Identification of biomarkers for MND, and understanding the potential role of the cyanotoxin BMAA in neurodegeneration

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BAppSci – Biotechnology
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Preface

"Amyotrophic Lateral Sclerosis."

"Never heard of it before"

"It's also known as Motor Neuron Disease, Lou Gehrig's Disease and Charcot disease."

"Ohh, okay."

This is the typical reaction to my answers when asked: "What is your research about?"

It's a neurological disease in which patients gradually lose control of their muscles and thus their movements, and they become paralysed.

Motor Neuron Disease (MND) is a death sentence. Patients ultimately succumb to death, often due to the failure of their breathing muscles, or complications from the disease.

That's not the worst part. Cognition often remains intact, meaning that they are consciously aware of what is happening to their body. They can hear you, think, and feel emotions.

Occasionally, I get: "Oh, it's like Multiple Sclerosis, or Huntington's Disease?"

And that's a pretty good guess. I explain that these are quite like MND. However, there is no remission, like in Multiple Sclerosis, and, unlike Huntington's, only 10% of MNDs are hereditary.

The irony is that this happens all the time in the clinic as well. There are no biochemical tests used to diagnose MND and as several other diseases present with similar symptoms, a clinician's only way to diagnose MND is through elimination of all other possible diseases. This is something I cannot fathom, when we live in a time of technological advances. Very little is known about this devastating disease and there is only one approved treatment that results in an extension of life by three months.

As a researcher, I was used to working on the bench and understanding what cells did, how animals responded, and reading about disease statistics. This changed when I was invited to help start up the MND Biobank at Macquarie Neurology with Prof Rowe, Prof Guillemin, and Dr Siu. Meeting patients made MND real for me. I saw how patients and their families were changed by this diagnosis and how the disease progresses over time. This was not just a disease I was conducting research on. It was something that affected people's lives.

Statement of Candidature

I certify that the work in this thesis entitled "**Identification of biomarkers for MND, and understanding the potential role of the cyanotoxin BMAA in neurodegeneration**" has not previously been submitted for a degree nor has it been submitted as part of requirements for a degree to any other university or institution other than Macquarie University and University Pierre et Marie Curie under a cotutelle arrangement.

I also certify that the thesis is an original piece of research and it has been written by me. Any help and assistance that I have received in my research work and the preparation of the thesis itself has been appropriately acknowledged.

In addition, I certify that all information sources and literature used are indicated in the thesis.

The research presented in this thesis was approved by:

Macquarie University Ethics Review Committee

Reference Numbers:

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Vanessa Xiuwen Tan

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Importantly, Sonia, thank you for imparting your valuable knowledge and technical skills in GC/MS. Your insistence on perfection and your warm personality has guided me well.

Et les français, Benjamin, Pauline, Maxime, Philippe, Josquin, Eric, Ségolène, Elodie, Gullen, merci beaucoup pour votre aide, et votre chaleureuse amitié. Je passe un bon temps à Paris parce que vous. Particulièrement, Benjamin et Philippe, je vous remercie pour votre patience et aide! Cet PhD n'aurait pas été possible sans votre aide et conseil. Et Maxime, merci pour toujours "pratique" ton anglaise avec moi. Tu es la raison ma française est encore très mauvais. Le ver vert sur un verre verte. Vraiment, française…. Merci aussi, des petites souris pour votre sacrifice.

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Thank you to more friends, Mou + Ben, who have been on this journey with me since my Masters. You did a bad job in reminding me not to write a thesis again! ;) Gilles, for endless chocolates and candy, and little random chats; Ed for Nespresso, yummy snacks, and random conversations; my favourite Angry Guy David, for always allowing me to be your most annoying girl; and Ben (AGAIN???), for being an awesome work-husband - you have been most dependable and trustworthy. I will always cherish the times we've had in the lab since 2012!

Finally, deepest gratitude to my family, Victor, Mum, Dad, Grandma, and my brother Dom, for your support always, and during my PhD. Particularly Victor, for not only bearing with the weird work hours, but even taking me to the lab at night to switch off machines or stop an incubation experiment. Your support has been invaluable in my career, and I truly appreciate you. I'm still not sure my family has an idea of what I spend all my time doing, but that's okay. You guys at least know it's got some sorts to do with brains, right? ;)

Abstract

Motor Neuron Disease (MND) or Amyotrophic Lateral Sclerosis (ALS) is a devastating neurological disease with no biological diagnostic markers, no effective treatment, and no cure. We investigate the immune related Kynurenine Pathway (KP) for a role in ALS. The production of neuroactive metabolites during the KP indicate that there is an overlap with the mechanisms of ALS, particularly with the neurotoxin quinolinic acid. Subsequently, we investigate the KP metabolome, analysing 10 metabolites using biochemical analyses including High Performance Liquid Chromatography and Gas Chromatography/Mass Spectrometry. Using serum from a longitudinal cohort of 66 ALS patients, we establish a potential for KP metabolomics to be used a biomarker for ALS. To increase specificity and reliability of these results, in collaboration with Macquarie University Neurology, we established a Neurodegenerative Diseases Biobank to collect patient biological samples. These samples would facilitate future investigations into the mechanisms, genetics, biomarkers, and to detect the presence of toxic compounds such as metals, or β-methylamino-L-alanine (BMAA). We describe the establishment of the biobank as a case study for future references. BMAA is known to be neurotoxic, and we investigate its role ALS. We reveal its role in promoting axonal degeneration and neuronal death, and show for the first time, its ability to spread transcellularly.

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Abbreviations

3HK	3hydroxykynurenine
AA	Anthranilic Acid
AD	Alzheimer's Disease
AHR	aryl-hydrocarbon receptor
ALS	Amyotrophic Lateral Sclerosis
ALS/PDC	Amyotrophic Lateral Sclerosis/Parkinson's dementia complex
ALSFRS	ALS Functional Rating Scale
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
APOE	Apolipoprotein E
ARE	Antioxidant Response Elements
ATP	Adenosine Triphosphate
BBB	Blood Brain Barrier
BH4	Tetrahydrobiopterin
BMAA	β-methylamino-L-alanine
BSC	Biological Safety Cabinet
CCL5	Chemokine Ligand 5
CHMP2B	Charged Multi-vesicular Body Protein 2B
CM	Culture Media
CNS	Central Nervous System
CoE	Council of Europe
COX-2	cycloxigenase-2
CSF	Cerebrospinal Fluid
D-1-MT	D-1-methyl tryptophan
DCTN1	Dynactin 1
DIV	Days in vitro
DNA	Deoxyribonucleic Acid
EAAT	Excitatory Amino Acid Transporters
ELN	Electronic Lab Book
ER	Endoplasmic Reticulum
fALS	familial ALS
FI	Fragmentation Index
FLTD-U	Frontotemporal lobar degeneration with ubiquinated inclusion
FTD	Fronto-Temporal Dementia
FUS	Fused in Sarcoma
GC	Gas Chromatography
GC/MS	Gas Chromatography / Mass Spectrometry
GFAP	Glial Fibrillary Acidic Protein
GTP	Guanosine 5' triphosphate
HAA	3 hydroxy anthranilic acid
HFP	1,1,1,3,3,3-hexafluoroisopropanol
HPLC	High Performance Liquid Chromatography
HREC	Human Research Ethics Committee (Macquarie University)

ID	Identification
IDO	Indoleamine2,3-dioxygenase
IFN-γ	Interferon-γ
IL	Interleukin
IL-1b	Interleukin-1b
IL-6	Interleukin 6
iNOS	inducible Nitric Oxide synthase
iPSC	induced Pluripotent Stem Cells
ISBER	International Society for Biological and Environmental Repositories
K/T ratio	Kynurenine/Tryptophan ratio
KIF1A	Kinesin family member 1A
KMO	Kynurenine monoxygenase
KP	Kynurenine Pathway
KT	Kynurenine/Tryptophan (ratio)
KYN	Kynurenine
KYNA	Kynurenic Acid
LC	Liquid Chromatography
LC-MS/MS	Liquid Chromatography coupled with tandem Mass Spectrometry
LIMS	Laboratory Information Management System
MAP2	Microtubule associated protein 2
MCP1	Monocyte Chemo-attractant Protein
MIP1-α	Macrophage Inflammatory Protein 1-α
MMT	Manual Muscle Testing
MND	Motor Neuron Disease
MS	Multiple Sclerosis
MS	Mass Spectrometry
MTA	Material Transfer Agreements
NAD	Nicotinamide Adenine Dinucleotide
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NDB	Neurodegenerative Disease Biobank
NDD	Neurodegenerative Disease
NEO	Neopterin
NGF	Nerve Growth Factor
NHMRC	National Health and Medical Research Council
NMDA	N-methyl-d-aspartate
nNOS	neuronal Nitric Oxide Synthase
NO	Nitric Oxide
NR	NMDA receptor
Nrf-2	Nuclear Erythroid 2 Related Factor 2
OECD	Organization for Economic Co-operation and Development
PA	Picolinic Acid
PD	Parkinson's Disease
PIC	Picolinic Acid
PICF	Patient Information and Consent Form

QLD	Quinaldic Acid
QUIN	Quinolinic Acid
RNA	Ribonucleic Acid
ROS	Reactive Oxygen Species
RPMI	Roswell Park Memorial Institute
sALS	sporadic ALS
SDNS	Scientific Data Management Systems
SMA	Spinal Muscular Atrophy
SOD1	Superoxide Dismutase 1
SVC	Slow Vital Capacity
tBHQ	t-Butyl Hydroquinone
TCA	Tricholoroacetic Acid
TDO	Tryptophan 2,3-dioxygenase
TDO-2	Tryptophan 2,3-dioxygenase
TDP-43	Transactive response DNA binding protein
TFA	Trifluoroacetic anhydride
TNF-α	Tumour Necrosis Factor - α
TRP	Trpolinic Acid
UBQLN2	Ubiquilin 2
UHPLC	ultra High Performance Liquid Chromatography
VAPB	Vesicle-associated Membrane Protein
VCP	Valosin-containing protein
WHO	World Health Organization
wt	Wildtype
ZnAc	Zinc Acetate

Publications arising from research during candidature

2017 **Neurotoxicity Research**: Neurotoxicity of the Cyanotoxin BMAA Through Axonal Degeneration and Intercellular Spreading
Vanessa X. Tan, Benjamin Lassus, Chai K. Lim, Philippe Tixador, Josquin Courte, Alban Bessede, Gilles J. Guillemin & Jean-Michel Peyrin (Accepted 21 July 2017)

2017 **Neurotoxicity Research**: Detection of the Cyanotoxin L-NMAA Uptake and Accumulation in Primary Neurons and Astrocytes
Vanessa X. Tan, and Claire Mazzocco (Co-First), Bianca Varney, Dominique Bodet, Tristan A. Guillemin, Alban Bessede & Gilles J. Guillemin (Accepted 21 July 2017)

2017 **Scientific Reports**: Kynurenine Pathway Metabolomics Predict and Provide Mechanistic Insight into Multiple Sclerosis Progression 2017
Chai K. Lim, Ayse Bilfin, David B. Lovejoy, **Vanessa X. Tan**, Sonia Bustamante, Bruce V. Taylor, Alban Bessede, Bruce J. Brew, Gilles J. Guillemin (Accepted 20 December 2016)

2017 **Neuropharmacology**: Involvement of Quinolinic acid in the Neuropathogenesis of ALS
Jong-Min Lee and **Vanessa X. Tan (Co-First)**, David Lovejoy, Nady Braidy, Dominic B. Rowe, Bruce J. Brew, Gilles J. Guillemin (Accepted 17 May 2016)

2015 **Human Molecular Genetics**: Defects in Optineurin and Myosin VI Mediated Cellular Trafficking in ALS
Vinod Sundaramoorthy, Adam K. Walker, **Vanessa Tan**, Jennifer A. Fifita, Emily P. McCann, Kelly L. Williams, Ian P. Blair, Gilles J Guillemin, Manal A. Farg, Julie D. Atkin (Accepted 9 April 2015)

Prepared Manuscripts

2017 **Journal of Visualized Experiments**: Quantification of the Kynurenine Pathway Metabolites Using ultra-High Performance Liquid Chromatography and Gas Chromatography/Mass Spectrometry (Under Revisions)
Kelly R. Jacobs, **Vanessa X. Tan**, Chai K. Lim

2017 **Journal of Neuroinflammation:** The Kynurenine Pathway as a Biomarker for ALS Progression (To be Submitted)
Vanessa X. Tan, Maeva Khyeng, Chai K. Lim, Laura Bourbon, Vincent Meninger, Lucette Lacomblez, Alain Duhamel, Pierre-Francois Pradat, Gilles J Guillemin

Awards

2016 INAUGURAL ENCOURAGE SYMPOSIUM 2016
PhD Student Oral Presentation Award
1st Annual Outstanding Biomedical Image Prize:
First place, People's Choice Category Award, and, Second Place, Postgraduate student Category

2015 LAUREATE: CAMPUS FRANCE EIFFEL EXCELLENCE DOCTORANTE
Only nominated candidate from University of Pierre and Marie Curie, and one of 42 international candidates, awarded international return travel and EUR 12,000.

2015 POST GRADUATE RESEARCH FUNDS, MACQUARIE UNIVERSITY
Modelling the ALS dying back process: awarded AUD 5000.

2013 MACQUARIE UNIVERSITY RESEARCH EXCELLENCE SCHOLARSHIP (INTERNATIONAL)
Scholarship: Tuition and Stipend (3.5 years)

Conferences & Presentations

2017 Invited Talk: Neuroimmunology Australia Workshop 2017
Kynurenine Pathway Metabolites as Progression Markers for MND

2017 Invited Talk: University of Queensland, AIBN
Using Microfluidics for Motor Neuron Disease: The Neurotoxin BMAA

2017 Invited Talk: Griffiths University
Using Microfluidics for Motor Neuron Disease: The Neurotoxin BMAA

2016 Inaugural EnCouRage Symposium 2016
Oral Presentation: Use of Microfluidic chambers to elucidate effects of BMAA on Neurons

2016 BMAA Conference
Poster Presentation: Use of Microfluidic chambers to elucidate effects of BMAA on Neurons

2016 Inter University Neuroscience and Mental Health Conference
Oral Presentation: Kynurenine Pathway as a Biomarker for Motor Neuron Disease

2016 Three Minute Thesis Competition
Fingerprinting MND

2015 Motor Neuron Disease and the Kynurenine Pathway
Oral Presentation. Hosted by Professor Robin Reed, Department of Cell Biology, Harvard Medical School

2015 Motor Neuron Disease and the Kynurenine Pathway
Oral Presentation. Hosted by Professor Nicholas Maragakis, Department of Neurology, Johns Hopkins University School of Medicine

2015 International Society for Tryptophan Research Meeting 2015
Poster Presentation: The Kynurenine Pathway and Motor Neuron Disease

2014 Motor Neuron Disease Research Institute of Australia Research Meeting 2014
Poster Presentation: Changes in Kynurenine Pathway metabolites as potential prognostic biomarker

2013 Macquarie University BioFocus Research Center Conference 2013
Poster Presentation: The Kynurenine Pathway and ALS

2013 Motor Neuron Disease Research Institute of Australia Research Meeting 2013
Poster Presentation: The Kynurenine Pathway and ALS: A Significant Target for Theragnosis

2010 Australian Society for Medical Research Medical Research Week 2010 (NSW)
Poster Presentation: Human Papillomavirus is Associated with Breast Cancer

2010 The University of New South Wales Faculty of Science Postgraduate Research Competition
Poster Presentations 2010: Human Papillomavirus is Associated with Breast Cancer

Section A

Problem Statement & Research Approach

Motor Neuron Disease (MND) is a cruel disease in which motor functions are lost leading to muscle wasting, paralysis and ultimately death, usually in 3 to5 years. This project focuses on the most common form of MND, Amyotrophic Lateral Sclerosis (ALS). There are no routinely used biochemical markers for diagnosis, monitoring progression, or prognosis of disease course. ALS is difficult to diagnose as symptoms often overlap with other diseases, resulting in patients seeing multiple specialists before receiving a conclusive diagnosis. The average time from symptom onset to diagnosis is 18 months. The disease has only one known cause, familial heritability, which accounts for only 10% of ALS. The mechanisms of ALS are well researched; however, much remains unclear about environmental contributions to the disease such as iron, or β-methylamino-L-alanine (BMAA) exposure.

We take a three-pronged approach to elucidate this disease. First, we investigate biomarker discovery by profiling the immune related Kynurenine pathway in patient serum samples. Secondly, we establish a biobank for the prospective collection and storage of patient samples for future use. Finally, we elucidate the role of the cyanobacterial neurotoxin BMAA *in vitro*, in causing neurodegeneration.

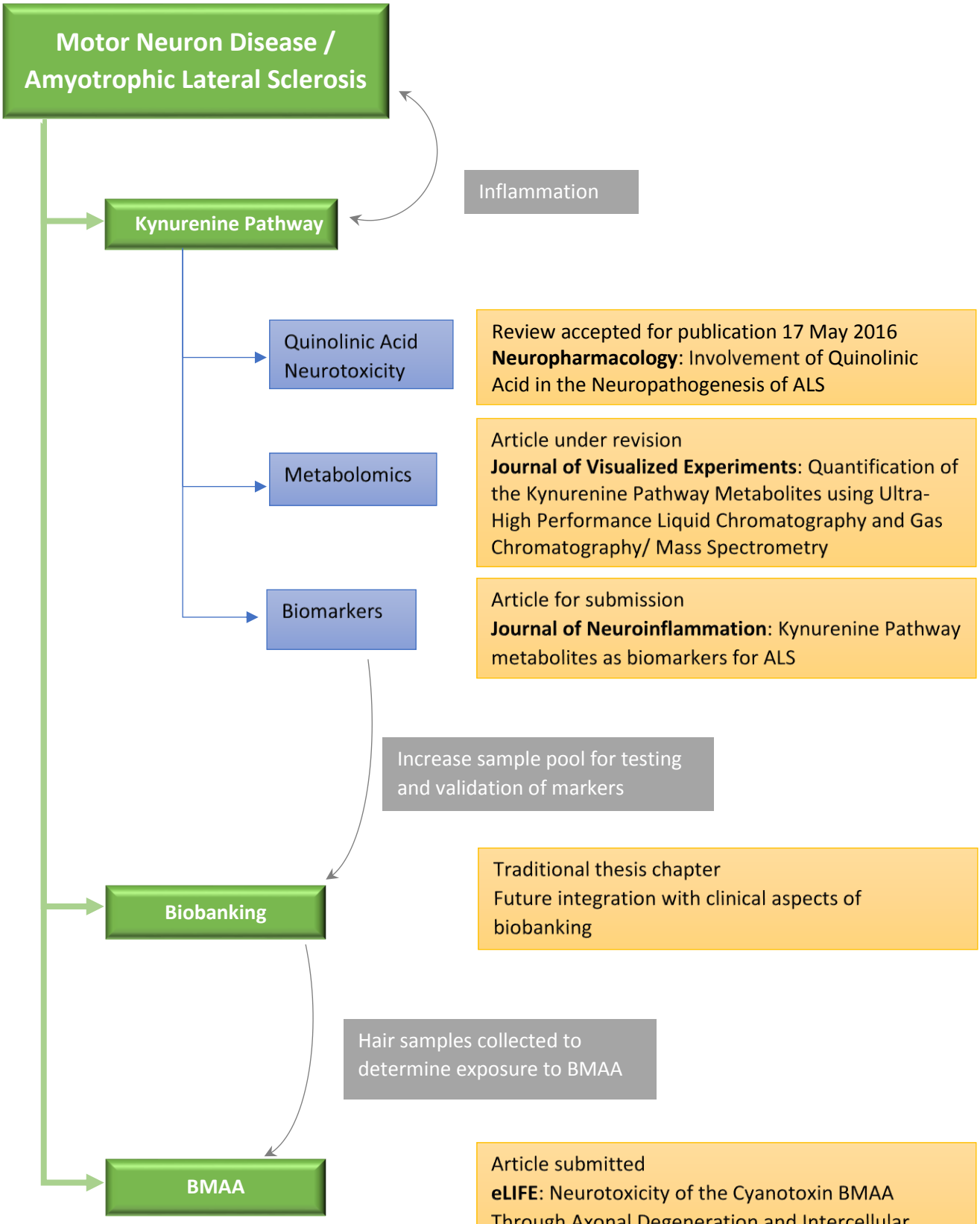
The overall hypothesis of this thesis is that:

- Neuroactive KP metabolites can be used as biomarkers to track progression or be a prognostic marker for ALS
- BMAA has a pathological role in neurodegeneration.

The specific aims of this study are to:

1. Evaluate the KP metabolites in longitudinal serum samples as a biomarker for MND using ultra High Performance Liquid Chromatography (uHPLC) and Gas Chromatography Mass Spectrometry (GC/MS)
2. Establish a biobank for long term storage of patient samples for future use
3. Examine if BMAA has a pathological role in neurodegeneration.

Summary of Research Approach



Section B: Findings

ALS is a devastating disease that is under-researched. There is no biochemical marker for diagnosis, prognosis, or monitoring of the disease, and no effective treatments or cure. Although there are several mechanisms for neuronal degeneration in ALS, few treatment options have been translated through to the clinic, and environmental causes have been debated.

This thesis will examine the role of a key neurotoxic metabolite in the KP, Quinolinic Acid, and its overlapping roles that reflect the mechanisms of neurotoxicity observed in ALS. The review provides evidence for the KP having a pathological role in ALS, highlighting two seminal research articles, directly showing dysregulation of the KP in ALS patients, leading to this thesis assessing the KP as a biomarker for ALS.

The candidate, with colleagues, refined analytical chemistry techniques for analysis of the KP metabolome in serum and plasma samples. These techniques using uHPLC, HPLC, and GC/MS, allowed the multiplexed analysis of multiple KP metabolites, decreasing sample volume requirements, and processing and laboratory time and consumables.

The candidate applies these techniques to determine the longitudinal KP metabolomic, and tryptophan (TRP) catabolite profile of ALS patients, revealing that they show potential as a disease progression biomarker for ALS. Specifically, metabolites of the KP were shown to be associated with clinical outcome measures, ALS Functional Rating Scale (ALSFRS) and Manual Muscle Testing (MMT), indicating that the KP can be used as a definitive biochemical marker to measure clinical outcomes. This has important ramifications for monitoring disease progression, particularly in clinical trials to allow for unbiased assessment of disease progression. Furthermore, we find that the TRP catabolite, neopterin, is a good prognostic indicator for rate of disease progression. To increase the reliability and sensitivity of these findings, the study cohort is to be expanded for verification of results.

As such, the Neurodegenerative Diseases Biobank (NDB) was set up in collaboration with Macquarie Neurology. The NDB was established to store various types of samples from both controls and ALS patients for future analyses. The establishment of the NDB has been presented as a case study for future references which includes key aspects of biobanking

such as governance, safety, and laboratory processes, the processes used at the NDB, a reflection on these processes and how they can be improved. As the candidate was less involved in the clinical considerations of the biobank, this section of the thesis will be reworked for publication upon the inclusion of key clinical aspects of establishing the biobank.

As part of a cotutelle degree, the candidate utilised a cutting edge microfluidic platform to examine a potential pathological role of the environmental neurotoxin BMAA in ALS. Although BMAA has been implicated in ALS, the role of BMAA has not been widely accepted. This is mainly due to the large, non-physiological concentrations of BMAA used to demonstrate neurotoxicity. The research presented bridges the conflicting opinions by demonstrating that low levels of BMAA can cause axonal disruptions. It also shows for the first time, the transmission of BMAA from cell-to-cell, implicating BMAA as a possible mechanism for the focal spread of ALS pathology.

This thesis (1) reviewed the roles of the KP and quinolinic acid in ALS; (2) refined the use of analytical chemistry methods for detection of KP and TRP catabolites; (3) determined the KP as a suitable candidate for a biomarker and prognosis marker for disease progression; (4) established a biobank for future investigations; (5) presented evidence for BMAA causing axonal toxicity; (6) demonstrated the transmission of BMAA from cell-to-cell.

Future studies are needed to explore the roles of the KP metabolites and TRP catabolites as a biomarker for ALS, and investigate if modulating KP activity can treat or improve disease outcome. The establishment of the biobank has allowed the current, ongoing investigation of multiple biomarkers for ALS, and is an immense resource for investigating time-based changes through the course of disease. Lastly, the finding that BMAA can be spread from cell-to-cell opens a new paradigm for understanding the role of BMAA in causing ALS, and future work would investigate the prion-like properties of BMAA.

This section details the core findings and publications arising or to result from the work done as part of this research. Research presented has been allocated to three categories, (1) investigating the KP in ALS, (2) the establishment of the Neurodegenerative Diseases

Biobank as a case study, and (3) unravelling the neurotoxicity of β-methylamino-L-alanine (BMAA) and its possible role in ALS.

Chapters 1 and 3 are presented in article format, while Chapter 2 is presented in a traditional thesis format for future integration into an article with the inclusion of clinical aspects of biobanking.

In each Chapter, a brief background identifying the research gap is addressed, followed by presentation of key results and implications of these findings. Articles will be noted if they have been published, submitted for peer review, or are under revision for submission.

Chapter 1

Kynurenine Pathway and ALS

Three manuscripts have resulted from this work

2016 **NEUROPHARMACOLOGY**:
INVOLVEMENT OF QUINOLINIC ACID IN THE NEUROPATHOGENESIS OF ALS
Jong-Min Lee and **Vanessa X. Tan (Co-First)**, David Lovejoy, Nady Braidy, Dominic B. Rowe, Bruce J. Brew, Gilles J. Guillemin (Accepted 17 May 2016)

2017 **JOURNAL OF VISUALIZED EXPERIMENTS**:
QUANTIFICATION OF THE KYNURENINE PATHWAY METABOLITES USING ULTRA-HIGH PERFORMANCE LIQUID CHROMATOGRAPHY AND GAS CHROMATOGRAPHY/MASS SPECTROMETRY (FINAL REVISIONS)
Kelly R. Jacobs, **Vanessa X. Tan**, Chai K. Lim

2017 **JOURNAL OF NEUROINFLAMMATION:**
THE KYNURENINE PATHWAY AS A BIOMARKER FOR ALS PROGRESSION (UNDER REVISION)
Vanessa X. Tan, Maeva Khyeng, Chai K. Lim, Laura Bourbon, Vincent Meninger, Lucette Lacomblez, Alain Duhamel, Pierre-Francois Pradat, Gilles J Guillemin

Chapter 1: Kynurenine Pathway and ALS

1.1 Research gap:

Motor neuron disease (MND) is a spectrum of neurodegenerative diseases that present with progressive loss of motor function due to loss of upper and/or lower motor neurons. It includes Amyotrophic Lateral Sclerosis (ALS) as the major type of MND, Spinal Muscular Atrophy (SMA), Primary Lateral Sclerosis (PLS), Progressive Muscular Atrophy (PMA) (Al-Chalabi & Hardiman, 2013; Ravits et al., 2013). In Australia, MND has an incidence of 7.9-9.6 per 100,000 Australians, with slightly more males than females, and mortality estimated to be 3.14 per 100,000 in the population. MND costs an estimated $1.13 million per person, significantly higher than diseases such as stroke ($133,108), and chronic kidney disease ($758) (Deloitte Access Economics, 2015).

Clinical features of ALS are well characterised, encompassing facial weakness, tongue fasciculation; symptoms in upper limbs with weak gripping to shoulder abduction; and in lower limbs, foot drop as well as tripping (Andersen, 2006; Turner & Talbot, 2013). However, there are many mimic diseases that present with similar symptoms. There is no specific test for ALS, and with only 5-10% of ALS being genetically linked (Al-Chalabi & Hardiman, 2013; Andersen, 2006; Li, Alberman, & Swash, 1988; Ravits et al., 2013; Zou et al., 2017), diagnosis of ALS relies largely on a clinician's ability to identify MND (Calvo et al., 2014; Mitchell & Borasio, 2007; Turner & Talbot, 2013). Furthermore, there are up to five different subtypes that may predict survival, and phenotype stratification based on hierarchical classification, and site of symptom onset (Al-Chalabi & Hardiman, 2013; Ganesalingam et al., 2009).

