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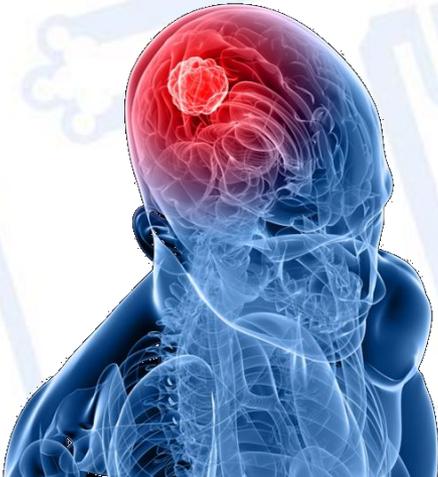
The Association for

Mass Spectrometry:
Applications to the Clinical Lab

Identification of Novel Biomarkers of Brain Injury by Integrating Bioinformatics and Mass Spectrometry-based Proteomics

MSACL 2014 EU – Salzburg, Austria
Thursday, September 4th, 2014

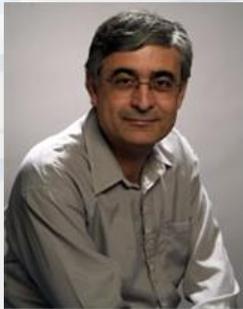
Eduardo Martínez-Morillo, PhD
Clinical Biochemist
Specialist in Mass Spectrometry



Collaborative Study

Lunenfeld-Tanenbaum
Research Institute

MOUNT SINAI HOSPITAL 
Joseph and Wolf Lebovic Health Complex

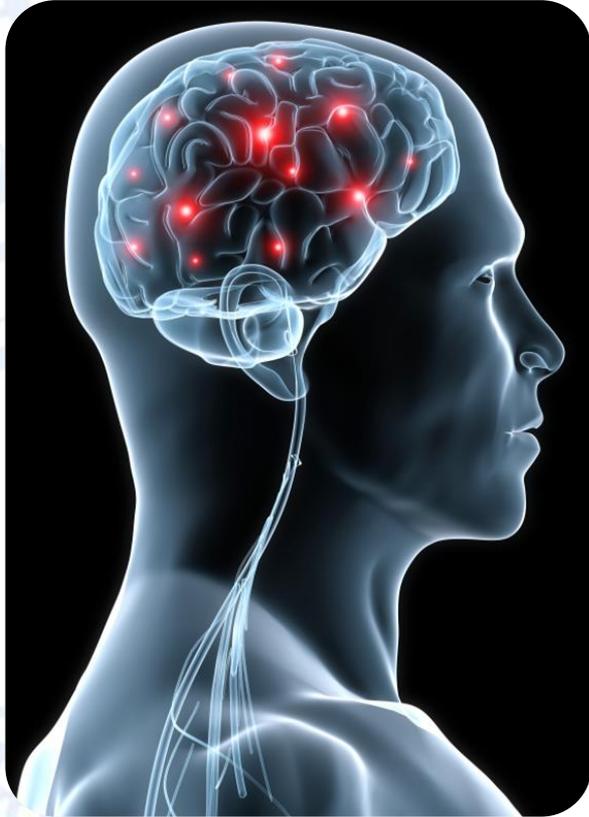


**Eleftherios P.
Diamandis**



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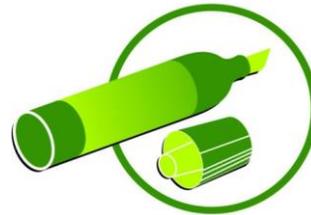
□ Brain injury:

1. Non-traumatic

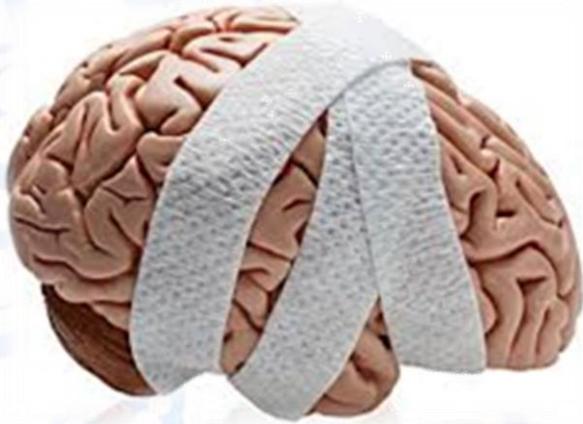
Infection

Stroke

2. Traumatic (TBI)



Introduction (II)



Approximately **1.7 million** Americans suffer a **TBI** each year, and of those 75-90% are classified as minor head injury (**MHI**)¹

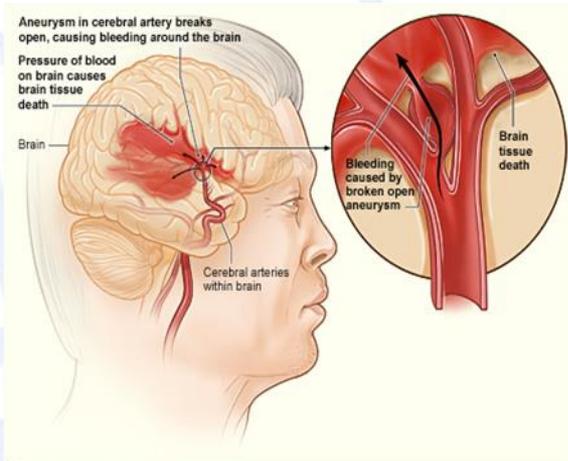
Stroke is the most common form of non-TBI, with **795,000** people in the United States suffering a new or recurrent stroke each year²



Differentiating between hemorrhagic and ischemic stroke is critical to determine treatment options

Hemorrhagic stroke

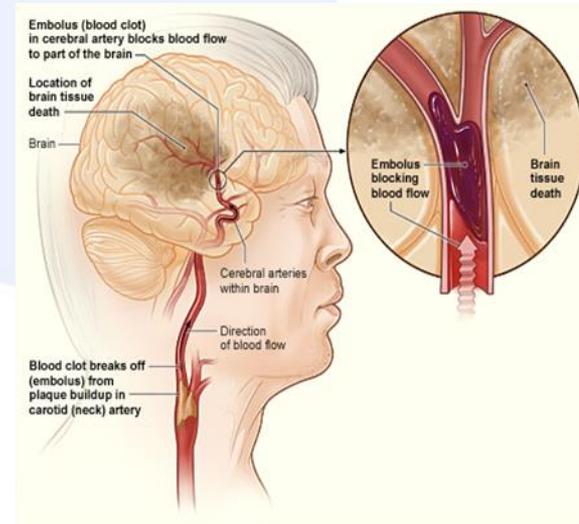
13%



Homeostatic therapy

Ischemic stroke

87%



Thrombolytic therapy

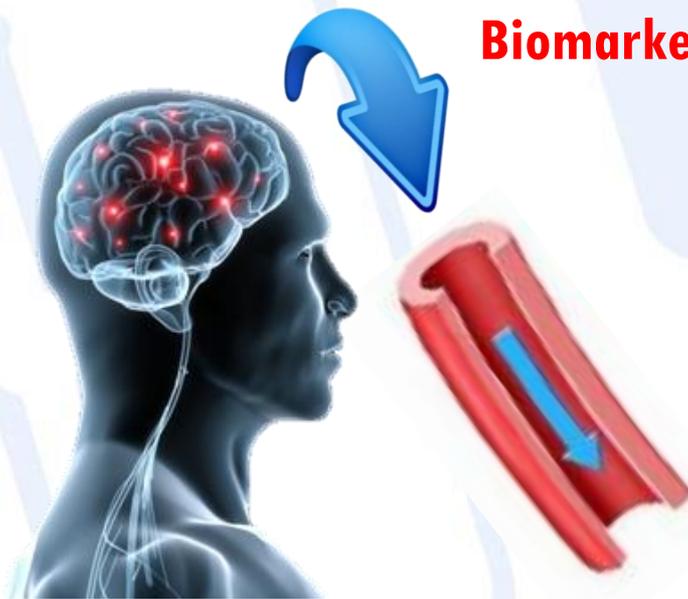


Rapid diagnosis and prompt medical attention is important because deterioration of patients is common in the first few hours after symptoms onset

