Reference measurement procedure for Aβ1-42 in CSF
"Highest level of expertise and a firm focus on the patient"

Sahlgrenska University Hospital
On the health care map

• One of the largest hospitals in Europe
• A specialist hospital for the Västra Götaland Region
• Seven national specialised medical care assignments
• Pioneering research conducted in collaboration with the Sahlgrenska Academy, Chalmers University of Technology, industry and other bodies
One single day at the hospital

- 30 deliveries
- 550 emergency visits
- 3,400 outpatient visits
- 17,000 laboratory analyses
Clinical Chemistry Laboratory

• This laboratory is accredited in accordance with the International Standards
  - ISO 15189:2012 - Medical laboratories - Requirements for quality and competence
  - ISO 22870:2006 - Point-of-care testing - Requirements for quality and competence

• First hospital laboratory in Sweden to be accredited (1992).
Alzheimer’s disease (AD)

• The most common cause of dementia
  - Accounts for 60-80% of all cases of dementia
• > 40 million people worldwide affected
• First described in 1906 by Alois Alzheimer
Characteristic clinical symptoms

• Impaired episodic memory
• aphasia
  - disturbance in formulation and comprehension of language
• apraxia
  - loss of the ability to execute or carry out learned purposeful movements
• agnosia
  - loss of ability to recognize objects, persons, sounds, shapes, or smells
• general cognitive symptoms
  - impaired judgment, decision-making and orientation
Neuropathology

• Deposits of extracellular plaques
  - mainly Aβ peptides
• Intracellular neurofibrillary tangles
  - phosphorylated tau protein
APP processing

- Aβ peptides - natural metabolic products of the transmembrane glycoprotein APP.
- Generated through the amyloidogenic pathway by consecutive actions of β- & γ-secretase.
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- Generated through the amyloidogenic pathway by consecutive actions of β- & γ-secretase.
Aβ misfolding and oligomerisation

• A conformational change of Aβ into high β-sheet content is believed to increase its propensity to self-aggregate.

• Which of these forms that are neurotoxic is still uncertain, but
  - levels of soluble Aβ dimers and oligomers has been shown to correlate with clinical symptoms and synaptic loss
  - Fibrils have been shown to induce neuronal loss
The amyloid cascade hypothesis

- Imbalance in $A\beta$ production & clearance
- Deposition of $A\beta$ in the brain
- Neuro-degeneration
- AD dementia

20-30 years

Clinical trials (current)
The amyloid cascade hypothesis

Imbalance in Aβ production & clearance → deposition of Aβ in the brain → neurodegeneration → AD dementia

Clinical trials (desired) → 20-30 years
Diagnosis

• Medical history, cognitive tests and mental state exams
• Post mortem neuropathological examination required for definitive diagnosis
• Ongoing process of including biomarkers
  - The International Working Group (IWG)-2 criteria for typical AD now include
    – increased tracer retention on amyloid PET
    – decreased $\text{A}\beta_{1-42}$ together with increased tau in CSF
Biomarkers

<table>
<thead>
<tr>
<th>Type</th>
<th>Biomarker</th>
<th>Change in AD</th>
</tr>
</thead>
<tbody>
<tr>
<td>CSF</td>
<td>$A\beta_{1-42}$</td>
<td>↓ concentration</td>
</tr>
<tr>
<td>CSF</td>
<td>$A\beta_{1-42}/A\beta_{1-40}$ ratio</td>
<td>↓ ratio</td>
</tr>
<tr>
<td>CSF</td>
<td>T-tau</td>
<td>↑ concentration</td>
</tr>
<tr>
<td>CSF</td>
<td>P-tau</td>
<td>↑ concentration</td>
</tr>
<tr>
<td>Imaging</td>
<td>Structural MRI</td>
<td>↓ volume</td>
</tr>
<tr>
<td>Imaging</td>
<td>Functional MRI</td>
<td>↓ functional connectivity</td>
</tr>
<tr>
<td>Imaging</td>
<td>FDG-PET</td>
<td>↓ glucose metabolism</td>
</tr>
<tr>
<td>Imaging</td>
<td>Amyloid PET</td>
<td>↑ $A\beta$ retention</td>
</tr>
<tr>
<td>Imaging</td>
<td>Tau PET</td>
<td>↑ intracellular tau</td>
</tr>
<tr>
<td>Type</td>
<td>Biomarker</td>
<td>Change in AD</td>
</tr>
<tr>
<td>-------</td>
<td>-----------</td>
<td>--------------</td>
</tr>
<tr>
<td>CSF</td>
<td>Aβ$_{1-42}$</td>
<td>↓ concentration</td>
</tr>
</tbody>
</table>

