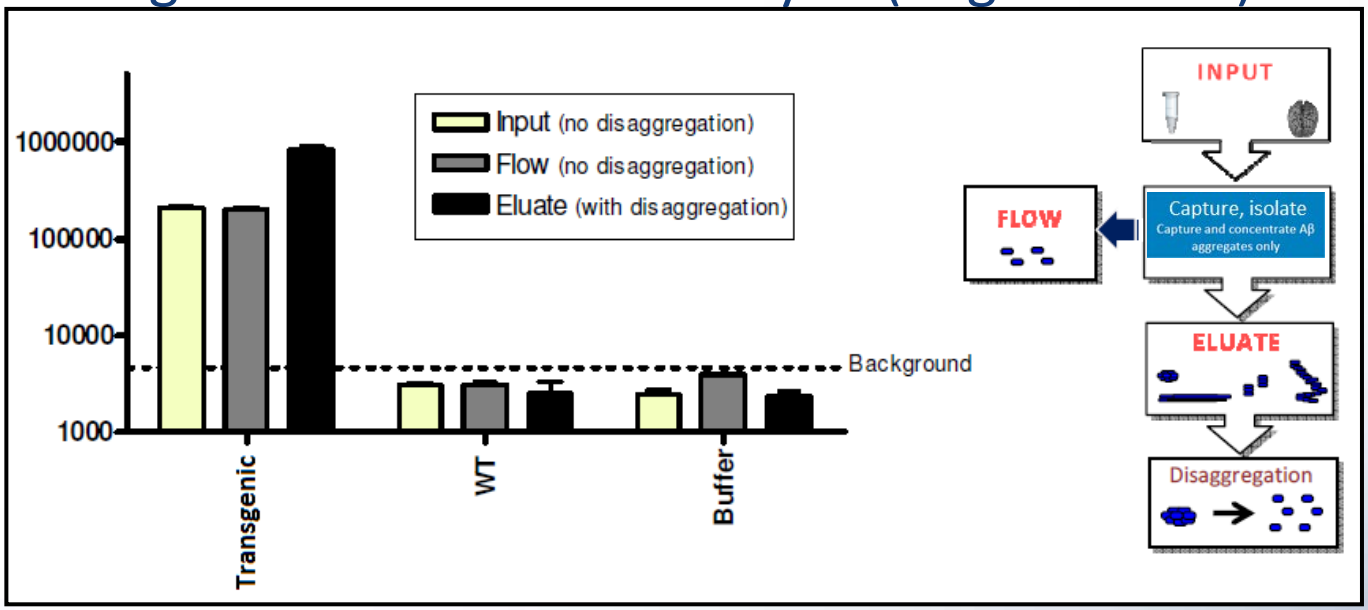


No Signal from monomeric Aß in eluates, aggregated Aß captured

Specifics for Aß aggregates

Additional validation

- Validation of Aß aggregates capture, isolation
  - Transgenic mice brain bioanalysis (bkgd= buffer)