Several proteins such as S100B, NSE and GFAP have been proposed as diagnostic and prognostic biomarkers of TBI and stroke



Proteins specifically expressed at high levels in the brain may be released and detected in the CSF of patients with hemorrhagic stroke



Biomarkers of cellular death

These proteins may be detected in circulation and act as biomarkers

1. **Select “brain-specific” proteins using a bioinformatic approach.**
2. **Develop selected reaction monitoring (SRM) assays for candidate protein biomarkers using brain tissue extracts.**
3. **Quantify these proteins in CSF samples from patients with hemorrhagic stroke, ischemic stroke and controls.**

Protein Selection

THE HUMAN PROTEIN ATLAS  (Version: 10.0)

Aim 1

14079



High expression in brain cell types
Absent or low expression in other cell types

390

PeptideAtlas 



Human Brain Proteome 2012-09
High number of observations

Human Plasma Proteome 2012-08
Zero or low number of observations

76

The Human Protein Atlas (www.proteinatlas.org)

Peptide Atlas (www.peptideatlas.org)

Protein Selection (II)

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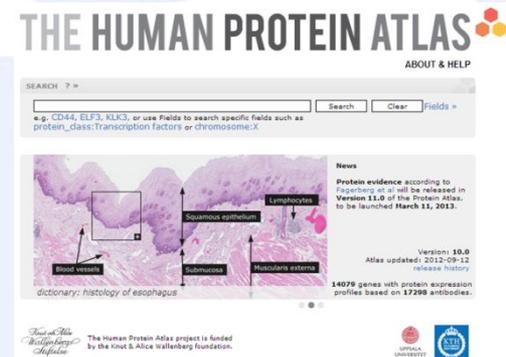
PeptideAtlas




Human Brain Proteome 2012-09
High number of observations

Human Plasma Proteome 2012-08
Zero or low number of observations

76



(17298 antibodies)

**Protein expression
profiles based on
immunohistochemistry**

The Human Protein Atlas (www.proteinatlas.org)

Peptide Atlas (www.peptideatlas.org)

Protein Selection (III)

THE HUMAN PROTEIN ATLAS  (Version: 10.0)

Aim 1

14079



High expression in brain cell types
Absent or low expression in other cell types

390

PeptideAtlas




Human Brain Proteome 2012-09
High number of observations

Human Plasma Proteome 2012-08
Zero or low number of observations

76

Human Brain Proteome 2012-09

Build Overview

Build Name	Human Brain 2012-09
Build Description	Update to ENS67
Reference Database	Ensembl Human Protein
Build Date	2012-09-13
# Samples	19
PSM FDR threshold (applied to each experiment)	0.0035000
Probability threshold	N/A
Canonical Proteins	2471
Distinct Peptides	15890
Total Observations	253253

Human Plasma Proteome 2012-08

Build Overview

Build Name	Human Plasma PeptideA 08
Build Description	Includes all plasma data
Reference Database	Ensembl Human Protein
Build Date	2012-08-11
# Samples	151
PSM FDR threshold (applied to each experiment)	0.0000500
Probability threshold	N/A
Canonical Proteins	3628
Distinct Peptides	49459
Total Observations	27629049

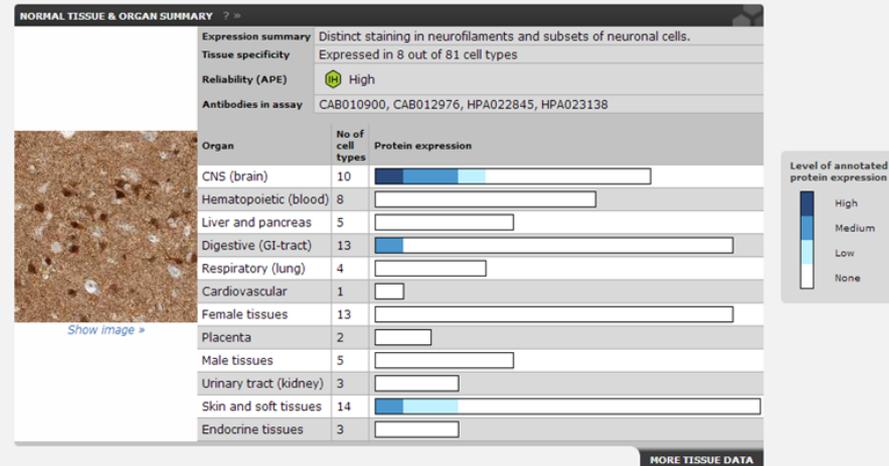
The Human Protein Atlas (www.proteinatlas.org)

Peptide Atlas (www.peptideatlas.org)

Protein Selection (IV)

THE HUMAN PROTEIN ATLAS

Neurofilament, medium polipeptide (NFM)



Positive Staining = 8 out of 81 cell types

Brain cell types = 4

Other cell types = 4



Brain Proteome (N Observations = 942)

Plasma Proteome (N Observations = 16)

Example:

NFM

Tissue	Antibody staining	Annotated expression
Central nervous system (Brain)		
Cerebral cortex	Neuronal cells	High
Cerebral cortex	Endothelial cells	None
Cerebral cortex	Glial cells	None
Hippocampus	Neuronal cells	Medium
Hippocampus	Glial cells	None
Lateral ventricle	Neuronal cells	High
Lateral ventricle	Glial cells	None
Cerebellum	Purkinje cells	High
Cerebellum	Cells in granular layer	None
Cerebellum	Cells in molecular layer	None

Specific expression in neuronal cells



Peptide Selection



Aim 2

Peptides previously observed in, at least, one of these three databases were selected

205



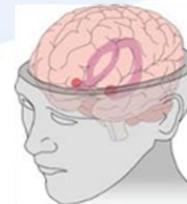
BLAST®

Uniqueness was confirmed
Peptides containing M residues or N-terminus C or Q residues were avoided if possible

Peptides were identified in brain tissue extracts (hippocampus)

127

(from 68 out of 76 proteins)



SRM atlas (www.srmatlas.org)

GPM database (gpmdb.thegpm.org)

Scaffold software (www.proteomesoftware.com)

Basic Local Alignment Search Tool (blast.ncbi.nlm.nih.gov/Blast.cgi)

