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Pathology biomarkers in animal models of amyotrophic lateral sclerosis: microsampling-based analytical investigation

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Pharmaco-Toxicological Analysis Laboratory

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AMYOTROPHIC LATERAL SCLEROSIS (ALS)

A type of motor neurone disease

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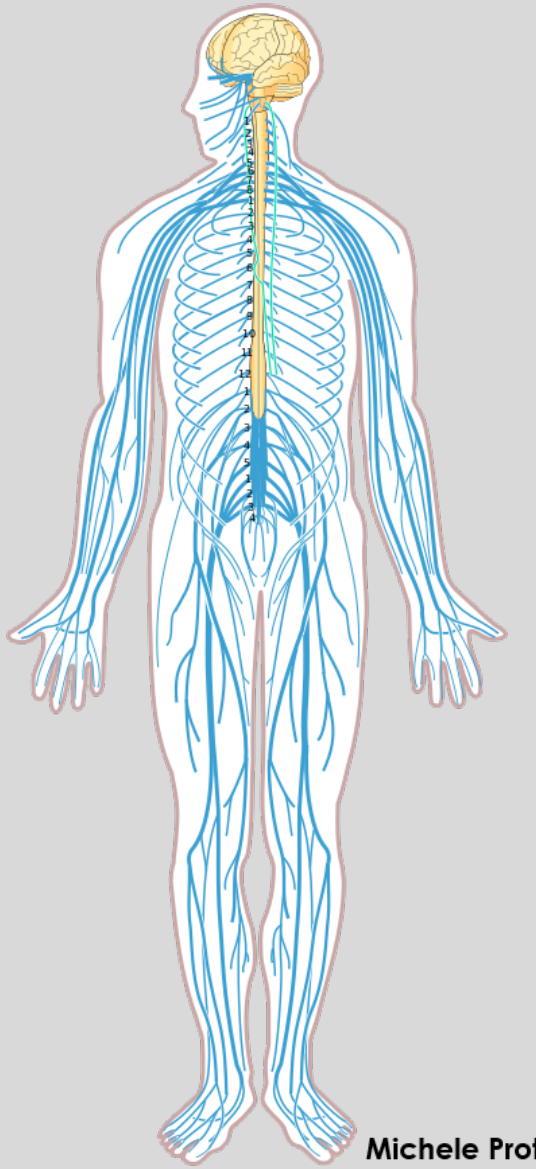
- ALS is a rare neurological condition
- Progressive - worsen with time – and with no cure
- Gradually muscles under voluntary control are affected, individual lose strength, ability to speak, eat, move, breath
- Most people with ALS die of respiratory failure within 3 to 5 years of first symptoms



- Affects nerve cells responsible for controlling voluntary muscle movement
- Motor neurons link between brain and voluntary muscles
- In ALS, the upper motor neuron and lower motor neurons degenerate and die
- Unable to function, the muscles degenerate

Stephen Hawking was diagnosed in his early 20s

He defied predictions he would only live a few years but was wheelchair-bound and spoke through a computerised voice system

National institute of Neurological Disorders and Stroke, NIH Publication No. 17-916, 2017

