Blood Biomarkers in Ischemic Stroke

A window to the brain...

Teresa García-Berrocoso
Neurovascular Research Laboratory, Vall d’Hebron Institute of Research (VHIR)
Barcelona
Stroke: a widespread disease

Death & disability
(socioeconomic burden)

World Stroke Day
October 29, 2011

Join the campaign to prevent stroke now:
WWW.WORLDSTROKECAMPAIGN.ORG

Mitsubishi Tanabe Pharma Corporation
Allergan
Stroke symptoms and signs

Face: Does the face look uneven? Ask them to smile.

Arm: Does one arm drift down? Ask them to raise both arms.

Speech: Does their speech sound strange? Ask them to repeat a phrase.

Time: Every second brain cells die. Call 9-1-1 at any sign of stroke.

Is it a stroke? Check these signs FAST!

Call 9-1-1 at any sign of stroke.
**What ischemic stroke is?**

Reduction of cerebral blood flow by occlusion

Because atherosclerosis

From the heart
Stroke treatment: recombinant tissular Plasminogen Activator

< 4.5 h from onset

Exogenous tPA

Thrombus Embolism

Activation of endogenous tPA

Cerebral Vessel Occlusion

Acute Ischemic Stroke

No Resolution

Resolution

Spontaneous Resolution

clot lysis

Desired Outcome in Acute Stroke
Same situation, different ending

- **Difussion**
- **Perfusion**
- **Angiography**

2 hours

3 days

rt-PA

3 days

What’s happening at the molecular & cellular level?

García Berrocoso T et al. AIT. Marge Médica Books, BCN. 2009
In total 5,190 references, only 1,043 for plasma biomarkers (2011/12/07)

But...any biomarker has not yet been applied to the clinical practice
Biomarkers in Acute Myocardial Infarction

2,264 references CK-MB [first publications in 1973]
4,020 references on Troponin [first publications in 1987]
The complexity of Stroke

Are cardiologists smarter than neurologists?

- **Barrier level**: BBB and protein release
- **Organ level**: other diseases than stroke produce acute brain damage
- **Cellular level**: Neurons, astrocytes…
Going further in discovery of biomarkers for stroke

Different approaches used in large-scale proteomics studies

Brain Samples
Cerebro Spinal Fluid
Microdialysate
Plasma/serum
CSF proteomics: close to the brain

Brain damage model

2-DE of Post-mortem CSF  2-DE of Ante-mortem CSF

Differentially expressed proteins

Mass spectrometry identification  Immunoassay validation (Plasma)
### Post-mortem CSF: 11 brain damage candidates

From over-expressed spots in 2DE-gels

<table>
<thead>
<tr>
<th>Protein Name</th>
<th>Abbreviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calcyphosin</td>
<td>CAYP</td>
</tr>
<tr>
<td>Cathepsin D</td>
<td>CATD</td>
</tr>
<tr>
<td>Protein DJ-1</td>
<td>PARK7</td>
</tr>
<tr>
<td><strong>Fatty Acid Binding Protein (FABP)</strong></td>
<td></td>
</tr>
<tr>
<td><strong>Glial Fibrillary acidic protein (GFAP)</strong></td>
<td></td>
</tr>
<tr>
<td>Glutathione S-transferase P</td>
<td>GSTP-1</td>
</tr>
<tr>
<td>Nucleoside Diphosphatase Kinase A</td>
<td>NDKA</td>
</tr>
<tr>
<td>Peroxiredoxin-5</td>
<td>PRDX5</td>
</tr>
<tr>
<td>Peptidyl-prolyl cis-trans isomerase A</td>
<td>PPIA</td>
</tr>
<tr>
<td>Ubiquitin carboxyl-terminal hydrolase isozyme L1</td>
<td>UCH-L1</td>
</tr>
<tr>
<td>Ubiquitin fusion degradation protein 1</td>
<td>UFD1</td>
</tr>
</tbody>
</table>