~50% lower concentration of Aβ$_{1-42}$ in CSF in AD patients compared to healthy controls

- Peptide accumulation in plaques in the brain → less in CSF
Combined with the microtubule-stabilizing tau protein → high diagnostic accuracy of AD

- t-tau – cortical axonal degeneration
- p-tau – tangle pathology

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<td>↑ concentration</td>
</tr>
<tr>
<td>CSF</td>
<td>P-tau</td>
<td>↑ concentration</td>
</tr>
<tr>
<td>CSF</td>
<td>Aβ1-42/Aβ1-40 ratio</td>
<td>↓ ratio</td>
</tr>
</tbody>
</table>

Low & high Aβ producers

- When using only Aβ1-42
  - Low producers might be false positive for AD
  - High producers might be false negative for AD

- Using the ratio of Aβ1-42/Aβ1-40 improve diagnostic accuracy
  - Aβ1-40 levels in CSF are relatively unchanged in AD compared to controls

Treatment

• Existing drugs temporarily improve symptoms
• There is no therapy that slows or stops the progression of AD
• Treatment strategies currently evaluated
  - Active immunotherapy: immunization with Aβ peptides
  - Passive immunotherapy: treatment with anti-Aβ antibodies
  - Inhibition of the β-secretase BACE1

*BACE1 = beta-site amyloid precursor protein cleaving enzyme 1*
AD QC program
Methods & results
180 µL CSF

IS

GdnHCl

H₃PO₄

SPE

Dry & resuspend eluate

RP LC

MS/MS
Quadrupole-orbitrap hybrid MS - parallel reaction monitoring (PRM)

Quadrupole: precursor ion selection

Collision cell: fragmentation

Orbitrap: detection

Output: chromatogram (top) MS/MS spectrum (bottom)
Round Robin study

- Perform an inter-laboratory study involving other laboratories using similar LC-MS methods
- Determine the inter-laboratory variation using these methods
- Examine if these methods are suitable to set the level of a certified reference material.

**Abstract**

Introduction: Cerebrospinal fluid (CSF) amyloid-β 1–42 (Aβ42) is an important biomarker for Alzheimer’s disease, both in diagnostics and to monitor disease-modifying therapies. However, there is a great need for standardization of methods used for quantification. To overcome problems associated with immunoassays, liquid chromatography-tandem mass spectrometry (LC-MS/MS) has emerged as a critical orthogonal alternative.