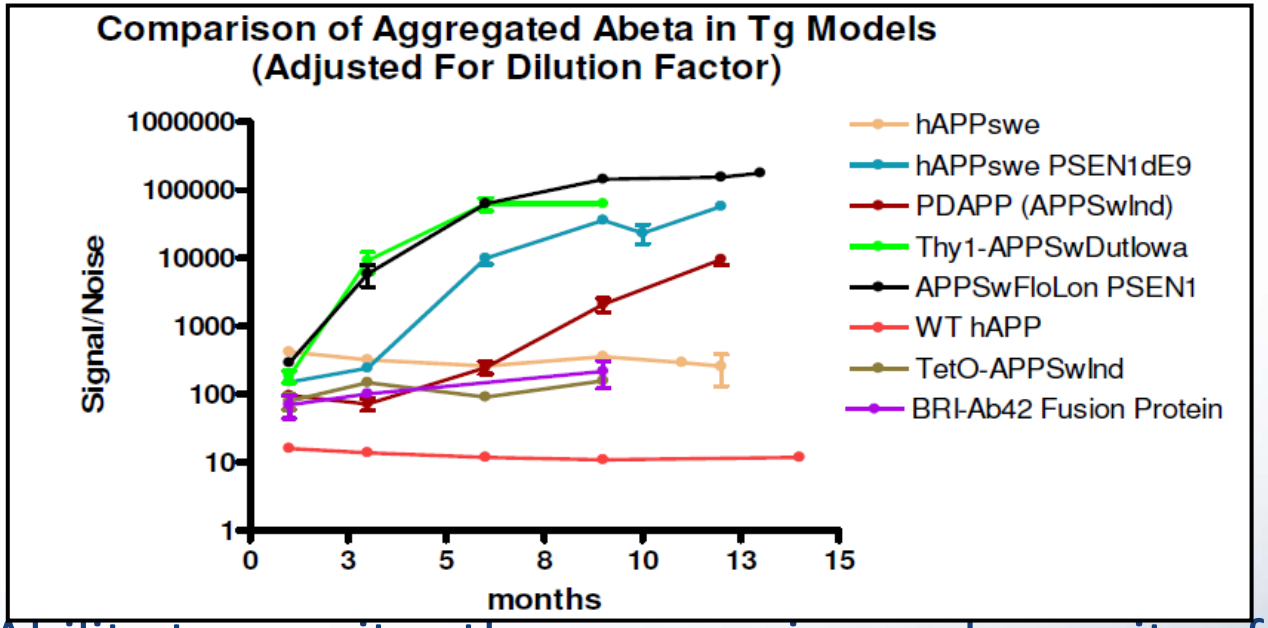


Monomeric Aß from transgenic mice brains not captured

Specifics for Aß aggregates

Additional validation

- Application in disease monitoring and treatment evaluation

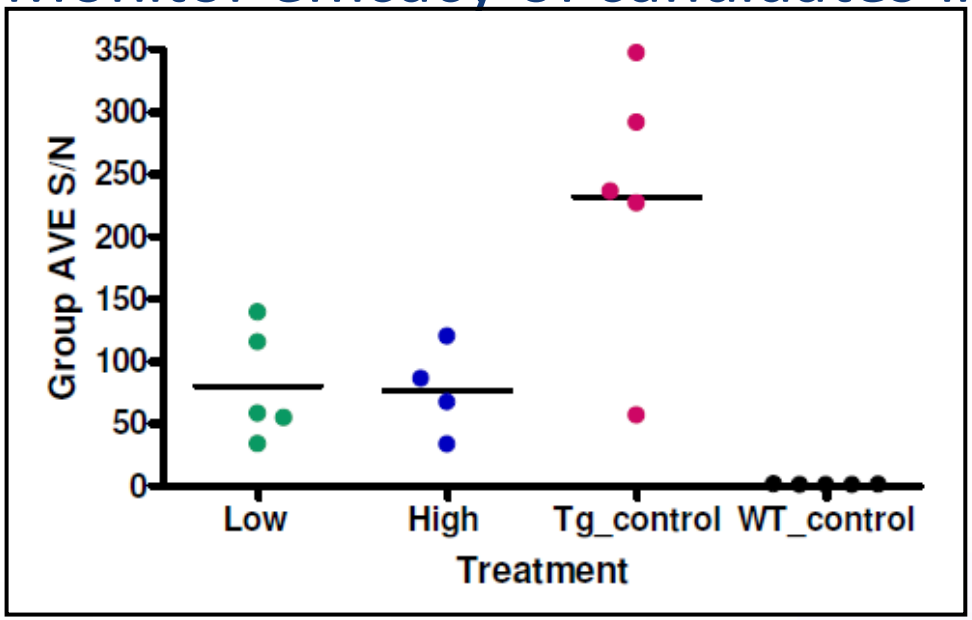


Ability to monitor the progression and severity of Aß aggregation in various models of AD, and evaluate treatments

Specifics for Aß aggregates

Additional validation

- Monitor efficacy of candidates in preclinical trials



Aß aggregates decrease brains of transgenic mice following treatment with -secretase modulator

Bioanalysis of Aß aggregates

What can be improved, what is needed

- Better biochemical profiles of actual tissue and actual markers, not derivatives or surrogates
  - intact aggregates profile
  - not just the sum of all monomer fragments
- Currently looking for and setting-up methods to detect intact aggregates
- Goal: to better link biochemical pattern with disease status, and evaluate specific treatment focused on Amyloid-ß aggregate pattern