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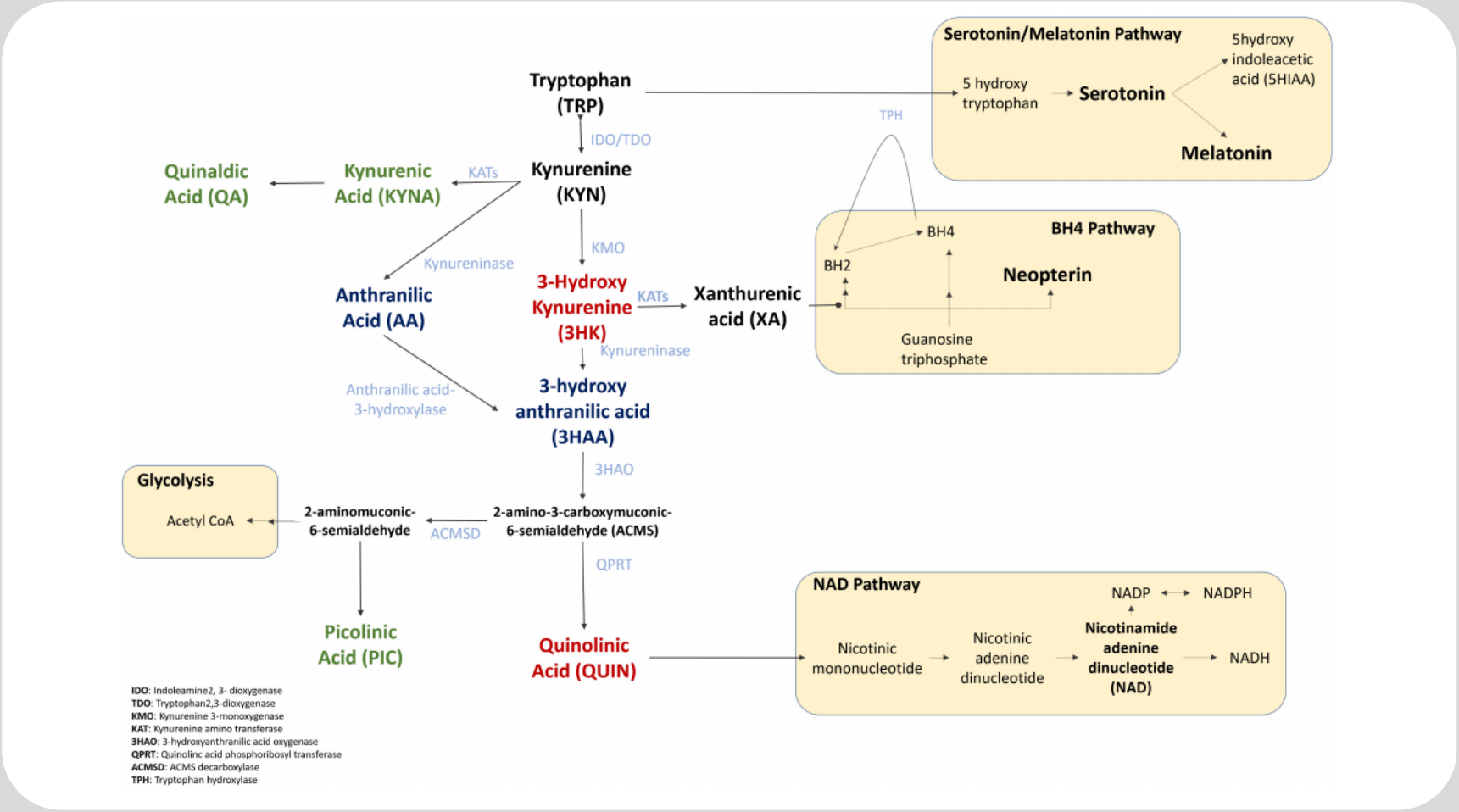
TRP METABOLISM INVOLVED IN NEUROPATHOLOGY

Through kynurenine pathway



The Kynurenine pathway and its downstream pathways of Serotonin/Melatonin, BH4, Glycolysis, and NAD+

Key metabolites

Neurotoxic metabolites

Neuroprotective metabolites

Dual-functioning metabolites

V.X. Tan, G.J. Guillemin, Kynurenine pathway metabolites as biomarkers for amyotrophic lateral sclerosis. Front Neurosci. 2019; 13: 1013.

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TRP METABOLISM INVOLVED IN NEUROPATHOLOGY

Through kynurenine pathway

- "Kynurenines, neurodegeneration and Alzheimer's disease"
  Z.T. Kincses, J. Toldi, L. Vécsei, J. Cell. Mol. Med. 14 (2010) 2045-2054.