Three ways:

- 1) Prediction of retention times using SRRCalc 3.0 (Skyline software).**
- 2) Co-elution of, at least, 6 transitions per peptide (from y_3 to y_{n-1}).**
- 3) Comparison of the observed fragmentation pattern with the fragmentation pattern displayed in publicly available databases (SRM atlas and GPM database) or in our in-house brain tissue proteome.**

Peptide Identification (II)

Example:



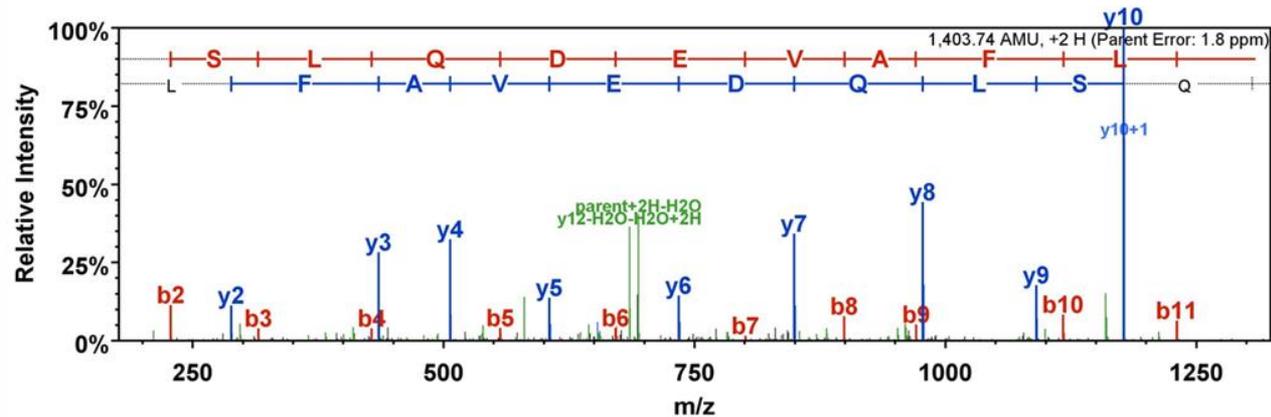
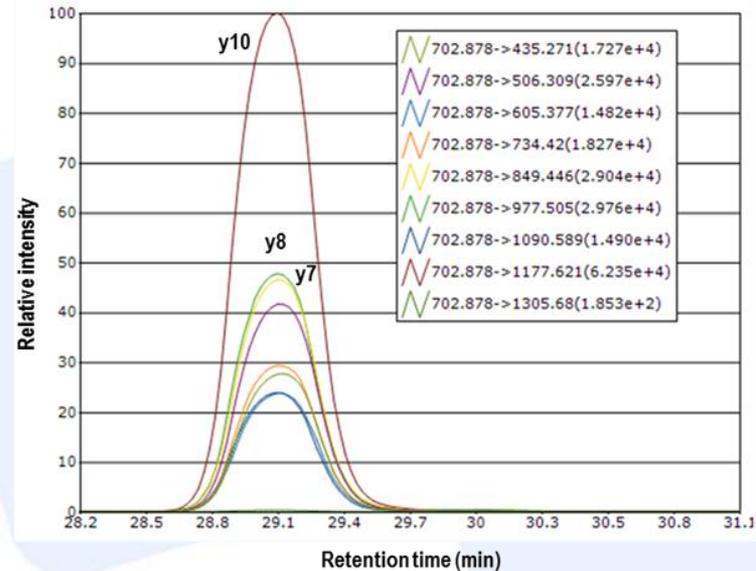
Peptide VQSLQDEVAFLR (from NFM)



SRRCalc 3.0

Predicted RT: **28.2 min** 95% CI (24.0 – 32.4)

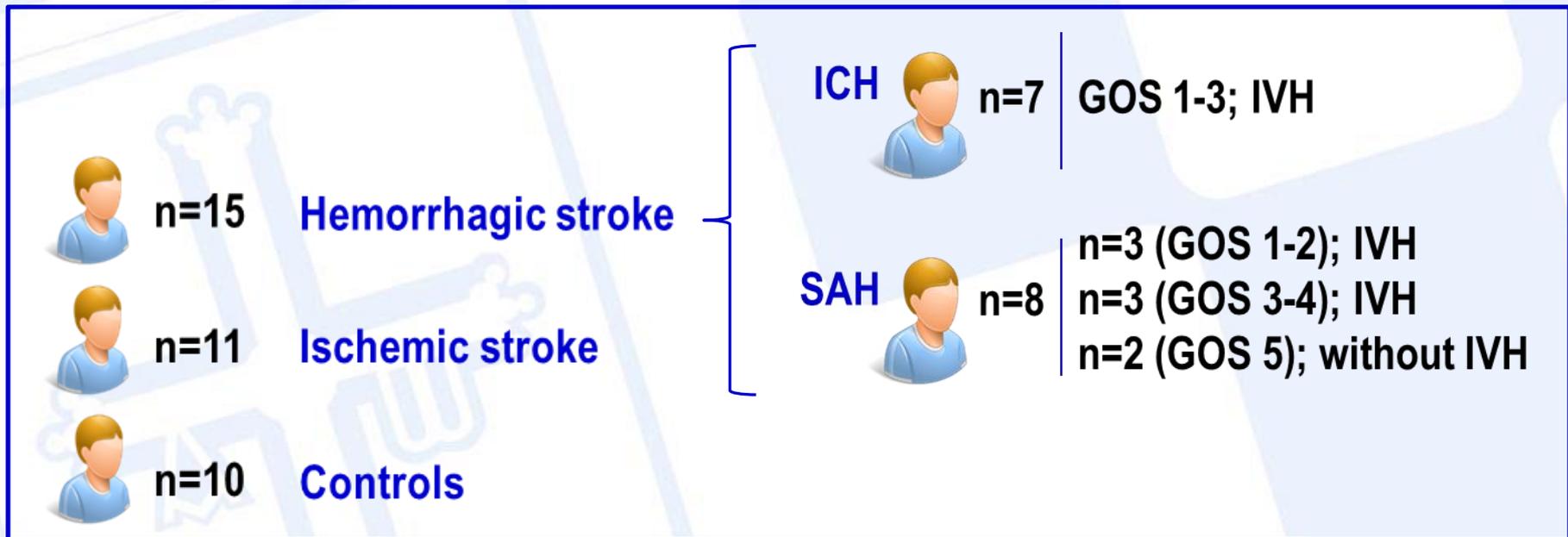
Observed RT: **29.1 min**



CSF sample collection

Aim 3

Age-matched CSF samples (n=36) were obtained from the department of Clinical Biochemistry at Hospital Universitario Central de Asturias (Spain)



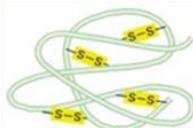
S100B protein was measured using a fully-automated immunoassay (Roche Diagnostics)

Sample preparation

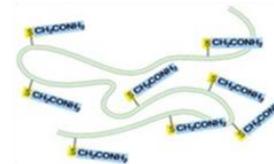
10 µg TP
Brain tissue extract or
CSF sample