Mechanistically, ALS has been associated with protein dysfunction, oxidative stress, glutamate toxicity, mitochondrial dysfunction, energy depletion, neuroinflammation, autophagy, and apoptosis (Bruijn, Miller, & Cleveland, 2004; Eisen, Kiernan, Mitsumoto, & Swash, 2014; Picher-Martel, Valdmanis, Gould, Julien, & Dupré, 2016).

The Kynurenine Pathway (KP) is an attractive candidate as a biomarker for ALS. It has been associated with several neurodegenerative diseases (Beal, Matson, Swartz, Gamache, & Bird, 1990; Chen et al., 2010; Chen & Guillemin, 2009; Lim et al., 2017; Ogawa et al., 1992), and has been shown to be involved in causing neurodegeneration. Most recently, it has been shown to be predictive for Multiple Sclerosis (MS) subtypes (Lim et al., 2017). The role

of the KP in ALS has been detailed in the review *Involvement of quinolinic acid in the neuropathogenesis of amyotrophic lateral sclerosis.*

Bibliography

Al-Chalabi, A., & Hardiman, O. (2013). The epidemiology of ALS: a conspiracy of genes, environment and time. *Nature Reviews Neurology*, *9*(11), 617–628. https://doi.org/10.1038/nrneurol.2013.203

Andersen, P. M. (2006). Amyotrophic lateral sclerosis associated with mutations in the CuZn superoxide dismutase gene. *Curr Neurol Neurosci Rep*, *6*(1), 37–46.

Beal, M. F., Matson, W. R., Swartz, K. J., Gamache, P. H., & Bird, E. D. (1990). Kynurenine Pathway Measurements in Huntington's Disease Striatum: Evidence for Reduced Formation of Kynurenic Acid. *Journal of Neurochemistry*, *55*(4), 1327–1339. https://doi.org/10.1111/j.1471-4159.1990.tb03143.x

Bruijn, L. I., Miller, T. M., & Cleveland, D. W. (2004). Unraveling the Mechanisms Involved in Motor Neuron Degeneration in Als. *Annual Review of Neuroscience*, *27*(1), 723–749. https://doi.org/10.1146/annurev.neuro.27.070203.144244

Calvo, A. C., Manzano, R., Mendonça, D. M. F., Muñoz, M. J., Zaragoza, P., & Osta, R. (2014). Amyotrophic lateral sclerosis: a focus on disease progression. *BioMed Research International*, *2014*, 925101. https://doi.org/10.1155/2014/925101

Chen, Y., & Guillemin, G. J. (2009). Kynurenine Pathway Metabolites in Humans : Disease and Healthy States. *Int J Tryptophan Res*, *61*(2), 1–19.

Chen, Y., Stankovic, R., Cullen, K. M., Meininger, V., Garner, B., Coggan, S., … Guillemin, G. J. (2010). The Kynurenine Pathway and Inflammation in Amyotropic Lateral Sclerosis. *Neurotoxicity Research*, *18*(2), 132–142. https://doi.org/10.1007/s12640-009-9129-7

Deloitte Access Economics. (2015). *Economic analysis of motor neurone disease in Australia, report for Motor Neuron Disease Australia*. Canberra.

Eisen, A., Kiernan, M., Mitsumoto, H., & Swash, M. (2014). Amyotrophic lateral sclerosis: a long preclinical period? *Journal of Neurology, Neurosurgery, and Psychiatry*, *85*(11), 1232–8. https://doi.org/10.1136/jnnp-2013-307135

Ganesalingam, J., Stahl, D., Wijesekera, L., Galtrey, C., Shaw, C. E., Leigh, P. N., … Rubinsztein, D. C. (2009). Latent Cluster Analysis of ALS Phenotypes Identifies Prognostically Differing Groups. *PLoS ONE*, *4*(9). https://doi.org/10.1371/journal.pone.0007107

Li, T. M., Alberman, E., & Swash, M. (1988). Comparison of sporadic and familial disease amongst 580 cases of motor neuron disease. *Journal of Neurology, Neurosurgery, and Psychiatry*, *51*(6), 778–84. https://doi.org/10.1136/JNNP.51.6.778

Lim, C. K., Bilgin, A., Lovejoy, D. B., Tan, V., Bustamante, S., Taylor, B. V., … Guillemin, G. J. (2017). Kynurenine pathway metabolomics predicts and provides mechanistic insight into multiple sclerosis progression. *Scientific Reports*, *7*(February), 41473. https://doi.org/10.1038/srep41473

Mitchell, J. D., & Borasio, G. D. (2007). Amyotrophic lateral sclerosis. *Lancet (London, England)*, *369*(9578), 2031–41. https://doi.org/10.1016/S0140-6736(07)60944-1

Ogawa, T., Matson, W. R., Beal, M. F., Myers, R. H., Bird, E. D., Milbury, P., & Saso, S. (1992). Kynurenine pathway abnormalities in Parkinson's disease. *Neurology*, *42*(9), 1702–6. https://doi.org/10.1212/WNL.42.9.1702

Picher-Martel, V., Valdmanis, P. N., Gould, P. V., Julien, J.-P., & Dupré, N. (2016). From animal models to human disease: a genetic approach for personalized medicine in ALS. *Acta Neuropathologica Communications*, *4*(1), 70. https://doi.org/10.1186/s40478-016-0340-5

Ravits, J., Appel, S., Baloh, R. H., Barohn, R., Brooks, B. R., Elman, L., … Ringel, S. (2013). Deciphering amyotrophic lateral sclerosis: what phenotype, neuropathology and genetics are telling us about pathogenesis. *Amyotrophic Lateral Sclerosis & Frontotemporal Degeneration*, *14 Suppl 1*(0 1), 5–18. https://doi.org/10.3109/21678421.2013.778548

Turner, M. R., & Talbot, K. (2013). Mimics and chameleons in motor neurone disease. *Practical Neurology*, *13*(3), 153–64. https://doi.org/10.1136/practneurol-2013-000557

Zou, Z.-Y., Zhou, Z.-R., Che, C.-H., Liu, C.-Y., He, R.-L., & Huang, H.-P. (2017). Genetic epidemiology of amyotrophic lateral sclerosis: a systematic review and meta-analysis. *Journal of Neurology, Neurosurgery, and Psychiatry*, jnnp-2016-315018. https://doi.org/10.1136/jnnp-2016-315018

1.2 Key Results and Implications

The work from this candidature resulted in a systemic review presenting evidence supporting the role of the KP metabolite, quinolinic acid (QUIN) in ALS. The review examines key mechanisms associated with ALS such as toxic protein aggregation, oxidative damage, and glutamate toxicity. It highlights the KP and its neuroactivity, focusing on QUIN toxicity mechanisms, and how these effects overlap with known mechanisms of ALS degeneration. Finally, the review provides insight into the KP as a potential therapeutic candidate for ALS, by modulating the neuroactivity of the KP using KP-altering chemicals and analogues.

Subsequently, the candidate, with colleagues, have refined methods for quantification of KP metabolites using analytical chemistry methods high performance liquid chromatography (HPLC) and Gas Chromatography / Mass Spectrometry (GC/MS). This method allows for the multiplexed analysis of (1) Tryptophan and Kynurenine, via HPLC, (2) Kynurenic Acid via ultra HPLC (uHPLC), and (3) Picolinic Acid and Quinolinic Acid, via GC/MS. The use of a multiplexed assay results in a decreased amount of sample and reagents required for analysis, while maintaining sensitivity and specificity.

Finally, this candidature presents evidence for the use of KP metabolites as a candidate biomarker for ALS. Longitudinal samples of 66 ALS patients from a clinical trial were obtained and KP metabolome analysed. Statistical analyses were performed to (1) observe evolution of KP metabolites and clinical outcomes over time, and (2) determine the associations of KP with clinical outcomes as (a) a biomarker for disease progression, (b) disease prognosis, and (c) survival prognosis. Analyses determined (i) 3hydroxykynurenine, to be associated with time; (ii) kynurenine and tryptophan, and quinaldic acid and kynurenic acid; to be indicative of disease progression; (iii) and neopterin to be associated with a lowered disease progression slope. These results indicate that the KP metabolome has potential as a candidate as a biomarker for ALS.

1.3 Original Research Article: Involvement of Quinolinic Acid in the Neuropathogenesis of ALS

2016 **NEUROPHARMACOLOGY**: INVOLVEMENT OF QUINOLINIC ACID IN THE NEUROPATHOGENESIS OF ALS

Jong-Min Lee and **Vanessa X. Tan (Co-First)**, David Lovejoy, Nady Braidy, Dominic B. Rowe, Bruce J. Brew, Gilles J. Guillemin

This paper has been published to Neuropharmacology 2016. JML assisted with draft. VXT wrote manuscript. DL, NB, DBR, BJB, and GJG reviewed manuscript. All other work is my own.

Abbreviations

3HAA	3 hydroxy anthranilic acid
AD	Alzheimer's Disease
ALS	Amyotrophic Lateral Sclerosis
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
APOE	Apolipoprotein E
ARE	Antioxidant Response Elements
ATP	Adenosine Triphosphate
CCL5	Chemokine Ligand 5
CHMP2B	Charged Multi-vesicular Body Protein 2B
CNS	Central Nervous System
COX-2	cycloxygenase-2
CSF	Cerebrospinal Fluid
D-1-MT	D-1-methyl tryptophan
DCTN1	Dynactin 1
EAAT	Excitatory Amino Acid Transporters
ER	Endoplasmic Reticulum
fALS	familial ALS
FLTD-U	Frontotemporal lobar degeneration with ubiquinated inclusion
FTD	Fronto-Temporal Dementia
FUS	Fused in Sarcoma
GFAP	Glial Fibrillary Acidic Protein
GTP	Guanosine 5' triphosphate
IDO	Indoleamine2,3-dioxygenase
IFN-γ	Interferon-γ
IL	Interleukin
IL-1b	Interleukin-1β
IL-6	Interleukin 6
iNOS	inducible Nitric Oxide synthase
iPSC	induced Pluripotent Stem Cells
KIF1A	Kinesin family member 1A
KP	Kynurenine Pathway
KYN	Kynurenine
KYNA	Kynurenic Acid
MCP1	Monocyte Chemo-attractant Protein
MIP1-α	Macrophage Inflammatory Protein 1-α
MND	Motor Neuron Disease
MS	Multiple Sclerosis
NAD	Nicotinamide Adenine Dinucleotide
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NDD	Neurodegenerative Disease

NGF	Nerve Growth Factor
NMDA	N-methyl-d-aspartate
nNOS	neuronal Nitric Oxide Synthase
NO	Nitric Oxide
NR	NMDA receptor
Nrf-2	Nuclear Erythroid 2 Related Factor 2
PD	Parkinson's Disease
PIC	Picolinic Acid
QUIN	Quinolinic Acid
ROS	Reactive Oxygen Species
sALS	sporadic ALS
SMA	Spinal Muscular Atrophy
SOD1	Superoxide Dismutase 1
tBHQ	t-Butyl Hydroquinone
TDO	Tryptophan 2,3-dioxygenase
TDP-43	TAR DNA BINDING PROTEIN-43
TNF-α	Tumour Necrosis Factor - α
TRP	Tryptophan
UBQLN2	Ubiquilin 2
VAPB	Vesicle-associated Membrane Protein
VCP	Valosin-containing protein
wt	Wildtype

Lee, J.-M., Tan, V., Lo e oy, D., Braidy, N., o e, D. B., Bre , B. J. & Guillemin, G. J. (2017). In ol ement of uinolinic acid in the neuropathogenesis of amyotrophic lateral sclerosis. *Neuro har aco o y*, 112 Part B, p. 346-364.

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1.4 Original Research Methodology Article: Quantification of the Kynurenine Pathway Metabolites Using Ultra High Performance Liquid Chromatography and Gas Chromatography/ Mass Spectrometry

2017 JOURNAL OF VISUALIZED EXPERIMENTS: QUANTIFICATION OF THE KYNURENINE PATHWAY METABOLITES USING ULTRA-HIGH PERFORMANCE LIQUID CHROMATOGRAPHY AND GAS CHROMATOGRAPHY/MASS SPECTROMETRY (FINAL REVISIONS)
Kelly R. Jacobs, **Vanessa X. Tan**, Chai K. Lim

This paper has been prepared for submission to the Journal of Visual Experiments (JOVE).

KJ, EL and VT contributed to the writing and running of samples, KJ and EL optimized methods.

Abbreviations

GC	Gas Chromatography
HPLC	High Performance Liquid Chromatography
IDO-1	Indoleamine 2,3-dioxygenase - 1
K/T ratio	Kynurenine/Tryptophan ratio
KP	Kynurenine Pathway
KYN	Kynurenine
KYNA	Kynurenic Acid
LC	Liquid Chromatography
LC-MS/MS	Liquid Chromatography coupled with tandem Mass Spectrometry
MS	Mass Spectrometry
PIC	Picolinic Acid
QUIN	Quinolinic Acid
TCA	Tricholoroacetic Acid
TDO-2	Tryptophan 2,3-dioxygenase
TRP	Trpolinic Acid
uHPLC	ultra High Performance Liquid Chromatography
ZnAc	Zinc Acetate

Original Research

Quantification of the kynurenine pathway metabolites using ultra-high performance liquid chromatography and gas-chromatography/mass spectrometry

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Keywords:

uHPLC, GC/MS, Kynurenine, Tryptophan, quantification

Abstract

The Kynurenine Pathway (KP) is associated with various diseased states such in neurological diseases, psychiatric illnesses, and HIV. Its metabolites have been recently investigated for its potential as a biomarker, and is the first blood biomarker to predict subtypes of multiple sclerosis. This has presented a novel method to facilitate early prognosis of multiple sclerosis subtypes, allowing clinicians to treat patients with medication suitable for each subtype. The accurate and validated analysis of metabolites of the KP is crucial for the development of the KP as diagnostic, prognostic, and progression biomarkers. We developed a reliable method for analysing the KP metabolome to analyse five KP metabolise using liquid, and gas chromatography, and describe the details.

Introduction

Kynurenine pathway (KP) metabolomics is an emerging field of research. The KP is the main catabolic route for tryptophan, one of the nine essential amino acids, and regulated physiological functions such as (1) protein synthesis; (2) regulation of the serotonin-melatonin pathway, regulating mood, behaviour, sleep and circadian rhythms; and (3) modulation of the Kynurenine-NAD pathway affecting energy balance, brain activity, and immune activation. Previously, the KP was presumed a passive catabolic route within the tryptophan metabolism, where the majority of tryptophan was utilised for the production of serotonin and melatonin. However, research advancements in immunology suggests that the KP is highly active, and competitive with the serotonin pathway for tryptophan during inflammation processes. It has been associated with various pathological conditions such as Alzheimer's disease [1], Parkinson's disease [2], amyotrophic lateral sclerosis [3] and multiple sclerosis [4], and psychiatric disorders including depression, schizophrenia and autism [5–7].

Activation of the KP, is reflected by an increased kynurenine/tryptophan (K/T) ratio, resulting from the upregulation of indoleamine dioxygenase (IDO-1) / TDO-2 activity, converting Tryptophan (TRP) to KynurFenine (KYN). IDO-1 has been suggested as a marker of immune activation since its increased activity is associated with proinflammatory mediators that are also present in the neurological diseases described above. Interestingly, it has been proposed that changes to the KP due to maternal immune activation, can result in development of behavioural and cognitive deficit in later life [8,9]. This is hypothesized to be due to mechanisms of disease tolerance and activation of the KP both acutely and chronically [10], resulting in the abnormal production of downstream KP metabolites, kynurenic acid (KYNA) and quinolinic acid (QUIN). Imbalance in the endogenous levels of KYNA and QUIN can be detrimental to

neuronal cells as described by Guillemin [11] and were also implicated in various neurological diseases [12]. Hence, profiling of the kynurenine pathway can provide insight into disease progression that enable the identification of new disease biomarkers, with implications for the development and use of therapeutics [3,4,13,14]. Therefore, the accurate and reproducible quantification of KP metabolites is important for clinical studies.

Several methods had been developed and validated for the quantification of TRP and its downstream derivatives in clinical samples. Typically, this includes the use of liquid chromatography (LC) and gas chromatography (GC) coupled with detection of ultraviolet/visible (UV/VIS) absorbance [15,16], fluorescence [15,17,18], electrochemical [19], and mass spectrometry (MS) detectors [20]. (Quantification of TRP, KYN, and KYNA are routinely achieved via high performance liquid chromatography (HPLC) coupled with absorbance and fluorescence detectors. However, detection and quantification of endogenous KYNA in clinical samples may be problematic due to its low native fluorescence and concentrations in the low nanomolar range. A common method used to enhance KYNA fluorescence is chelation with Zn^{2+} forming a fluorescent complex which absorbs and emits strongly at 344 nm and 398 nm respectively. This is usually achieved via post-column addition of zinc acetate (ZnAc). However, when post-column derivatisation is not possible due to limitations of available instrumentation, addition of ZnAc directly to the mobile phase has also been reported [5].

Although liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) is becoming increasingly popular for concurrent quantification of KP metabolites, like other MS methods, require additional processing. Sample preparation and purification steps such as solid phase extraction are often required to reduce noise from sample matrix. In addition, labelled internal standards increase the cost per sample. Exaggerated overhead costs for on-

going maintenance and prior knowledge for technical troubleshooting on the mass spectrometer limits its wide spread use. There are now HPLC systems that can withstand higher back pressure and higher sensitivity detectors (due to narrower flow path), and the use of sub two micron columns with greater resolution is increasingly popular. This means that we can enjoy the advantages of a mass spectrometry application, i.e. using less sample material and achieving similar sensitivity; without the limitation of a costly, and technical mass spectrometer. This allows the multiplexing of analyses, reducing sample volume and costs. This proposes the ultra HPLC (uHPLC) system as an economical choice, that provides sufficient detection sensitivity of KP metabolites in clinical samples.

Despite several methods using LC systems to separate QUIN, based on our experience, we find that using GC systems produce the best result in QUIN separation and quantification when considering for establishment of a routine application.

This study describes established, simple and sensitive methods used to quantify KYN and TRP via UHPLC, KYNA by HPLC, and picolinic acid (PIC) and QUIN via GC/MS in plasma and serum samples. Users should take note that method described herein are based on the specified analytical systems and columns. Some variation in signal intensity, analyte retention time and detection limits are expected between systems, and some in-house optimization may be required.

Protocol

1. Sample preparation

Note: All reagents used were of analytical grade and purchased from Sigma Aldrich (MO, USA) unless otherwise specified.

1.1 Deproteinise plasma and serum samples with the addition of equal volumes of 10% w/v trichloroacetic acid (TCA).

1.2 Centrifuge samples at 12,000 rpm, 4°C for 15 min.

1.3 Collect and filter the supernatants through 0.45 µm PTFE syringe filters (Merck-Millipore, CA, USA). Samples may be analysed immediately or stored at -20°C for up to 1 week prior to analysis.

2. TRP and KYN quantification via uHPLC

Note: The instrument used is an Agilent 1290 Ultra-high performance liquid chromatography system equipped with G4220A Binary pump, G1330B temperature-controlled auto-sampler, a G4212A diode-array detector, and a G1321B xenon flash lamp fluorescence detector.

2.1 Preparation of external standards.

2.1.1 Prepare 10 mM KYN and TRP stock standards in ultrapure water. Standards may be stored in aliquots at -20°C prior to use.

2.1.2 From stock standards prepare 6 working standards containing both TRP and KYN for the co-current quantification of TRP (50 to 0 µM) and KYN (10 to 0 µM), or to your working range.

2.2 Preparation of UHPLC mobile phase.

2.2.1 Prepare 1 L of mobile phase containing 0.1 M sodium acetate, pH 4.65.

2.2.2 Degas the buffer by filtering the mobile phase through a 0.2 µm PTFE membrane (Millipore).

2.3 Establish the UHPLC chromatography and analysis method.

2.3.1 A reverse phase C18 column (ZORBAX Rapid Resolution High Definition C18 reversed phase 2.1 x 150 mm column with pore size of 1.8 µm (Agilent Technologies, CA, USA)) was used for compound separation at 38°C. The mobile phase is as described above in section 2.2 and column equilibrated in mobile phase to a volume greater than 20 column void volumes prior to use. The isocratic mobile phase is run at 0.75 mL/min for 12 min/sample. System pressure after equilibration is approximately 800-900 mbar. Injection volume was set to 20 µL and autosampler tray set at 4°C to prevent sample degradation.

2.3.2 UV and Fluorescence detectors were equilibrated for at least 30 min prior to use. The UV detector was set to measure absorbance at 365 nm (reference signal off) for the detection of KYN. The FLD was set to excitation: 280 nm, emission: 438 nm, PMT: 11

Note: depending on your signal-to-noise ratio, set the gain accordingly. These settings may vary slightly for different detectors. In-house optimization may be required.

2.3.3 Set up the sample run sequence and run the sequence. Re-run a standard and blank every 20-30 samples to assess shifts in retention time, background and sample carry over.

3. Kynurenic acid (KA) quantification using HPLC

Note: The instrument used is an Agilent 1260 high performance liquid chromatography system equipped with G4220A Quaternary pump, G1330B temperature-controlled auto-sampler, and a G1321B xenon flash lamp fluorescence detector.

3.1 Preparation of external standards.

3.1.1 Prepare 10 mM KYNA in ultra-pure water. If compound solubility is an issue, add the stoichiometrically equivalent amount of 40% w/v sodium hydroxide. Standards may then be aliquoted and stored at -80°C prior to use.

3.1.2 From the stock standard prepare 6 working standards for KYNA with concentrations ranging from 100-0 nM, or to your working range.

3.2 Preparation of HPLC mobile phase

3.2.1 Prepare 1 L mobile phase containing 50 nM sodium acetate, 50 nM zinc acetate in 5% v/v HPLC grade acetonitrile, final pH 5.2.

3.2.2 Filter and degas the buffer by passing through a 0.2 µm PTFE membrane (Millipore).

3.3 Establish the HPLC chromatography and analysis method.

3.3.1 As with the UHPLC method a reverse phase C18 column (ZORBAX XDB C18 reversed phase 4.6 x 100 mm column with pore size of 3.5µm (Agilent Technologies, CA, USA)) was used for compound separation at 38°C. The mobile phase is as described above in section 3.2 and column allowed to equilibrate for a volume of more than 20 column void volumes. The isocratic mobile phase is run at 1.00 mL/min for 10 mins. System

pressure after equilibration is approximately 300 mbar. Injection volume was set at 10 µL and autosampler tray is kept at 4°C to prevent sample degradation.

3.3.2 The FLD is set to excitation: 344 nm and emssion: 388 nm. Set PMT gain set to 16. Note: depending on your signal-to-noise ratio, set the gain accordingly. These settings may vary slightly in different detectors. In-house optimization will be required.

3.3.3 Set up the sample run sequence and run the sequence. Every 20-30 samples re-run 1 of the working standards and a blank sample to assess shifts in retention time, background and sample carry over.

4. QUIN and PIC acid quantification using GCMS

Note: These analyses were performed using an Agilent 7890 gas chromatograph coupled with an Agilent 5975 mass spectrometer as previously described with some modification (Guillemin et al., 2007).

4.1 Preparation of external standards for standard curve.

4.1.1 Prepare working standards of QUIN and PIC. Sonication may be required to dissolve completely in solution. Standards may then be aliquoted and stored at -80°C prior to use.

4.1.2 Make a 250µM of combined QUIN and PIC to be used as working standards and prepare 6 standards ranging from 0 to 200 (e.g. 0, 10, 20, 50, 100 and 200 nM) in glass tube (13*100mm), or to your working range.

4.2 Preparation of internal standards.

4.2.1 Dissolve deuterated internal standards in deuterated water to make stock of 1 mM for QUIN and PIC. Aliquot and store standards at -20°C.

4.2.2 Add equal amount of combined deuterated PIC and QUIN into every tube including working standards and samples (note: do not confuse working standards, i.e. non-deuterated, with internal standards).

4.3 Drying of standards and samples.

4.3.1 Once the standards and samples (usually 50-100µL of deproteinized extract depending on the detection limit of your sample matrix) are added to the glass tube containing internal standards, dry the tubes in a SpeedVac Concentrator (Savant SC250EXP, Thermo Fisher)

4.3.2 Set the SpeedVac Concentrator for 1 hour run accounting for the vacuum to kick in and reach high vacuum pressure (preset settings high pressure). SpeedVac refrigerated vapour trap (Savant RVT 5105, Thermo Fisher) must be pre-cooled to full capacity for at least 2 hours.

4.4 GCMS pre-check and maintenance

It is good practise to do some quick maintenance check before each run. Ensure liner, inlet septa, and sample needle are in good working condition. Change as required. Run auto-tune of the MS to ensure optimal settings for detection.

4.5 Derivatization of PIC and QUIN.

4.5.1 After the drying step is completed, add 60µL of trifluoroacetic anhydride (TFAA) and 60µL of 1,1,1,3,3,3-hexafluoroisopropanol (HFP) into each tube and cap tight.

4.5.2 Heat the capped tubes for an hour on a preheated heating block at 60^{o}C to allow derivatization of PIC and QUIN into stable fluorinated esters.

4.5.3 After heating, remove from heat block and allow the tubes to cool to room temperature for 10-15min.

4.6 Toluene extract and clean up TFAA and HFP.

4.6.1 Remove cap and add 100 µL of toluene to extract the fluorinated esters.

4.6.2 Add 500µL of 5% w/v sodium bicarbonate to the toluene extract. This will two phases where the top layer consists of the toluene extract (toluene phase) and the bottom layer is sodium bicarbonate (aqueous phase). Shake the tube gently (the mixture will start to bubble).

4.6.3 Once the bubbling process ceases, use a glass pipette to remove the entire mixture, allowing the bilayer to settle inside the glass pipette. Dispose the aqueous phase while retaining the toluene phase, and place back into the original glass tube.

4.6.4 Add 1mL of ultrapure water into the glass tube to clean the toluene phase by shaking gently. Allow the mixture to settle. Remove the toluene phase and pass through an improvised filter (see **Figure 1** for details) containing sodium sulphate to remove any excess aqueous solution before collecting into a glass vial suitable for the GCMS.

4.7 GCMS setup and analysis

4.7.1 A DB-5MS, 0.25 µm film thickness, 0.25mm x 30m GC capillary column (Agilent Technologies, CA, USA) is installed in the oven maintained at 75°C, and 290°C during run.

4.7.2 Sample injection is achieved using splitless mode with 1 µL injection volume of the toluene extract.

4.7.3 The mass spec is set up with the following conditions:

Negative chemical ionization (NCI) mode, ramp 25°C/min, 2 min hold time, to 290°C, 5 min hold time. Runtime of 15.6 min with solvent delay of 3 minutes to preserve filament life. Selected ions (m/z 273 for PIC, m/z 277 for d4-PIC, m/z 467 for QUIN and m/z 470 for d3-QUIN) were simultaneously monitored.

5. Representative Results

The accuracy of the metabolite data is highly dependent on the UHPLC, HPLC and GC/MS instrument performance. The use of control charts assessing changes in the instrument performance over time is recommended. Representative chromatograms from the analysis of TRP and KYN standards are shown in **Figure 2 A and B** respectively while **Figure 2 C and D** highlight TRP and KYN from a representative plasma sample showing good resolution from surrounding peaks. A representative standard curve for each metabolite is shown in **Figure 3 A and B**.

Similarly, a representative KYNA standard chromatogram, sample chromatogram and standard curve are shown in **Figure 4 A-C**, respectively.

uHPLC Intra- and inter-assay coefficients of variation (CV) values were calculated by standards incorporated in the sequence run, and met a 5-8% acceptability criterion.

Finally, typical chromatograms and standard curves for PIC, QUIN and d4-PIC and d3-QUIN are shown in **Figure 5 A-J**, respectively.

GCMS intra- and inter-assay coefficients of variation (CV) values were calculated by standards incorporated in the sequence run, and met a 7-10% acceptability criterion.