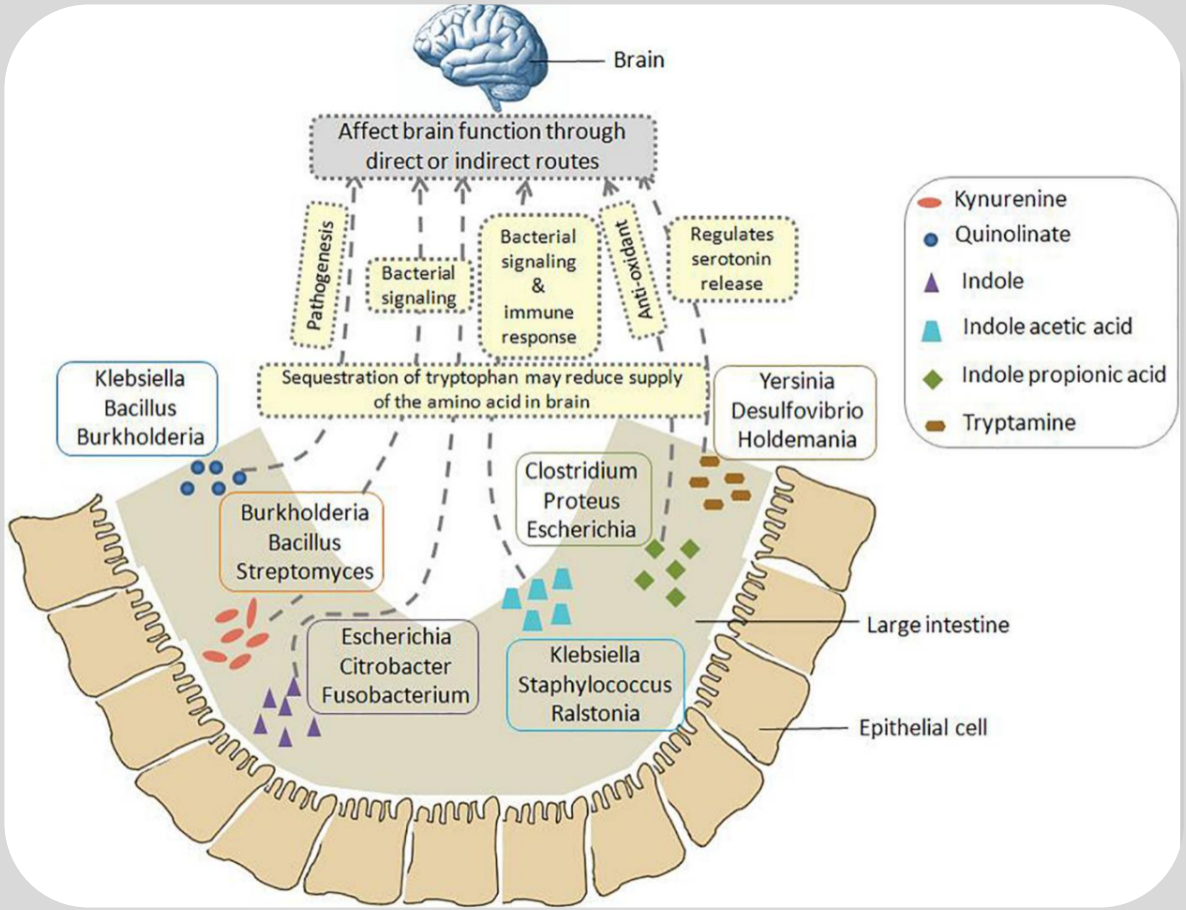
- "Stress, the autonomic nervous system, and the immune-kynurenine pathway in the etiology of depression" *E. Won, Y.K Kim, Curr. Neuropharmacol. 14 (2016) 665-673.*

- "Dysfunctional kynurenine pathway metabolism in the R6/2 mouse model of Huntington's disease" *K.W. Sathyasaikumar, E.K. Stachowski, L. Amori, P. Guidetti, P.J. Muchowski, R. Schwarcz, J. Neurochem. 113 (2010) 1416-1425.*

- "Involvement of the kynurenine pathway in the pathogenesis of Parkinson's disease" *C.K. Lim, F.J. Fernández-Gomez, N. Braidy, C. Estrada, C. Costa, S. Costa, A. Bessede, E. Fernandez-Villalba, A. Zinger, M.T. Herrero, G.J. Guillemin. Prog. Neurobiol. 155 (2017) 76-95.*

TRP METABOLISM IN GUT BACTERIA

Possible connection to brain functions



The **link between gut microbiome and brain** is being slowly acknowledged due to the speculated role of **resident gut microbial community in altering the functions of gut-brain axis** (GBA). Recently, a number of **microbial metabolites** (referred to as neuro-active metabolites) **produced through tryptophan metabolism** have been suggested to influence the GBA

H. Kaur, C. Bose, S.S. Mande, Tryptophan metabolism by gut microbiome and gut-brain-axis: An in silico analysis. Front Neurosci. 2019; 13: 1365.