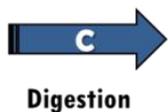
0.05% RapiGest®



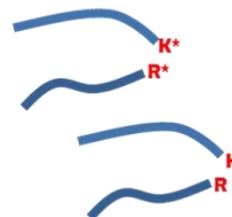
DTT (5 mM)
Iodoacetamide (15 mM)



Trypsin 1/10



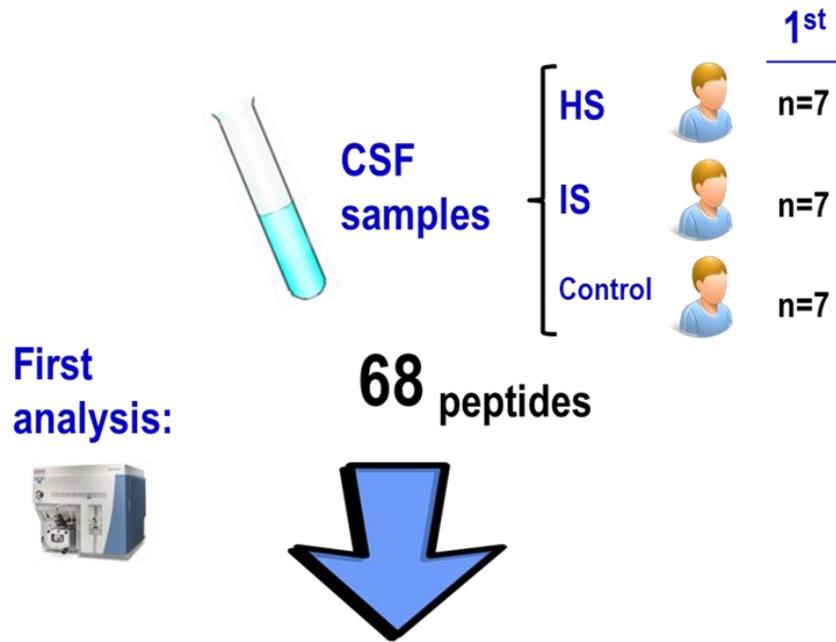
SpikeTides L

