Biomarker Assays for Alzheimer’s Disease
- An update for the GBSC Workgroup

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Ideally, we require the ability to measure biochemical markers within peripheral biofluids to:

- Assist early diagnosis and stratify patients for PET imaging
- Discriminate between MCI converters and non-converters
- Predict fast/slow rate of decline for individual patients
- Correlate with response to experimental medicines during clinical trials
We have developed several new mass spectrometry based multiplexed assays to measure key analytes associated with Alzheimer’s disease.

- Abeta isoforms
- CSF16plex
- Total CSF Tau
- TMTcalibrator IV (Hybrid)

TMT is covered by granted and pending patents in Europe, USA and Japan
TMT is licensed to and distributed by Pierce Biotechnology a Thermo Fisher Scientific Company

Thompson et. al
*Anal. Chem. 2003, 75, 1895-1904*
• CSF samples have kindly been provided by Dr. Henrik Zetterburg, Mölndal (Sweden)

• 31 non AD but memory impaired and 31 AD CSF samples

• Following metadata has been provided
  – Diagnosis, gender, age, and concentration of tau, beta-amyloid and phospho-tau

• Additional Metadata such as vial type and protein concentration after Bradford assay has also been considered
CSF samples were processed according to the Abeta-IP-MALDI process (adapted from Portelius et al with slight modifications)

200 µl of CSF spiked with 10 ng/ml heavy 1-40 and used for IP MALDI preparation in triplicate

Referencing of endogenous peptides to spike enables comparison of spectra in each batch of Immunoppts.

Aβ-IP-MALDI workflow. Enrichment from CSF and simultaneous MS-detection of different Aβ-peptides and heavy internal standard Aβ 1-40.
A mean ratio of [AD/non AD] and each endogenous peptide was calculated for each IP batch and the mean of these 4 ratios is displayed above for each of the peptides (error bars display the variation of the relative ratio between the 4 IP-batches.

- A clear upregulation of a factor up to ~2 is seen for peptides 1-40, 1-39, 1-38 and 1-37 in ADs.
- An upregulation trend is less pronounced but visible in 1-34, 1-33 and 1-19 in ADs.
- These IP-MALDI data do not demonstrate differences in 1-17 and 1-42 between AD cases and non AD subjects (1-17 has high variations, 1-42 is a very weak signal, close to background)
Measurement of Aβ-peptides in the IP-MALDI assay enables a clear separation between AD cases and non AD samples.

The PCA Scores Plot shown relates to single IP batch (8 vs 8) to illustrate typical outcome.

Overall the separation is mainly driven by higher levels of Aβ1-37, Aβ1-38, Aβ1-39 and Aβ1-40 isoforms in AD cases as indicated in the Loadings Plot.
A subset of Aβ-peptides deliver excellent p-values for the diagnosis of 31 vs 31 AD cases and non AD subjects.

<table>
<thead>
<tr>
<th>IP Batch 1</th>
<th>IP Batch 2</th>
<th>3</th>
<th>IP Batch 4</th>
<th>Gender</th>
<th>Age</th>
<th>Total protein conc.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-17</td>
<td>0.789</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.049</td>
<td>0.036</td>
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<tr>
<td>1-19</td>
<td>0.117</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.098</td>
<td>0.009</td>
</tr>
<tr>
<td>1-33</td>
<td>0.001</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.282</td>
<td>0.001</td>
</tr>
<tr>
<td>1-34</td>
<td>0.018</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.174</td>
<td>0.378</td>
</tr>
<tr>
<td>1-37</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.462</td>
<td>0.008</td>
</tr>
<tr>
<td>1-38</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.502</td>
<td>0.005</td>
</tr>
<tr>
<td>1-39</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.423</td>
<td>0.015</td>
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<tr>
<td>1-40</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.220</td>
<td>0.023</td>
</tr>
<tr>
<td>1-42</td>
<td>0.051</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.253</td>
<td>0.689</td>
</tr>
</tbody>
</table>
Content: 16 proteins represented by 31 peptides with 236 transitions

- Amyloid-like protein 1
- Amyloid beta A4 protein
- Beta-2-microglobulin
- Complement C3 alpha and beta
- Chromogranin A
- Complement factor H
- Cystatin C
- Serum amyloid P-component
- Clusterin alpha chain
- Clusterin beta chain
- Apolipoprotein E
- Alpha-2-macroglobulin
- Secretogranin-2
- Gelsolin
- Fibrinogen gamma chain

Isotopic TMTduplex workflow

- TMTzero: Individual samples (50 µL)
- TMT6-127: Universal reference
- (Seralab #CSF-123-S-26975)
- Volumetric 1:1 mixture
- QC samples: Universal reference
  - TMTzero and TMT6-127 1:1
- 1h nLC gradient
- TSQ Vantage
- Triplicate analysis
- 2 µL CSF o/c
- PinPoint 1.1 including manual editing
Assay Precision - All CV’s below 15% except three peptides

Decreased performance of peptides GLEVTITAR, ALQEYR and AVEVLPK due to reduced quality of SRM transitions
For this cohort, the 16plex CSF assay gives remarkable separation of AD cases and non AD subjects with most analytes having a strong influence on the differentiation of the clinical groups.
TMTcalibrator CSF-Tau