Replication of new identified markers in plasma

Home-made ELISAs for comparing strokes and healthy controls

3 different cohorts with similar se/sp %


Brain microdialysate proteomics

Directly from the lesion
# Brain extracellular fluid protein changes in stroke

List of proteins increased in IC vs PI areas from 2 patients

<table>
<thead>
<tr>
<th>Protein</th>
<th>Abbrev.</th>
<th>Ratio IC/PI (Exp_a)</th>
<th>Ratio IC/PI (Exp_b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acyl-CoA-binding protein</td>
<td>ACBP</td>
<td>1.95</td>
<td>2.67</td>
</tr>
<tr>
<td>Beta-2-microglobulin precursor</td>
<td>B2MG</td>
<td>1.49</td>
<td>2.09</td>
</tr>
<tr>
<td>Coactivin-like protein</td>
<td>COTL1</td>
<td>1.72</td>
<td>2.04</td>
</tr>
<tr>
<td>Complement C4-A precursor</td>
<td>P0C0L4</td>
<td>2.50</td>
<td>1.10</td>
</tr>
<tr>
<td>Cystatin-B</td>
<td>CYTB</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cystatin-C</td>
<td>CYSC</td>
<td>1.73</td>
<td>1.66</td>
</tr>
<tr>
<td>Cysteine and glycine-rich protein 1</td>
<td>CSRP1</td>
<td>3.33</td>
<td>2.88</td>
</tr>
<tr>
<td>Fatty acid-binding protein, brain</td>
<td>FABP</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fibrinogen alpha chain precursor</td>
<td>FIBA</td>
<td>2.97</td>
<td>0.61</td>
</tr>
<tr>
<td>Glutathione S-transferase P</td>
<td>GSTP1</td>
<td>2.79</td>
<td></td>
</tr>
<tr>
<td>Heterogeneous nuclear ribonucleoprotein G</td>
<td>HNRPG</td>
<td>2.35</td>
<td></td>
</tr>
<tr>
<td>Metallothionein-3</td>
<td>MT3</td>
<td>2.10</td>
<td>2.79</td>
</tr>
<tr>
<td>Myelin basic protein [ISOFORM 3 or 4]</td>
<td>MBP</td>
<td>1.71</td>
<td>3.11</td>
</tr>
<tr>
<td>Neutrophil defensin 1 precursor</td>
<td>DEF1</td>
<td></td>
<td>2.45</td>
</tr>
<tr>
<td>Paraldehyde</td>
<td>PALM</td>
<td>3.52</td>
<td></td>
</tr>
<tr>
<td>Peptidyl-prolyl cis-trans isomerase A</td>
<td>PPIA</td>
<td>2.45</td>
<td>1.60</td>
</tr>
<tr>
<td>Peroxiredoxin-1</td>
<td>PRDX1</td>
<td></td>
<td>1.93</td>
</tr>
<tr>
<td>Peroxiredoxin-2</td>
<td>PRDX2</td>
<td>2.72</td>
<td></td>
</tr>
<tr>
<td>Peroxiredoxin-6</td>
<td>PRDX6</td>
<td>2.15</td>
<td>2.16</td>
</tr>
<tr>
<td>Phosphatidylethanolamine-binding protein 1</td>
<td>PEBP1</td>
<td>2.06</td>
<td>1.60</td>
</tr>
<tr>
<td>Plasma retino-binding protein precursor</td>
<td>RETBP</td>
<td>2.83</td>
<td>1.63</td>
</tr>
<tr>
<td>Plasminogen precursor</td>
<td>PLMN</td>
<td>2.27</td>
<td></td>
</tr>
<tr>
<td>Platelet basic protein precursor</td>
<td>SCYB7</td>
<td>2.51</td>
<td>0.85</td>
</tr>
<tr>
<td>Profilin-1</td>
<td>PROF1</td>
<td>2.40</td>
<td>0.91</td>
</tr>
<tr>
<td>SH3 domain-binding glutamic acid-rich-like protein</td>
<td>SH3L1</td>
<td>2.17</td>
<td>1.92</td>
</tr>
<tr>
<td>Thioredoxin</td>
<td>THIO</td>
<td>2.17</td>
<td></td>
</tr>
<tr>
<td>Ubiquitin</td>
<td>UBIQ</td>
<td>2.09</td>
<td>1.50</td>
</tr>
</tbody>
</table>

From brain to blood: replication of candidates

Peroxiredoxin family


- **Brain microdialysate levels** (MS quantification)
- **Serum levels** (ELISA)

![Graph showing increase in PRDX1 and PRDX6](image)
So, a very interesting list of potential stroke biomarkers coming from close to the human brain has been generated through proteomics techniques.