Discussion

The use of HPLC separation has been routinely used, and method development has been well established based on sample characteristics such as UV spectra, pK_a values, chemical structures, and molecular weights (Snyder 2012); and its interactions with the column, and mobile liquid phase. Here, we describe the **development/use** of HPLC, and GC/MS in analysing five KP metabolites, TRP, KYN, KYNA, PIC, and QUIN. These methods include the multiplexing of metabolite analysis with good separation, recovery, and reproducibility with intra- and inter- assay %CV of 7-10%.

There are several critical steps in the protocol that must be adhered to ensure reproducible data, and minimize batch-to-batch variation. These steps include (1) preparation of stock standards at high concentration (10mM) in small aliquots to minimize freeze-thaw cycles. For consistency and cost-effectiveness, two freeze-thaw cycles are acceptable, and standards are stable in storage at -20^{o}C for up to 3 months as reported by several other labs [18,21–23]. Data from standards of previous runs should be kept for comparison purposes during significant differences in read out values, which indicate that standards have degraded; (2) Buffer that has not entered the machine can be retained, and re-filtered before use. We find that re-filter buffers tend to give more consistent retention times in comparison to the previous runs; (3) TFAA and HFP are highly volatile, and thus prone to inaccurate pipetting volumes. Filtered tips should be used to protect the pipette, and air in the tip should always be saturated by pipetting with the solution several times before actual pipetting of the liquid into your samples. Tips should always be pre-saturated in this manner each time they are changed; (4) a sodium sulphate filter is made in-house by using a pipette tip plugged with silane treated

wool, then filled with sodium sulphate. This ensures that there is no water remaining in the toluene. Water present in toluene injected will affect the chromatogram.

Based on our past experiences, there are several troubleshooting issues that should be taken note of by the user when running these methods routinely, and are summarized in Table 1.

It was commonly observed in our run that the zinc acetate in the buffer can precipitate causing increase in the pump pressure during each run which can limit the run and hence the need to use gradient elution to minimize the zinc acetate in the system [5]. We found that maintaining the pH at 5.2 kept zinc acetate soluble in the buffer, and does not precipitate even after extensive runs of over 72 hours.

Here, we present a reliable method that can be used to routinely measure five KP metabolites, namely, TRP, KYN, KA, PA, and QUIN in serum samples. We have recently extended this application to other biological fluid in CSF [4] and also cell culture media [24]. We have also expanded this method to include other KP metabolites such as 3HK and 3HAA, however only achieve a LOD of 20nM. The development of this method for quantification of KP in urinary samples confer great potential for KP metabolomics in biomarker discovery.

Bibliography

1. Guillemin, G. J. & Brew, B. J. Implications of the kynurenine pathway and quinolinic acid in Alzheimer's disease. *Redox Report* **7** (4), 199–206, doi:10.1179/135100002125000550 (2002).
2. Lim, C. K. *et al.* Involvement of the kynurenine pathway in the pathogenesis of Parkinson's disease. *Progress in Neurobiology* , doi:10.1016/j.pneurobio.2015.12.009 (2016).
3. Lee, J.-M. M. *et al.* Involvement of quinolinic acid in the neuropathogenesis of amyotrophic lateral sclerosis. *Neuropharmacology* **112** (B), 346–364, doi:10.1016/j.neuropharm.2016.05.011 (2017).
4. Lim, C. K. *et al.* Kynurenine pathway metabolomics predicts and provides mechanistic insight into multiple sclerosis progression. *Scientific Reports* **7** (February), 41473, doi:10.1038/srep41473 (2017).
5. Lim, C. K. *et al.* Altered kynurenine pathway metabolism in autism: Implication for immune-induced glutamatergic activity. *Autism Research* **9** (6), 621–631, doi:10.1002/aur.1565 (2016).
6. Steiner, J. *et al.* Severe depression is associated with increased microglial quinolinic acid in subregions of the anterior cingulate gyrus: evidence for an immune-modulated glutamatergic neurotransmission? *Journal of neuroinflammation* **8** (1), 94, doi:10.1186/1742-2094-8-94 (2011).
7. Miller, C. L. The Evolution of Schizophrenia: A Model for Selection by Infection, with a Focus on NAD. *Current Pharmaceutical Design* **15**, 100–108 (2009).
8. Zavitsanou, K. *et al.* Effect of maternal immune activation on the kynurenine pathway in preadolescent rat offspring and on MK801-induced hyperlocomotion in adulthood: Amelioration by COX-2 inhibition. *Brain, Behavior, and Immunity* **41**, 173–181, doi:10.1016/j.bbi.2014.05.011 (2014).
9. Knuesel, I. *et al.* Maternal immune activation and abnormal brain development across CNS disorders. *Nature Publishing Group* **10** (10), doi:10.1038/nrneurol.2014.187 (2014).
10. Bessede, A. *et al.* Aryl hydrocarbon receptor control of a disease tolerance defence pathway. *Nature* **511**, doi:10.1038/nature13323 (2014).
11. Guillemin, G. J. Quinolinic acid, the inescapable neurotoxin. *FEBS Journal* **279**, 1356–1365, doi:10.1111/j.1742-4658.2012.08485.x (2012).
12. Chen, Y. & Guillemin, G. J. Kynurenine Pathway Metabolites in Humans : Disease and Healthy States. *Int J Tryptophan Res* **61** (2), 1–19 (2009).
13. Bohár, Z., Toldi, J., Fülöp, F. & Vécsei, L. Changing the Face of Kynurenines and Neurotoxicity: Therapeutic Considerations. *International Journal of Molecular Sciences* **16** (5), 9772–9793, doi:10.3390/ijms16059772 (2015).
14. Schwarcz, R. The kynurenine pathway of tryptophan degradation as a drug target. *Current opinion in pharmacology* **4** (1), 12–7, doi:10.1016/j.coph.2003.10.006 (2004).
15. Laich, A., Neurauter, G., Widner, B. & Fuchs, D. More Rapid Method for Simultaneous Measurement of Tryptophan and Kynurenine by HPLC. *Clinical Chemistry* **48** (3) (2002).
16. Lim, C. K. *et al.* Characterization of the kynurenine pathway and quinolinic Acid production in macaque macrophages. *International journal of tryptophan research : IJTR* **6**, 7–19, doi:10.4137/IJTR.S11789 (2013).
17. Lim, C. K. *et al.* Characterization of the kynurenine pathway and quinolinic Acid production in macaque macrophages. *International journal of tryptophan research : IJTR* **6**, 7–19, doi:10.4137/IJTR.S11789 (2013).

18. Shibata, K. Fluorimetric micro-determination of kynurenic acid, an endogenous blocker of neurotoxicity, by high-performance liquid chromatography. *Journal of Chromatography B: Biomedical Sciences and Applications* **430**, 376–380, doi:10.1016/S0378-4347(00)83173-4 (1988).
19. Guillemin, G. J. *et al.* Characterization of the kynurenine pathway in human neurons. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **27** (47), 12884–92, doi:10.1523/JNEUROSCI.4101-07.2007 (2007).
20. Smythe, G. A. *et al.* ECNI GC-MS analysis of picolinic and quinolinic acids and their amides in human plasma, CSF, and brain tissue. *Advances in experimental medicine and biology* **527**, 705–12 (2003).
21. de Jong, W. H. A., Smit, R., Bakker, S. J. L., de Vries, E. G. E. & Kema, I. P. Plasma tryptophan, kynurenine and 3-hydroxykynurenine measurement using automated on-line solid-phase extraction HPLC–tandem mass spectrometry. *Journal of Chromatography B* **877** (7), 603–609, doi:10.1016/j.jchromb.2009.01.015 (2009).
22. Mawatari, K., Oshida, K., Iinuma, F. & Watanabe, M. Determination of quinolinic acid in human urine by liquid chromatography with fluorimetric detection. *Analytica Chimica Acta* **302** (2), 179–183, doi:10.1016/0003-2670(94)00493-6 (1995).
23. Bizzarri, M., Catizone, A., Pompei, M., Chiappini, L., Curini, L. & Lagana, A. Determination of urinary tryptophan and its metabolites along the nicotinic acid pathway by high performance liquid chromatography with ultraviolet detection. *Biomedical Chromatography* **4** (1), 24–27, doi:10.1002/bmc.1130040104 (1990).
24. Jones, S. P. *et al.* Expression of the Kynurenine Pathway in Human Peripheral Blood Mononuclear Cells: Implications for Inflammatory and Neurodegenerative Disease. *PloS one* **10** (6), e0131389, doi:10.1371/journal.pone.0131389 (2015).

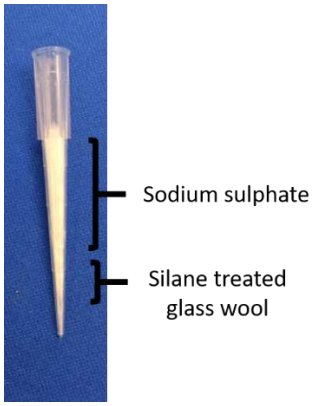

Figure 1: In-house sodium sulphate filter. P20 pipette tip plugged with silane treated glass wool, then filled with sodium sulphate.

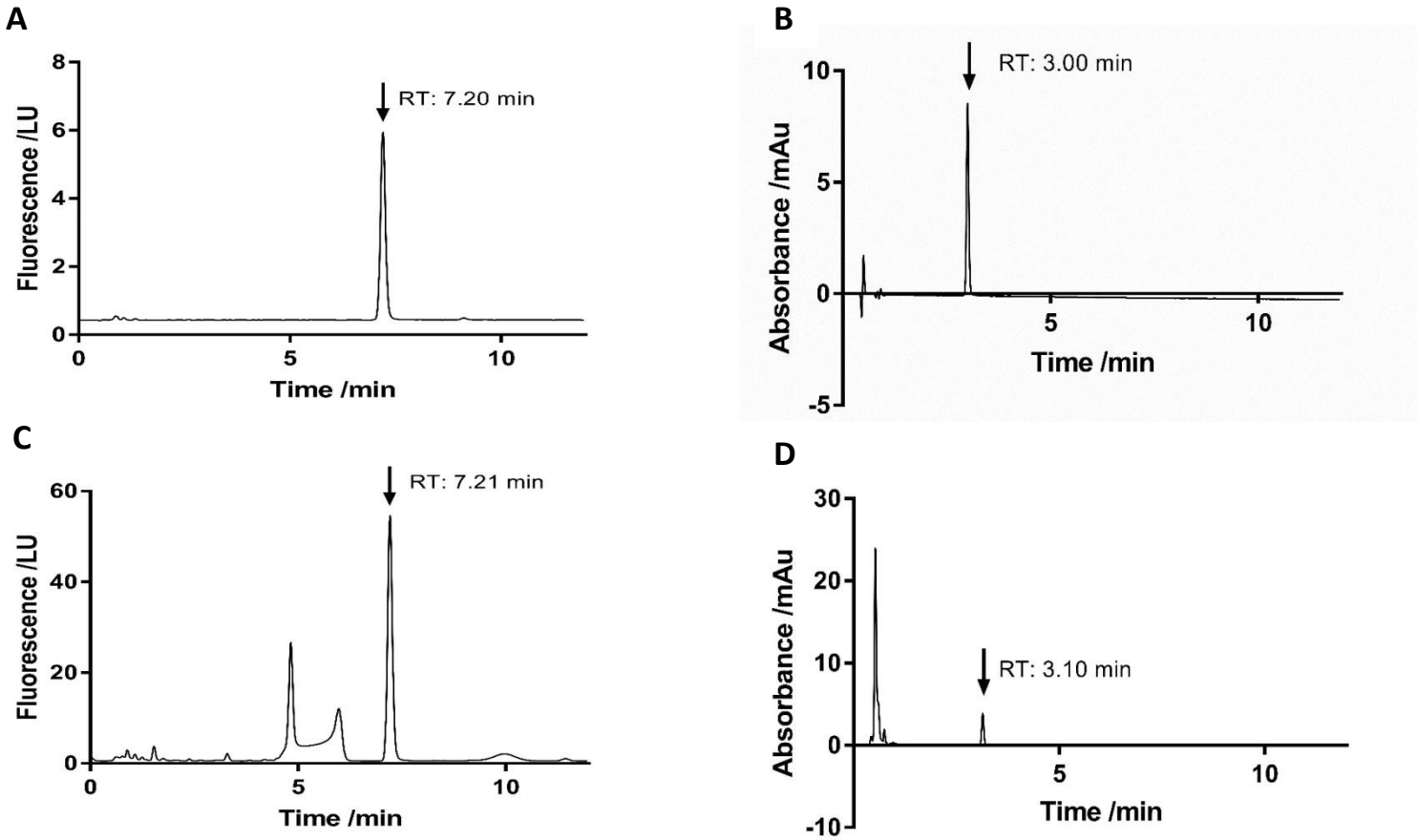

Figure 2: Representative chromatograms for TRP **(A)** 2 µM TRP chromatogram, excitation: 285 nm and emission: 365 nm with retention time at 7.20 min; **(B)** 1 µM KYN chromatogram, absorbance 365 nm and retention time at 3.00 min; **(C)** Plasma sample chromatogram, excitation: 285 nm emission: 365 nm and the TRP retention time at 7.21 min; **(D)** Plasma sample chromatogram, absorbance 365 nm and KYN retention time at 3.10 min.

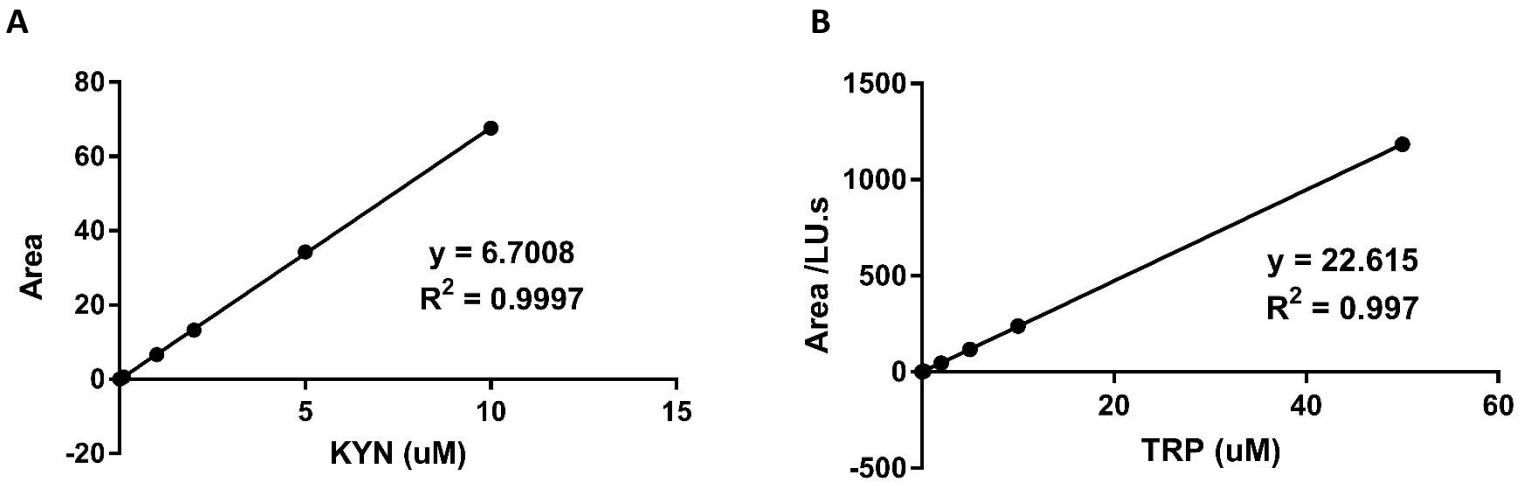

Figure 3: Representative chromatograms for KYN **(A)** Standard curve for KYN with a 20 µL injection; **(B)** Standard curve for TRP, 20 µL injection.

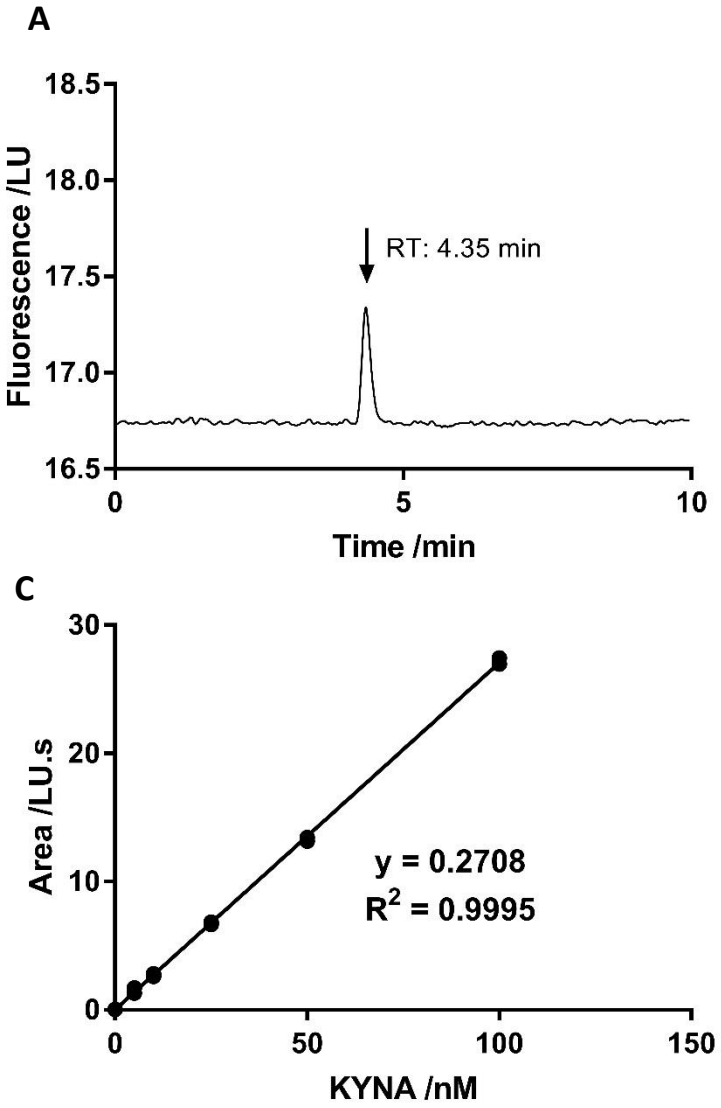

Figure 4: Representative chromatograms for KYNA **(A)** 25 nM KYNA chromatogram, Ex: 344 Em: 388 nm and retention time at 4.35 min;
(B) Plasma sample chromatogram, Ex: 344 nm Em: 388 nm with KYNA retention time at 4.23 min;
(C) Standard curve for KYNA, 10 µL injection, PMT gain: 14.

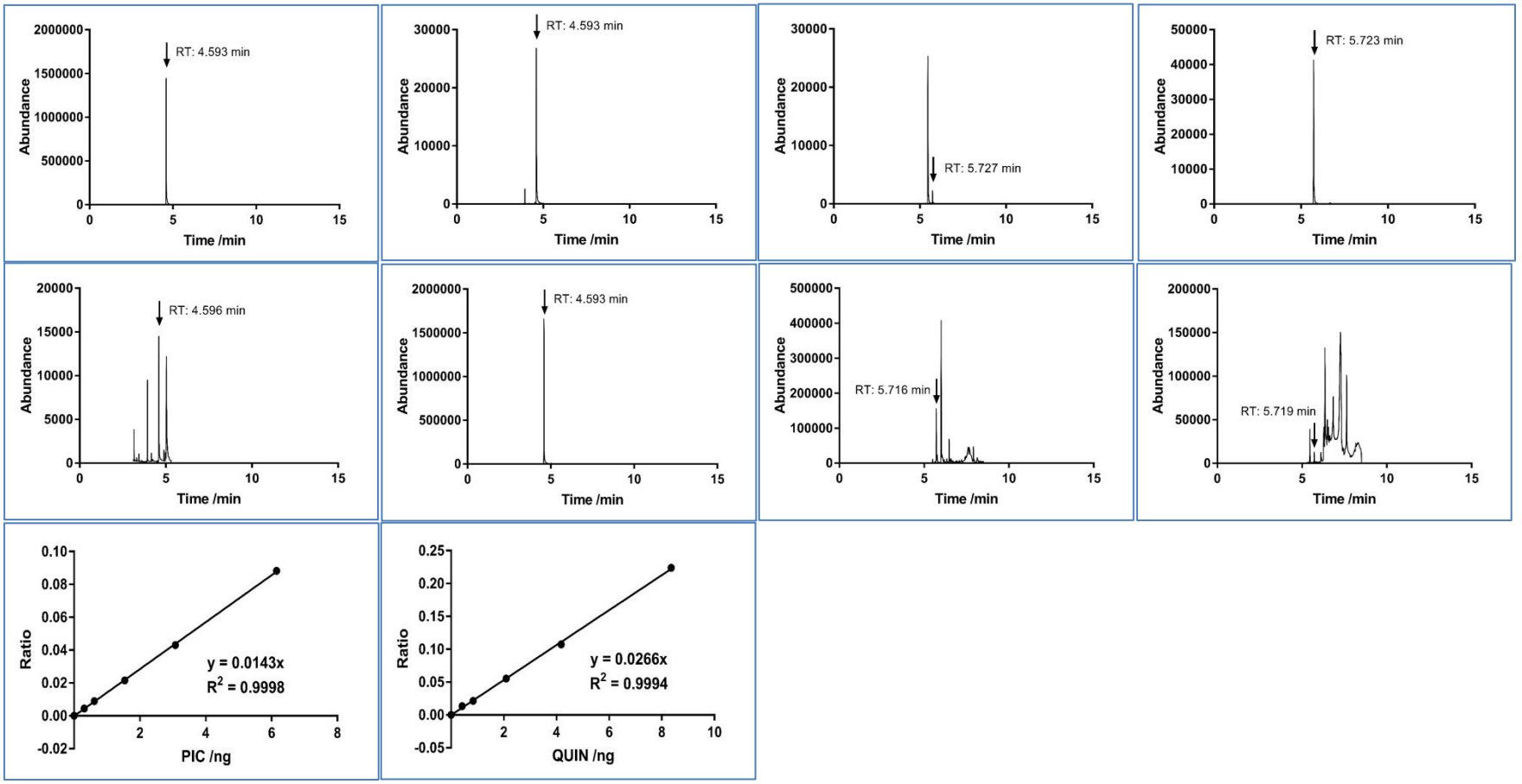

Figure 5: Representative chromatograms for PIC and QUIN **(A)** 0.05 µg/µL PIC chromatogram. m/z 273. Retention time 4.593 min; **(B)** d4-PIC chromatogram. m/z 277, Retention time 4.593 min; **(C)** 0.05 µg/µL QUIN chromatogram. m/ 467 Retention time 5.727 min; **(D)** d3-QUIN chromatogram, m/z 470 Retention time 5.723 min; **(E)** Plasma sample chromatogram. m/z 273, PIC retention time 4.596 min; **(F)** d4-PIC internal standard spiked into plasma sample. m/z 277, retention time 4.593 min; **(G)** Plasma sample chromatogram. m/z 467, QUIN retention time 5.719

min; **(H)** d3-QUIN internal standard spiked into plasma sample. m/z 470, retention time 5.716 min; **(I)** Standard curve for PIC; **(J)** Standard curve for QUIN.

Problem	Cause	Action	Comments
Double shouldered peaks, or broadening peaks	Column collapse due to long exposure to highly aqueous buffers	Replacement with a new column	Column regeneration is often ineffective
Gradual increase in pump pressure	Blocked column	Use in-line filter and guard column. Regular monitoring of buffer pH, especially during reuse of left-over buffer,	Important in high salt buffers such as Zinc Acetate, which are prone to precipitation. Regular monitoring of pump pressure is crucial.
Difficulty in extracting toluene phase effectively from aqueous phase	Inexperience, and different technique when working with small volume derivatisation.	Draw up mixture completely into glass pasture pipette, and eliminate aqueous phase after separation in pipette	Fine control of rubber dropper required

Table 1: Troubleshooting symptoms and methods to resolve

1.5 Original Research Article: The Kynurenine Pathway as a Biomarker for ALS Progression

2017 **JOURNAL OF NEUROINFLAMMATION:** THE KYNURENINE PATHWAY AS A BIOMARKER FOR ALS PROGRESSION (UNDER REVISION)

Vanessa X. Tan, Alain Duhamel, Chai K. Lim, Laura Bourbon, Maeva Khyeng, Vincent Meninger, Lucette Lacomblez, Pierre-Francois Pradat, Gilles J Guillemin

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VXT, MK, LB, AD, CKL, contributed to statistical analyses. GG, CKL, VXT assisted with experimental design. VM, LL, PFP collected samples and clinical information. VXT wrote the manuscript. All other work is my own.

Abbreviations

3HAA	3-hydroxyanthranilic acid
3HK	3-hydroxykynurenine
AA	Anthranilic Acid
AHR	aryl-hydrocarbon receptor
ALS	Amyotrophic Lateral Sclerosis
ALSFRS	ALS Functional Rating Scale
BBB	Blood Brain Barrier
BH4	Tetrahydrobiopterin
CNS	Central Nervous System
CSF	Cerebrospinal Fluid
DNA	Deoxyribonucleic Acid
FUS	Fused in Sarcoma
GC/MS	Gas Chromatography / Mass Spectrometry
HFP	1,1,1,3,3,3-hexafluoroisopropanol
HPLC	High Performance Liquid Chromatography
IDO	Indoleamine 2, 3-Dioxygenase
KMO	Kynurenine monoxygenase
KP	Kynurenine Pathway
KT	Kynurenine/Tryptophan (ratio)
KYN	Kynurenine
KYNA	Kynurenic Acid
MMT	Manual Muscle Testing
MND	Motor Neuron Disease
NAD	Nicotinamide Adenine Dinucleotide
NEO	Neopterin
PA	Picolinic Acid
QLD	Quinaldic Acid
QUIN	Quinolinic Acid
SVC	Slow Vital Capacity
TDP-43	Transactive response DNA binding protein-43
TFA	Trifluoroacetic anhydride
TRP	Tryptophan

UHPLC	Ultra High Performance Liquid Chromatography

Original Research

The Kynurenine Pathway as an indicator for ALS Progression and Prognosis

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PFP, LL, VM collected samples and clinical information. VXT analysed samples. MK, AD, VXT did the statistical analysis. VXT, CKL, LB did the interpretations of statistical analyses. VXT wrote the research article. All authors contributed to reviewing the research article.

Abstract

The diagnosis of amyotrophic lateral sclerosis (ALS) takes on average 13-18 months, and there are no indicators of clinical progression, nor biomarkers to track progression of disease. We conduct a pilot study to investigate the longitudinal Kynurenine Pathway (KP) profile, and neopterin in a subset of ALS patients in a clinical trial to determine if the KP is a suitable candidate as an objective biomarker or prognostic indicator of ALS. The KP is activated by inflammation, and has been shown to be dysregulated in ALS. We show that together with clinical outcomes, ALS Functional Rating Scale (ALSFRS) and Manual Muscle Testing (MMT), KP metabolite 3hydroxyanthranilic acid is increased over time (-1.20, -3.74, and -0.46 units over time). Tryptophan and Kynurenine are both positively associated with ALSFRS; and Kynurenic Acid and Quinaldic Acid are associated with MMT, indicating that they are good candidates as biomarkers for ALS. Lastly, we show that neopterin is a significant prognostic marker for disease progression, with an increased neopterin level being associated with slower disease progression. These findings suggest that the KP may be a useful marker of disease progression and prognosis in ALS, and that further research to integrate the KP with other known biomarkers, and larger datasets may produce a clinically relevant and specific biomarker for ALS.

Keywords:

Kynurenine, tryptophan, biomarker, metabolomics, ALS, longitudinal, prognosis

Introduction

Amyotrophic Lateral Sclerosis (ALS) is the main form of Motor Neuron Disease (MND) in adults, associated with progressive motor deficits such as muscular fasciculation, weak limbs and slurred speech, resulting in paralysis and death [1]. Even though ALS was described almost 150 years ago, the aetiology of the disease is still poorly understood. The hereditary forms of ALS account for only 10% of ALS cases, but are clinically indistinguishable from the sporadic forms. Some patients progress rapidly, succumbing to the disease in 2-3 years, while others survive more than 5 years and sometimes for few decades [2]. There is no treatment or cure for ALS. ALS affects 1 in 11000 Australians. In 2015, 752 Australians died from MND, and the estimated cost of MND to the Australian economy is AUD 430.9 million including health care, productivity, care, financial, and deadweight costs. Deloitte Access Economics [3] estimate the individual cost of MND to be AUD 1.13 million per person, significantly greater than diseases such as stroke and chronic kidney disease.