Methods: We compared results for CSF Aβ42 quantification in a round robin study performed in four laboratories using similar sample preparation methods and LC-MS instrumentation.
<table>
<thead>
<tr>
<th></th>
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<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>IS concentration, ng/mL</td>
<td>1</td>
<td>2</td>
<td>2</td>
<td>1.6</td>
</tr>
<tr>
<td>CSF volume, μL</td>
<td>200</td>
<td>100</td>
<td>250</td>
<td>200</td>
</tr>
<tr>
<td>Calibrator matrix</td>
<td>aCSF with 5% rat plasma</td>
<td>aCSF with 4 mg/mL HSA + IgG, glucose</td>
<td>aCSF with 4 mg/mL BSA</td>
<td>Human CSF</td>
</tr>
<tr>
<td>LC System</td>
<td>ACQUITY, 1D</td>
<td>ACQUITY; 2D trapping/eluting</td>
<td>ACQUITY; 2D trapping/eluting</td>
<td>Accela 1250</td>
</tr>
<tr>
<td>Dilution (injection)</td>
<td>50 μL + 25 μL H₂O (10 μL)</td>
<td>50 μL + 50 μL H₂O (30 μL)</td>
<td>50 μL + 50 μL H₂O (50 μL)</td>
<td>None. Dried eluate resuspended in 25 μL 79:20:1 H₂O/ACN/NH₄OH (20 μL)</td>
</tr>
<tr>
<td>LC mobile phases</td>
<td>A: 0.3% NH₄OH B: 90:10 ACN/MP A</td>
<td>A: 0.3% NH₄OH B: 90:5:5 ACN/TFE/H₂O</td>
<td>A: 0.1% NH₄OH B: 75:25:5 ACN/MeOH/TFE</td>
<td>A: 0.1% NH₄OH, 5% ACN B: 0.03% NH₄OH, 95% ACN</td>
</tr>
<tr>
<td>Column</td>
<td>Waters BEH 300</td>
<td>Waters BEH 300</td>
<td>Waters BEH 300</td>
<td>Thermo ProSwift RP-4H</td>
</tr>
<tr>
<td></td>
<td>2.1 × 150 mm, 1.7 μm, 50°C</td>
<td>2.1 × 150 mm, 1.7 μm, 50°C</td>
<td>2.1 × 50 mm, 1.7 μm, 60°C</td>
<td>1 × 250 mm, 50°C</td>
</tr>
<tr>
<td>Flow rate, μL/min</td>
<td>200</td>
<td>300</td>
<td>200</td>
<td>300</td>
</tr>
<tr>
<td>MS</td>
<td>Waters Xevo TQ-S</td>
<td>Waters Xevo TQ-S</td>
<td>ABSciex API 5000</td>
<td>Thermo TSQ Vantage</td>
</tr>
<tr>
<td>Transitions, m/z</td>
<td>1129.0→1078.5</td>
<td>1129.0→1078.5</td>
<td>1129.0→1078.5</td>
<td>1129.58→1054.03, 1078.79, 1107.06</td>
</tr>
<tr>
<td>Run time</td>
<td>8.5 minutes</td>
<td>8.5 minutes</td>
<td>12 minutes</td>
<td>14 minutes</td>
</tr>
</tbody>
</table>
Twelve pools of human CSF were analyzed at four different laboratories. Using sample 11 as a reference, the measurements for the other samples were adjusted.
Interlab CV%

Average inter-lab CV = 12 %

Average inter-lab CV = 8 %
Conclusions

• A good agreement was seen between the laboratories, with an average inter-laboratory CV of 12.2%
  - despite the different methods and instrumentations used

• Using a common reference sample significantly decreased the average inter-laboratory CV (to 8.3%)
# Mass Spectrometry-Based Candidate Reference Measurement Procedure for Quantification of Amyloid-β in Cerebrospinal Fluid

**Andreas Leinenbach,¹¹ Josef Panne,²¹ Thomas Dülfger,¹ Andreas Huber,¹ Tobias Bittner,¹ Ulf Andreasson,² Johan Gobom,² Henrik Zetterberg,²,³ Uwe Kobold,¹ Erik Portelius,² and Kaj Blennow² on behalf of the IFCC Scientific Division Working Group on CSF proteins**

**BACKGROUND:***
Cerebrospinal fluid (CSF) is a well-conserved body fluid. Several immunoassays target amyloid-β (Aβ) in absolute concentration, free from matrix interference, there are no matrix effects (2, 3). The broadscale use of immunoassays for Aβ in CSF is limited, due to a lack of high-quality, internationally recognized Aβ reference standards.

**METHODS:***
The analyte Aβ42 (Aβ42) was based on sandwich immunoassays, because of the lack of matrix-free calibrators and lack of stable isotopes. The reference matrix solution was produced by the Biomarker Research Laboratory of the Perelman School of Medicine, University of Pennsylvania, USA. The technical details can be found in the reference.