MULTIDISCIPLINARY RESEARCH PROJECT:

Amyotrophic lateral sclerosis and tryptophan metabolism

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Ministero dell'Istruzione, dell'Università e della Ricerca

Research Projects of National Interest (PRIN) - Call 2017

Italian Ministry of Education, Universities and Research (MIUR)

PRIN 2017

Linking tryptophan catabolism to amyotrophic lateral sclerosis: from the pathogenesis to the pharmacological treatment

20173EAZ2Z - 2019-2023

University of Perugia

University of Bologna

University of Naples

University of Milan

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PTA Lab

MULTIDISCIPLINARY RESEARCH PROJECT:

Aim of the study

Linking tryptophan catabolism to amyotrophic lateral sclerosis: from the pathogenesis to the pharmacological treatment

20173EAZ2Z

- **Studying Trp catabolism, both in the host and in the gut microbiota, in a murine model of ALS** (G93A SOD1 mice), as a pathogenetic mechanism of the disease
  - analyzing the expression of IDO1 and IDO2 enzymes
  - quantifying Trp metabolites in plasma, CSF, spinal cord and feces of G93A ALS mice
- **Developing new "chemical tools"** able to interfere with Trp catabolism
  - examining the molecular mechanism of action of edaravone, a recently approved drug for the treatment of ALS, and its potential correlation with the Trp degradation pathways

BIOANALYTICAL APPROACH

Target metabolites

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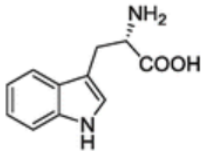
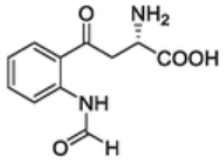
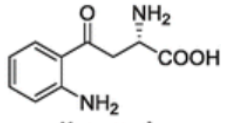
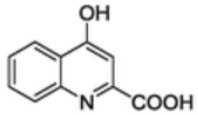
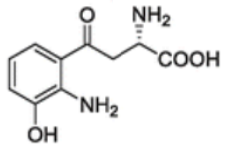
Kynurenine pathway

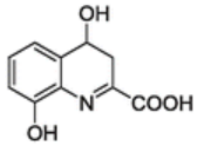

L-tryptophan

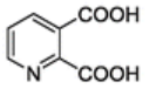

N-formyl-L-kynurenine

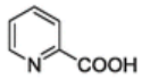

L-kynurenine

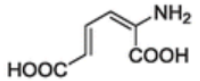

Kynurenic acid


3-hydroxy-L-kynurenine

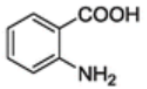

Xanthurenic acid

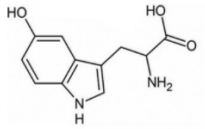

Quinolinic acid

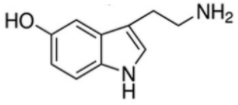

Picolinic acid

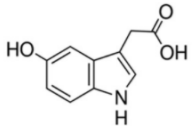

3-aminomuconic acid

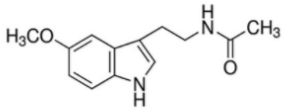
Methoxyindole pathway


Anthranilic acid

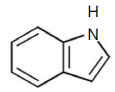

5-hydroxytryptophan

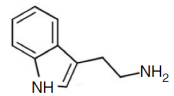

Serotonin

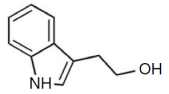

5-hydroxyindole acetic acid

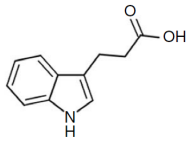

Melatonin

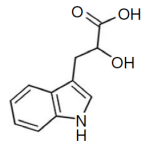
Gut microbiota TRP metabolism

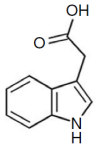

Indole

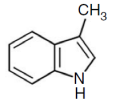

Tryptamine

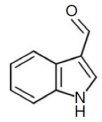

Indoleethanol

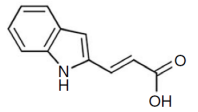

Indolepropionic acid


Indolelactic acid


Indoleacetic acid


Skatole


Indolealdehyde


Indoleacrylic acid

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BIOANALYTICAL APPROACH

Instrumentation and analytical conditions

CE-DAD
HP 3D CE

LC-MS/MS
Waters
Quattro Micro

UHPLC-QTOF
Waters Xevo G2-X2

CE-DAD (preliminary screening)