Patients often see multiple specialists before being correctly diagnosed, and the average time from onset of symptoms to an official diagnosis is 18 months [4–6], a very long time for a disease with a prognosis of death within 3 years from onset [7]. The absence of valid biochemical markers hampers the diagnosis and/or prognosis of ALS. Biomarkers would also allow **1)** an affirmative prognosis (fast versus slow progressors) after diagnosis; **2)** differentiate between subtypes of MND; **3)** assess response to potential treatments (clinical trials), and **4)** better manage supportive interventions such as the effective use of non-invasive ventilation or management of cognitive changes (Ganesalingam & Bowser, 2010; Otto et al., 2012; Turner, Bowser, et al., 2013; Hu, Loo, & Wong, 2006).

Neuroinflammation is a hallmark of ALS [11], and the Kynurenine Pathway (KP) metabolites (**Fig. 1**), and neopterin (NEO) (a TRP catabolite), are considered markers of activation of the immune system [12–14]. The KP is the main pathway catabolising the essential amino acid tryptophan (TRP). The KP is activated during neuroinflammatory conditions, and the metabolites generated can be either **1)** neurotoxic such as quinolinic acid (QUIN), 3-hydroxykynurenine (3HK); **2)** neuroprotective such as kynurenic acid (KYNA), picolinic acid (PA), quinaldic acid (QLD); **3)** have dual neuroactive properties such as 3-Hydroxyanthranilinc acid (HAA), which is toxic at higher concentrations, and protective at lower concentrations; or **4)** potent immunomodulatory properties, especially kynurenine (KYN) [15–19].

The involvement of the KP in ALS has been described, finding significant changes in the KP between patient and control subjects [27–30], and the overlap in the neurotoxic effects of QUIN and the neuropathology of ALS [15]. Dysregulation of the KP and NEO are known to be associated with several other neurological diseases such as autism [20], multiple sclerosis [21,22], Parkinson's disease [23,24]

and Alzheimer's [25], among others [26]. These studies support the role of neuroinflammation in neurodegenerative diseases, and the use of KP metabolites as a potential panel of biomarkers.

Highly sensitive gas and liquid chromatography methods have allowed us to perform multiplex KP metabolomic quantitation assays, with low sample volumes. It should be highlighted that the KP is a "generic" marker for inflammation, and is not disease specific. However, the KP metabolomic profile provides an important insight into molecular mechanisms contributing to disease aetiology and progression [31–33]. We hypothesize that the KP metabolome, and neopterin could be used for the prognosis and/or monitoring of progression of ALS. In this study, we conducted a pilot study on serum from a retrospective longitudinal cohort of ALS patients and controls to assess the KP metabolome as a potential biomarker for disease progression, and prognosis.

Methods

Subjects

Subjects used in this pilot study were a cohort of 66 patients from GH Pitié-Salpêtrière in a double-blind, randomized, placebo-controlled, multicentre trial (clinicaltrials.gov, number NCT 00868166) conducted according to the European Guidelines for Good Clinical Practice. Results from this trial have been published in [34]. Patients enrolled were aged 18-80 years with El Escorial definite or probably ALS of between 6 and 36 months' duration treated with 50mg riluzole twice a day for at least 1 month and had a slow vital capacity (SVC) of 70% or more. Written consent was given and study was approved by all relevant ethics committees and national regulatory authorities (CPP IDF IV – GH Pitié-Salpêtrière). Macquarie University Human Research Ethics Committee approved research (5201600401). Clinical samples and data were collected 6 monthly for 18 months. Current study was conducted using only samples from GH Pitié-Salpêtrière, from patients who were on either placebo or control.

TRP catabolite and KP metabolite Profiling

Reagents. Analytical grade acetonitrile, methanol, sodium acetate, toluene, trichloro acetic acid (TCA), trifluoracetic anhydride (TFAA), 1,1,1,3,3,3-hexafluoroisopropanol (HFP), sodium bicarbonate, sodium sulphate, metabolite standards (Tryptophan, TRP; Kynurenine, KYN; Kynurenic Acid, KYNA; 3-hydroxykynurenine, 3HK; 3hydroxy-anthranilic acid, 3HAA; anthranilic acid, AA; picolinic acid, PA; quinolinic acid, QUIN; quinaldic acid, QLD; and NEO, NEO) suitable for UHPLC and GC/MS were purchased from Sigma-Aldrich (USA), unless otherwise stated. Deuterated internal standards for PA, QLD, and QUIN ($^{2}H_{4}$-PA,$^{2}H_{6}$-QLD, and $^{2}H_{3}$-QUIN) were purchased from Medical Isotopes, Inc (USA).

Sample Preparation. Protocol was carried out in accordance to previously described methods [22]. Briefly, serum samples were thawed on ice and mixed thoroughly with a vortex. Samples were deproteinized using 10% TCA (*w/v*) to an equal volume of serum. The samples were vortexed briefly before centrifugation at 12,000 rpm at 4^{o}C for 10min. Supernatant was collected and filtered through a 0.22µm syringe filter (Merck-Millipore, USA) and then transferred into a glass vial for analysis in the uHPLC.

For GC/MS, 50µL of the filtered samples were transferred into a fresh Pyrex glass tube (Sigma-Aldrich, USA) containing deuterated internal standards for both PIC, QUIN, and QLD. Mixtures were then dried under vacuum using Savant SpeedVac (Thermo Fisher, USA). The dried samples were reconstituted with 60 µL each of TFAA and HFP for derivatization at 45^{o}C for 40 mins. The esterified samples were dissolved in 130µL toluene, washed with 5% (*w/v*) sodium bicarbonate, and then ultrapure water. Samples were passed through a filter with a silane-treated glass wool plug (Grace, USA) filled with sodium sulphate to remove contaminants and/or water, and collected into a pulled point glass vial for GC/MS analysis.

GC/MS Analysis. Analysis was carried out as described by Smythe et al (2003) with slight modifications using an Agilent 7890A GC system coupled with Agilent 5975C mass spectrometry detector and Agilent 7693A autosampler (Agilent Technologies, USA). 1µL of sample is injected and chromatographic separation occurs under helium (BOC, AUS), and methane (Air Liquide, AUS) as reagent gas. A negative chemical ionisation detector detected selected m/z ratio (273, 277; 329, 323; 467 and 470) to identify PA, QLD, QUIN, and their corresponding deuterated internal standards. Agilent GC/MSD ChemStation software was used to read data, and concentrations extrapolated from standard curves against deuterated internal standards.

uHPLC Analysis. TRP, KYN, AA, 3HK, 3HAA, NEO, and KYNA were analysed as previously described [22]. Briefly, TRP, KYN, AA, 3HA, 3HAA, and NEO were simultaneously detected on the Agilent uHPLC 1290 Infinity series on an isocratic ammonium acetate mobile phase. KYNA was detected on an Agilent 1200 Infinity series with mobile phase of sodium and zinc acetate. Agilent OpenLAB CDS ChemStation was used to analyse chromatograms and concentrations extrapolated from standard curves.

Statistics

Data was tested for normality, and log transformations conducted where required (S1). Age, gender, location of disease onset, and treatment were assessed for effects on KP at baseline.

Quantitative variables are expressed as mean (standard deviation) in the case of normal distribution or median (interquartile range) otherwise. Categorical variables are expressed as number (percentage). Normality of distributions was assessed using histograms and Shapiro-Wilk test.

Baseline values for Amyotrophic Lateral Sclerosis Functional Rating Scale (ALSFRS) and KP metabolites were described in each treatment group (Olesoxyme and Placebo) and absolute standardized differences were calculated to evaluate baseline imbalance [36].

The evolution of each metabolite over time was analysed using a linear mixed model with a fixed time effect and we included a random intercept and a random slope for time effect.

We investigated the association between the baseline values of each metabolite and the ALSFRS, FVC and the Manual Muscle Testing (MMT) progression respectively, with linear mixed models. In these models, we considered the time, the metabolite and their interactions with time as fixed effect. As in the previous analysis, the random effects were the intercept and the slope for time.

The association between each metabolite and the ALSFRS at any time was investigated using a linear mixed model with the repeated measures of ALSFRS as independent variable and the time and the repeated measures of the metabolite as fixed dependent variables. In this model, we also incorporated a random intercept and a random slope for time effect. The same model was used to analyse the association between the metabolites and the MMT.

All the statistical analyses were adjusted for treatment group and disease duration. The statistical tests were performed at the 2-tailed α level of 0.05 and the data were analysed using SAS version 9.4 [SAS Institute Inc., Cary, NC 27513, USA].

Results

Descriptive statistics

This study was conducted on serum samples obtained from GH Pitié-Salpêtrière, a subset of the larger clinical trial study [34]. In this study, 66 patients were included with KP metabolomics information. 68% of this population was female, and 50% took placebo. At baseline, age at weakness onset was 56±10.3, ALSFRs score was 37.3±4.6, and FVC was 2.9±1.0. The age of population at baseline was 59.2±10.4. Overall, the two treatment groups are well balanced regarding most of the baseline KP metabolites values (**Table 1**), with an absolute standardized difference greater than 30% for PA, KYNA, and ratio KYNA/QUIN.

Factor changes across time

As expected, we observe a significant decrease in ALSFRS and MMT, with a mean of decreasing estimated at -1.20 ±0.23 point per month for ALSFRS (p<0.0001) and -3.74±0.96 points per month for MMT (p=0.0007) (**Table 2**), characterizing the change in clinical outcomes as disease progresses. Interestingly, the KP metabolite HAA decreases significantly over time (-0.46±0.18) unit per month, p=0.033).

KP Metabolites as prognostic markers for disease progression

The KP metabolites values measured at baseline are not associated with a modification of the decline of ALSFR score (**Table 3**; p>0.05) except the HAA/AA ratio (p=0.031).). The results are similar for MMT except for NEO, which is associated to a lower decline of MMT. An increase of one unit of NEO at baseline results in an increasing in the slope of MMT per month of 0.12±0.05 (**Table 4**; p=0.023). None of KP metabolites except HAA/AA ratio is significantly associated to overall survival (**Table 5**). HAA/AA ratio is a protective factor of survival (HR=0.48 [0.29 ; 0.81], p=0.006). The p value of TRYP is near to significant level (p=0.051) and that means that an increase in TRP value could be a protective factor of survival.

KP Metabolites as a marker for progression of ALS

The results concerning the associations between the KP metabolites and ALSFRS at any time are presented in **Table 6**. During the follow up, an increase at a given time in KYN and/or TRP values results in a greater value of the ALSFRS score (+2.29±1.01 per unit of KYN, p=0.027 and +0.07±0.03 per unit of TRP, p=0.022). For the MMT (**Table 7**), an increase in KYNA is favourable (+0.07±0.02 per unit of KYNA, p=0.009) while an increase in QLD is unfavourable (-6.57±2.20 per unit of QLD, p=0.006).

Discussion

This study describes the quantification of 10 KP metabolites and a TRP catabolite, neopterin, in the serum of 66 ALS patients from a comprehensive and retrospective longitudinal study. The data presented in this paper show that out of the 10 KP metabolites, significant correlations were observed in 1) HAA with time; 2) HAA/AA ratio as a predictor of better prognosis through decreased ALSFRS progression, and is protective from death; 3) NEO indicates better prognosis through decreased MMT progression; 3) TRP and KYN with as a biomarker for ALSFRS scores; and 4) QLD and KYNA as a biomarker for MMT. These key findings have been collated into **Table 8**. The range of concentrations of some of the KP metabolites quantified are in accordance with those from previous studies [27,28]. We discuss the possible contributions of the KP metabolites and NEO in ALS based on these changes.

The KP is involved in many neurodegenerative diseases and psychiatric disorders including Huntington's, Parkinson's, Alzheimer's, and multiple sclerosis [22,24,37–40], but also in brain cancers [41,42]. We and others have demonstrated the KP is also significantly dysregulated in ALS. Ilzecka et al. [28] reported an increase in the neuroprotective KP metabolite KYNA in CSF of ALS patients, but lower in serum. Chen et al (2010) demonstrated that in CSF, the K/T ratio indicating inflammation, and the neurotoxin QUIN is increased in ALS patients as compared to controls. We recently demonstrated that KP metabolites can be used as a biomarker for the prognosis of MS subtypes [22]. With strength of this expertise, we have applied a similar approach for ALS.

Descriptive statistics confirmed normality of data, and transformations performed where required. We confirmed that age, gender, location of disease onset, and treatment did not influence KP at baseline. Further, the standardized differences of the KP metabolites and clinical measures at baseline, revealed that there were no significant differences in the factors assessed (KP metabolites and clinical outcomes), between the treatment (Olesoxyme) and placebo groups (**Table 1**) except in PA, KYNA, and their associated ratios. Change in PA between treatment and placebo groups is mostly due to the small concentrations of PA. No significant changes were expected as the treatment was concluded to not have an effect on ALS patients treated with riluzole [34].

A linear mixed model with a fixed time effect observed the evolution of factors including KP metabolites and clinical outcomes in ALS patients over time (**Table 2**). Although the model showed an expected significant decrease in both ALSFRS (-1.20 ±0.23 points per month) and MMT (-3.74±0.96 points per month) over time, this is not observed in SVC. SVC is expected, and known to

progressively decrease [43,44]. Pulmonary function is an important predictor for survival in ALS, death of ALS patents is largely attributed to respiratory failure [45–47]. This can be explained by the variance in measurements such as SVC and FVC, and the use of non-invasive ventilation may be a confounding factor [48–51]. This lack of change in SVC over time may be due to the inclusion criteria for SVC ≥ 70%, which may unintentionally select for patients who have better pulmonary function.

More interestingly, the linear mixed model also revealed that HAA is changed through time in a negative relationship (-0.46±0.18) (**Table 3**). Within the CNS, HAA displays a Janus face, depending on its concentration. At high concentrations (pathological range of greater than 100μM) HAA can generate reactive oxygen species [52–55], but antioxidant properties have also been determined [17,56–61]. Interestingly, superoxide dismutase (SOD), of which SOD1 is dysregulated in ALS, increases the auto-oxidative properties of HAA, augmenting conversion of TRP to KYN [62,63]. The decrease in HAA may be explained by an increase in the activity of enzyme 3hydroxyanthranilic oxygenase, which results in the production of neurotoxic QUIN [64], followed by the synthesis of NAD+ [65]. Finally, HAA has an immunosuppressive role, inhibiting T cell function [66–69], which may regulate chronic inflammation in ALS.

HAA, in a ratio with its upstream metabolite, AA, predicts survival, and a decreased rate of ALSFRS progression.

In measuring the KP and ALSFRS as a function of time, the mixed model showed positive associations of ALSFRS with KYN and TRP (**Table 6**). This is a significant discovery, as they are two of the key, and stable molecules in the KP [26,70]. A previous study from our group on a different cohort of ALS patients also found concordant results, observing significant increases in both TRP and KYN in comparison to control patients [27]. The association between TRP and KYN with ALSFRS over time indicates that they have strong potentials to be used as biomarkers for ALS. TRP was also close borderline significant in predicting survival (**Table 9**).

The increase in serum TRP may be a result of the homeostasis response to the activation of IDO1, which has been demonstrated during neuroinflammation, and in spinal cords of ALS [27], reducing the amount of TRP available in the in the central nervous system (CNS). This may trigger the dissociation of TRP from albumin, to allow transport of TRP across the BBB in response to an increase in IDO activity.

KYN is increased in Alzheimer's disease and other neuroinflammatory diseases. The increase in KYN in ALS patients is likely to be associated with its neuroprotective properties that are increased over time, as it is a potent immunomodulator that can cross the blood brain barrier [64,71,72]. KYN has is

also an endogenous ligand for the human aryl-hydrocarbon receptor (AhR), which has a role responding to toxic compounds such as the ALS associated proteins transactive response DNA binding protein (TDP-43) and fused in sarcoma (FUS), AhR has DNA binding properties [72–74]. KYN is also a point of divergence in the KP, it can be converted into the neuroprotective KYNA; or the neurotoxic AA; and 3HK which is neurotoxic, and whose enzyme kynurenine monooxygenase (KMO) has the highest affinity for KYN [72].

Similarly, the mixed model showed that QLD and KYNA are associated with MMT as a function of time (**Table 6**). Both QLD and KYNA have neuroprotective properties [18,75,76], thus it was surprising that QLD was inversely associated with MMT.

KYNA is a potent regulator of the NMDA and AMPA receptors, and can attenuate excitotoxicity of QUIN [15,18,41,75,77–79]. However, KYNA is decreased in patients with MS, AD, PD, Huntington's, chronic brain injury, and in ALS [18,28,30,80–86]. This is often accompanied by an increase of the neurotoxin QUIN. QUIN is a neurotoxin that has toxicity mechanisms closely associated with ALS, such as oxidative stress, protein and mitochondrial dysfunction [15]. Although QUIN was not shown to be negatively associated with MMT changes, we have previously demonstrated that QUIN is increased in serum and CSF of ALS patients as compared to controls [27], which may explain the increase in KYNA for its neuroprotective properties.

The scientific literature about QLD is very limited. QLD is neuroprotective, lacking the ability to generate ROS due to the absence of a metal binding 8-hydroxyl group [87] and like KYN, is a ligand for AhR [72–74]. However its neuroprotective effects are inferior to KYNA and other KP metabolites [55,88]. Outside of the nervous system, quinaldic acid is implicated in diabetes, through the regulation of insulin release from islet of Langerhans cells [89]; and inhibition of bacterial growth [90,91]. Most significantly, Duleu et al reported an increase in circulating antibodies against QLD in the serum of ALS, PD, and MS, compared to healthy controls [92]. Since QLD is the main downstream metabolite derived from KYNA [93], but both are inversely correlated with MMT as a function of time, these results indicate that there may be a dysfunction in the conversion of KYNA to QLD, for which the enzymatic process is still unknown.

To determine the prognostic capability of variables in predicting clinical outcomes, we use the variable values in mixed model with clinical outcomes as a dependent variable. NEO was the only TRP metabolite that showed an influence on prognosis (**Tables 3 and 4**). An increase in neopterin levels are associated with a lowered ALSFRS disease progression slope.

NEO is a small molecule that is produced by macrophages and monocytes [94], and has recently been shown to be produced by astrocytes [95]. It can cross the BBB, and levels in plasma/serum correlate with those found in the CSF, although at a different magnitude [12,96–99]. NEO is increased during neuroinflammation [95], which is one of the major hallmarks of ALS [2,11,100–102].. ALS [103] and other neuroinflammatory diseases such as encephalitis, Huntington's AD and PD, have been shown to have elevated concentrations of NEO [99,104–106]. The increase in NEO confirms the inflammatory status of ALS, and may be attributed to the role of NEO neuroprotection against oxidative stress due to mitochondrial dysfunction [95], a key pathology in ALS [1,8]

An increase in NEO is expected to be accompanied by an increase in the K/T ratio, since they both are indicators of inflammation. Thus, a lack of change in the KT ratio is perplexing, since both NEO and KP activation are associated with inflammatory mediators such as IFN-γ [14,107,108]. However, we previously observe similar results with a different ALS cohort [27]. Although both serum and CSF TRP and KYN were higher in ALS patients, increased IDO activity (as indicated by the K/T ratio) was present in only the CNS [27]. This indicates that IDO, and thus inflammation, is active in the CNS, while being likely mitigated in the periphery.

Limitations of this study include the absence of control population for comparison. However, we have a large number of data on KP in controls and diseased states [30], and specifically in ALS and control populations [27,28]. Future studies would require the inclusion of other neurodegenerative disorders, inflammatory and non-inflammatory; as well as other MND-mimic disease, which may assist in providing a biochemical method for disease distinction and diagnosis. The second limitation, is the difference in duration from onset of disease to first collection of blood ranged from 7-36 months (mean 19.5 months) considered in the analysis through adjustments. As such, we cannot currently comment on changes in the KP during the earlier stages of disease, particularly due to the delayed diagnosis of ALS, and recall biases that may affect date of symptom onset. Finally, we also acknowledge that analysis of the KP metabolome was performed with blood samples, and that changes in blood may not directly reflect changes in the CNS. The most relevant samples for ALS would be CSF, which is difficult to obtain and is regarded as semi-invasive, rendering collections of CSF much less viable, and less common in longitudinal samples [10,109].

Further work needs to be done to characterize changes in TRP catabolites between the different clinical phenotypes of ALS subtypes that present with similar symptoms, but have different rates of disease progression [110]. The ability to differentiate these subtypes will be critical when drugs are target different subtypes or different symptoms, as in MS, can be better prescribed to patients for personalised treatment. Such biomarkers are also essential for assessing the efficacy of new drugs in

clinical trials, as not all drugs may have the same effect on all subtypes of patients. A single biomarker is often insufficient to meet stringent criteria as biomarkers, and the use of combination of biomarkers is likely to enhance sensitivity and specificity without continual clinical monitoring, increasing translation of the biomarkers as a prognostic tool for the clinicians [4,8,10]. Some existing single biomarkers should be considered for combination such as p75-ECD, Nogo, neurofilament, and complement components [45,109,111–113].

Conclusion

The results presented in this pilot study examined the association of TRP and its derived metabolites together with the disease progression and prognosis of ALS in 66 patients. Cumulatively, our data suggest that the TRP catabolites may provide sensitive biomarkers for 1) the prognostic of ALS (neopterin) and 2) to monitor clinical progression of ALS (KP metabolites HAA, TRP, KYN, QLD, KYNA). We found that an increased neopterin concentration is associated with a better prognosis of the disease. The levels of KP metabolites represent a potential biomarker for prognosis and assessing ALS progression. Even though the results are already significant, we believe that this study would need to be replicated and validated with other, and larger cohorts. Finally, the quantification of KP metabolites in urine should be exploited as a less invasive method to look for biomarkers, as for p75 ECD [93,114–116]. These changes in the KP metabolites also unveil the potential of new therapeutical targets for ALS. KP metabolite analogues such as 4-chlorokynurenine (AV-101), or KP enzymatic modulators can be explored [67,117–119].

References

1. Bruijn LI, Miller TM, Cleveland DW. Unraveling the Mechanisms Involved in Motor Neuron Degeneration in Als. Annu. Rev. Neurosci. Annual Reviews; 2004;27:723–49.

2. Calvo AC, Manzano R, Mendonça DMF, Muñoz MJ, Zaragoza P, Osta R. Amyotrophic lateral sclerosis: a focus on disease progression. Biomed Res. Int. 2014;2014:925101.

3. Deloitte Access Economics. Economic analysis of motor neurone disease in Australia, report for Motor Neuron Disease Australia. Canberra; 2015.

4. Robelin L, Gonzalez De Aguilar JL. Blood biomarkers for amyotrophic lateral sclerosis: myth or reality? Biomed Res. Int. 2014;2014:525097.

5. Donaghy C, Dick A, Hardiman O, Patterson V. Timeliness of diagnosis in motor neurone disease: a population-based study. Ulster Med. J. 2008;77:18–21.

6. Wood-Allum C, Shaw PJ. Motor neurone disease: a practical update on diagnosis and management. Clin. Med. 2010;10:252–8.

7. Al-Chalabi A, Hardiman O. The epidemiology of ALS: a conspiracy of genes, environment and time. Nat. Rev. Neurol. Nature Publishing Group; 2013;9:617–28.

8. Turner MR, Bowser R, Bruijn L, Dupuis L, Ludolph A, McGrath M, et al. Mechanisms, models and biomarkers in amyotrophic lateral sclerosis. Amyotroph. Lateral Scler. Frontotemporal Degener. 2013;14 Suppl 1:19–32.

9. Ganesalingam J, Bowser R. The application of biomarkers in clinical trials for motor neuron disease. Biomark. Med. NIH Public Access; 2010;4:281–97.

10. Otto M, Bowser R, Turner M, Berry J, Brettschneider J, Connor J, et al. Roadmap and standard operating procedures for biobanking and discovery of neurochemical markers in ALS. Amyotroph. Lateral Scler. Taylor & Francis; 2012;13:1–10.

11. McGeer PL, McGeer EG. Inflammatory processes in amyotrophic lateral sclerosis. Muscle Nerve. 2002;26:459–70.

12. Fuchs D, Spira TJ, Hausen A, Reibnegger G, Werner ER, Felmayer GW, et al. Neopterin as a predictive marker for disease progression in human immunodeficiency virus type 1 infection. Clin.

Chem. 1989;35.

13. Zelante T, Fallarino F, Bistoni F, Puccetti P, Romani L. Indoleamine 2,3-dioxygenase in infection: the paradox of an evasive strategy that benefits the host. Microbes Infect. 2009;11:133–41.

14. Huber C, Batchelor JR, Fuchs D, Hausen A, Lang A, Niederwieser D, et al. Immune response-associated production of neopterin. Release from macrophages primarily under control of interferon-gamma. The Rockefeller University Press; 1984;160:310–6.

15. Lee J-MM, Tan V, Lovejoy D, Braidy N, Rowe DB, Brew BJ, et al. Involvement of quinolinic acid in the neuropathogenesis of amyotrophic lateral sclerosis. Neuropharmacology. Elsevier Ltd; 2017;112:346–64.

16. Behan WM, Stone TW. Enhanced neuronal damage by co-administration of quinolinic acid and free radicals, and protection by adenosine A2A receptor antagonists. Br J Pharmacol. 2002;135:1435–42.

17. Colín-González AL, Maldonado PD, Santamaría A. 3-Hydroxykynurenine: an intriguing molecule exerting dual actions in the central nervous system. Neurotoxicology. 2013;34:189–204.

18. Stone T. Neuropharmacology of quinolinic and kynurenic acids. Pharmacol. Rev. 1993;45:309–79.

19. Guillemin GJ. Quinolinic acid, the inescapable neurotoxin. FEBS J. 2012. p. 1356–65.

20. Lim CK, Essa MM, de Paula Martins R, Lovejoy DB, Bilgin AA, Waly MI, et al. Altered kynurenine pathway metabolism in autism: Implication for immune-induced glutamatergic activity. Autism Res. 2016;9:621–31.

21. Lim CK, Brew BJ, Sundaram G, Guillemin GJ. Understanding the roles of the kynurenine pathway in multiple sclerosis progression. Int. J. Tryptophan Res. 2010;3:157–67.

22. Lim CK, Bilgin A, Lovejoy DB, Tan V, Bustamante S, Taylor B V., et al. Kynurenine pathway metabolomics predicts and provides mechanistic insight into multiple sclerosis progression. Sci. Rep. Nature Publishing Group; 2017;7:41473.

23. Szabó N, Kincses ZT, Toldi J, Vécsei L. Altered tryptophan metabolism in Parkinson's disease: a possible novel therapeutic approach. J. Neurol. Sci. 2011;310:256–60.

24. Lim CK, Fernández-Gomez FJ, Braidy N, Estrada C, Costa C, Costa S, et al. Involvement of the kynurenine pathway in the pathogenesis of Parkinson's disease. Prog. Neurobiol. 2016;

25. Guillemin GJ, Brew BJ. Implications of the kynurenine pathway and quinolinic acid in Alzheimer's disease. Redox Rep. Maney Publishing; 2002;7:199–206.

26. Maddison DC, Giorgini F. The kynurenine pathway and neurodegenerative disease. Semin. Cell Dev. Biol. 2015;40:134–41.

27. Chen Y, Stankovic R, Cullen KM, Meininger V, Garner B, Coggan S, et al. The Kynurenine Pathway and Inflammation in Amyotropic Lateral Sclerosis. Neurotox. Res. 2009/11/19. 2010;18:132–42.

28. Ilzecka J, Kocki T, Stelmasiak Z, Turski WA. Endogenous protectant kynurenic acid in amyotrophic lateral sclerosis. Acta Neurol Scand. Munksgaard International Publishers; 2003;107:412–8.