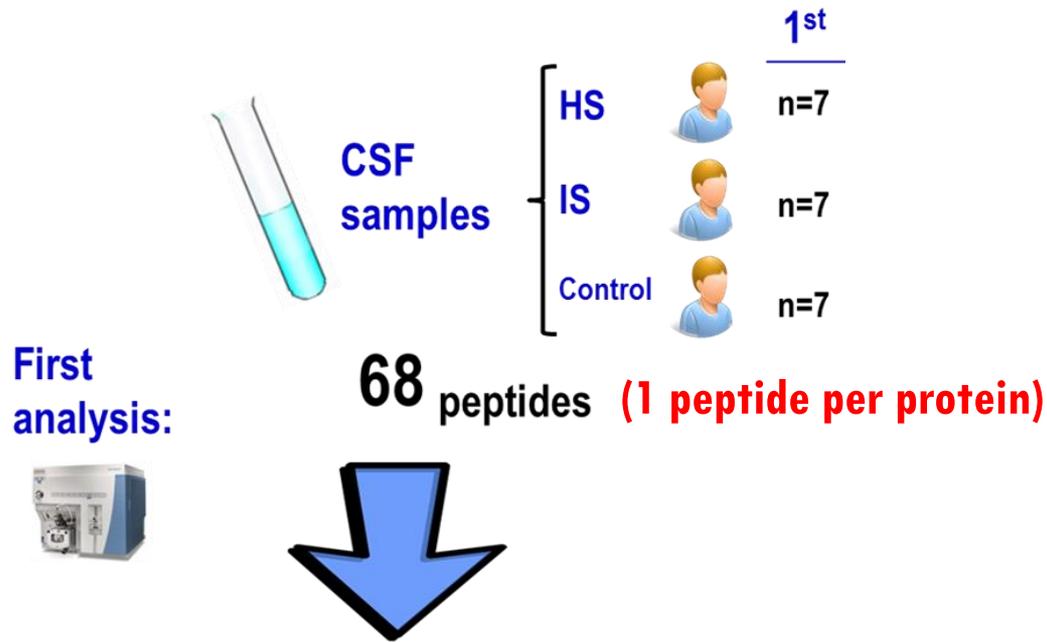


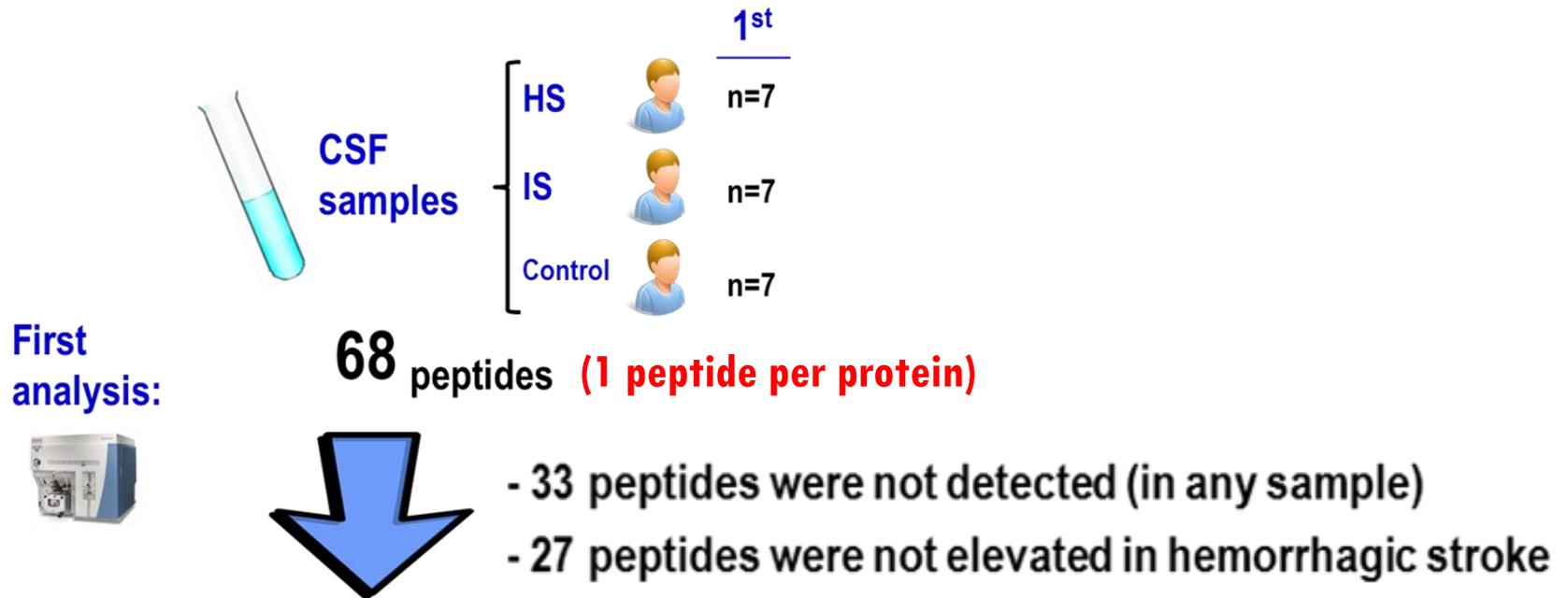
OMIX C18 tips
E →
Micro-extraction

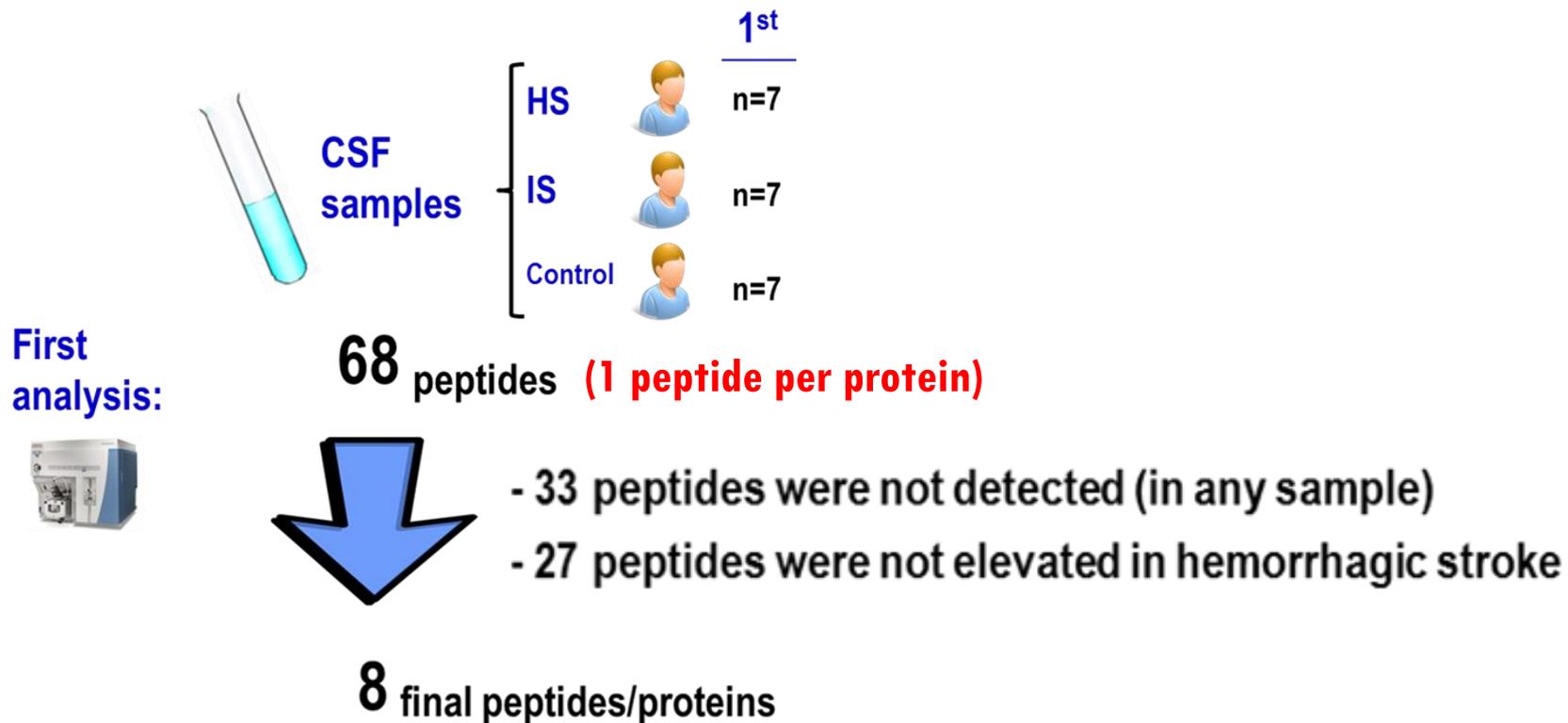


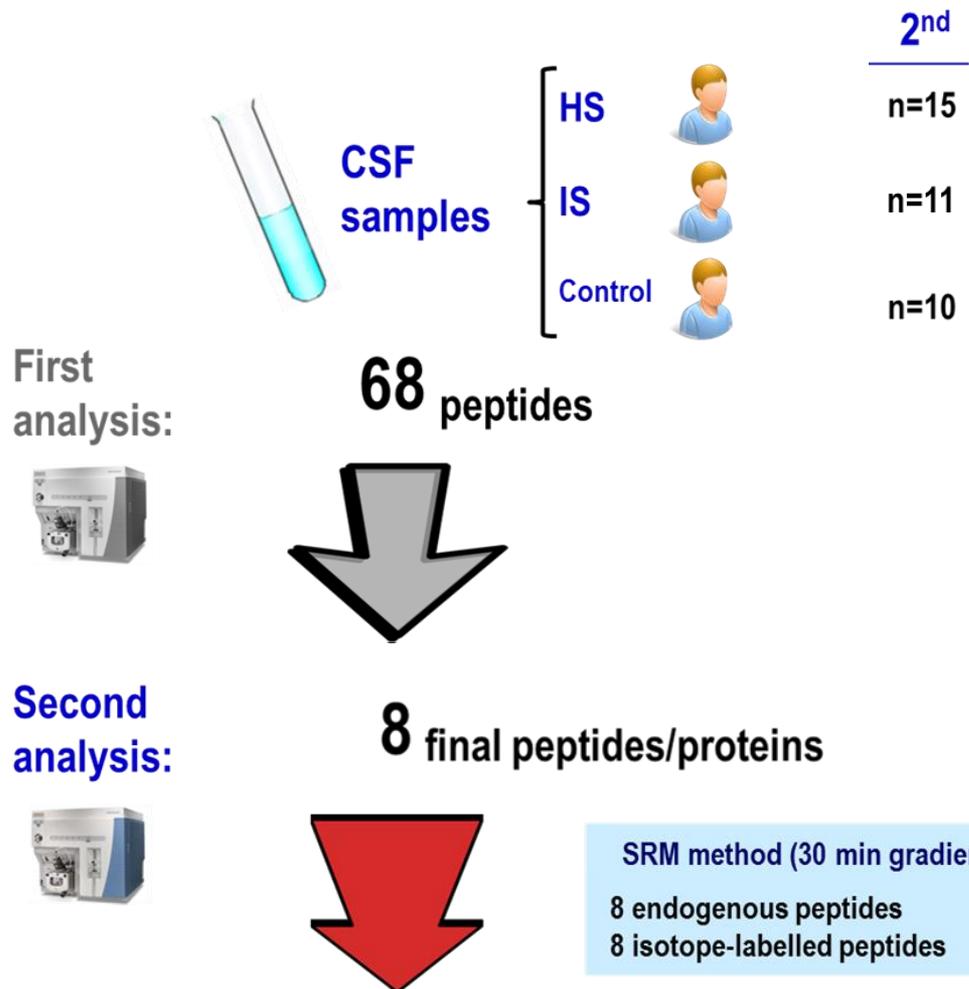
EASY-nLC 1000 + TSQ Vantage (Thermo Fisher)