### Isotope Dilution Mass Spectrometry Methods for Aβ42 in other matrices

<table>
<thead>
<tr>
<th>Method</th>
<th>Applicable matrix(s)</th>
<th>Full description of technique(s)</th>
<th>Quantity</th>
<th>Applicable range</th>
<th>Expected uncertainty (level of confidence 95%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mass spectrometry</td>
<td>Human cerebrospinal fluid</td>
<td>Isotope dilution mass spectrometry</td>
<td>Mass concentration</td>
<td>150 pg/mL to 4000 pg/mL</td>
<td>15.7%</td>
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**ACKNOWLEDGEMENTS:**
The authors acknowledge the support of the IFCC Scientific Division Working Group on CSF proteins and the Biomarker Research Laboratory of the Perelman School of Medicine, University of Pennsylvania, USA.

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**Sahlgrenska University Hospital**

**Clinical Chemistry 60:7**
**987–994 (2014)**

**Proteomics and Protein Markers**

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**JCTLM approved 2015-10-21**
Calibration in human CSF

• Surrogate matrix such as artificial CSF might lead to low recoveries of the analyte → sensitivity issue

• Calibrators were prepared in human CSF using the surrogate analyte approach
Surrogate analyte approach

• $^{15}\text{N-}\text{A}\beta_{1-42}$ was used as a surrogate for the native $\text{A}\beta_{1-42}$ for calibration.
• $^{13}\text{C-}\text{A}\beta_{1-42}$ was used as IS in both calibrators and unknowns.
• A response factor ($f$) for $^{15}\text{N-}\text{A}\beta_{1-42}$ to native $\text{A}\beta_{1-42}$ was determined in artificial CSF.
• When determining the concentration of endogenous $\text{A}\beta_{1-42}$ in unknown CSF samples, the concentration of $^{15}\text{N-}\text{A}\beta_{1-42}$ used in the calibration curve was multiplied by $f$, which was measured before and after each set of unknown samples.
Imprecision

$^{15}$N-A$\beta_{1-42}$

- LLOQ: 150 pg/mL
- Truenesss: 100%±15%*
- No matrix-dependent ion suppression

Native A$\beta_{1-42}$ in CSF

- Interassay imprecision for endogenous A$\beta_{1-42}$ <5.6% (6 different CSF pools over 6 days)

<table>
<thead>
<tr>
<th>Imprecision</th>
<th>Intra-assay</th>
<th>Inter-assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>250 pg/mL</td>
<td>5.0%</td>
<td>6.4%</td>
</tr>
<tr>
<td>1000 pg/mL</td>
<td>2.2%</td>
<td>5.6%</td>
</tr>
</tbody>
</table>

*Use of spiking and recovery
Response factor of native Aβ$_{1-42}$ and $^{15}$N-Aβ$_{1-42}$ in artificial CSF at different concentrations (150–4000 pg/mL, n=2 at each concentration).

Relative response of endogenous Aβ$_{1-42}$ and $^{15}$N-Aβ$_{1-42}$ in human CSF as well as human CSF diluted with artificial CSF (volume CSF : artificial CSF)
CSF Aβ1–42 – an excellent but complicated Alzheimer's biomarker – a route to standardisation

Julia Kuhlmann b,c, Ulf Andreasson b,c, Josef Pannee b,c, Maria Bjerke b,c, Erik Portelius b,c, Andreas Leinenbach d, Tobias Bittner d, Magdalena Korecka e, Rand G. Jenkins f, Hugo Vanderstichele g, Erik Stoops g, Piotr Lewczuk h,i, Leslie M. Shaw g, Ingrid Zegers a, Heinz Schimmel a, Henrik Zetterberg b,c,i, Kaj Blennow b,c,i, on behalf of the IFCC Working Group on Standardization of CSF proteins (WG-CSF)

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ABSTRACT

The 42 amino acid form of amyloid β (Aβ42) in cerebrospinal fluid (CSF) has been widely accepted as a central biomarker for Alzheimer’s disease. Current immunometric assays for CSF Aβ42 are commercially available. But can ref
CSF Aβ₁₋₄₂ – an excellent but complicated Alzheimer’s disease route to standardisation