29. Chen Y, Meininger V, Guillemin GJ. Recent advances in the treatment of amyotrophic lateral sclerosis. Emphasis on kynurenine pathway inhibitors. Cent. Nerv. Syst. Agents Med. Chem. 2009;9:32–9.

30. Chen Y, Guillemin GJ. Kynurenine Pathway Metabolites in Humans : Disease and Healthy States. Int J Tryptophan Res. 2009/01/01. 2009;61:1–19.

31. Fiehn O, Robertson D, Griffin J, van der Werf M, Nikolau B, Morrison N, et al. The metabolomics standards initiative (MSI). Metabolomics. Springer US; 2007;3:175–8.

32. Kumar A, Ghosh D, Singh RL. Amyotrophic Lateral Sclerosis and Metabolomics: Clinical Implication and Therapeutic Approach. J. biomarkers. Hindawi Publishing Corporation; 2013;2013:538765.

33. Wuolikainen A, Hedenström M, Moritz T, Marklund SL, Antti H, Andersen PM, et al. Amyotrophic Lateral Sclerosis Optimization of procedures for collecting and storing of CSF for studying the metabolome in ALS Optimization of procedures for collecting and storing of CSF for studying the metabolome in ALS. Amyotroph. Lateral Scler. 2017;10:229–36.

34. Lenglet T, Lacomblez L, Abitbol JL, Ludolph A, Mora JS, Robberecht W, et al. A phase II-III trial of olesoxime in subjects with amyotrophic lateral sclerosis. Eur. J. Neurol. 2014;21:529–36.

35. Smythe GA, Poljak A, Bustamante S, Braga O, Maxwell A, Grant R, et al. ECNI GC-MS analysis of picolinic and quinolinic acids and their amides in human plasma, CSF, and brain tissue. Adv. Exp. Med. Biol. 2003;527:705–12.

36. Cohen J. A Power Primer. Psychol. Bull. 1992;112:155–9.

37. Umbach JA, Adams KL, Gundersen CB, Novitch BG. Functional neuromuscular junctions formed

by embryonic stem cell-derived motor neurons. PLoS One. Public Library of Science; 2012;7:e36049.

38. Majláth Z, Toldi J, Vécsei L, Majláth Z, Toldi J, Vécsei L. The Role of the Kynurenine Pathway in Neurodegenerative Diseases. Target. Broadly Pathog. Kynurenine Pathw. 2015. p. 193–204.

39. Schwarcz R, Okuno E, White RJ, Birdt ED, Whetsell W 0. 3-Hydroxyanthranilate oxygenase activity is increased in the brains of Huntington disease victims (kynurenines/quinolinic acid/excitotoins/neurodegenerative disorders). Neurobiology. 1988;85:4079–81.

40. Ting KK, Brew BJ, Guillemin GJ. Effect of quinolinic acid on human astrocytes morphology and functions: implications in Alzheimer's disease. J Neuroinflammation. 2009/12/17. 2009;6:36.

41. Adams S, Braidy N, Bessesde A, Brew BJ, Grant R, Teo C, et al. The Kynurenine Pathway in Brain Tumor Pathogenesis. Cancer Res. 2012;72:5649–57.

42. Opitz CA, Litzenburger UM, Sahm F, Ott M, Tritschler I, Trump S, et al. An endogenous tumour-promoting ligand of the human aryl hydrocarbon receptor. 2011;

43. Clavelou P, Blanquet M, Peyrol F, Ouchchane L, Gerbaud L. Rates of progression of weight and forced vital capacity as relevant measurement to adapt amyotrophic lateral sclerosis management for patient Result of a French multicentre cohort survey. J. Neurol. Sci. Elsevier B.V.; 2013;331:126–31.

44. Pinto S, De Carvalho M. Amyotrophic Lateral Sclerosis and Frontotemporal Degeneration Correlation between Forced Vital Capacity and Slow Vital Capacity for the assessment of respiratory involvement in Amyotrophic Lateral Sclerosis: a prospective study Correlation between Forced . Amyotroph. Lateral Scler. Front. Degener. 2017;18:86–91.

45. Dupuis L, Gonzalez de Aguilar J-L, di Scala F, Rene F, de Tapia M, Pradat P-F, et al. Nogo Provides a Molecular Marker for Diagnosis of Amyotrophic Lateral Sclerosis. Neurobiol. Dis. 2002;10:358–65.

46. Ilieva H, Polymenidou M, Cleveland DW. Non-cell autonomous toxicity in neurodegenerative disorders: ALS and beyond. J. Cell Biol. 2009;187:761–72.

47. Turner MR, Hardiman O, Benatar M, Brooks BR, Chio A, de Carvalho M, et al. Controversies and priorities in amyotrophic lateral sclerosis. Lancet Neurol. Elsevier Ltd; 2013;12:310–22.

48. Lee S, Kim H-J. Prion-like Mechanism in Amyotrophic Lateral Sclerosis: are Protein Aggregates the Key? Exp. Neurobiol. 2015;24:1–7.

49. Kleopa KA, Sherman M, Neal B, Romano GJ, Heiman-Patterson T. Bipap improves survival and

rate of pulmonary function decline in patients with ALS. J. Neurol. Sci. 1999;164:82–8.

50. Lechtzin N, Wiener CM, Clawson L, Chaudhry V. Hospitalization in amyotrophic Causes , costs , and outcomes. Neurology. 2001;753–7.

51. Lee JR, Annegers JF, Appel SH. Prognosis of amyotrophic lateral sclerosis and the effect of referral selection. J. Neurol. Sci. BMJ Publishing Group Ltd; 1995;132:207–15.

52. Goldstein LE, Leopold MC, Huang X, Atwood CS, Saunders AJ, Hartshorn M, et al. 3-Hydroxykynurenine and 3-hydroxyanthranilic acid generate hydrogen peroxide and promote alpha-crystallin cross-linking by metal ion reduction. Biochemistry. 2000;39:7266–75.

53. Shoki O, Nobuyoshi N, Hiroshi S, Hiroshi K, Okuda S, Nishiyama N, et al. 3-Hydroxykynurenine, an Endogenous Oxidative Stress Generator, Causes Neuronal Cell Death with Apoptotic Features and Region Selectivity. J. Neurochem. 1998;70:299–307.

54. Perez-De La Cruz V, Carrillo-Mora P, Santamaria A, Pérez-De La Cruz V, Carrillo-Mora P, Santamaría A. Quinolinic Acid, an endogenous molecule combining excitotoxicity, oxidative stress and other toxic mechanisms. Int. J. Tryptophan Res. 2012/03/13. 2012;5:1–8.

55. Jhamandas K, Boegman RJJ, Beninger RJJ, Bialik M. Quinolinate-induced cortical cholinergic damage: modulation by tryptophan metabolites. Brain Res. 1990/10/08. 1990;529:185–91.

56. Thomas SR, Witting PK, Roland S, Stocker R. 3-Hydroxyanthranilic Acid Is an Efficient, Cell-derived Co-antioxidant for alpha -Tocopherol, Inhibiting Human Low Density Lipoprotein and Plasma Lipid Peroxidation. J. Biol. Chem. American Society for Biochemistry and Molecular Biology; 1996;271:32714–21.

57. Christen S, Peterhans E, Stocker R. Antioxidant activities of some tryptophan metabolites: Possible implication for inflammatory diseases. Proc. Natl. Acad. Sci. U. S. A. 1990;87:2506–10.

58. Krause D, Suh H-S, Tarassishin L, Cui QL, Durafourt BA, Choi N, et al. The Tryptophan Metabolite 3-Hydroxyanthranilic Acid Plays Anti-Inflammatory and Neuroprotective Roles During Inflammation: Role of Hemeoxygenase-1. Am. J. Pathol. 2011;179:1360–72.

59. Leipnitz G, Schumacher C, Dalcin KB, Scussiato K, Solano A, Funchal C, et al. In vitro evidence for an antioxidant role of 3-hydroxykynurenine and 3-hydroxyanthranilic acid in the brain. Neurochem. Int. 2007;50:83–94.

60. Guidetti P, Schwarcz R. 3-Hydroxykynurenine potentiates quinolinate but not NMDA toxicity in

the rat striatum. Eur. J. Neurosci. Blackwell Science Ltd; 1999;11:3857–63.

61. Pérez-González A, Raú Alvarez-Idaboy J, Galano A. Dual antioxidant/pro-oxidant behavior of the tryptophan metabolite 3-hydroxyanthranilic acid: a theoretical investigation of reaction mechanisms and kinetics. New J. Chem. 2017;

62. Owe-Young R, Webster NL, Mukhtar M, Pomerantz RJ, Smythe G, Walker D, et al. Kynurenine pathway metabolism in human blood-brain-barrier cells: implications for immune tolerance and neurotoxicity. J Neurochem. 2008/01/29. 2008;105:1346–57.

63. Iwahashi H, Ishii T, Sugata R, Kido R. Superoxide dismutase enhances the formation of hydroxyl radicals in the reaction of 3-hydroxyanthranilic acid with molecular oxygen. Biochem. J. 1988;251.

64. Baran H, Jellinger K, Deecke L. Kynurenine metabolism in Alzheimer's disease. J Neural Transm. 1999;106:165–81.

65. Braidy N, Grant R, Adams S, Brew BJ, Guillemin GJ. Mechanism for quinolinic acid cytotoxicity in human astrocytes and neurons. Neurotox Res. 2009/06/16. 2009;16:77–86.

66. Hayashi T, Mo J-H, Gong X, Rossetto C, Jang A, Beck L, et al. 3-Hydroxyanthranilic acid inhibits PDK1 activation and suppresses experimental asthma by inducing T cell apoptosis. Proc. Natl. Acad. Sci. U. S. A. National Academy of Sciences; 2007;104:18619–24.

67. Platten M, Ho PP, Youssef S, Fontoura P, Garren H, Hur EM, et al. Treatment of Autoimmune Neuroinflammation with a Synthetic Tryptophan Metabolite. Source Sci. New Ser. Syst. Brain Dev. 2005;310:850–5.

68. Morita T, Saito K, Takemura M, Maekawa N, Fujigaki S, Fujii H, et al. 3-Hydroxyanthranilic acid, an L-tryptophan metabolite, induces apoptosis in monocyte-derived cells stimulated by interferon-. Ann. Clin. Biochem. SAGE PublicationsSage UK: London, England; 2001;38:242–51.

69. Fallarino F, Grohmann U, Vacca C, Orabona C, Spreca A, Fioretti MC, et al. T cell apoptosis by kynurenines. Adv Exp Med Biol. 2003;527:183–90.

70. Lovelace MD, Varney B, Sundaram G, Lennon MJ, Lim CK, Jacobs K, et al. Recent evidence for an expanded role of the Kynurenine pathway of tryptophan metabolism in neurological diseases. Neuropharmacology. 2016;

71. Gál EM, Sherman AD. SYNTHESIS AND METABOLISM OF L-KYNURENINE IN RAT BRAIN. J. Neurochem. 1978;30:607–13.

72. Bohár Z, Toldi J, Fülöp F, Vécsei L. Changing the Face of Kynurenines and Neurotoxicity: Therapeutic Considerations. Int. J. Mol. Sci. Multidisciplinary Digital Publishing Institute; 2015;16:9772–93.

73. Neumann M, Sampathu DM, Kwong LK, Truax AC, Micsenyi MC, Chou TT, et al. Ubiquitinated TDP-43 in frontotemporal lobar degeneration and amyotrophic lateral sclerosis. Science. American Association for the Advancement of Science; 2006;314:130–3.

74. Kwiatkowski TJ, Bosco DA, Leclerc AL, Tamrazian E, Vanderburg CR, Russ C, et al. Mutations in the FUS/TLS gene on chromosome 16 cause familial amyotrophic lateral sclerosis. Sci. (New York, N Y). 2009/03/03. American Association for the Advancement of Science; 2009;323:1205–8.

75. Foster AC, Vezzani I, French ED. KYNURENIC ACID BLOCKS NEUROTOXICITY AND SEIZURES INDUC-ED IN RATS BY THE RELATED BRAIN METABOLITE QUINOLINIC ACID. Neurosci. Lett. 1984;48:273–8.

76. García-Lara L, Pérez-Severiano F, González-Esquivel D, Elizondo G, Segovia J. Absence of aryl hydrocarbon receptors increases endogenous kynurenic acid levels and protects mouse brain against excitotoxic insult and oxidative stress. J. Neurosci. Res. 2015;93:1423–33.

77. Guillemin GJ, Kerr SJ, Smythe GA, Smith DG, Kapoor V, Armati PJ, et al. Kynurenine pathway metabolism in human astrocytes: a paradox for neuronal protection. J Neurochem. 2001/08/25. 2001;78:842–53.

78. Schwarcz R, Guidetti P, Sathyasaikumar K V, Muchowski PJ. Of mice, rats and men: Revisiting the quinolinic acid hypothesis of Huntington's disease. Prog. Neurobiol. 2010;90:230–45.

79. Chen Y, Brew BJ, Guillemin GJ. Characterization of the kynurenine pathway in NSC-34 cell line: Implications for amyotrophic lateral sclerosis. J. Neurochem. 2010/12/25. 2011;118:816–25.

80. Hartai Z, Juhász A, Rimanóczy Á, Janáky T, Donkó T, Dux L, et al. Decreased serum and red blood cell kynurenic acid levels in Alzheimer's disease. Neurochem. Int. 2007.

81. Rejdak K, Bartosik-Psujek H, Dobosz B, Kocki T, Grieb P, Giovannoni G, et al. Decreased level of kynurenic acid in cerebrospinal fluid of relapsing-onset multiple sclerosis patients. Neurosci. Lett. 2002.

82. Heyes MP, Brew BJ, Saito K, Quearry BJ, Price RW, Lee K, et al. Inter-relationships between quinolinic acid, neuroactive kynurenines, neopterin and β2-microglobulin in cerebrospinal fluid and serum of HIV-1-infected patients. J. Neuroimmunol. 1992;40:71–80.

83. Ogawa T, Matson WR, Beal MF, Myers RH, Bird ED, Milbury P, et al. Kynurenine pathway abnormalities in Parkinson's disease. Neurology. Lippincott Williams & Wilkins; 1992;42:1702–6.

84. Jauch D, Urbańska EM, Guidetti P, Bird ED, Vonsattel J-PG, Whetsell WO, et al. Dysfunction of brain kynurenic acid metabolism in Huntington's disease: focus on kynurenine aminotransferases. J. Neurol. Sci. 1995;130:39–47.

85. Beal MF, Matson WR, Swartz KJ, Gamache PH, Bird ED. Kynurenine Pathway Measurements in Huntington's Disease Striatum: Evidence for Reduced Formation of Kynurenic Acid. J. Neurochem. Blackwell Publishing Ltd; 1990;55:1327–39.

86. Mackay GM, Forrest CM, Stoy N, Christofides J, Egerton M, Stone TW, et al. Tryptophan metabolism and oxidative stress in patients with chronic brain injury. Eur. J. Neurol. 2006;13:30–42.

87. Murakami K, Haneda M, Yoshino M. Prooxidant action of xanthurenic acid and quinoline compounds: role of transition metals in the generation of reactive oxygen species and enhanced formation of 8-hydroxy-2¢-deoxyguanosine in DNA. BioMetals. 2006;19:429–35.

88. Robinson MB, Schulte MK, Freund RK, Johnson RL, Koerner JF. Structure-Function Relationships for Kynurenic Acid Analogues at Excitatory Pathways in the Rat Hippocampal Slice. Brain Res. 1985;361:19–24.

89. Okamoto H, Miyamoto S, Mabuchi H, Takeda R. INHIBITORY EFFECT OF QUINALDIC ACID ON GLUCOSE-INDUCED INSULIN RELEASE FROM ISOLATED LANGERHANS ISLETS OF THE RAT*. Biochem. Biophys. Res. Commun. 1974;59.

90. Lee C-H, Lee H-S. Growth Inhibiting Activity of Quinaldic Acid Isolated from Ephedra pachyclada against Intestinal Bacteria. J. Korean Soc. Appl. Biol. Chem. Springer-Verlag; 2009;52:331–5.

91. Schneidert DR, Parker CD. Effect of Pyridines on Phenotypic Properties of Bordetella pertussis. Infect. Immun. 1982;38:548–53.

92. Duleu S, Mangas A, Poulletier de Gannes F, Tranchant M-C, Geffard M. Circulating antibodies to conjugated tryptophan derivatives of the IDO pathway in amyotrophic lateral sclerosis, Alzheimer's, Parkinson's and multiple sclerosis patients. Immuno-analyse Biol. Spécialisée. 2008.

93. Takahashi H, Kaihara M, Pricet JM. THE CONVERSION OF KYNURENIC ACID TO QUINALDIC ACID BY HUMANS AND RATS. J. Biol. Chem. 1956;223:705–8.

94. Werner ER, Werner-Felmayerg G, Fuchs D, Hause A, Reibneggers G, Yim JJ, et al.

Tetrahydrobiopterin Biosynthetic Activities in Human Macrophages, Fibroblasts, THP-1, and T 24 Cells. J. Blo~o~lca~ Chem. 1990;265:3189–92.

95. Ghisoni K, Martins R de P, Barbeito L, Latini A. Neopterin as a potential cytoprotective brain molecule. J. Psychiatr. Res. 2015;71:134–9.

96. Hagberg L, Dotevall L, Norkrans G, Larsson M, Wachter H, Fuchs D. Cerebrospinal Fluid Neopterin Concentrations in Central Nervous System Infection. Source J. Infect. Dis. 1993;168:1285–8.

97. Kuehne LK, Reiber H, Bechter K, Hagberg L, Fuchs D. Cerebrospinal fluid neopterin is brain-derived and not associated with blood-CSF barrier dysfunction in non-inflammatory affective and schizophrenic spectrum disorders. J. Psychiatr. Res. 2013;47:1417–22.

98. Suh J, Sinclair E, Peterson J, Lee E, Kyriakides TC, Li F-Y, et al. Progressive increase in central nervous system immune activation in untreated primary HIV-1 infection. J. Neuroinflammation. BioMed Central; 2014;11:199.

99. Widner B, Leblhuber F, Fuchs D. Increased neopterin production and tryptophan degradation in advanced Parkinson's disease. J Neural Transm. Springer-Verlag; 2002;109:181–9.

100. McCombe PA, Henderson RD. The Role of immune and inflammatory mechanisms in ALS. Curr. Mol. Med. 2011;11:246–54.

101. Lima IVDA, Bastos LFS, Limborço-Filho M, Fiebich BL, De Oliveira ACP. Role of prostaglandins in neuroinflammatory and neurodegenerative diseases. Mediators Inflamm. 2012;2012.

102. Ravits J, Appel S, Baloh RH, Barohn R, Brooks BR, Elman L, et al. Deciphering amyotrophic lateral sclerosis: what phenotype, neuropathology and genetics are telling us about pathogenesis. Amyotroph. Lateral Scler. Frontotemporal Degener. NIH Public Access; 2013;14 Suppl 1:5–18.

103. Yoshida Y, Une F, Utatsu Y, Nomoto M, Furukawa Y, Maruyama Y, et al. Adenosine and Neopterin Levels in Cerebrospinal Fluid of Patients with Neurological Disorders. Intern. Med. 1989;38:133–9.

104. Azumagawa K, Suzuki S, Tanabe T, Wakamiya E, Kawamura N, Tamai H. Neopterin, biopterin, and nitric oxide concentrations in the cerebrospinal fluid of children with central nervous system infections. Brain Dev. 2003;25:200–2.

105. Parker DC, Mielke MM, Yu Q, Rosenberg PB, Jain A, Lyketsos CG, et al. Plasma neopterin level as a marker of peripheral immune activation in amnestic mild cognitive impairment and Alzheimer's

disease. Int. J. Geriatr. Psychiatry. 2013;28:149–54.

106. Stoy N, Mackay GM, Forrest CM, Christofides J, Egerton M, Stone TW, et al. Tryptophan metabolism and oxidative stress in patients with Huntington's disease. J. Neurochem. Blackwell Science Ltd; 2005;93:611–23.

107. Takikawa O, Yoshida R, Kido R, Hayaishi O. Tryptophan degradation in mice initiated by indoleamine 2,3-dioxygenase. J Biol Chem. 1986/03/15. 1986;261:3648–53.

108. Dai W, Gupta SL. Regulation of indoleamine 2,3-dioxygenase gene expression in human fibroblasts by interferon-gamma. Upstream control region discriminates between interferon-gamma and interferon-alpha. J Biol Chem. 1990/11/15. 1990;265:19871–7.

109. Lu C-H, Macdonald-Wallis C, Gray E, Pearce N, Petzold A, Norgren N, et al. Neurofilament light chain: A prognostic biomarker in amyotrophic lateral sclerosis. Neurology. 2015;84:2247–57.

110. Talman P, Duong T, Vucic S, Mathers S, Venkatesh S, Henderson R, et al. Identification and outcomes of clinical phenotypes in amyotrophic lateral sclerosis/motor neuron disease: Australian National Motor Neuron Disease observational cohort. BMJ Open. British Medical Journal Publishing Group; 2016;6:e012054.

111. Lawton K a, Cudkowicz ME, Brown M V, Alexander D, Caffrey R, Wulff JE, et al. Biochemical alterations associated with ALS. Amyotroph. Lateral Scler. 2012;13:110–8.

112. Shepheard SR, Wuu J, Cardoso M, Wiklendt L, Dinning PG, Chataway T, et al. Urinary p75(ECD): A prognostic, disease progression, and pharmacodynamic biomarker in ALS. Neurology. Lippincott Williams & Wilkins; 2017;10.1212/WNL.0000000000003741.

113. Ganesalingam J, An J, Shaw CE, Shaw G, Lacomis D, Bowser R. Combination of neurofilament heavy chain and complement C3 as CSF biomarkers for ALS. J. Neurochem. 2011;117:528–37.

114. Tompsett SL. The determination in urine of some metabolites of tryptophan-kynurenine, anthranilic acid and 3-hydroxyanthranilic acid-and reference to the presence of o-aminophenol in urine. Clin. Chim. Acta. 1959;4:411–9.

115. McMillan M. THE IDENTIFICATION OF A FLUORESCENT REDUCING SUBSTANCE IN THE URINE OF PATIENTS WITH RHEUMATOID ARTHRITIS: THE EXCRETION OF 3-HYDROXYANTHRANILIC ACID IN THIS AND OTHER CONDITIONS. J. Clin. Pathol. BMJ Publishing Group; 1960;13:140–8.

116. Price SA, Rose DP, Toseland PA. Effects of dietary vitamin B 6 deficiency and oral contraceptives

on the spontaneous urinary excretion of 3-hydroxyanthranilic acid. Am. J. Clin. Nutr. American Society for Nutrition; 1972;25:494–8.

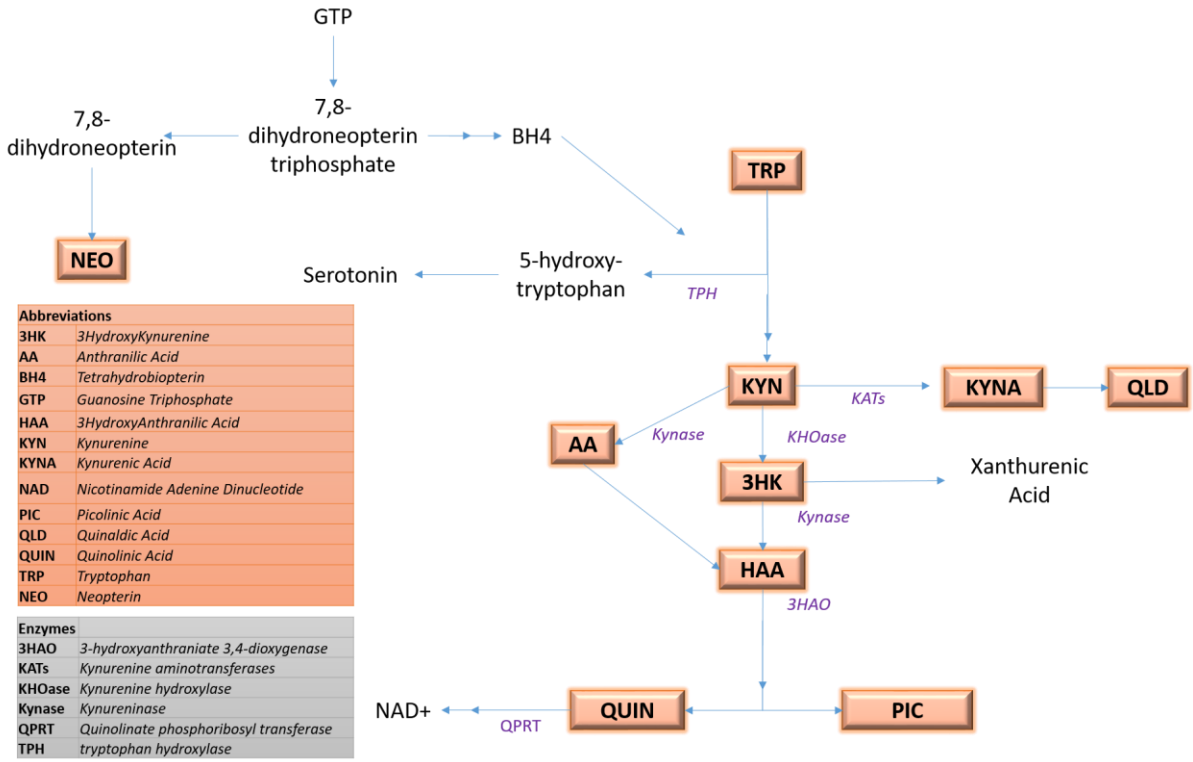
117. Dounay AB, Tuttle JB, Verhoest PR. Challenges and Opportunities in the Discovery of New Therapeutics Targeting the Kynurenine Pathway. J. Med. Chem. American Chemical Society; 2015;58:8762–82.

118. Wu H-Q, Lee S-C, Schwarcz R. Systemic administration of 4-chlorokynurenine prevents quinolinate neurotoxicity in the rat hippocampus. Eur. J. Pharmacol. 2000;390:267–74.

119. Erhardt S, Olsson SK, Engberg G. Pharmacological manipulation of kynurenic acid: Potential in the treatment of psychiatric disorders. CNS Drugs. 2009;23:91–101.