The detection in higher levels in blood samples from stroke patients of these proteins pointed out its promising use to the biochemical monitoring of stroke patients.
Stroke biomarkers applications

- Risk prediction: Lp-PLA2
- Diagnosis
- Treatment effectiveness
- Prognosis
- Recurrence
Diagnosis of stroke

- Stroke has to be distinguished from ‘mimics’ QUICKLY
- % ‘mimics’ depends on the healthcare system level

But... few publications so far comparing strokes with mimics, but with healthy controls
## Diagnosis of stroke: Screening of an antibodies library by multiple ELISAs in plasma samples

### Angiogenesis Factors
- ANG-2
- FGF basic
- HB-EGF
- HGF
- KGF
- NGAL
- PDGF-AA
- PDGF-BB
- TIMP-1
- TIMP-2
- TPO
- VEGF
- VEGF-C
- VEGF-D
- VEGF-R1
- VEGF-R2

### Cell Adhesion Molecules
- E-Cadherin
- E-Selectin
- ICAM-1
- L-Selectin
- P-Selectin
- VCAM-1

### Chemokines
- EDA
- Eotaxin
- Eotaxin-2
- Eotaxin-3
- GROα
- GROβ
- HCC-4 (CCL-16)
- I-300
- IP-10
- ITAC
- Lymphectatin
- MCP-1
- MCP-2
- MCP-3
- MCP-4
- MDC
- MIF
- MG21
- MMP-1
- MMP-2
- MMP-3
- MMP-4
- MMP-5
- MMP-6
- MMP-7
- MMP-9
- MMP-10
- MMP-13

### Cytokines
- GM-CSF
- G-CSF
- IFNα
- IFNβ
- IL-1α
- IL-1β
- IL-1ra
- IL-2
- IL-3
- IL-4
- IL-5
- IL-6
- IL-7
- IL-8
- IL-9
- IL-10
- IL-11
- IL-12(p40, p70), (heterodimer)
- IL-13
- IL-15
- IL-16
- IL-17
- IL-18
- IL-23
- MCP-3
- TNFα
- TNFβ

### Trimer Specific Cytokine Receptor

### Growth Factors

### Immunoglobulins
- IgG

### Matrix Metalloproteinases
- MMP-1
- MMP-2
- MMP-3
- MMP-7
- MMP-8
- MMP-9
- MMP-10
- MMP-13

### Neurotrophic Factors
- β - NGF
- BDNF
- CNTF
- GDNF
- NT3

### Other Biomarkers
- 2-Macroglobulin
- A-SAA
- Actn-30 (Adiponectin)
- AR (Ampullae of Retzius)
- Apo A-1
- Apo B-100
- β-APP
- Cathespin-D
- C-peptide
- CD14
- CD30 (TNFRSF8)
- CD40L
- CGBP
- CCL2
- CRP (C-reactive protein)
- D-Dimer
- ER (Epiregulin)
- FSHα
- FasL
- Fibronogen
- Fibropeptin
- IFGBP-1
- IFGBP-3
- Inhibin A
- Leptin
- LIF
- MPO (Myeloperoxidase)
- MRP-8/14
- NGAL
- NT-proBNP
- OPN (Osteoprotegrin)
- OPN (Osteopontin)
- PAA-1 Active
- PAA-1 Total
- PAP-A
- PEPF
- PLGF (Placental Growth Factor)
- Prolactin
- Protein C
- RAGE
- RANK
- RANKL
- Resistin
- S1Bβ
- SIRP
- SP-D
- Thrombomodulin
- Tissue Factor
- TRAIL
- TSP-1 (Thrombospondin-1)
- TSP-2 (Thrombospondin-2)
- TWEAK
- Viscatun
- VorWillebrand Factor

---

### III SCIENTIFIC MEETING

Searchlight library: 178 human proteins
Stroke diagnostic test

SCREENING

178 proteins
9 Stroke
2 Mimics
4 Healthy controls

CANDIDATES VALIDATION

22 first candidates
146 Stroke
61 Mimics
23 Healthy controls

6 DIAGNOSTIC BIOMARKERS FOR STROKE


www.lin-bcn.com
### A new diagnostic model

Combination of clinical & biomarkers data

<table>
<thead>
<tr>
<th>Clinical Factors + Biomarkers</th>
<th>Odds Ratio (I.C. 95%)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein A</td>
<td>114.766 (3.496-3767.611)</td>
<td>0.008</td>
</tr>
<tr>
<td>Protein B</td>
<td>9.193 (2.264-37.323)</td>
<td>0.002</td>
</tr>
<tr>
<td>Protein C</td>
<td>34.552 (2.613-456.953)</td>
<td>0.007</td>
</tr>
<tr>
<td>Protein D</td>
<td>8.699 (2.877-26.299)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Hypertension</td>
<td>3.467 (1.125-10.687)</td>
<td>0.03</td>
</tr>
<tr>
<td>Atrial Fibrillation</td>
<td>26.72 (4.766-149.805)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Dyslipidemia</td>
<td>8.116 (1.795-36.697)</td>
<td>0.007</td>
</tr>
</tbody>
</table>

Montaner J et al. Manuscript in preparation

Now we’re preparing a multicenter study which will include **1,500 stroke suspicions** to validate this **DIAGNOSTIC model**
Prognosis of stroke

How to predict outcome?