LC-MS/MS (quantitative analysis)
UHPLC-HRMS (confirmation)

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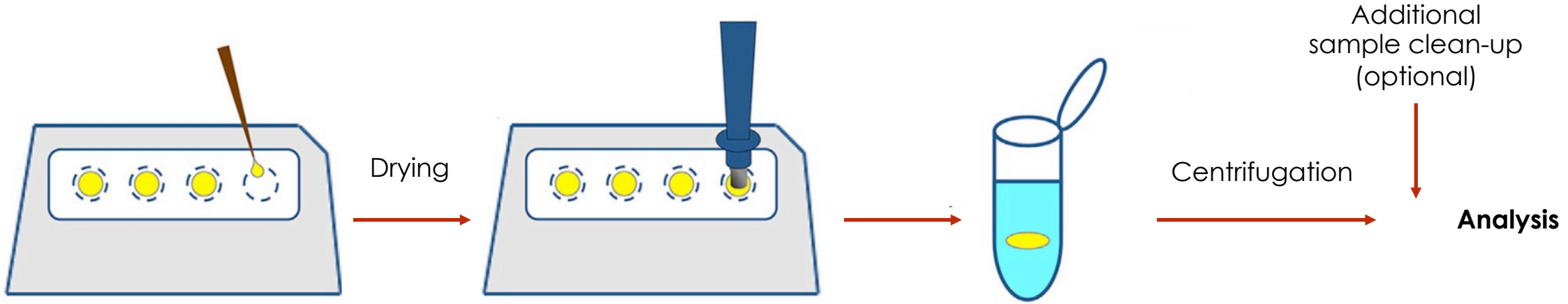
BIOANALYTICAL APPROACH

DMS microsampling

Dried matrix spots (DMS)



Optimised parameters:
- Sampling volume
- Drying time, temperature, humidity
- Storage conditions
- Mid-term stability
- Extraction solvent, mode, time

Developed on:
- Simulated samples
- Real whole blood samples
- Real plasma/serum samples
- Real homogenate brain tissue

Applicability:
- Plasma/serum/whole blood
- CSF
- (homogenate) brain tissue
- (homogenate) spinal cord
- (homogenate) fecal samples

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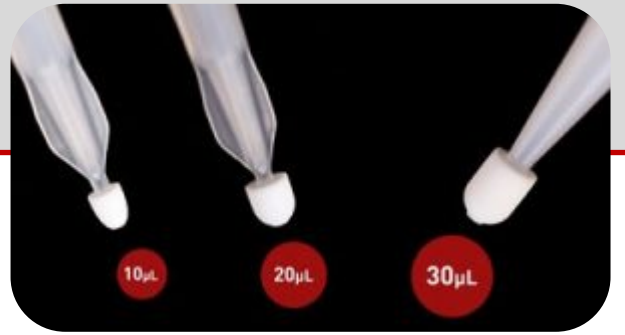
BIOANALYTICAL APPROACH

VAMS microsampling



Volumetric absorptive microsampling (VAMS)

Sample collection (10/20/30 µL) → Drying → Tip detachment & extraction → Centrifugation → **Analysis**

Additional sample clean-up (optional) ↓

Optimised parameters:
- Volume accuracy
- Sampling time
- Drying time, temperature, humidity
- Storage conditions
- Mid-term stability
- Extraction solvent, mode, time

Developed on:
- Simulated samples
- Real plasma, serum, whole blood samples

Applicability:
- Plasma/serum
- CSF
- Whole blood directly from mice

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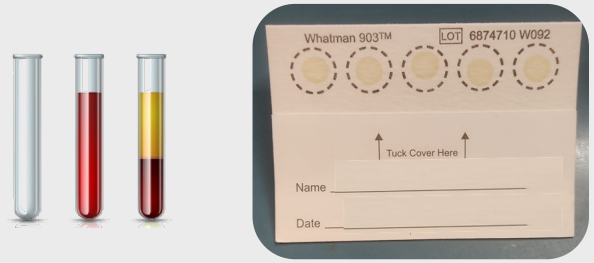
METHOD VALIDATION