ABSTRACT

The 42 amino acid form of amyloid β (Aβ₁₋₄₂) in cerebrospinal fluid (CSF) has been widely accepted as a central biomarker for Alzheimer’s disease. Current immunoassays for CSF Aβ₁₋₄₂ can now commonly be found in clinical laboratories. However, as with any biomarker, standardisation is paramount to ensure clinical validity.
Fig. 3. Correlation of the results on 10 CSF pools measured with the 2 reference measurement procedures (RMPs) for CSF Aβ1-42 quantification by LC-MS/MS. Over the course of 3 days, 2 aliquots per CSF pool were measured in duplicate. Both procedures were calibrated with a common Aβ1-42 Calibrator provided by JRC-IRMM. Error bars indicate standard deviations of the daily averages measured with RMP2.
The certification of Amyloid β_{1-42} in CSF in ERM®-DA480/IFCC, ERM®-DA481/IFCC and ERM®-DA482/IFCC

CRM released 1/DEC/2017

High, middle and low concentrations

Julia Kuhlmann¹, Sébastien Boulo¹, Ulf Andreasson², Maria Bjerke², Josef Panneè², Jean Charoud-Got¹, Guy Auclair¹, Stéphane Mazoua¹, Stefanie Trapmann¹, Heinz Schimmel¹, Hendrik Emons¹, Doris Florian¹, Milena Quaglia³, Erik Portelius², Magdalena Korecka³, Leslie M. Shaw⁴, Mary Lame⁵, Erin Chambers⁶, Hugo Vanderstichele⁶, Erik Stoops⁶, Andreas Leinenbach⁷, Tobias Bittner⁷, Rand G. Jenkins⁸, Vesna Kostanjavecki⁹, Piotr Lewczuk¹⁰, Henrik Zetterberg², Ingrid Zegers¹, Kaj Blennow²
<table>
<thead>
<tr>
<th>Amyloid β_{1-42} peptide in human CSF&lt;sup&gt;1)&lt;/sup&gt;</th>
<th>Mass concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Certified value&lt;sup&gt;2)&lt;/sup&gt; [µg/L]</td>
</tr>
<tr>
<td>ERM-DA480/IFCC</td>
<td>0.45</td>
</tr>
<tr>
<td>ERM-DA481/IFCC</td>
<td>0.72</td>
</tr>
<tr>
<td>ERM-DA482/IFCC</td>
<td>1.22</td>
</tr>
</tbody>
</table>

<sup>1)</sup> As obtained by solid phase extraction and subsequent quantification by liquid chromatography with mass spectrometry detection, according to the reference methods (Leinenbach et al. Clin. Chem. 60 (2014) 987-94; Korecka et al. J. Alzheimers Dis. 41 (2014) 441-451) [5,6].

<sup>2)</sup> Certified values are values that fulfil the highest standards of accuracy and represent the unweighted mean value of the means of 5 accepted sets of data, each set being obtained in a different laboratory. The certified value and its uncertainty are traceable to the International System of Units (SI).

<sup>3)</sup> The uncertainty is the expanded uncertainty of the certified value with a coverage factor $k = 2$ corresponding to a level of confidence of about 95% estimated in accordance with ISO/IEC Guide 98-3, Guide to the Expression of Uncertainty in Measurement (GUM:1995), ISO, 2008 [4].
Commutability

"A property of a reference material, demonstrated by the equivalence of the mathematical relationships among the results of different measurement procedures for a reference material and for representative samples of the type intended to be measured."

Three commutability studies show good commutability of the three materials

- Manuscript in preparation (III)
Homogeneity

Averages / vial number and their 95 % CI
based on the within-group standard deviation as derived from a one-way ANOVA of all data grouped by vial number after correction of the analysis trend.
Average Aβ1-42 concentrations in CSF the three CRMs as measured with the RMPs.