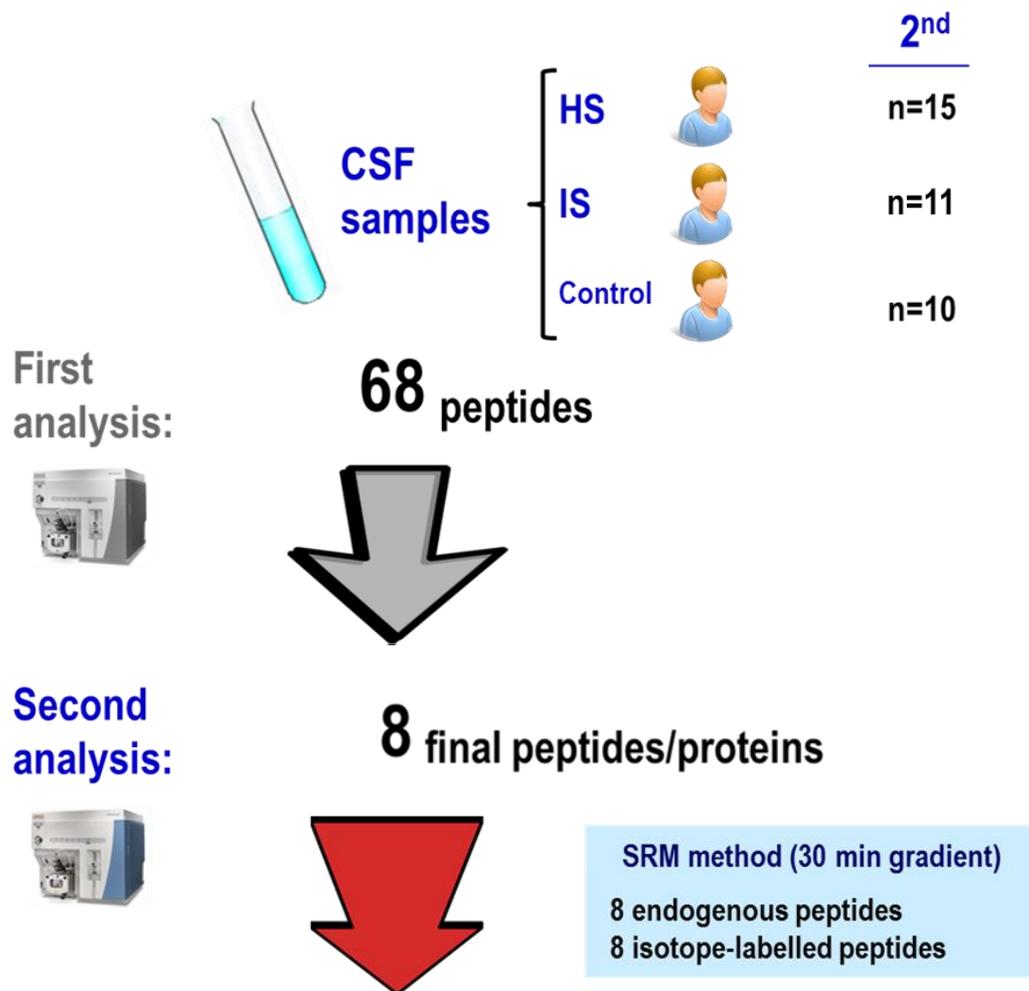




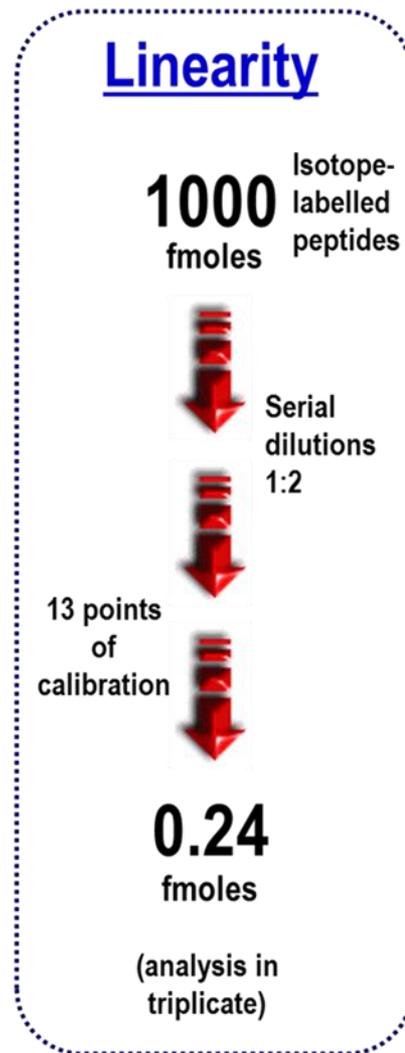




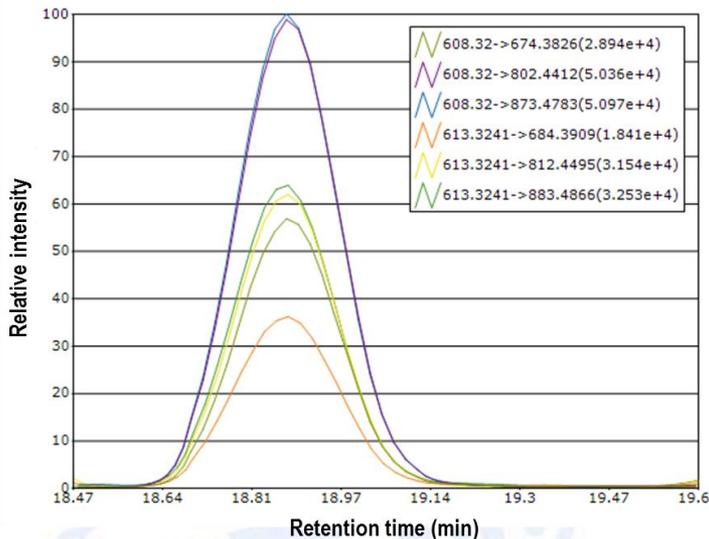
Selection of Candidate Protein Biomarkers



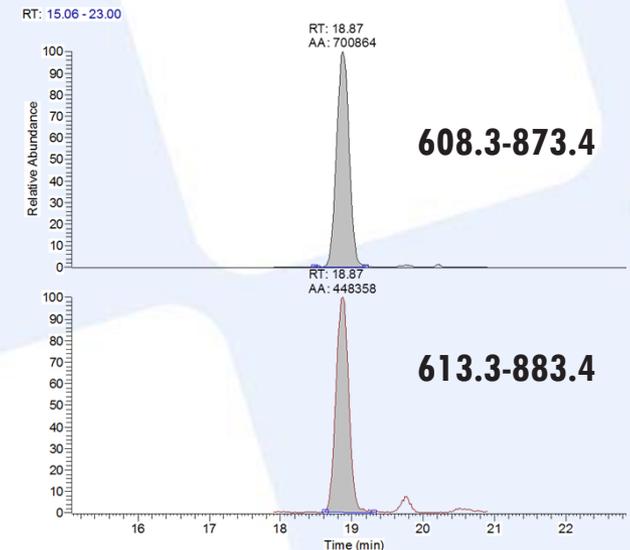
Linearity and LOQ were studied



Multiplex SRM method with 16 peptides (8 endogenous and 8 isotope-labelled)



Co-elution of six transitions for endogenous and isotope-labelled peptide DNLAQDLATVR