- Patient and relatives information
- Risk/benefit of treatment options
- Rationing decisions to safe sources (patient allocation)
The proteome of human brain after stroke

Brain tissue obtained <6 h post-mortem

3 Strokes

Periinfarct (PI)

Infarct Core (IC)

Contralateral (CH)

3 Controls (C)
Brain proteome: 2DE-DIGE + MALDI-TOF/TOF MS

About 1,500 spots in 2DE-DIGE gels.
132 spots differentially expressed between areas.

42 spots identified by MALDI-TOF/TOF MS, corresponding to 39 different proteins

Tannu NS, Hemby SE. *Nat Protoc.* 2006;1:1732-42.
39 proteins identified from stroke brains

Proteins mainly involved in:
- Immunity pathways
- Axonal growth
- Energy and structure

90 missing spots

Orbitrap MS: higher resolution & accuracy

20 spots identified by ESI LTQ-OT MS, corresponding to 12 NEW proteins

García-Berrocoso T et al. Manuscript in preparation

From brain to blood
Replication study with selected candidates

51 proteins identified in brain samples
- Biological function
- Levels in IC
- Commercial ELISA

8 protein candidates
- Overexpressed in IC
- Underexpressed in IC

60 Stroke blood samples (ELISAs)
- Clinical data
- Neurological outcome (NIHSS)
- Functional state (mRS) (3rd month)
Biomarkers associated to long-term prognosis

3 candidates have not been detected in blood samples

Proteins A & B are higher expressed in patients functionally disabled at 3rd month

Protein D is lower expressed in patients functionally disabled at 3rd month

García-Berrocoso T et al. Manuscript in preparation
Protein A > 19.87 ng/mL improves significantly the predictive value of the clinical data for the prognosis of disability at 3rd month.
What about in-hospital mortality?

Garcia-Berrocoso T et al. Manuscript in preparation

Protein B > 34.35 ng/mL improves significantly the prediction of in-hospital mortality in comparison with only clinical data

<table>
<thead>
<tr>
<th>Model</th>
<th>AUC</th>
<th>SE</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>NIHSS, previous stroke</td>
<td>0.876</td>
<td>0.051</td>
<td>0.776 – 0.976</td>
</tr>
<tr>
<td>NIHSS, previous stroke, protein B</td>
<td>0.968</td>
<td>0.025</td>
<td>0.919 – 1.016</td>
</tr>
</tbody>
</table>
To explore the presence of hundreds of proteins (low scale) directly in the plasma has allowed us to create a Diagnostic model for stroke that hopefully will help clinicians in the future.

At a large scale, the screening of the proteome of brain tissue has also given very good candidates for the prognosis of stroke when measured in blood within the first hours after the symptoms’ onset.
What’s happening at the cellular level?

Cellular complexity: Neurovascular Unit (NVU)
Metalloproteinases: a familiar face in stroke

Protein array Searchlight

Cuadrado E, et al. J Proteome Res. 2009; 8: 3191-7
**Immuno-guide laser microdissection (LMD)**

Neurons & vessels from stroke human brains

Before LMD

After LMD

<table>
<thead>
<tr>
<th>Protein</th>
<th>Vessels</th>
<th>Neurons</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD31</td>
<td></td>
<td>130 kDa</td>
</tr>
<tr>
<td>NeuN</td>
<td>48 kDa</td>
<td></td>
</tr>
<tr>
<td>GfAP</td>
<td>50 kDa</td>
<td></td>
</tr>
</tbody>
</table>

MMP-9/TIMP-2 from vessels; MMP-10 from neurons

Cuadrado E, et al. J Proteome Res. 2009; 8: 3191-7
To study the **cellular proteomes** might help in finding the **origin** of some protein candidates to be biomarkers for stroke, but also finding **new proteins** that were hidden within the dynamic range of whole brain homogenates usually used.

Now we’re working on this cellular approach at proteomics large scale.
Conclusions

Now we know better what we want from biomarkers to stroke:

- Specific from the disease
- Highly sensitive to help in decision-making processes
- Measurable in blood
- Enough power to improve clinical models

We need:

- Go-on discovering new blood biomarkers
- Verification of known biomarkers (multicenter & multinacional studies)
- Put all pieces together (e.g. metanalysis)