Microsampling approaches

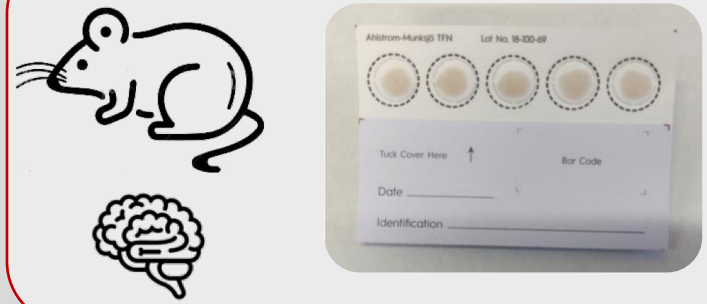
Whole blood VAMS

Sensitivity (LOQ):	0.5 ÷ 15 ng/mL
Linearity (R^2):	> 0.9986
Precision (RSD%):	< 4.1 %
Accuracy:	> 96 %
Extraction yield:	> 94 %
Matrix effect:	< 6.2 %



Plasma DMS

Sensitivity (LOQ):	0.5 ÷ 15 ng/mL
Linearity (R^2):	> 0.9990
Precision (RSD%):	< 4.4 %
Accuracy:	> 93 %
Extraction yield:	> 92 %
Matrix effect:	< 5.9 %



Homogenated brain tissue DMS

Sensitivity (LOQ):	1 ÷ 20 ng/mL
Linearity (R^2):	> 0.9982
Precision (RSD%):	< 7.1 %
Accuracy:	> 88 %
Extraction yield:	> 85 %
Matrix effect:	< 8.4 %

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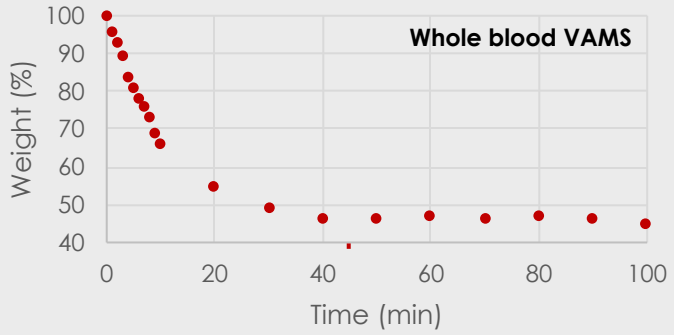
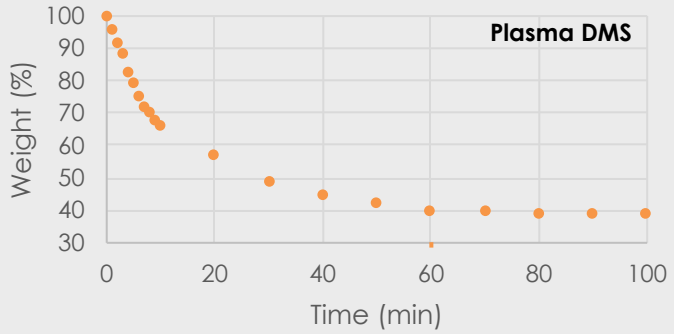
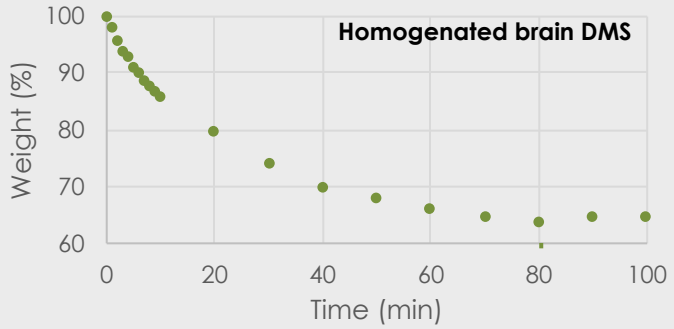
METHOD DEVELOPMENT

Optimised microsampling parameters

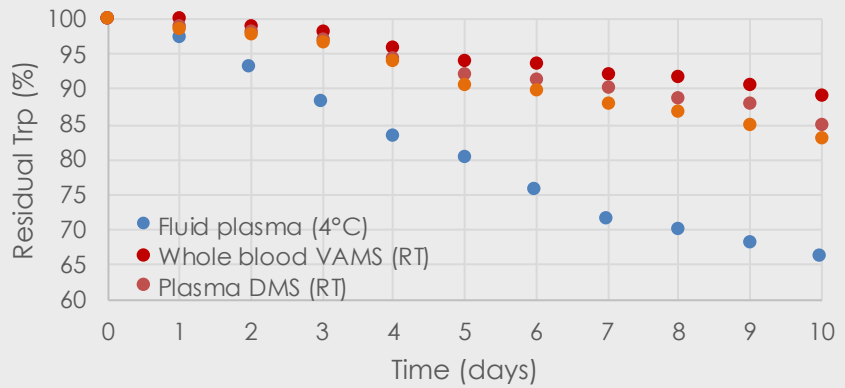
Drying time (@ RT)