Figures and Tables

Figure 1 The Kynurenine Pathway Tryptophan metabolism and catabolites



Abbreviations	
3HK	*3HydroxyKynurenine*
AA	*Anthranilic Acid*
BH4	*Tetrahydrobiopterin*
GTP	*Guanosine Triphosphate*
HAA	*3HydroxyAnthranilic Acid*
KYN	*Kynurenine*
KYNA	*Kynurenic Acid*
NAD	*Nicotinamide Adenine Dinucleotide*
PIC	*Picolinic Acid*
QLD	*Quinaldic Acid*
QUIN	*Quinolinic Acid*
TRP	*Tryptophan*
NEO	*Neopterin*

Enzymes	
3HAO	*3-hydroxyanthraniate 3,4-dioxygenase*
KATs	*Kynurenine aminotransferases*
KHOase	*Kynurenine hydroxylase*
Kynase	*Kynureninase*
QPRT	*Quinolinate phosphoribosyl transferase*
TPH	*tryptophan hydroxylase*

Table 1 Comparison of KP metabolites and clinical outcomes at baseline according to the Olesoxyme and placebo groups

KP metabolites	Olesoxyme Group n=33	Placebo Group n=33	Standardized difference % (+)
3HK nM	67.7 [41.9 to 109.7]	58.3 [40.5 to 100.0]	19
KYN µM	1.2±0.3	1.1±0.3	16
HAA nM	14.0±7.6	12.3±6.5	23
AA nM	102.1 [77.5 to 125.3]	87.0 [67.9 to 126.4]	10
TRP µM	53.6±9.8	53.9±14.8	2
Neo nM	5.4 [2.8 to 12.0]	7.0 [4.2 to 13.0]	24
KT ratio	0.02 [0.02 to 0.03]	0.02 [0.02 to 0.02]	29
PA nM	0.10 [0.09 to 0.12]	0.10 [0.09 to 0.12]	**41**
QA nM	0.09 [0.08 to 0.10]	0.09 [0.08 to 0.10]	29
QLD nM	0.004 [0.003 to 0.007]	0.005 [0.003 to 0.006]	7
KYNA nM	72.8 [51.8 to 112.6]	64.5 [40.5 to 97.2]	**31**
HAA/AA ratio	0.13 [0.10 to 0.19]	0.11 [0.05 to 0.17]	2
KYNA/QUIN ratio	574 [420 to 996]	578 [287 to 706]	**50**
QLD/QA ratio	0.04 [0.03 to 0.07]	0.04 [0.03 to 0.06]	2
KYNA/QLD ratio	14208 [7745 to 24914]	15302 [7957 to 24772]	**37**
ALSFRS	37.9±4.9	36.8±4.4	24

Values are expressed as means ± standard deviation in case of normal distribution (**KYN**, **HAA**, **TRP**, and **ALSFRS**) or as median [interquartile range] otherwise. Standardized difference of <30% are regarded as not different between placebo and treatment groups. **+:** Calculated after rank transformation for non-normal distribution. Abbreviations: **3HK** = 3-hydroxykynurenine; **KYN** = kynurenine; **HAA** = 3-hydroxyanthranilic acid; **AA** = anthranilic acid; **TRP** = tryptophan; **NEO** = neopterin; **KT ratio** = Kynurenine/Tryptophan ratio; **PA** = picolinic acid; **QA** = quinolinic acid; **QLD** = quinaldic acid; **KYNA** = kynurenic acid; **ALSFRS** = ALS Functional Rating Scale; **MMT** = Manual Muscle Testing;

Table 2 Evolution of KP profile in ALS patients over time (n=66)

KP metabolites over time	Changes over time β_1± standard error (#)	p-value (•)
3HK (+)	0.002 ± 0.01	0.61
KYN (+)	-0.002 ±0.008	0.57
HAA	-0.46 ±0.18	**0.033**
AA (+)	-0.0006 ±0.0002	0.91
TRP	-0.23 ±0.34	0.36
Neo (+)	0.02 ±0.02	0.37
KT ratio (+)	-0.003 ±0.009	0.71
PA (+)	-0.01 ±0.009	0.26
QA (+)	-0.01 ±0.01	0.11
QLD (+)	-0.04 ±0.02	0.054
KYNA (+)	-0.02 ±0.01	0.09
HAA/AA (+)	-0.03±0.02	0.22
KYNA/QUIN (+)	0.03±0.02	0.08
QLD/QA (+)	0.02±0.02	0.35
KYNA/QLD (+)	-0.01±0.02	0.54
ALSFRS	-1.20 ±0.23	**<.0001**
MMT	-3.74 ± 0.96	**0.0007**
SVC	0.04±0.03	0.11

#: Coefficient of Mixed Model with the KP metabolite as dependent variable. $KP_{ij} = \beta_0 + \beta_1 t_{ij} + \beta_2 TRTi + \beta_3 TRTi * t_{ij} + \beta_4 DiseaseDuration_i + \beta_5 DiseaseDuration_i * t_{ij} + \gamma_{oi} + \gamma_{1ij} + \varepsilon_{ij}$.
The model is adjusted on treatment and disease duration. **(+):** The parameter has been log transformed before using in linear mixed model. Abbreviation: **TRT** = treatment group (1=Olesoxyme, 0=Placebo); $\mathbf{t_{ij}}$= for any subject *i*, time in months elapsed from baseline to the current measure *j* of parameters. **3HK** = 3-hydroxykynurenine; **KYN** = kynurenine; **HAA** = 3-hydroxyanthranilic acid; **AA** = anthranilic acid; **TRP** = tryptophan; **NEO** = neopterin; **KT ratio** = Kynurenine/Tryptophan ratio; **PA** = picolinic acid; **QA** = quinolinic acid; **QLD** = quinaldic acid; **KYNA** = kynurenic acid; **ALSFRS** = ALS Functional Rating Scale; **MMT** = Manual Muscle Testing; **SVC** = slow vital capacity. •: p-values tagged in bold are significant at the 0.05 level.

Table 3 Effect of baseline values of KP metabolites on ALSFRS progression (n=66)

KP metabolites at baseline	Effects on ALSFRS changes over time β_7 ± standard error (#)	p-value
3HK	0.0006 ± 0.001	0.66
KYN	-0.02±0.21	0.94
HAA	0.008±0.01	0.42
AA	-0.002±0.002	0.33
TRP	0.0007±0.006	0.90
Neo	0.01±0.01	0.40
KT ratio (¤)	-0.02±0.12	0.84
PA (¤)	-0.03±0.03	0.31
QA (¤)	0.004±0.02	0.81
QLD (¤)	-0.06 ±0.16	0.71
KYNA	-0.0001±0.0008	0.87
HAA/AA	1.61±0.73	**0.031**
KYNA/QUIN	-0.02±0.02	0.27
QLD/QA	-3.11±1.78	0.08
KYNA/QLD	0.0009±0.003	0.73

#: Coefficient of Mixed Model with the dependent variable ALSFRS. $ALSFRS_{ij} = \beta_0 + \beta_1 t_{ij} + \beta_2 TRT_i + \beta_3 TRT_i * t_{ij} + \beta_4 DiseaseDuration_i + \beta_5 DiseaseDuration_i * t_{ij} + \beta_6 KP_{0i} + \beta_7 KP_{0i} * t_{ij} + \gamma_{oi} + \gamma_{1ij} + \varepsilon_{ij}$. The model is adjusted on treatment and disease duration (in months). Abbreviation: **ALSFRS** = Amyotrophic Lateral Sclerosis Functional Rating Scale; **TRT** = treatment group (1= Olesoxyme, 0=Placebo); $\mathbf{t_{ij}}$= for any subject *i*, time in months elapsed from baseline to the current measure *j* of parameters; **3HK** = 3-hydroxykynurenine; **KYN** = kynurenine; **HAA** = 3-hydroxyanthranilic acid; **AA** = anthranilic acid; **TRP** = tryptophan; **NEO** = neopterin; **KT ratio** = Kynurenine/Tryptophan ratio; **PA** = picolinic acid; **QA** = quinolinic acid; **QLD** = quinaldic acid; **KYNA** = kynurenic acid. **(¤)** in unit*100: The coefficient $\boldsymbol{\beta_7}$ corresponds to an increase in the ALSFRS value per increasing of the metabolite of one unit*100.

Table 4 Effect of KP metabolites at baseline on MMT progression (n=66)

KP Metabolites at baseline	Effects on MMT changes over time β_7± standard error (#)	p-value (•)
3HK	-0.003±0.006	0.61
KYN	0.37±0.90	0.68
HAA	-0.006±0.04	0.88
AA	-0.004±0.008	0.60
TRP	0.02±0.03	0.49
Neo	0.12±0.05	**0.023**
KT ratio (¤)	0.21 ± 0.49	0.67
PA (¤)	-0.04 ± 0.13	0.73
QA (¤)	0.06±0.07	0.47
QLD (¤)	-0.20±0.67	0.77
KYNA	0.0003±0.003	0.92
HAA/AA	1.83±3.07	0.56
KYNA/QUIN	-0.01±0.09	0.90
QLD/QA	-13.4±7.4	0.08
KYNA/QLD	0.003±0.003	0.28

#: Coefficient of Mixed Model with the dependent variable MMT. $MMT_{ij} = \beta_0 + \beta_1 t_{ij} + \beta_2 TRT_i + \beta_3 TRT_i * t_{ij} + \beta_4 DiseaseDuration_i + \beta_5 DiseaseDuration_i * t_{ij} + \beta_6 KP_{0i} + \beta_7 KP_{0i} * t_{ij} + \gamma_{oi} + \gamma_{1ij} + \varepsilon_{ij}$. The model is adjusted on treatment and disease duration (in months). Abbreviation: **MMT**= Manual Muscle Testing; **TRT** = treatment group (1= Olesoxyme, 0=Placebo); $\mathbf{t_{ij}}$= for any subject *i*, time in months elapsed from baseline to the current measure *j* of parameters; **3HK** = 3-hydroxykynurenine; **KYN** = kynurenine; **HAA** = 3-hydroxyanthranilic acid; **AA** = anthranilic acid; **TRP** = tryptophan; **NEO** = neopterin; **KT ratio** = Kynurenine/Tryptophan ratio; **PA** = picolinic acid; **QA** = quinolinic acid; **QLD** = quinaldic acid; **KYNA** = kynurenic acid •: p-values tagged in bold are significant at the 0.05 level. **(¤)** in unit*100: The coefficient $\boldsymbol{\beta_7}$ corresponds to an increase in the MMT value per increasing of the metabolite of one unit*100.

Table 5 Effect of KP metabolites at baseline on survival (n=54, number of deaths = 43)

KP Metabolites at baseline	Effects on survival HR[95%CI] (#)	p-value
3HK (+)	0.86 [0.52 ; 1.43]	0.57
KYN (+)	0.50 [0.15 ; 1.72]	0.27
HAA	0.98 [0.93 ; 1.023]	0.35
AA (+)	1.53 [0.65 ; 3.59]	0.33
TRP	0.98 [0.95 ; 1.00]	0.051
Neo (+)	0.94 [0.64 ; 1.37]	0.75
KT ratio (+)	1.13 [0.34 ; 3.74]	0.84
PA (+)	1.58 [0.42 ; 6.00]	0.50
QA (+)	0.57 [0.19 ; 1.70]	0.31
QLD (+)	0.93 [0.56 ; 1.53]	0.76
KYNA (+)	0.85 [0.56 ; 1.29]	0.44
HAA/AA (+)	0.48 [0.29 ; 0.81]	**0.006**
KYNA/QUIN (+)	0.99 [0.60 ; 1.63]	0.97
QLD/QA (+)	1.01 [0.60 ; 1.70]	0.97
KYNA/QLD (+)	0.99 [0.70 ; 1.43]	0.99

HR= hazard ratio; 95%CI: 95% confidence interval. #: The Cox proportional hazards model was adjusted for treatment effect and disease duration (in months). **(+):** the parameter has been log transformed before using in Cox proportional hazard model. Abbreviations: **3HK** = 3-hydroxykynurenine; **KYN** = kynurenine; **HAA** = 3-hydroxyanthranilic acid; **AA** = anthranilic acid; **TRP** = tryptophan; **NEO** = neopterin; **KT ratio** = Kynurenine/Tryptophan ratio; **PA** = picolinic acid; **QA** = quinolinic acid; **QLD** = quinaldic acid; KYNA = kynurenic acid

Table 6 Association between KP metabolites and ALSFRS at any time (n=66)

KP metabolites over time	Effects on ALSFRS at any time $\boldsymbol{\beta_6}$ ±standard error (#)	p-value (•)
3HK	0.01 ±0.008	0.22
KYN	2.29±1.01	**0.027**
HAA	0.01±0.05	0.84
AA	-0.01±0.008	0.18
TRP	0.07±0.03	**0.022**
Neo	-0.12±0.47	0.80
KT ratio (¤)	-0.21±0.24	0.39
PA (¤)	0.07±0.11	0.52
QA (¤)	-0.01±0.08	0.86
QLD (¤)	-0.85±0.61	0.17
KYNA	0.009±0.005	0.08
HAA/AA	3.96±3.22	0.22
KYNA/QUIN	0.07±0.08	0.38
QLD/QA	0.98±5.09	0.85
KYNA/QLD	0.0002±0.002	0.92

#: Coefficient of Mixed Model with the dependent variable ALSFRS. $ALSFRS_{ij} = \beta_0 + \beta_1 t_{ij} + \beta_2 TRT_i + \beta_3 TRT_i * t_{ij} + \beta_4 DiseaseDuration_i + \beta_5 DiseaseDuration_i * t_{ij} + \beta_6 KP_{ij} + \gamma_{oi} + \gamma_{1ij} + \varepsilon_{ij}$. The model is adjusted on treatment and disease duration (in months). Abbreviation: **ALSFRS** = Amyotrophic Lateral Sclerosis Functional Rating Scale; **TRT** = treatment group (1= Olesoxyme, 0=Placebo); $\mathbf{t_{ij}}$= for any subject *i*, time in months elapsed from baseline to the current measure *j* of parameters; **3HK** = 3-hydroxykynurenine; **KYN** = kynurenine; **HAA** = 3-hydroxyanthranilic acid; **AA** = anthranilic acid; **TRP** = tryptophan; **NEO** = neopterin; **KT ratio** = Kynurenine/Tryptophan ratio; **PA** = picolinic acid; **QA** = quinolinic acid; **QLD** = quinaldic acid; **KYNA** = kynurenic acid. •: P-values tagged in bold are significant at the 0.05 level. (¤) in unit*100: The coefficient $\boldsymbol{\beta_6}$ corresponds to an increase in the ALSFRS value per increasing of the metabolite of one unit*100.

Table 7 Association between KP metabolites and MMT at any time (n=66)

KP metabolites over time	Effects on MMT over time $\boldsymbol{\beta_6}$ ± standard error (#)	p-value (•)
3HK	0.03±0.04	0.50
KYN	5.03±4.62	0.29
HAA	0.31±0.21	0.15
AA	-0.05±0.04	0.17
TRP	0.007±0.12	0.96
Neo	-0.12±0.25	0.63
KT ratio (¤)	0.79±0.91	0.40
PA (¤)	0.46±0.40	0.26
QA (¤)	0.26±0.39	0.52
QLD (¤)	-6.57±2.20	**0.006**
KYNA	0.07±0.02	**0.009**
HAA/AA	-4.90±12.7	0.70
KYNA/QUIN	0.05±0.31	0.86
QLD/QA	-21.3±14.9	0.17
KYNA/QLD	-0.007±0.007	0.32

#: Coefficient of Mixed Model with the dependent variable MMT. $MMT_{ij} = \beta_0 + \beta_1 t_{ij} + \beta_2 TRT_i + \beta_3 TRT_i * t_{ij} + \beta_4 DiseaseDuration_i + \beta_5 DiseaseDuration_i * t_{ij} + \beta_6 KP_{ij} + \gamma_{oi} + \gamma_{1ij} + \varepsilon_{ij}$. The model is adjusted on treatment and disease duration (in months). Abbreviation: **MMT**= Manual Muscle Testing; **TRT** = treatment group (1= Olesoxyme, 0=Placebo); $\mathbf{t_{ij}}$= for any subject *i*, time in months elapsed from baseline to the current measure *j* of parameters; **3HK** = 3-hydroxykynurenine; **KYN** = kynurenine; **HAA** = 3-hydroxyanthranilic acid; **AA** = anthranilic acid; **TRP** = tryptophan; **NEO** = neopterin; **KT ratio** = Kynurenine/Tryptophan ratio; **PA** = picolinic acid; **QA** = quinolinic acid; **QLD** = quinaldic acid; **KYNA** = kynurenic acid. •: P-values tagged in bold are significant at the 0.05 level. **(¤)** in unit*100: The coefficient $\boldsymbol{\beta_6}$ corresponds to an increase in the MMT value per increasing of the metabolite of one unit*100.

Table 8 Significant findings summary

KP metabolites	Biomarker or Prognostic marker	Clinical Scale of impact	Effect in coefficient of Mixed Model and dependant variable	p-value (•)	Reference Table
3HK	Biomarker	Changes over time	-0.46 ±0.18	0.033	2
HAA/AA	Prognostic	ALSFRS	1.61±0.73	0.031	3
Neo	Prognostic	MMT	0.12±0.05	0.023	4
HAA/AA	Prognostic	Survival	0.48 [0.29 ; 0.81] HR[95%CI]	0.006	5
KYN	Biomarker	ALSFRS	2.29±1.01	0.027	6
TRP	Biomarker	ALSFRS	0.07±0.03	0.022	6
QLD	Biomarker	MMT	-6.57±2.20	0.006	7
KYNA	Biomarker	MMT	0.07±0.02	0.009	7

Summary of key significant findings from study highlighting potential KP metabolites as biomarkers or prognostic markers for ALS, where biomarker indicates changes over time following progression of disease; and prognostic refers to the baseline measure of KP Metabolites having an indicative alteration in clinical scale from predicted effects on clinical measurements (ALSFRS or MMT). Abbreviation: **ALSFRS** = Amyotrophic Lateral Sclerosis Functional Rating Scale; **MMT**= Manual Muscle Testing; **3HK** = 3-hydroxykynurenine; **HAA** = 3-hydroxyanthranilic acid; **AA** = anthranilic acid; **NEO** = neopterin; **KYN** = kynurenine; **TRP** = tryptophan; **QLD** = quinaldic acid; **KYNA** = kynurenic acid; **HR**= hazard ratio; **95%CI**: 95% confidence interval.

Chapter 2

Case-study: The Neurodegenerative Diseases Biobank at Macquarie University, the establishment of a resource for MND research

This section has been written in the format of a traditional thesis. It will be integrated into a larger case study, after inclusion of a comprehensive description of clinical processes involved in establishing a Biobank.

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The author thanks Dr Sarah Furlong and Ms Lorel Adams for their assistance in providing updates on Biobank processes. Figures 3 and 4, updated Biobank statistics, and SOPs were kindly provided by Dr Furlong. The author recognizes the patience and generosity of participants of the Neurodegenerative Diseases Biobank.

Abbreviations

BMAA	β-methylamino-L-alanine
BSC	Biological Safety Cabinet
CoE	Council of Europe
CSF	Cerebrospinal Fluid
DNA	Deoxyribonucleic Acid
ELN	Electronic Lab Book
HREC	Human Research Ethics Committee (Macquarie University)
ID	Identification
iPSC	induced Pluripotent Stem Cells
ISBER	International Society for Biological and Environmental Repositories
LIMS	Laboratory Information Management System
MND	Motor Neuron Disease
MTA	Material Transfer Agreements
NDB	Neurodegenerative Disease Biobank
NHMRC	National Health and Medical Research Council
OECD	Organization for Economic Co-operation and Development
PICF	Patient Information and Consent Form
RNA	Ribonucleic Acid
RPMI	Roswell Park Memorial Institute
SOP	Standard Operating Procedures
SDNS	Scientific Data Management Systems
WHO	World Health Organization

Contents

1. Need for Biobanking

A biobank is a repository facility for the organised, collection and long-term storage of biological samples, corresponding information, and the dissemination of samples and information for research and clinical purposes. The careful organisation of the collection process of high quality specimens allows for the study of disease pathogenesis, biomarker discovery, and to correlate genetic and environmental information in retrospective studies. Furthermore, the collection of baseline and longitudinal follow-up samples allows not only for cross-sectional studies, but also the observation of time-based changes in diseases. [1–4]

The Neurodegenerative Diseases Biobank (NDB) was established to collect biological samples from patients with neurodegenerative diseases, with a focus on Motor Neuron Disease (MND). It is a collaborative effort between the Macquarie University Hospital Neurology Unit lead by Professor Dominic Rowe, and Professor Gilles Guillemin, one of the scientists at the MND Research Centre.

To date, there are more than 522 patients enrolled, and 1,200 collections annually, that include samples of serum, plasma, DNA, RNA, urine, hair, and fibroblast samples banked at this medium-sized biobank [3].

2. Considerations for Biobanking

There are many factors to consider in establishing a Biobank, such as the partnership between the clinic and scientists, ethics, governance, and financing, which will be discussed further, and in context to the establishment and evolution of the Neurodegenerative Diseases Biobank (NDB) set up at Macquarie University.

2.1 Personnel

Personnel involved in biobanking are broadly divided into two categories, clinic based, and laboratory based staff. Clinic based staff require specific expertise such as being proficient in phlebotomy, performing skin biopsies, removal of whole organs such as the brain and spinal cord, as well as attention to detail in matching patient IDs with collection IDs. Clinic staff may also double-up as recruitment officers, seeking consent and enrolling patients to the Biobank. Laboratory based staff maintain processing of samples according to specific requirements compatible with specific research uses, technical scientific skills, storage, and data management.

Clinical collection of biofluids at the NDB was initially performed by Neurology Registrar Dr Ronald Siu and Professor Gilles Guillemin, assisted by myself. As these samples were critical for my PhD project on the ALS biomarker, I have also performed all sample processing, aliquoting, storage, data management, development of procedures, biosafety, and administration for almost 18 months. Subsequently, personnel were engaged to spearhead various aspects of laboratory processing. Ms Ariel Seaton cultured fibroblasts from skin biopsies under the guidance of Dr Shu Yang, and Dr Kelly Williams led sample processing of blood for DNA and RNA with Ms Emily McCann. Serum, plasma, and urine samples were managed by myself with assistance from Ms Serene Gwee.

Following the expansion of the NDB, Ms Lorel Adams, a clinical nurse, was recruited as the Biobank Coordinator in 2015. Ms Adams manages most of the clinical aspects such as patient recruitment, phlebotomy, sample collection and clinical scoring. In 2016, my biobanking roles were handed over to Dr Sarah Furlong and Ms Elisa James, after they joined as the MND Biobank Manager and Biobank Technician respectively. Dr Furlong maintains the database, processes, and proceedings of the biobank, as well as policies and governance of the biobank. Ms James has a crucial dual role, being well versed in

phlebotomy, which is vital for the NDB; and is highly skilled in laboratory techniques, managing the laboratory aspects of the biobank.

2.2 Ethics

Human research ethics approval is a core component of biobanking, whereby a regulator external to the biobank committee, provides a broader view on biobank processes. Ethics approval ensures that participants are enrolled in a programme that has been well thought out with scientific merit and community consultation, whilst still protecting the moral integrity of donors [2]. It ensures that participants are not exposed to harm due to the sensitive nature of information collected as part of the biobank [5, 6]. The ethics committee also ensures that the proceedings of the biobank are in compliance with the appropriate laws and legislations relevant to local jurisdiction, as well as the interests of the broader community [4].

The NDB has received ethical and scientific approval from the Macquarie University Human Research Ethics Committee (HREC) Medical Sciences (Appendix 1). The committee assessed a range of elements of the biobank including the list of investigators, funding, methods and nature of research, recruitment and consent, risks and benefits, publication of results, and conflicts of interest.

2.3 Recruitment

Participant consent and recruitment generally consists of referral for contact, preliminary interview, and informed consent discussion. It is key that recruitment is conducted in a manner that is non-coercive, such that it is the free choice of an individual [7]. Participants must be informed of the information collected, assurance to safeguard their privacy, and their right to withdraw consent if they wish [6, 8, 9].

Scientifically, it is important to 1) well define the population being studied, and 2) to maintain a cohort that reflects the demography and diversity of the population, while maintaining significance for the research question [4, 10, 11].

At the NDB, clinic staff external to the consulting clinician facilitated the recruitment process to prevent a conflict of interest. As outlined in the ethics application, patients are informed

of their right to decline to participate and that their participation or non-participation does not affect their treatment. Participants are also given an information and consent form (Appendix 2, PICF), which include details of the study such as the samples taken, research being carried out, risks and privacy statements, and instructions on how to withdraw consent.

There is a broad recruitment criterion for participation in the NDB. The population under study comprises patients with neurodegenerative diseases, and in particular, ALS. Control participants include volunteers and family members, who form the demographics of the general population, and at-risk family members (such as in familial forms of disease), who may allow a larger catchment of rare genetic variants of the disease. There is a high participation rate at the NDB, with most patients electing to participate, attributed both to the reputation of the clinic and research centre, and the good rapport between patients and clinic staff members (personal communication, Dr Sarah Furlong).

2.4 Biological Samples

Biological samples that can be collected include blood, urine, cerebrospinal fluid (CSF), saliva, and tissue biopsies including skin, muscle, and hair. Participants may also opt to donate organs *post mortem*. Each type of biological sample has its own strengths and weaknesses, and is well summarised in a table published by Otto et al (**Table 1**), including its relevance to neurology (by proximity to the Central Nervous System (CNS) pathology), and sampling practicality. In addition to these characteristics, the collection of *post mortem* tissue requires specific expertise. Organs or tissues must be preserved soon after death, and in a specific manner to allow for high quality preservation so a range of techniques can be later applied to the samples [12].This was not achievable at commencement of the biobank.

Table V. Strengths and weaknesses among biomarker sources.

Characteristic	Blood[1]	CSF	Urine	Saliva	Skin	Muscle
Proximity to CNS pathology	+ +	+ + +	+	+	+	+
Less molecular complexity	+	+	+ +	+ + +	+ +	+ +
Less invasive	+ +	+	+ + +	+ + +	+	+
Practicality of sampling	+ + +	+ +	+ + +	+ +	+	+
Ease of handling for storage	+ +	+[2]	+ +	+	+	+
Resistance to exogenous drug contamination	+	+ + +	+	+ +	+ +	+ +
Candidate molecules to date	+ +	+ + +	+	+	+	+
Potential for DNA/RNA analysis	+ + +	+	+	+ +	+ + +	+ + +

+++: highly significant; ++: significant; +: low significance.
[1]Plasma versus serum needs to be specified; serum may have advantages for the stability of some proteins, e.g. immunoglobulins. EDTA sample will be needed for DNA or RNA studies.
[2]Bloody tap contamination potential and the particular need for rapid centrifugation.

Table 1. Strengths and Weaknesses among biomarker sources. Reproduced from [12].

In the NDB, the core samples initially collected were urine, skin biopsies for fibroblasts, and blood for serum, deoxyribonucleic acid (DNA), and ribonucleic acid (RNA) (**Fig 1**). It was decided that CSF samples would not be collected as it is a relatively invasive procedure that may cause minor side effects [13]. These samples were collected as a reflection of the needs of the scientists on the biobank committee, for example metabolomics, genomics, and proteomics. Shortly after, the list of biological samples was expanded to include plasma, and hair samples. Hair is not a typical sample type collected by biobanks, but is used in analysing the presence of the neurotoxin β-methylamino-L-alanine (BMAA), a particular interest of some researchers in the team [14].

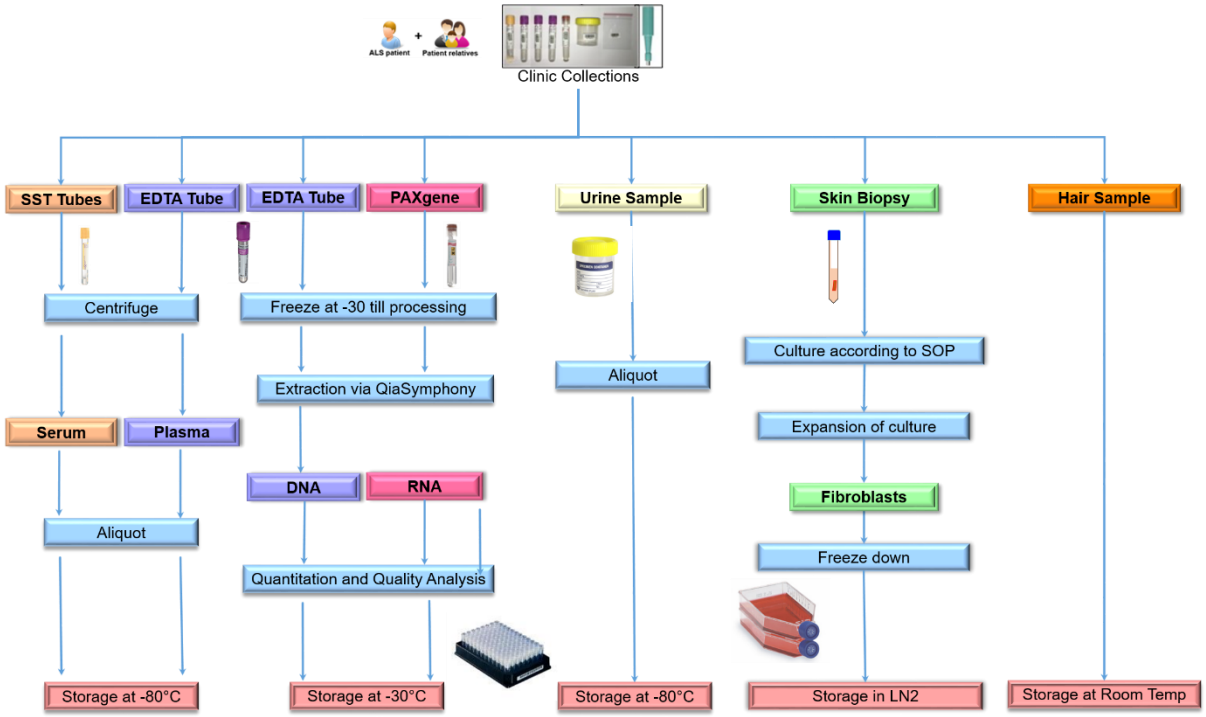

Fig 1. Laboratory Processes Flow Chart outlining the workflow of lab processes

Ideally, participants would be fasting prior to blood collections to minimize variability in blood analyte levels [12]. However, this was deemed impractical and too burdensome on participants as appointments are often scheduled in the afternoon, and neurodegenerative disease patients may face obstacles such as mobility issues, in getting to the clinic. However, it was noted if patients had anything to eat in the past 8 hours, or had taken any medications.