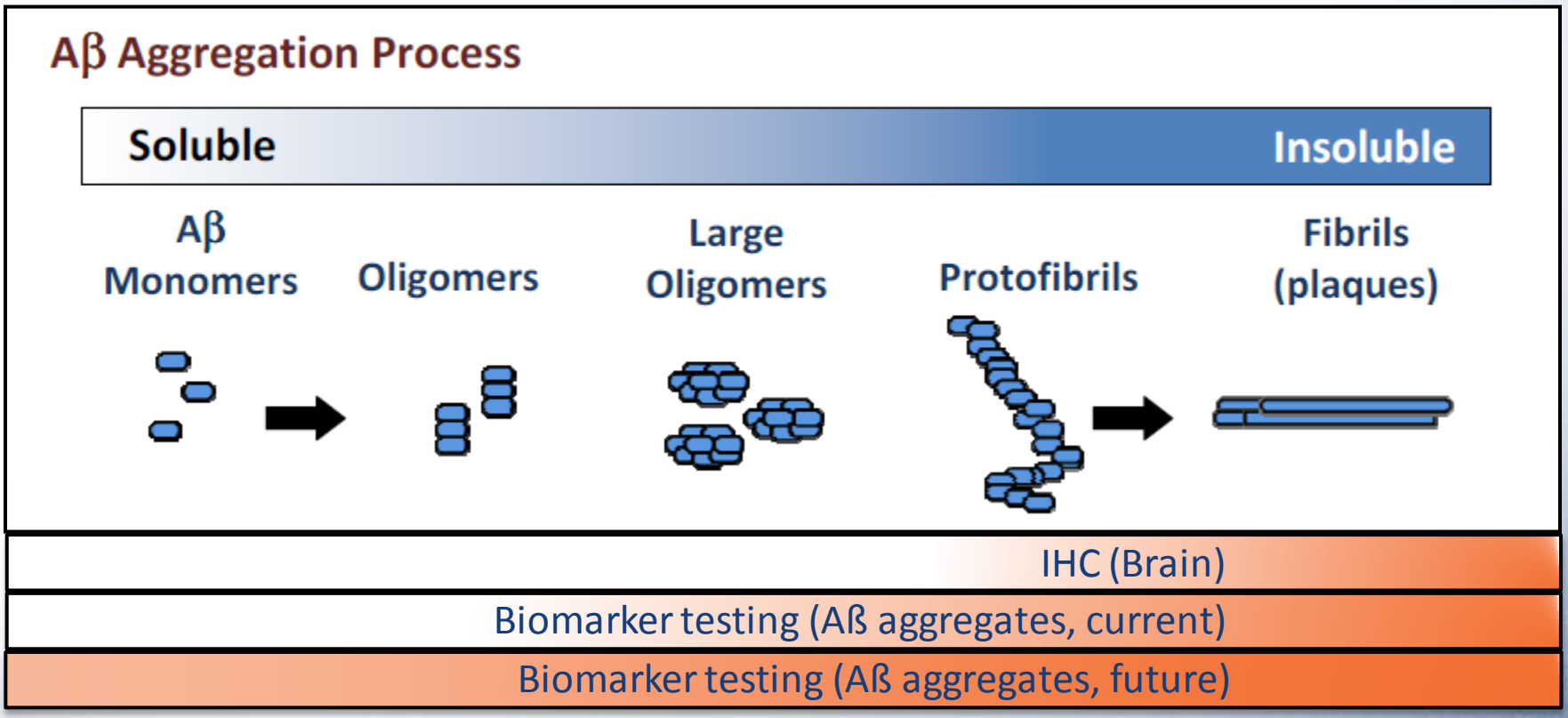
Future developments

Assays to simultaneously detect protofibrils, aggregates, monomers

- identity of analyte(s) unknown
- initially need insight in stability, 'soft' sample prep approach
- electrophoretic techniques, e.g. SDS-PAGE, combined with immunodetection e.g. Western blot)
  - soluble aggregates sensitive to detergents and denaturing agents utilized, causing aggregate decomposition
- size exclusion chromatography (SEC)
  - limited resolution and dilution of sample (sensitivity problem due to very low concentrations)
- MALDI / SELDI – high expertise and low throughput in production setting

Future developments

Final goal: full Aß pattern (monomers to (proto)fibrils)



Conclusions

- Complex mixture assay development and validation challenging if nature and composition not fixed
- To maximize information on efficacy and safety look for alternatives, e.g.
  - pattern recognition in LC-MS/MS peak profiles, and/or MALDI/SELDI spectra
  - signature compounds/peaks
- Not for every application there is a bioanalytical method, let alone a validation
- Specifically for Amyloid-ß aggregates
  - we need to fundamentally and interdisciplinary look at the challenge and find solutions
  - full analytical and clinical validation is needed.

Thank you for your attention: Questions?