Fluid Samples

Control

Disease

Reduce, Alkylate, Digest, Label

TMT<sup>10.126</sup> -127e -127 -128e -128 -129e -129

TMT<sup>10.129</sup> -130 -130e -131

Mix & Analyse

Four point calibration curve generated from tissue reporter ion intensities allows endogenous fluid peptide quantitation.
TMTcalibrator for CSF Tau

1 mL CSF

Perchloric Acid Precipitation

Neutralisation of supernatant (with KOH)

Vivaspin filtration (concentration & desalting)

Trypsin digestion and labelling with TMT6-126, 127, 127e, and 128 respectively

Addition of 0.8ng Tau131 peptide and 0.2ng (129) and 0.4ng (129e) and 0.6ng (130) phospho-peptide mixture as a 4 point spike with total of 2ng

SCX fractionation (10 fractions)

HCD and MS3, include list

2 mL CSF

MARS6 depletion

Vivaspin filtration (concentration & desalting)

Trypsin digestion and labelling with TMT6-126, 127, 127e, and 128 respectively

Addition of 0.8ng Tau131 peptide and 0.2ng (129) and 0.4ng (129e) and 0.6ng (130) phospho-peptide mixture as a 4 point spike with total of 2ng

SCX fractionation (10 fractions)
Several Tau peptides as surrogates for Total Tau measurement each with a four-point internal calibration curve
Illustration of TMTcalibrator results

**TPPSSGEPPK**

![Graph showing reporter intensity against different sample volumes and spike mixes.](image)

<table>
<thead>
<tr>
<th>Sample</th>
<th>Slope</th>
<th>Y axis Offset</th>
<th>$R^2$</th>
<th>Intensity Average</th>
<th>Amount Peptide in Sample [ng]</th>
<th>Used Volume in Preparation [mL]</th>
<th>Amount Peptide per mL [ng/mL]</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.0 ml 540</td>
<td>1451.9000</td>
<td>26.4500</td>
<td>0.9383</td>
<td>208.70</td>
<td>0.13</td>
<td>1.00</td>
<td>0.13</td>
</tr>
<tr>
<td>2.0 ml 540</td>
<td>1451.9000</td>
<td>26.4500</td>
<td>0.9383</td>
<td>613.20</td>
<td>0.40</td>
<td>2.00</td>
<td>0.20</td>
</tr>
<tr>
<td>1.0 ml 550</td>
<td>1451.9000</td>
<td>26.4500</td>
<td>0.9383</td>
<td>190.60</td>
<td>0.11</td>
<td>1.00</td>
<td>0.11</td>
</tr>
<tr>
<td>2.0 ml 550</td>
<td>1451.9000</td>
<td>26.4500</td>
<td>0.9383</td>
<td>463.70</td>
<td>0.30</td>
<td>2.00</td>
<td>0.15</td>
</tr>
</tbody>
</table>
Our hunt for Tau phospho-peptides in CSF continues…

- Further exploration of Tau/pTau enrichment prior to MS
  - Immuno-ppt and TiO$_2$
- Create optimal TMTcalibrator standard
  - Post mortem AD brain or RecTau+Kinase(s) + ATP
- Use of additional TMT related fragment ions for quantitation purposes (TMT$^C$ ions Wühr & Gygi 2012)
- We are still looking for additional partners to help
  - Access to CSF cohorts
  - Advice on tau fragments for inclusion
  - Funding
A hybrid targeted and non targeted screening approach
**Input A:** Samples (n=90)

**Input B:** Include List Content

**Output:** New Biomarker Panels

Group modeling of data handling (GMDH)

<table>
<thead>
<tr>
<th>Protein</th>
<th>Number of peptides</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alpha-2-macroglobulin</td>
<td>15</td>
</tr>
<tr>
<td>Apolipoprotein E</td>
<td>13</td>
</tr>
<tr>
<td>Complement C3</td>
<td>14</td>
</tr>
<tr>
<td>Complement factor H</td>
<td>10</td>
</tr>
<tr>
<td>Gelsolin</td>
<td>12</td>
</tr>
<tr>
<td>Clusterin</td>
<td>11</td>
</tr>
<tr>
<td>Fibrinogen gamma chain</td>
<td>12</td>
</tr>
<tr>
<td>Serum amyloid P-component</td>
<td>8</td>
</tr>
</tbody>
</table>

Table 1: AD SRM proteins and number of peptides included in the LTQ Orbitrap Velos method

2089 distinct peptides corresponding to 199 identified protein groups

Reference for GMDH: Alexey G. Ivakhnenko 1968
To Conclude

• Use of MS combined with TMT facilitates rapid and inexpensive set up and assay validation

• Multiple analytes are measured with high quantitative precision and accuracy

• Several AD Biomarker Assays are “Ready to Use”

• Further details on our posters at AAIC tomorrow

# P1-141: Quantitative mass spectrometry assays for Amyloid beta, tau and phospho-tau in CSF

# P1-233: Further exploration of Plasma Biomarkers for Alzheimer’s disease using isotopic Tandem Mass Tags and a combined targeted /non-targeted LC/MS/MS method