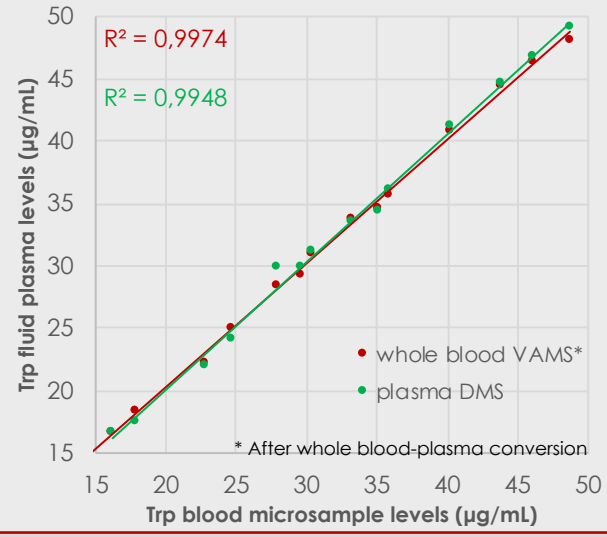




Sample stability (test compound: tryptophan)



Conventional plasma analysis vs. microsampling result comparison (test compound: tryptophan)



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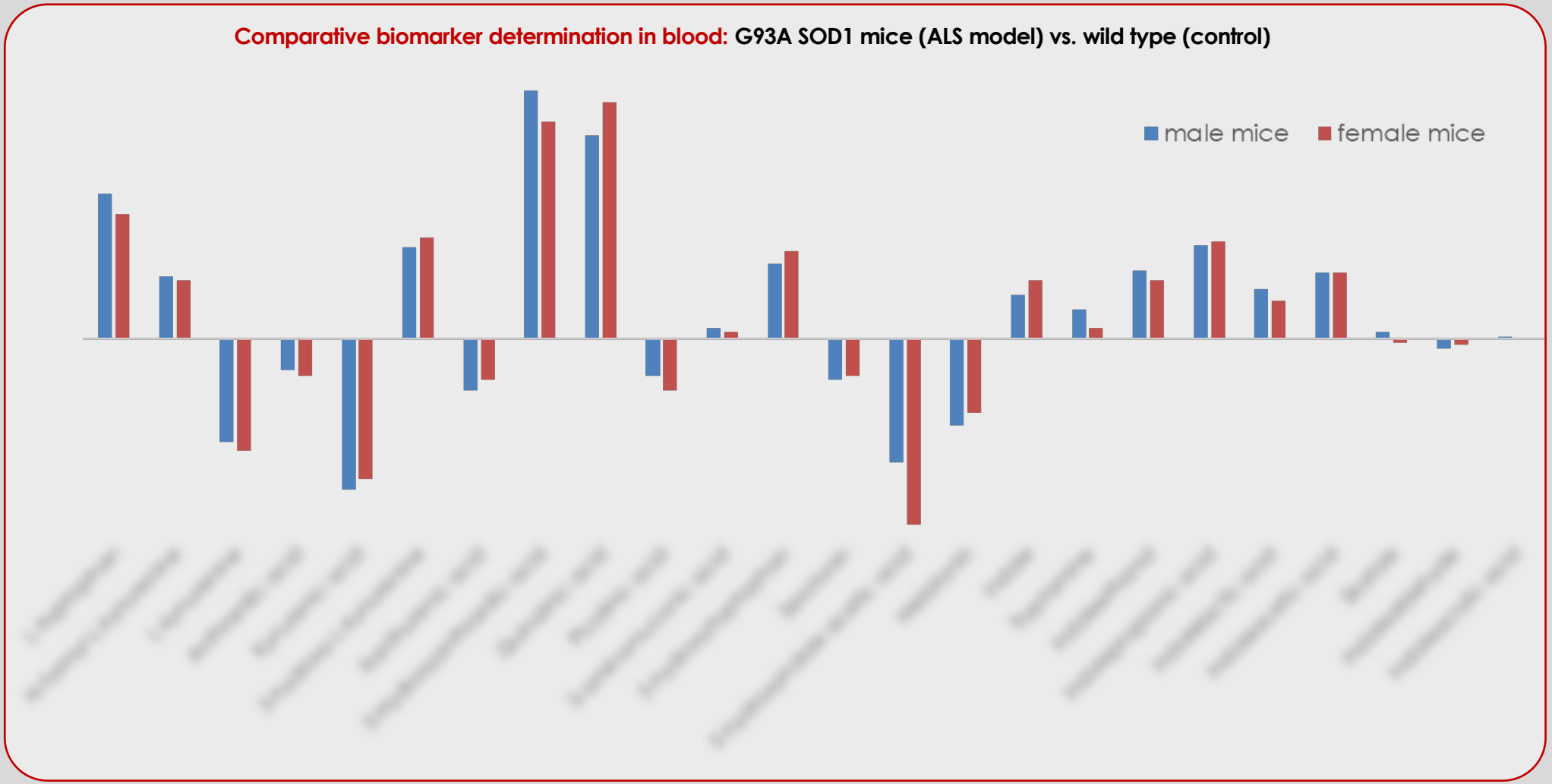
METHOD DEVELOPMENT