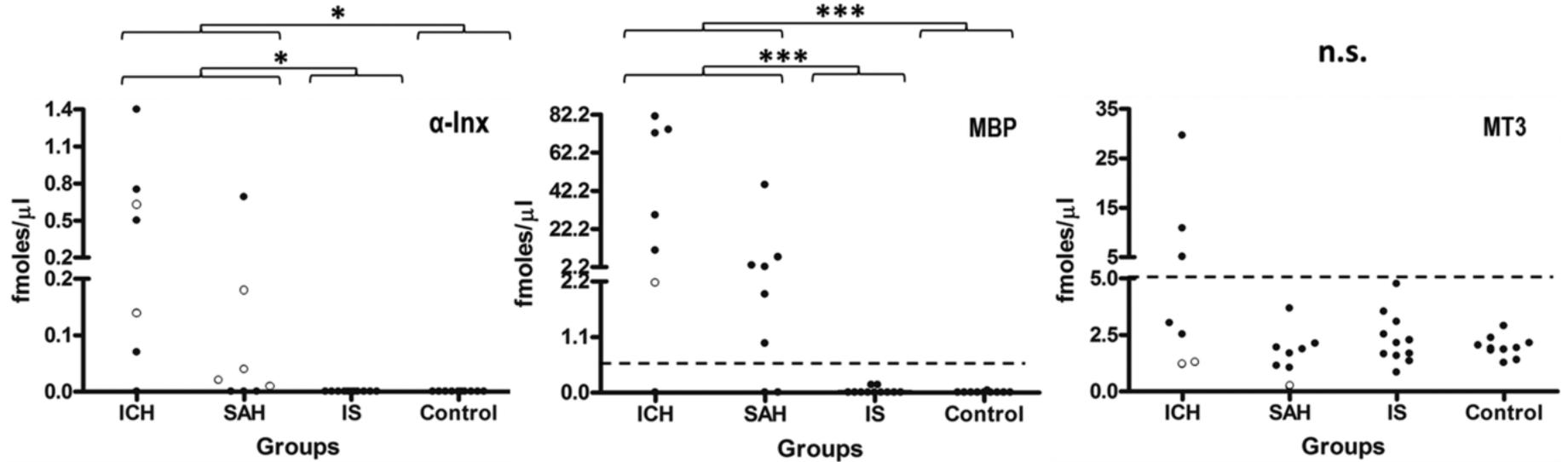
Three transitions per peptide were monitored
(two qualifiers and one quantifier)

Linearity

Protein	Peptide	Transitions	LOQ	CV	Linearity	R ²
NSE	IEEELGDEAR	580.7-918.4	0.24	4%	0.24 - 1000	0.9999
		585.7-928.4			0.24 - 3.9	0.9997
GFAP	DNLAQDLATVR	608.3-873.4	0.24	5%	0.24 - 1000	0.9999
		613.3-883.4			0.24 - 3.9	0.9999
α-Inx	ALEAELAALR	528.8-872.4	0.49	9%	0.49 - 1000	0.9999
		533.8-882.4			0.49 - 7.8	0.9987
MBP	GVDAQGTLK	488.2-819.4	0.24	12%	0.24 - 1000	0.9999
		492.2-827.4			0.24 - 3.9	0.9996
MT3	GGEAAEAEAEK	531.2-747.3	0.98	6%	0.98 - 1000	0.9998
		535.2-755.3			0.98 - 15.3	0.9996
NFM	VQSLQDEVAFLR	702.8-1177.6	0.49	13%	0.49 - 1000	0.9998
		707.8-1187.6			0.49 - 7.8	0.9992
β-Syn	EGVVQGVASVAEK	636.8-760.4	0.24	10%	0.24 - 1000	0.9999
		640.8-768.4			0.24 - 3.9	0.9999
γ-Syn	TVEEAENIAVTSGVVR	837.4-788.4	1.95	7%	1.9 - 1000	0.9997
		842.4-798.4			1.9 - 31.2	0.9971

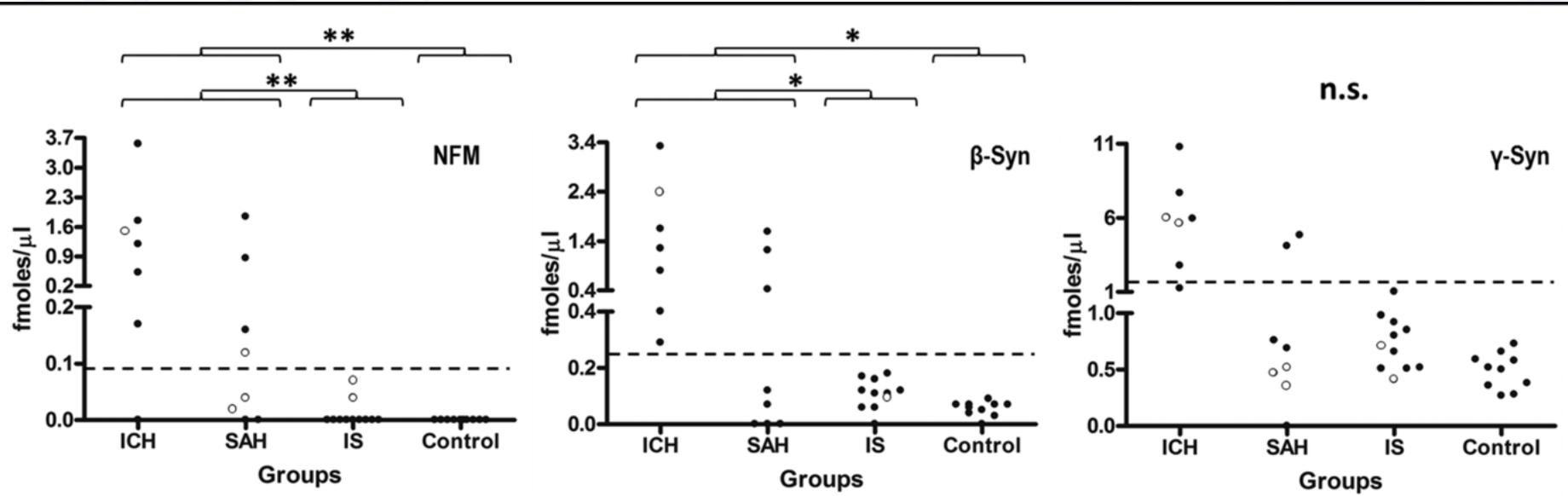
LOQ = Limit of quantification (fmol); CV = Coefficient of variation (triplicates); R² = Coefficient of determination

Results (II)



***) $p < 0.001$
**) $p < 0.01$
) $p < 0.05$
n.s.) not significant

Results (III)