Bars = laboratory means ± 2s.
Full line = mean of the means
Dotted lines = the mean of the means ± 2s.
Next steps
Concordance between CSF Aβ & amyloid PET

Pittsburgh compound B imaging and cerebrospinal fluid amyloid-β in a multicentre European memory clinic study

Antoine Leuzu, Konstantinos Chiotis, Steen G. Hasselbalch, Juha O. Rinne, Alexandre de Mendonça, Markus Otto, Alberto Lleo, Miguel Castelo-Branco, Isabel Santana, Jarkko Johansson, Sarah Andersen-Brahe, Christine A. F. von Arnim, Ambros Beer, Rafael Blasa, Juan Fortea, Saskia Berl, Erik Portellus, Josef Pannek, Henrik Zetterberg, Kaj Blennow, and Agnete Nordberg

The aim of this study was to assess the agreement between data on cerebral amyloidosis, derived using Pittsburgh compound B positron emission tomography and (i) multi-laboratory INNOTEST enzyme-linked immunosorbent assay derived cerebrospinal fluid concentrations of amyloid-β1-42; (ii) cerebrospinal fluid amyloid-β1-42, centrally measured using an antibody-independent mass spectrometry-based reference method. Moreover, we examined the hypothesis that discordance between amyloid biomarker measurements may be due to interindividual differences in total amyloid-β production, by using the ratio of amyloid-β1-42 to amyloid-β42. Our study population consisted of 245 subjects from seven centres belonging to the Biocrates for Alzheimer’s and Parkinson’s Disease Initiative, and included subjects with normal cognition and patients with mild cognitive impairment, Alzheimer’s disease dementia, frontotemporal dementia, and vascular dementia. All had Pittsburgh compound B positron emission tomography data, cerebrospinal fluid INNOTEST amyloid-β1-42 values, and cerebrospinal fluid samples available for reanalysis. Cerebrospinal fluid samples were reanalyzed (amyloid-β1-42 and amyloid-β1-40) using Meso Scale Discovery electrochemiluminescence enzyme-linked immunosorbent assay technology, and a novel, antibody-independent, mass spectrometry reference method. Pittsburgh compound B standardized uptake value ratio results were scaled using the Centiloid method. Concordance between Meso Scale Discovery mass spectrometry reference measurement procedure findings and Pittsburgh compound B was high in subjects with mild cognitive impairment and Alzheimer’s disease, while more variable results were observed for cognitively normal and non-Alzheimer’s disease groups. Agreement between Pittsburgh compound B classification and Meso Scale Discovery mass spectrometry reference measurement procedure findings was further improved when using amyloid-β1-40.
$A\beta_{1-38}$, $A\beta_{1-40}$ and the $A\beta_{1-42}/A\beta_{1-40}$ ratio

- Validation of LC-MS $A\beta_{1-38}$ & $A\beta_{1-40}$
- Comparison to amyloid PET
Validation results - Aβ₁-₄₀

<table>
<thead>
<tr>
<th>Sample</th>
<th>Average concentration (pg/mL)</th>
<th>sᵣ (pg/mL)</th>
<th>CVᵣ (%)</th>
<th>sᵦW (pg/mL)</th>
<th>CVᵦW (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HIGH</td>
<td>4197</td>
<td>172</td>
<td>4.1</td>
<td>252</td>
<td>6.0</td>
</tr>
<tr>
<td>LOW</td>
<td>2738</td>
<td>94</td>
<td>3.4</td>
<td>166</td>
<td>6.1</td>
</tr>
</tbody>
</table>

Imprecision - Repeatability (CVᵣ) <10% and reproducibility (CVᵦW) <15% for high & low QC samples.

Measurement range - The relative errors for the back-calculated calibrators <15% in the whole measurement range defined by the calibrator curve (1 500 – 40 000 pg/mL).

Sample stability - A sample can go through up to five freeze/thaw cycles with no statistically significant effect on the measured concentration of the analyte. Storage in -80 ºC is preferred while storage in -20 ºC is acceptable.
Validation results - $\text{A} \beta_{1-40}$

** Carry over - No carry-over was detected. 

Highest calibrator (top panel) followed by a blank injection (bottom panel, Y-axis range adjusted to typical LLOQ-level). No analyte was detected in the blank injection.

** Dilution linearity - Human CSF serially diluted with a-CSF (2, 5, 10 & 20 fold)

** Parallelism - Calibrators prepared in human CSF & artificial CSF.

Both matrices can be used
Results show that the CSF $A\beta_{1-42}/A\beta_{1-40}$ ratio using LC-MS is strongly associated with cortical $A\beta$ fibrils measured by $^{18}$F-flutemetamol PET.
Thank you for your attention