Optimised microsampling parameters

Comparative biomarker determination in blood: G93A SOD1 mice (ALS model) vs. wild type (control)



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CONCLUSION

- A multidisciplinary project is ongoing with the aim of **studying the role of tryptophan metabolism** on the onset and progression of **amyotrophic lateral sclerosis (ALS)**
- An effective, straight-forward and fully validated **LC-MS/MS** method (+HRMS confirmation) is being exploited for the **analysis of several Trp-related metabolites in blood and tissues** of **animal models of ALS.**
- **Miniaturised analytical approaches** (namely whole blood VAMS, plasma DMS and homogenated brain tissue DMS) are currently being applied in order to study **quali-quantitative correlations**, to expand the knowledge of **physiopathological mechanisms** and to investigate possible **novel therapeutic targets**
- The **samples can be stored and shipped without special precautions** to the analytical lab as demonstrated by comparative stability assays.

ACKNOWLEDGEMENTS

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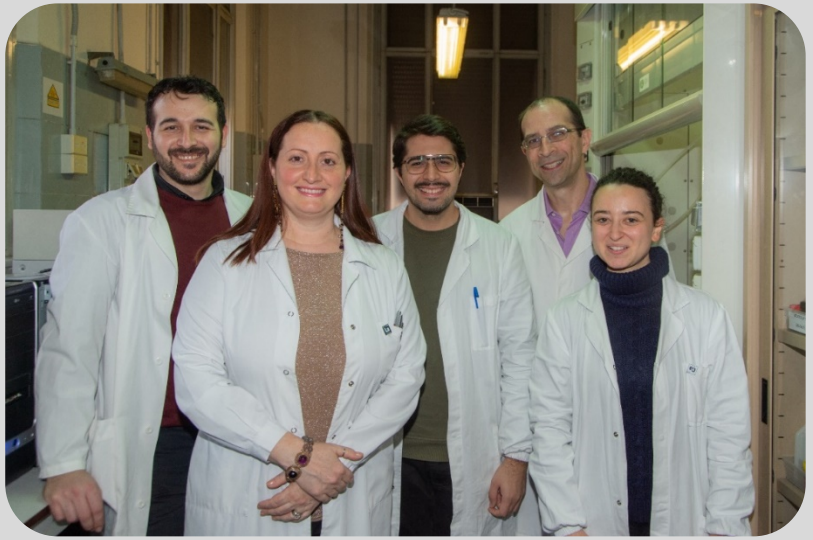
Dr. Claudia Volpi
Department of Medicine and Surgery
University of Perugia

Dr. Valeria Valsecchi
Department of Neuroscience and Reproductive and Odontostomatological Sciences
University of Naples Federico II

Prof. Luca Regazzoni
Department of Pharmaceutical Sciences
University of Milan



The PTA Lab at Unibo

Prof. Laura Mercolini

Prof. Roberto Mandrioli

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Stay connected with PTA Lab

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