2.5 Banking Processes and SOPs

Processes and Standard Operating Procedures (SOPs) are of crucial importance in maintaining a well characterized Biobank, with optimal collection, processing, and storage procedures. SOPs ensure procedures are carried out methodically, and are recorded to ensure consistency and reduce variables [2, 12].

The procedures and protocols relating to biobanking processes were established where required, with the fundamental procedures of collection process, core patient information, labelling of tubes and samples, processing, storage, data management, and biosafety, which will be discussed individually.

Biosafety

As with all processes, it is important to have procedures in place to address biosafety issues. In a biobank, biosafety matters are present in predictable, but unlikely risks of exposure to infectious agents and chemicals [2, 5]. Specimens, biofluids, and biological samples are all treated as biohazards, and must be handled with care. Personnel safety is of upmost importance, and personnel are offered vaccinations by the Faculty including Tetanus/Diphtheria/Pertussis, Hepatitis B, Measles/Mumps/Rubella (MMR), and Varicella.

Establishing safety protocols was a crucial first step in setting up the biobank. Clinically, gloves were used during phlebotomy, and handling of all biological samples including blood collection tubes and jars. Samples were transported to the laboratory in double containment, even though the laboratory was only one level below the clinic. Venepuncture was performed according to the World Health Organization (WHO) Guidelines [15].

Risk Assessments were also done for use of chemicals, such as with Qiagen PAXgene RNA tubes, as well as SOPs for DNA and RNA extraction. Safe work procedures had been previously established by the Faculty of Medicine and Health Sciences (formerly known as the Australian School of Advanced Medicine) for handling of human samples and cultures (Appendix 3), and a decontamination protocol (Appendix 4) was available for biobank specific cleaning up processes.

Collection Pipeline Processes

The first SOPs created were basic, with paper-based collection forms recording core information such as the unique patient Biobank ID code, collection ID, date and time of sample collection, processing, and storage, and medication taken.

Each patient was assigned a unique biobank ID code, which allowed for identification of the patient, and connection to each of their follow up collections. The patients were also given a unique collection ID. Each sample collection vessel was identified only by the collection ID to maintain patient privacy, and was labelled using a bar code printer (Brady LABXPERT Labelling system).

To familiarise personnel with the procedures before commencing the collections, a trial run was conducted to ensure that these processes were suitable. The collection pipeline commenced with pre-collection administration such as printing of consent and collection forms, labelling of collection tubes, jars, and bags. Collection procedures were split into two segments of clinical and laboratory (**Fig 2**). Post collection processes included filing and entering of information into the relevant databases.

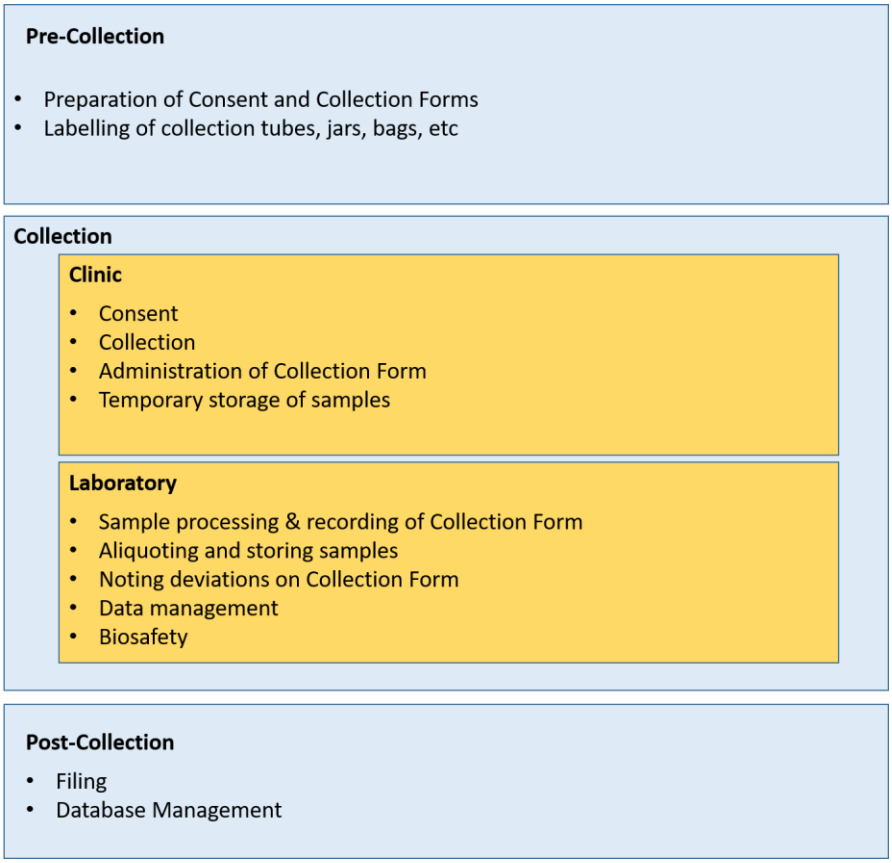

Fig 2. NDB Collection flow chart. The flow chart illustrates the general collection procedures of the biobank

Clinical Processes

A basic SOP was included in the collection form to ensure the correct order of tubes to use in order to prevent cross contamination of blood collection tubes, and adequate inversion of tubes. The collection order was established to be SST Gel Separator tubes, EDTA tubes, and lastly RNA tubes according to the BD Vacutainer Order of Draw for Multiple Tube Collections, and as required by the PAXgene blood RNA tube [16]. This was written into the collection form so that the information was readily available to clinic staff.

It was also important to maintain consistency in the storage of samples until they were ready for processing. Processing was not performed immediately as more collections may have beeen carried out during the day, and the laboratory was separate to the clinic. However, processing was carried out twice daily, once at mid-day, and subsequently at the end of the collection day. Therefore, blood tubes, urine samples, and skin biopsies were required to remain on ice until processing, apart from SST tubes, which were required to remain at room temperature for 30 minutes to clot before setting on ice. RNA tubes remained at room temperature for 24h before being frozen and extracted when required.

The 2mm skin biopsies were taken only at the first collection from the forearm of patients under local anaesthetic, and placed into ice-cold RPMI (Roswell Park Memorial Institute) media before being processed in the laboratory. Hair was cut from both the left and right sides of the head, placed into a small zip lock bag and stored at room temperature.

Laboratory Processes

As mentioned, processing of biological samples was carried out twice a day to prevent samples from sitting on ice for an extended period. Laboratory processes were handled under biosafety cabinets as outlined in the Safe Work Procedure.

Laboratory Processes are outlined in **Fig 2**. Urine samples were aliquot into 1ml vials 2D coded vials (Nunc™ Bank-It™ Vial Systems), and stored at -80°C. Blood tubes for serum and plasma were spun as per the manufacturer's instructions and stored in small volume

aliquots in 2D coded vials. Vials were also labelled with the collection number in freezer-safe labels for human-decoding (non-barcode, non 2D). DNA and RNA tubes were frozen at -30°C as recommended by manufacturers until ready for extraction. Extraction was facilitated by the QiaSymphony automated instrument (Qiagen), thereby reducing processing time to aliquoting of samples. DNA and RNA quantitation and quality assessment were completed and samples banked in 2D coded vials. Skin biopsies were cultured according to the SOP, and allowed to expand before being frozen down and stored in liquid nitrogen.

Storage

Samples must be stored at appropriate conditions to maintain integrity. For example, the storage of serum or plasms samples at -20°C or -80°C can affect samples as some neurologically relevant chemical process such as glutamate and serine retain some form of activity at -20°C [17].

After processing, aliquots of serum, and plasma, were stored at -80°C. After extraction and aliquoting, DNA and RNA were stored at -30°C. Skin biopsies were cultured and expanded before storage in liquid nitrogen (**Fig 1**). All -80°C freezers were also monitored, and temperature logs stored electronically on the freezer. This was also relevant to data management of samples, such as locating samples, records, and even transportation conditions when samples were sent to other laboratories for analysis. Currently, this information is not being stored as no storage provisions have been made. This could be further improved by backing up data onto a server either manually through retrieval of data and logging, or the automated back-up to server systems.

This is particularly important during occasions of power outages or freezer failure, which may lead to thawing of samples. In addition, it is recommended that multiples of the same samples are banked in separate locations or freezers to ensure that in the event of failure, there will not be a global loss of all banked samples [2].

Tubes used for sample storage are an important consideration as they are expected to be stored in the biobank for an extended period. The Bank-It™ Vial System was used as it had a 2D bar code on the bottom of the tube to ensure the vials could still be identified even if the stuck-on labels fell off. These 2D barcodes had lower scanning errors, a small footprint that

could encode denser information, and were compatible formats for database management systems [1, 18]

Post collection Administration

Post sample collection, data entry is required to upload the information in the FileMaker database to ensure that patient consent was digitised, clinical information updated, and collection information keyed in. This ensures that biological samples and corresponding data can be located.

Process Review

Development of banking processes and SOPs are always a work in progress as new information and new technologies emerge. In establishing the NDB, these processes were developed when a situation was predicted or had happened, accompanied by the gradual changes through feedback and advice from staff directly involved with the biobank, and external experts such as other biobanks and companies. For example, the Collection Processes (**Fig 2**) were in place before the Biobank Committee was officially established. These processes were reviewed when the committee was formed, and deliberation by the committee resulted in amendment of the processes to include additional sample collection types (e.g. plasma and hair samples). This resulted in the implementation of Project Request forms (discussed under Governance, and Appendix 5).

It should be noted that in the early stages, many of these reviewing processes did not happen on an official level. End-user scientists, and personnel managing the clinic would discuss and manage many of these processes, adapting them where required to suit each situation to the best of their ability and resources.

For instance, the biobank conducted a trial run for sample collection on a volunteer before commencing collections from patients. This allowed us to understand aspects that cannot efficiently be predicted, such as the time required, logistics of collections and processing, and if processes could be streamlined. After the trial, we reorganised collection logistics into two sections, phlebotomy, and skin biopsy, as patients would only have a biopsy taken on their first visit. It was further reorganised with the use of a trolley which enabled a mobile

laboratory where clinic staff could go from room to room, rather than patients going into the sampling room. This was particularly useful for patients with limited mobility.

As the biobank evolved, the requirements for various processes changed, and improvements were continually being sought to achieve better quality samples. This took place on a diverse scale, reducing processing times, the type of vial used for storage, or even type of needle used during venepuncture as this may affect haemolysis. Documentation of these processes and updates should be recorded where possible, to establish quality assurance. Where assurance is not practical, the recording of these changes from normal processing allows for tracing of information to trace a possible explanation for unexpected deviation in results, such as processing of samples by different staff, storage in different locations, or freeze thaw cycles.

2.6 Facilities & Equipment

An array of facilities are required for biobanking, ranging from basics such as biological safety cabinets (BSC) and laboratory space, to freezers, and specialised equipment such as the QiaSymphony, that allows for rapid liquid handling.

At the NDB, the clinic and laboratories are located in the same building on different levels. This is an advantageous circumstance that allows for cohesive collaboration between the clinic and laboratory, facilitating both communication and logistics. The clinic provides facilities for collection of biological samples, and freezer storage, while the laboratory provides access to pre-existing BSCs, centrifuges, and freezer storage space.

Equipment may facilitate the efficiency of sample processing. Use of equipment such as a decapper for removing screw caps off storage tubes was found to decrease the possibility of repetitive strain injury, and to increase efficiency in aliquoting. The use of barcode labellers and generators reduced the errors in writing/reading collection numbers on collection vessels, and sample vials. At the NDB, DNA and RNA extractions were eased by specialty equipment hosted by the laboratory (**Fig 3**). The workflow commences with blood tubes being loaded onto the QiaSymphony (Qiagen) for extraction, which greatly reduces manual labour in extracting samples. The QiaSymphony is an immense asset in which these processes are automated and can potentially be applied for other liquid handling processes.

The genetic material is then quantitated, 16 at a time, by the QIAxpert (Qiagen), followed by quality analysis by the Bioanalyzer (Agilent) (**Fig 3**).

Fig. 3 Equipment facilitating DNA/RNA Workflow Expedition of processing by specialist equipment that reduces time and increases quality and reliability of samples

Automation such as in this workflow can be costly, but comes with benefits such as reducing error and time spent by personnel going through these laborious processes. On a larger scale, automation of storage and retrieval processes are also available in terms of the CryoPlus ™ Storage System [19] or the BioStore™ II [20]. Consideration of these storage systems may be worthwhile, particularly in larger biobanks, and where financially viable.

2.7 Data Management

Another aspect where automation is crucial in biobanking is in data management, a process that is vital for maintaining patient, consent, sample storage, handling, and retrieval.

There are several core data management blueprints that must be established as priorities in new biobanks. For example, collation of patient consent forms, collection forms, allocation of unique patient and collection IDs, storage locations, notes on deviations from normal processes, and sample information such as concentration and purity of DNA/RNA. Where possible, these should be digitalised, to reduce the administration required to input this information. Where paper based administration is used, a filing system should be organised, for easy tracking of collection IDs and patient consent forms.

In addition, the use of programmes to automate or to better manage this data is infinitely valuable. Data management systems such as Laboratory Information Management Systems (LIMS) provide an organised inventory system, recording diverse sets of information, while at the same time, ensure patient privacy and data security [21, 22]. These systems can

manage change and laboratory data, track storage conditions, highlight deviations from normal, and retrieve calibration and maintenance data, therefore supporting the organisation, access, and control of laboratory processes. [23, 24]

Furthermore, LIMS can also be integrated with other digital systems such as the Electronic Lab Book (ELN) and Scientific Data Management Systems (SDNS), and laboratory equipment [21, 25]. For example, integration with the QiaSymphony will allow storage of sample information such as sample tracking, and the quality of nucleic acids, as well as information on the kit used during the extraction process. Much of this information can be important in times of predicament, but is often neglected in day-to-day processes. For example, noting a change in product batch numbers can provide an extra dimension of verification and checks when problems arise.

At the NDB, several levels and types of data management were implemented. Paper-based patient consent forms were filed sequentially by collection numbers, together with a digitalised copy saved into the clinical database Genie, and subsequently entered into a research information FileMaker database designed by Prof Rowe. As described under Laboratory Processes, bar codes were printed and applied on sample collection vessels, facilitating processing. The QiaSymphony can read barcodes and consolidate this information with output of extracted nucleic acid into pre-labelled sample storage vials. After collection and processing, details of samples were stored separately in an Excel database, and were subsequently integrated into the FileMaker database. The databases were hosted on the secured University Hospital network, with different levels of access assigned to different personnel and researchers according to their needs. Access to the unique patient ID was highly restricted to protect patient privacy and data security.

Samples were stored in Nunc™ Bank-It™ Vial Systems, which were 2D coded. 2D coding was an advantageous system for sample data and tracking as it allowed for secure tracking, and were laser etched onto the tubes. However, the main drawback with the Bank-It™ Vial Systems was that 2D codes could not be read by humans, and therefore, the tubes required manual labelling for readability.

Other biobanking vials such as the Micronic storage tubes could be customised, such as with a study ID, to avoid duplication of the 2D code, and stringent validation processes applied

such as sample evaporation and direct laser etching onto the tubes. Specialised storage tubes like these can facilitate the banking and storage processes as they can be compatible with freezers and LIMS systems. They can also use scanning codes to replace and accelerate mundane data entry.

Data management systems can be a source of complication. These systems often require customisation to suit the needs of the individual biobank and its processes, where current practices may not easily integrate with other programmes such as LIMS or clinical databases [22, 24, 25]. They need to be flexible to allow customisation for bar code reading, filtering of information, dynamic searches, ease of use, compatibility with other programmes and equipment, and most importantly, automatically backed up and secured with modifiable access levels and audit trails. In addition to software costs, there are also the added costs of hardware such as barcode readers, database maintenance and technical support that are required to sustain the seamless use of data management systems [22].

After four years of sample banking, the data and samples collected in this biobank have grown to a point where manual entries are highly impractical, and are susceptible to human errors. Overall, systems would benefit from increased automation, which would increase the integrity of stored information and databases, quality assurance, and reporting. This would also allow for further streamlining of the data collection process, reducing errors due to the monotonous work required in data entry, especially if it is replicated through both paper recording and subsequent entry into digital databases. An integrated clinical and LIMS database is being investigated for modification and application at the NDB. The implementation and adaptation of a biobank data management system, such as the ALSBank [1] will prove useful for securely managing increased amounts of information.

2.8 Policies & Legal

Legislation, policies, and guidelines are required to ensure that biological materials donated to the biobanks will be used in research that is compliant with legislation and protects the interest of participants [4]. The legal framework surrounding biobanking has not always been direct, there is not yet an official definition for a biobank. Based the type of biological specimens being collected, and its application in research, there are differing rules which complicate an already difficult to navigate landscape [4, 6].

Furthermore, if the biological sample is modified, as in induced pluripotent stem cells (iPSCs), the legal policies and context is modified, and the consequences are unclear [6]. These guidelines must maintain patient autonomy and privacy, especially as information collected pertains to personal health information. This leads to the issue of the identification of a person linked to data.

Initially, it seems that data must not be linked to an identifiable person (such as the use of a social security number), and should instead be irretrievably unlinked to maintain absolute anonymity. However, studies may require the data to be linked to the individual, particularly in longitudinal data sets, where collections must be linked to individuals to study changes that occur across time [6, 26].

Clearly, there are many aspects of the legalities of biobanking that vary from biobank to biobank, with differences in national regulations, developments, and taking into consideration religious and cultural differences [5, 6, 27, 28]. Although the legal framework is a work in progress, there are existing Australian legislations such as the *Commonwealth Privacy Act 1988*and the *Genetic Register Guidelines*, that can help form the backbone of future policies. These relevant legal topics have been discussed in the Biobanks Information Paper 2010 [4], and in an international context, by the Organization for Economic Co-operation and Development (OECD) Guidelines [7]. Therefore, they will not be reproduced here.

Ethics for the NDB was obtained from Macquarie University (Appendix 1), reviewing the scientific basis and need for establishment and continuation of the NDB. It has reviewed the protocols on obtaining consent and withdrawal, sample collections and procedures, governance, risks and benefits for participants and the wider community, data security, finances, and conflicts of interests.

As there is a potential conflict of interest such as a doctor/patient relationship, potential participants are invited to enrol in the study by biobank representatives. It is made clear to them that the decision to participate in the research is voluntary, and will not affect their medical treatment.

Patients are also informed in the participant informed consent form (PICF; Appendix 2) that they are at liberty to withdraw their consent at any point by informing the biobank

representatives. As such, samples are de-identified and linked to a random, sequential collection number that does not contain any personal information, which is the only information given to research scientists. Information linkage to the specific donor is restricted and available only to clinical staff and biobank representatives. This allows scientists to conduct research independently, without knowledge of the donor, but provides the ability for the donor to be traced where required, such as in the case of withdrawal of consent, or where particular information from research results may be important for the donor [12, 29].

This brings about an important issue on a patient's access to health information that is collected about them. It was decided that the results of studies would be published in journals and at conferences, but no research results would be returned to individual participants except for circumstances when genetic testing indicated important information about the participant which may have consequences for family health. After consulting with the MQ HREC Committee, the NDB committee, decided that patients would be consulted on their choice to access this information. As a result, the consent form was edited to include a section on the PICF to indicate a participant's choice of knowledge if a significant genetic finding was uncovered (Appendix 2, Page 5). These cases would then be referred to a genetic counsellor to facilitate information sharing and further accredited diagnostic tests.

The diversity in biobanking results in difficulties for scientist and biobank management to determine specific legal requirements that they are obliged to follow. An equilibrium needs to be established between the rights of donors, the community value of research and research independence to achieve sound legislation. Nonetheless, the best practice guidelines outlined by the OECD, National Health and Medical Research Council (NHMRC), and International Society for Biological and Environmental Repositories (ISBER) provide good guidance on the most important functions of biobanking and should be applied where reasonably possible [4, 7, 28].

2.9 Financing a Biobank

The establishment and continued running of a biobank can be costly and needs to be well managed for long term sustainability. Costs contributing to the management of a biobank

are vast, and include staffing, equipment, facilities, supplies, inventory equipment and databases [28].

The main sources of income for biobanks are usually grants from national and international sources which may be supplemented by institutions [30]. Biobanking is a long-term process, and the receipt of grants for short periods results in difficulties for them to be financially self-supporting and sustainable. Supplementary income generating avenues such as cost recovery and provision of additional services such as sample processing and storage should be considered [8, 30].

Financial limits of biobanks evoke an effective use of resources [31], but can be a limiting constraint in terms of management. For instance, significant financial investment is required to employ skilled staff, obtain equipment for process automation and engage an effective LIMS programme. However, this will provide long-term benefits such as ensuring a high quality and standard of biobanking. Cost benefit analyses must be undertaken to evaluate the financial feasibility of the biobank and policies and procedures frequently reviewed and streamlined to ensure sustainable financing.

Notably, cost recovery such as recapturing costs such as labour, consumables, processes, and maintenance is a viable approach to safeguard a biobank's financial stability. A biobank would benefit from development of a business plan to ensure long term sustainability of its samples, equipment and professional staff [3, 9, 31].

The NDB was formed as a non-commercial entity funded by donations to MND research, the Macquarie University Department of Vice Chancellor and by research grants. The Faculty of Medicine and Health Sciences also supports the NDB through the provision of facility space, and administration support. The operation costs of the NDB are approximately $300,000 per annum including staff salaries and consumables. Currently, samples are limited for retrieval by groups internal to, or collaborating with, the MND Research Centre. Hence, there have been no cost recoveries in effect. However, it is planned for limited cost recovery to take place to ensure the sustainability of the biobank, as well as to encourage financial responsibility for sample usage. The detailed financial aspects of the biobank are not within the scope of a PhD thesis, and will not be further discussed.

2.10 Governance

Governance of biobanks is challenging as the varying types of biological specimens, the size of the biobank and its objectives need to comply with legislation and fit within a comprehensive regulation framework. Governance involves issues such as responsibility, ownership and control of data and samples provides guidance on whether data should be made publicly available, and ensures the rights of the participants take precedence over research interests [7, 27].

These decisions should be made by a governing body, such as a board that includes funding members and the host institution, and a scientific committee who will be held accountable for transparency, use, and best practice procedures of the biobank. Collectively, these committees are responsible for establishing policies and procedures that will determine outcomes and directions of the biobank such as internal controls and risk management [2, 7]. In addition, there have been recommendations for independent examinations of the biobanks for auditing purposes to determine oversights, compliance, and regulation [2, 27].

Governance is core in ensuring that there are no conflicts of interest that may result in sub-optimal use of banked material. The establishment of good policies with transparent criteria or guiding principles will allow the biobank to navigate these conflicts. Proposed research to be conducted on banked samples must be carefully assessed by a scientific committee, to ensure use of the material has the potential to result in a significant contribution to knowledge gaps or validation of projects.

The NDB has a scientific board which includes founding members of the biobank, as well as principle investigators of the MND Research Centre. It is led by Dr Sarah Furlong who oversees the proceedings of the biobank and chairs monthly meetings. The biobank is still growing and is only available for internal use or through collaborations with members of the biobank. Regardless, an approval process is required to review use of precious biobank resources (**Fig 4**). Research projects require an application form (Appendix 5),to indicate the scientific justification, description, and rationale for sample utilisation with supporting feasibility of experimental design and statistical power. There are 16 current projects, utilising all types of samples, and new projects being reviewed. The role of the board will evolve as the biobank progresses. Collaborations with external universities and institutions have led to the establishment of Material Transfer Agreements (MTAs), prepared by the

Macquarie University Legal and Contracts Team, and new protocols for the physical transfer of biological samples.

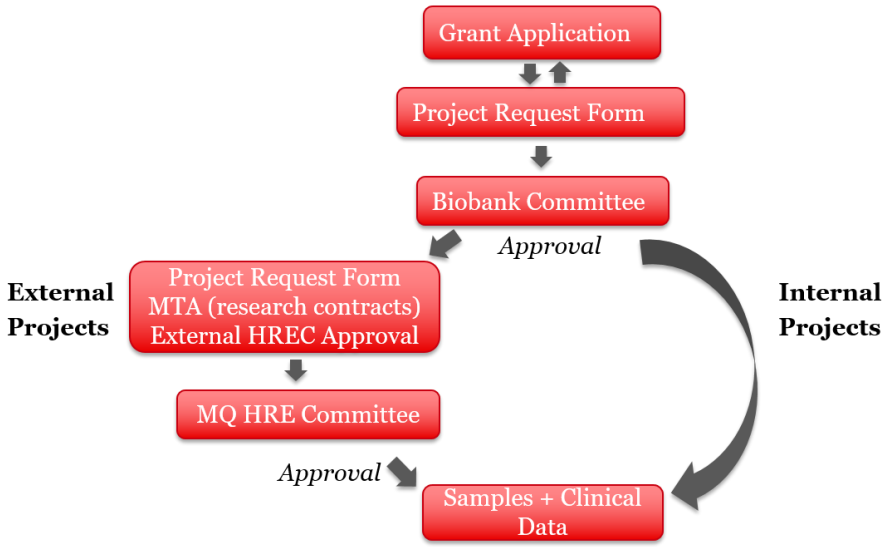

Fig 4. Project request system Flow chart of approval processes for projects requesting access to biobank samples

Governance of biobanks require the design and implementation of transparent policies and regulations, and are subject to evolution through adaptation and modifications. Guidelines that will assist the improvement of governance include resources such as the Council of Europe (CoE) Guide, EuroBiobank, NCI Best Practices for Biospecimen Resources, Swedish National Biobank Program, and ISBER

3. Lessons Learnt

Biobanking is an invaluable resource that creates opportunities to study processes and track changes in disease. The amalgamation of policies, governance, management, finances, research, and the clinic can be challenging, balancing practicality without over-administrating. However, careful consideration of all factors and continual evolution will allow for an ethical, efficient, and reliable resource for research.

Since the commencement of the NDB in 2013, the biobank has collected samples from more than 500 patients (**Fig 5**), including longitudinal samples which allow for tracking of changes through time (**Table 2**). Biobank statistics were kindly provided by Dr Furlong.

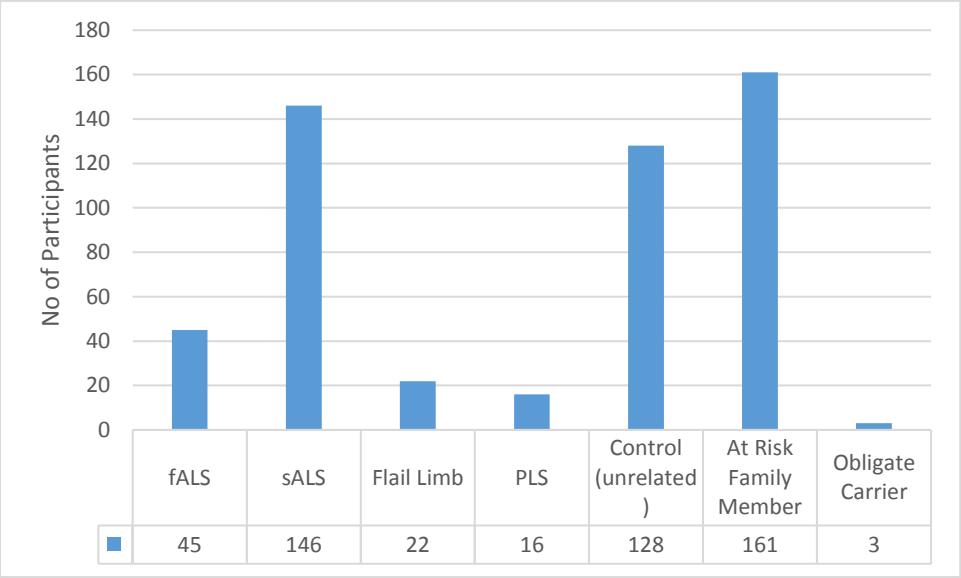

Fig 5 Classification of samples. Familial ALS (fALS), and sporadic ALS (sALS), Flail Limb, Primary Lateral Sclerosis, unrelated controls, at risk family members (genetically related to ALS patient), obligate carrier (known carrier of genetic mutation), and number of participants in each category.