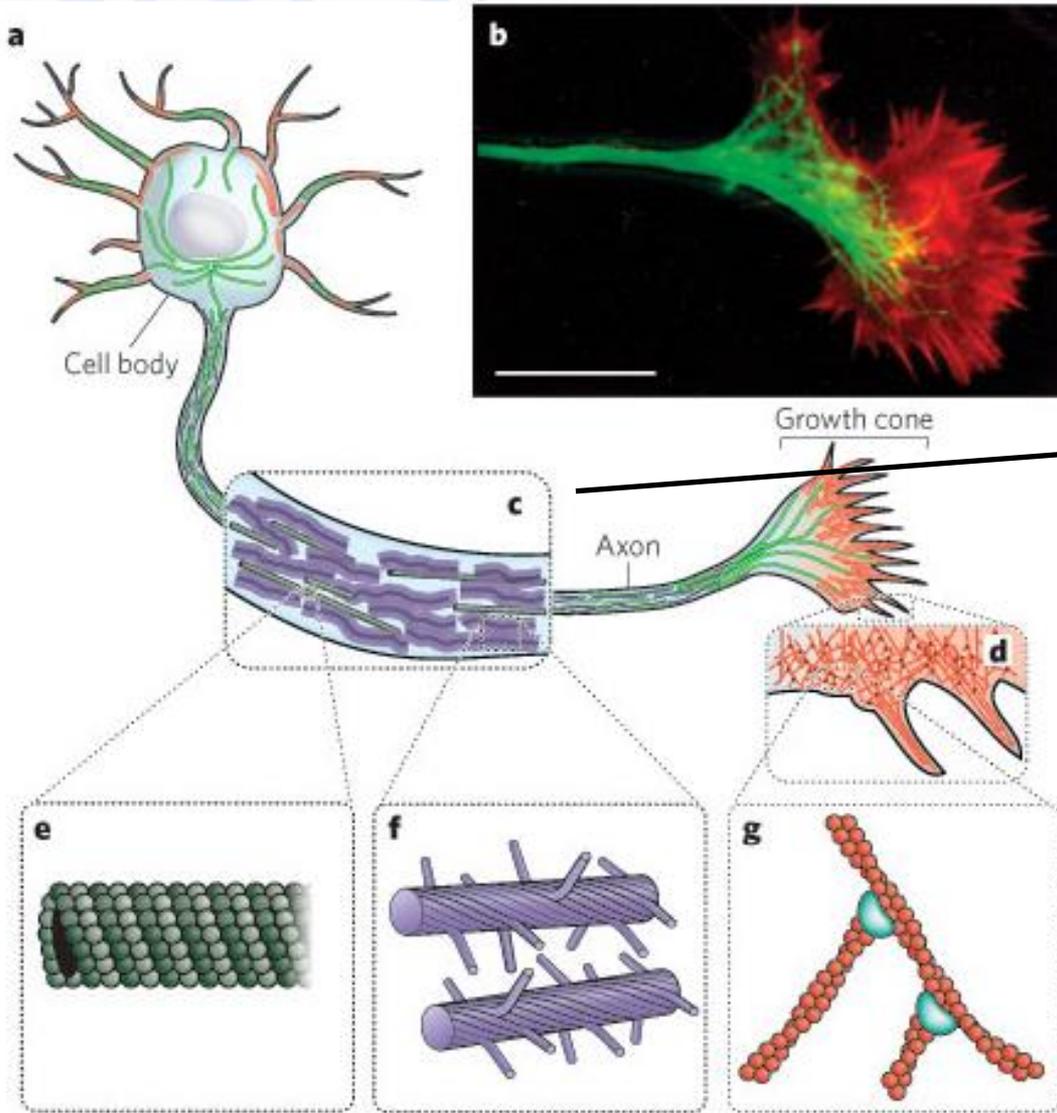
***) $p < 0.001$
**) $p < 0.01$
) $p < 0.05$
n.s.) not significant

Four known biomarkers: S100B, NSE, GFAP and MBP

Three novel biomarkers: NFM, α -Inx and β -Syn

Focus on **biomarkers of cell death** since necrosis, apoptosis and autophagy cell death pathways are activated early after hemorrhage

Discussion (II)



<http://www.nature.com/scitable/topicpage/microtubules-and-filaments-14052932>

→ Neurofilament triplet proteins (NFL, NFM and NFH) and α -Inx are the four major components of the neuronal intermediate filaments

Biomarkers of axonal degeneration

Microtubules Intermediate filaments Microfilaments

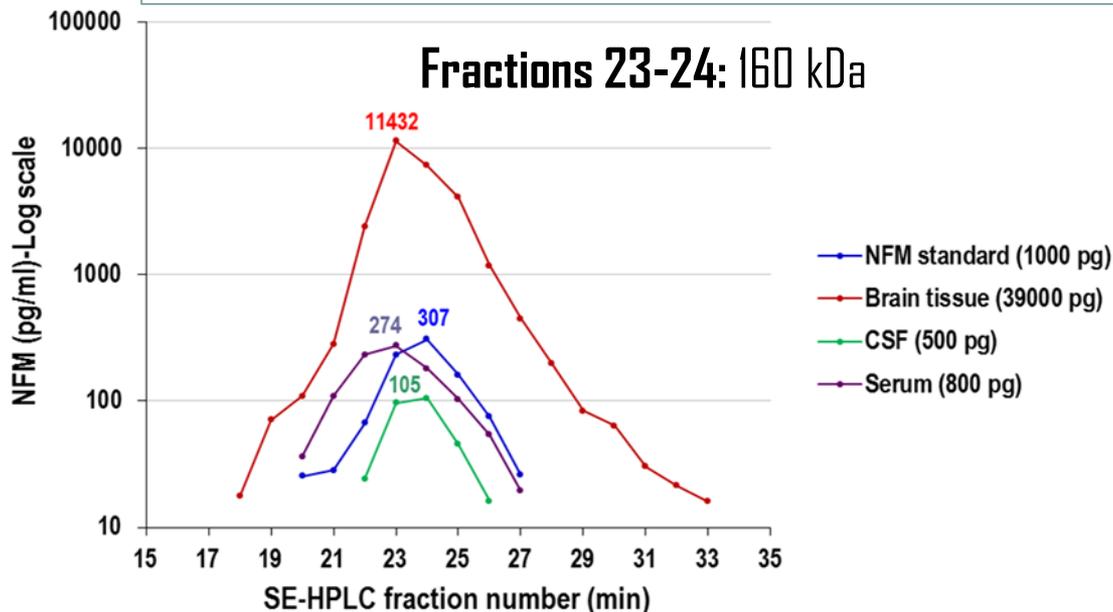
ELISA kit for NFM



Good linearity, sensitivity (LOD: 10 pg/ml) and precision
(CV intra-batch \leq 10%, CV inter-batch \leq 13%)

Evaluate the selectivity of the ELISA kit:
(using size-exclusion HPLC and mass spectrometry)

40 chromatographic fractions (from 18 to 58 min)



Mass spectrometry analysis:
Fractions 18, 23 and 34



NFM identified only in fraction 23

NFM in serum

Healthy individuals



n=46



n=46

NFM (ng/ml)

0.26-8.57 ng/ml

Hemorrhagic stroke



n=7



n=78

0.97-42.4 ng/ml

Traumatic brain injury (TBI)



n=12



n=106

3.48-45.4 ng/ml

Minor head Injury (MHI)



n=68



n=68

0.21-202.2 ng/ml

We found increased concentrations of NFM protein in CSF and serum of patients with brain injury

New studies are needed to elucidate the value of this protein in the diagnosis, prognosis and management of patients with brain injury and other neurological diseases with axonal degeneration

Acknowledgements

**Lunenfeld-Tanenbaum
Research Institute**

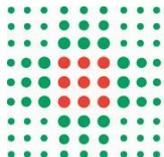
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