No. Longitudinal Collections	fALS	sALS	Unrelated Controls
1	29	67	65
2	10	47	38
3	8	25	14
4	3	19	7
5	4	6	5
6+	3	7	0

Table 2 Breakdown of samples by number of longitudinal collections stored and participant classification. Participants are sub-categorized to controls, familial ALS (fALS), and sporadic ALS (sALS), and number of longitudinal collections.

There have been several significant challenges during the establishment of the NDB. For example, the absence of a LIMS system during the commencement of the biobank resulted in the underutilisation of 2D barcodes on sample storage tubes, resulting in the manual organisation of sample IDs, and location of storage. Furthermore, a lack of full time staff managing the biobank during the initial period, resulted in a slow start. Nonetheless, this has allowed for the gradual evolution of the biobank, allowing for rapid adaptation where required, before increasing the quantity of sample collections.

Significantly, the largest setback for the NDB was in banking of fibroblast samples. Skin biopsies could only be performed by clinicians with local anaesthetic, and it is considered to be a moderately invasive procedure [12]. These were conducted by the Neurology Registrar, but were discontinued after he left the clinic in 2014 due to lack of manpower. Disappointingly, many frozen cultures of fibroblasts failed to be revived after storage in liquid nitrogen. This was attributed to use of unsuitable sample storage vials. Recently, the protocols for skin biopsy collection, processing, and storage were optimised, and a project submitted to the biobank committee for review. The project was approved, and skin biopsy collections have recommenced.

Finally, there was slight confusion in the classification of control participants, as those who did not present with disease were considered controls. However, this was inaccurate as some of these “control” participants were from the families of patients who may have carried a genetic mutation, and as such, their family members could not be accurately classified as controls. Participants were subsequently classified as in **Table 3**.

Table 3 Classification of biobank participants

Classification	Description
Affected	Individual diagnosed with neurodegenerative disease (including ALS, PLS, etc.)
Unaffected carriers	Individual carrying a known or candidate mutation, currently unaffected

Obligate carrier	Individual for whom DNA was unavailable but who carries the disease gene mutation based upon analysis of the family history.
"Married-in" control	Spouse of a patient, unaffected carrier, or obligate carrier, who is genetically unrelated to their partner as determined by family history
At risk	Individual who is unaffected at time of recruitment, but is at risk of developing disease due to their relationship with affected individuals in the family (e.g. child, or sibling of affected individual)

Nevertheless, there have been many positive outcomes from the establishment of the biobank. First, the altruism of participants to donate time, biological samples, and information, particularly after the knowledge that their donation was unlikely to result in outcomes that would help them. The NBD has also recruited several pairs of discordant twins, who will contribute to valuable insights into disease epidemiology, including genetics, environment and lifestyle factors, and other comorbidity traits [32, 33].

Dedicated biobank staff who are well trained and equipped with good facilities and automation equipment improve the quality assurance of the biobank. Staff have also allowed for development of protocols, often in consultation with other biobanks, and in-depth research, achieving an increasing number of biobanking standards such as custodianship, governance, and technical best practices.

The establishment of the NDB has progressed through various stages of evolution. From its establishment, the first trial run of the collections, ethics, recruitment, establishment of SOPs, increasing sample types, reviewing processes and best practices to improve quality, and fostering project collaborations.

Future directions of the NDB would be geared towards improving the quality of samples and efficiency, increasing the number of high quality projects utilizing samples, and facilitating collaborations both within the MND centre and with other Neurodegenerative Disease biobanks. In less common diseases such as ALS, biobanks will face difficulty in achieving sufficient patient numbers to be statistically significant [4, 34], making collaborations crucial for good scientific studies. Additionally, increased reporting to external stakeholders, donors and the public, will improve publicity of the biobank and aid in financial viability through charitable donations [3]. Evidently, continual reviews, improvement, and implementation

will allow the NDB to address these and future challenges to remain a viable, and applicable resource for MND research.

Bibliography

1. Sherman A, Bowser R, Grasso D, et al (2010) Proposed BioRepository platform solution for the ALS research community. Amyotroph Lateral Scler 12:11–16.
2. Hallmans G, Vaught JB (2011) Best Practices for Establishing a Biobank. In: Methods Mol. Biol. pp 241–259
3. Simeon-Dubach D, Watson P (2014) Biobanking 3.0: Evidence based and customer focused Biobanking. Clin Biochem 47:300–308. doi: 10.1016/j.clinbiochem.2013.12.018
4. NHMRC (2010) Biobanks Information Paper.
5. Eriksson S, Helgesson G (2005) Potential harms, anonymization, and the right to withdraw consent to Biobank research. Eur J Hum Genet 13:1071–1076. doi: 10.1038/sj.ejhg.5201458
6. Rynning E (2009) Legal Challenges and Strategies in the Regulation of Research Biobanking. Ethics Res Biobanking. doi: 10.1007/978-0-387-93872-1
7. OECD (2009) OECD Guidelines on Human Biobanks and Genetic Research Databases.
8. Chalmers D Biobanking and Privacy Laws in Australia.
9. LeBlanc J, Dee S, Braun L, et al (2013) Impact of a Permission to Contact (PTC) Platform on Biobank Enrollment and Efficiency. Biopreserv Biobank 11:144–148. doi: 10.1089/bio.2013.0004
10. Kaufmann P, Mitsumoto H (2009) ALS Research Group (ALSRG): Second meeting, a summary report. Amyotroph Lateral Scler 7:258–261. doi: 10.1080/07853890601012096
11. Toledo JB, Van Deerlin VM, Lee EB, et al (2014) A platform for discovery: The University of Pennsylvania Integrated Neurodegenerative Disease Biobank. Alzheimer's Dement 10:477–484.e1. doi: 10.1016/j.jalz.2013.06.003
12. Otto M, Bowser R, Turner M, et al (2012) Roadmap and standard operating procedures for Biobanking and discovery of neurochemical markers in ALS. Amyotroph Lateral Scler 13:1–10. doi: 10.3109/17482968.2011.627589
13. Teunissen CE, Tumani H, Engelborghs S, Mollenhauer B (2014) Biobanking of CSF: International standardization to optimize biomarker development. Clin Biochem 47:288–292. doi: 10.1016/j.clinbiochem.2013.12.024
14. Banack SA, Murch SJ (2009) Multiple Neurotoxic Items in the Chamorro diet link BMAA with ALS/PDC. Amyotroph Lateral Scler 10:34–40. doi: 10.3109/17482960903278451
15. World Health Organization (2010) Best practices in phlebotomy. WHO Guidel. Draw. Blood Best Pract. phelmbotomy
16. Beckton Dickinson and Company (2010) BD Vacutainer Order of Draw for Multiple Tube Collection.
17. Wuolikainen A, Hedenström M, Moritz T, et al (2017) Amyotrophic Lateral Sclerosis Optimization of procedures for collecting and storing of CSF for studying the metabolome in ALS Optimization of procedures for collecting and storing of CSF for studying the metabolome in ALS. Amyotroph Lateral Scler 10:229–236. doi: 10.1080/17482960902871009
18. Thermo Fisher Scientific Nunc™ Bank-It™ Vial Systems. http://www.thermofisher.com/order/catalog/product/374078. Accessed 13 Apr 2016

19. Thermo Scientific CryoPlus™ Storage Systems. https://www.thermofisher.com/order/catalog/product/7400. Accessed 20 Mar 2017

20. Brooks Life Science Solutions BioStore™ II Automated Sample Storage. http://www.brooks.com/products/life-science/storage-systems/biostore-minus-80c-automated-sample-storage. Accessed 20 Mar 2017

21. Voegele C, Tavtigian SV, de Silva D, et al (2007) A Laboratory Information Management System (LIMS) for a high throughput genetic platform aimed at candidate gene mutation screening. Bioinformatics 23:2504–2506. doi: 10.1093/bioinformatics/btm365

22. Chen Y, Lin Y, Yuan X, et al (2016) LIMS and Clinical Data Management. In: Transl. Biomed. Informatics. pp 225–239

23. Mahaffey RR (1990) LIMS : Applied Information Technology for the Laboratory. Springer US

24. Paszko C, Pugsley C Considerations in selecting a laboratory information management system (LIMS).

25. Machina HK, Wild DJ (2013) Laboratory Informatics Tools Integration Strategies for Drug Discovery. J Lab Autom 18:126–136. doi: 10.1177/2211068212454852

26. Kaufman DJ, Murphy-Bollinger J, Scott J, Hudson KL (2009) Public Opinion about the Importance of Privacy in Biobank Research. Am J Hum Genet 85:643–654. doi: 10.1016/j.ajhg.2009.10.002

27. Gottweis H, Zatloukal K (2007) Biobank governance: trends and perspectives. Pathobiology 74:206–11. doi: 10.1159/000104446

28. Campbell LD, Betsou F, Garcia DL, et al (2011) Best Practices for Repositories: International Society for Biological and Environmental Repositories Editor-in-Chief.

29. Barbour V (2003) UK Biobank: a project in search of a protocol? Health Policy (New York) 361:1734–1738. doi: 10.1016/S0140-6736(03)13377-6

30. Parry-Jones A Assessing the Financial, Operational, and Social Sustainability of a Biobank: The Wales Cancer Bank Case Study. doi: 10.1089/bio.2014.0044

31. Watson PH, Nussbeck SY, Carter C, et al A Framework for Biobank Sustainability. doi: 10.1089/bio.2013.0064

32. Boomsma D, Busjahn A, Peltonen L (2002) Classical twin studies and beyond. Nat Rev Genet 3:872–882. doi: 10.1038/nrg932

33. Al-Chalabi A, Fang F, Hanby MF, et al (2010) An estimate of amyotrophic lateral sclerosis heritability using twin data. J Neurol Neurosurg Psychiatry 81:1324–6. doi: 10.1136/jnnp.2010.207464

34. Chad DA, Bidichandani S, Bruijm L, et al (2013) Funding agencies and disease organizations: Resources and recommendations to facilitate ALS clinical research. Amyotroph Lateral Scler Front Degener 14:sup1:62–66. doi: 10.3109/21678421.2013.778588

Pages 125-127 (HREC approval) of this thesis has been removed as they may contain sensitive/confidential content

A2 Patient Information and Consent Form (PICF)

Issue Date: 09/06/16

MACQUARIE University

Neurodegenerative Disease Biobank

Participant Information & Consent Form

You are invited to participate in the Macquarie University neurodegenerative disease biobank, and its associated research, as a patient, family member or control. You will be asked to donate biological samples for research. This document provides you with information about why the research is being done and what it will involve. It will help you to decide whether you would like to participate. Please take the time to read the following information carefully and discuss it with others if you wish.

1. What is the Macquarie University Neurodegenerative Disease Biobank?

The Macquarie University neurodegenerative disease biobank is collection of biological samples such as blood, saliva, skin, urine or hair from patients with neurodegenerative disease, as well as from unaffected individuals. Samples are linked to clinical and lifestyle information. Samples are stored and made available for neurodegenerative disease research now and in the future. These are a valuable resource for researchers to increase our understanding of neurodegenerative disease with the overall goal to identify treatments and preventative measures.

2. What does taking part in the Biobank involve?

After you read this information sheet you will be given the opportunity to ask the biobank representative questions. Once you understand what the biobank is about and you agree to participate you will be asked to sign the consent form at the end of this document.

Your consent to the neurodegenerative disease biobank involves providing samples such as blood, urine, saliva, hair or skin. You may be asked to provide some or all of these samples. You may also be asked to provide samples on more than one occasion, as this allows research into biological changes over time.

You may also be asked to complete a questionnaire related to your lifestyle and environment, which you will take home to complete in your own time and can be returned on your next visit to your clinician or by mail.

We would also like to collect and store your associated clinical information and to get your permission to contact you regarding any future research projects that you may be interested in taking part in.

3. Who is asked to take part?

Patients are asked if they would like to participate in the neurodegenerative disease biobank whilst visiting their clinician. We may also request for family members, spouses or unrelated individuals to take part. Samples from these individuals may act as controls so that we can compare biological samples of patients with neurodegenerative disease to those without disease.

4. What will happen to my samples and health information?

Your samples and health information will be stored in Macquarie University indefinitely. This is so researchers can access your samples for any future research without having to re-contact you for more samples and information.

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It is common practice for collaboration in national and international medical research. As such your samples may be used in future research into neurodegenerative disease conducted by Macquarie University and its national and international collaborators. If your samples are sent to collaborators, only your coded information will be sent so that you cannot be identified.

Every research project involving biobank samples and data must be reviewed and approved by the Macquarie Human Research Ethics Committee (HREC). Permission to access biobank samples must also be granted by the neurodegenerative disease biobank committee. Only when both stages are complete can researchers access your coded samples and health information. This is to ensure we protect your rights and privacy.

5. Do I have to take part?

It is up to you whether or not you want your data and biological samples made available for future research. If you choose not to participate this will in no way affect the care or treatment that you receive.

By consenting you are telling us that you:

- Understand what you have read or been told.
- Consent to take part in the research that is described.
- Consent to the use of your information as described to you.

Participating in research is voluntary.

6. Can I change my mind?

Yes you may withdraw your permission to be contacted for future research and/or to access your data and samples at any time. You can withdraw your consent by contacting the biobank using the contact details below or by filling in a 'Withdrawal of Consent Form' provided to you.

If you withdraw your consent, the biobank will destroy your unused stored biological samples. However please note that any research that has happened up until the point that you withdraw may be kept, as the researcher may not be able to identify your specific sample or data once pooled.

7. What type of research will be conducted on my samples and information?

Your samples will be used in any current and future research into neurodegenerative disease by research teams at Macquarie University including those lead by Professor Dominic Rowe, Professor Gilles Guillemin, Professor Roger Chung, Associate Professor Ian Blair and Associate Professor Julie Atkin as well as their collaborators.

Some researchers will carry out genetic analysis on your samples. Genes are made of DNA – the chemical structure carrying your genetic information that determines many human characteristics such as the colour of your eyes or hair. DNA stores this genetic information in the form of a code and this is the code that you inherit from your parents and that you pass on to your children. Researchers might sequence your DNA to look at your genetic code, as well as using other types of genetic studies. Future genetic research on your samples may look not only at genes related to neurodegenerative diseases but also at

your whole genome and therefore other disease types. Studying genes along with health information helps to better understand what causes certain diseases. It also may help to understand prognosis, subtypes of disease and how different patients respond to treatment.

Research will also be carried out to identify and understand biomarkers of disease. Biomarkers are biological characteristics that can be measured and evaluated as an indicator of normal, or abnormal (disease) biological processes. Biomarkers can add to our understanding of disease cause and progression and can lead to new therapeutic targets.

Some researchers are investigating why particular proteins are abnormal in neurodegenerative diseases. These abnormal proteins interfere with the normal function of nerve cells, and the goal of this research is to identify measures that that prevent this process.

Research is also underway to identify and understand how environmental exposure and lifestyle factors contribute to neurodegenerative disease.

The overall aim of the research is to increase our understanding of neurodegenerative disease so that new effective treatment strategies can be developed.

8. Will results be returned to me?

Results of the research will be published in medical & scientific journals but this will not include any information identifying you. Much of the research done on your samples will be exploratory and will therefore not be relevant to your care. If genetic testing is carried out on your samples and shows results that could be of significance to you or your family, you will be asked at that time if you wish to know the results. You can also tell us now if you do not wish to know genetic results that could be of significance to you or your family by using the check box at the end of this form. You can change your mind at any time by contacting the biobank. As the genetic testing may be broad this could have significance to you or your family with respect to conditions unrelated neurodegenerative disorders. The results may be important to you as they may provide:

- Information about risk of an inherited condition
- Information that might influence a decision to have children
- Information that might affect your ability to obtain insurance or employment.

You will have the opportunity to talk to a genetic counsellor before making a decision whether to know the results. Such results will need to have the tests repeated and the results verified in an accredited testing laboratory.

9. Are there any risks to me?

If we collect a blood sample (typically 3-4 tablespoons), you may feel brief pain or have some bruising from the needle. There is also a small risk of infection, light-headedness, and fainting. If we collect a skin biopsy (where we take a small sample of your skin), this will be carried out under local anesthetic. This will result in local discomfort and minor bleeding which will be stopped with pressure and a bandage. There are no known risks for giving urine or saliva samples.

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MACQUARIE University

10. How will my privacy be protected?

Macquarie University will use, access, hold and store your data in accordance with relevant National and State privacy laws. You have the right to request access to your personal information that has been collected by the research team and you also have the right to request that any information with which you disagree be corrected.

Data held by the researchers may be audited for the purposes of verifying safety and compliance with Macquarie University policy and relevant legislation. Such inspection will be conducted by an authorized representative of Macquarie University or as required by law.

11. Will I be contacted again?

If you decide not to take part you will not be contacted by the biobank again. If you agree to take part you will be asked to provide samples on each visit to the Macquarie Neurology clinic, if applicable. Collection of samples at each visit allows for research into disease course. You will only be asked to provide consent on your first visit. You can agree to provide all or no samples on any visit without having to provide a reason.

At some point in the future, you and your family may be asked to participate in other research projects and you will be asked to go through a similar process of giving consent. You may, of course, refuse to participate in any future studies.

12. Will I benefit from taking part?

Participation in the biobank is unlikely to result in any direct benefit to the care and treatment that you currently receive, or any future care that you may receive. However, your samples are an invaluable resource for research into understanding how to prevent, detect, and treat neurodegenerative disease in the future.

13. Who has reviewed this study?

All research in Australia that involves human participants is reviewed by an independent group of people called a Human Research Ethics Committee (HREC). HRECs must review research in accordance with a set of ethical guidelines called the National Statement on Ethical Conduct in Human Research (2007 – Updated March 2014) and other relevant legislation and guidelines. This study has been reviewed and given ethical approval by the Macquarie University HREC (Medical Sciences). This research meets the requirements of the National Statement which is available at the following website:
http://www.nhmrc.gov.au/guidelines/publications/e72
If you have any complaints or reservations about any ethical aspect of your participation in this research or the way the research has been conducted, you may contact the Director, Research Ethics and Integrity (telephone (02) 9850 7854; email ethics@mq.edu.au). Any complaint you make will be treated in confidence and investigated, and you will be informed of the outcome.

Issue Date: 09/06/16

MACQUARIE University

Neurodegenerative Disease Biobank

Consent Form

If you wish to participate please sign this consent form

Declaration by Participant

- I have read the patient information sheet or have had read to me in my first language, and I understand it.
- I have been given the opportunity to ask any questions and I have received satisfactory answers.
- I understand that I can withdraw consent at any time without affecting any medical treatment or care now or in the future.
- I understand that my coded samples and information may be sent to Macquarie University's national and international collaborators to conduct future research
- I agree that research data gathered from the results of the biobank may be published, provided that I cannot be identified.
- I am aware that if I do not opt out of receiving genetic results below, I will be contacted if genetic testing shows important information about me, and asked if I wish to know the results. I will have the opportunity to talk to a genetic counsellor before making a decision whether to know the results. Such results will need to have the tests repeated and the results verified in an accredited testing laboratory. The results may be important as they may provide:
  - Information about risk of an inherited condition
  - Information that might influence a decision to have children
  - Information that might affect ability to obtain insurance or employment.
- I acknowledge receipt of a copy of the patient information sheet for my own records.

1. If a significant genetic finding is uncovered, I do not wish to be informed (you can change your mind at any time by contacting the biobank) ☐

In respect to the storage and use of my biological samples and related clinical information, I give permission for the use of these samples the purpose of

2. Current and future research into neurodegenerative diseases Yes ☐ No ☐

3. Future research projects that may or may not be related to neurodegenerative diseases. Yes ☐ No ☐

Issue Date: 09/06/16

MACQUARIE University

Name of Participant (please print) _____ Date of Birth _____

Signature _____ Date _____

Or if applicable

Name of Guardian/Power of Attorney (please print) _____

Signature _____ Date _____

Declaration by Biobank representative

I have given a verbal explanation of the research project, its procedures and risks and I believe that the participant has understood that explanation.

Name of Biobank representative (please print) _____

Signature _____ Date _____

Note: All parties signing the consent section must date their own signature.

Further information and contacts

If you would like any further information on the biobank please do not hesitate to contact:

Biobank Manager

Dr. Sarah Furlong,
Faculty of Medicine & Health Sciences,
F10A Building,
2 Technology Place,
Macquarie University,
NSW 2109.
Telephone: +61 2 9850 2766
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Biobank Custodian

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A3 Safe Work Procedure for Handling of Human Samples and Cultures

MACQUARIE UNIVERSITY | THE AUSTRALIAN SCHOOL OF ADVANCED MEDICINE

Handling of Human Samples and Cultures

SOP Reference: ASAM/SOP/004	
Version Number: DRAFT 1.4 (26th Feb 2013)	
Effective Date: 06 February 2010	Review Date: Feb 2013
Author: Prof Jacqueline Phillips	
Approved by: Prof Mark Connor	Date:

Version	Date	Reason for Change
Draft 1.4	26th Feb 2013	Update

MACQUARIE UNIVERSITY | THE AUSTRALIAN SCHOOL OF ADVANCED MEDICINE

TABLE OF CONTENTS

MACQUARIE UNIVERSITY | THE AUSTRALIAN SCHOOL OF ADVANCED MEDICINE

1. PURPOSE

This Standard Operating Procedure (SOP) provides Biosafety guidelines for procedures associated with the handling of specimens of human origin, specifically human tissue and blood samples, human primary cell cultures, and other potentially infectious or hazardous agents used at the Australian School of Advanced Medicine (ASAM).

2. EQUIPMENT

All work with human blood in the laboratories must be performed in a certified Class 2 Biohazard cabinet. Procedures that produce aerosols, such as sonication, mixing, washing, etc., should not be performed in the open laboratory. Where this is not possible, appropriate procedures and techniques should be discussed with the Macquarie University Biosafety Committee.

Where blood is being collected with minimal processing (e.g. when serum is being isolated), work may be conducted outside a certified Class 2 Biohazard cabinet.

Any centrifugation of human samples or cell cultures must be done in centrifuges fitted with sealed rotors, with samples in sealed tubes and observing standard precautions for handling blood.

Disposable equipment should be used wherever possible and discarded into yellow contaminated waste bags.

Vacuum aspiration should not be used. Waste supernatant should be transferred into a disposable tube and then autoclaved or chemically disinfected.

3. SUPPORTIVE DOCUMENTATION

3.1 Macquarie University Biosafety Website

http://www.research.mq.edu.au/for/researchers/how_to_obtain_ethics_approval/biosafety_research_ethics/application_process

The Institutional Biosafety website provides all recommended policy and guidelines, plus key contact information. Researchers and students must check with the Chair of the Biohazards Safety Committee if they are in any doubt as to whether their research needs Biohazard Safety Committee approval (biohazard@mq.edu.au).

4. SET UP

4.1 Ethics and Biosafety Approval

All work with specimens of human origin, whether teaching or research, requires prior approval from the Macquarie University Human Ethics Committee and Biohazard Safety Committees.

Work with human material must be carried out in designated areas as approved by the Biosafety Committee. Any taking of human blood samples must be done in a designated room following approval from the Human Ethics and Biosafety Committees.

4.2 Vaccinations

All workers handling human specimens are recommended to have a vaccination against Hepatitis B Virus (HBV). While risk associated with exposure to blood and tissues contaminated by HBV can be mitigated by vaccination, the potential risk of infection by other agents such as Hepatitis C, Human Immunodeficiency Virus (HIV) and Creutzfeldt-Jakob disease (CJD) can only be reduced by following prudent safety measures when handling specimens.

5. SAFETY PRECAUTIONS AND GENERAL INFORMATION

5.1 Standard Precautions

The following precautions must be adhered to at all times when working with specimens of human origin:

- All material of human origin should be treated as potentially infectious, irrespective of HIV or HBV status.
- Eating, drinking, smoking, application of cosmetics and storage of food or drink is prohibited in the laboratory or in any designated areas.
- Mechanical pipetting, not mouth pipetting, must be used for the manipulation of all liquids in the laboratory.
- All open cuts and abrasions must be covered.
- Hand/mouth contact should be kept to a minimum.
- Hands must be thoroughly washed with soap and water, and dried:
  - following completion of laboratory activities;
  - following removal of protective clothing and before leaving the laboratory; and
  - immediately if they become contaminated with blood or body fluids.

5.2 Training

All staff undertaking work using human samples and cultures must have undertaken appropriate training and been deemed proficient by their supervisor through such training or prior experience.

5.3 Personal Protection

5.3.1 Laboratory Coats

Laboratory coats or gowns must be worn while working with human material, and removed when leaving the laboratory. Garments which are known or are suspected to have been contaminated with human sample must be autoclaved before laundering.

5.3.2 Gloves

Disposable gloves must be worn to avoid skin contact with:

- human blood, tissue, other body fluids or human cell derived cultures;
- infectious or potentially infectious materials, and
- hazardous chemicals.

Care must be taken to avoid contaminating surfaces when wearing soiled gloves.

Gloves must be removed:

- following completion of laboratory activities;

- before leaving the laboratory;
- when using a telephone; or
- when performing any office work.

5.3.3 Face Masks and Eye Protection

Face masks and eye protection must be worn at all times when there is potential for procedures to cause blood and other body fluids to splash or spray into the eyes, nose or mouth. Eye protection must otherwise be worn at all times in the PC2 laboratory environment.

5.4 Storage of Samples

All samples must be:

- stored in leak proof containers with secure lids;
- labelled appropriately; and
- stored in a designated area e.g. fridge, freezer or portion of.

5.5 Decontamination

5.5.1 Glassware

Glassware must be decontaminated by soaking in 0.5% (5000ppm) sodium hypochlorite, a proprietary disinfectant or by autoclaving before washing up. Small numbers of glass Pasteur pipettes may be put in sharps containers. Larger numbers should be decontaminated by soaking in 0.5% (5000ppm) sodium hypochlorite, and disposed of as broken glass.

Many disinfectants are less effective in the presence of protein so where possible proteinaceous material should be removed before soaking.

5.5.2 Work Surfaces

All work surfaces must be decontaminated with sodium hypochlorite or a proprietary disinfectant. Sodium hypochlorite corrodes metal so 70% ethanol or a proprietary disinfectant should be used to disinfect metal equipment such as centrifuge rotors.

Surfaces must be decontaminated following any spills of infectious (or potentially infectious) material with 0.5% (5000ppm) sodium hypochlorite, and routinely with 0.05% (500ppm) sodium hypochlorite.

Contact time must be taken into consideration in order for the disinfectant to be effective. The hypochlorite solution should be in contact for at least 10 minutes.

5.5.3 Prion Contaminated Material[1]

Prions are infectious agents that produce slow, progressive and fatal diseases of the central nervous system. Prions are the causative agents for a number of degenerative brain diseases, including scrapie (a fatal disease of sheep and goats), mad cow disease, Creutzfeldt Jacob disease (CJD) and Gertsmann-Straeussler-Scheinker (GSS) disease.

Prions are resistant to most traditional methods of inactivation used for other microorganisms and therefore pose particular problems in the laboratory. Known prion infected tissue should only be handled in dedicated laboratories using dedicated equipment.

[1] Information obtained from the University of Sydney, Occupational Health and Safety Guidelines for the Decontamination of Clinical/Biological Waste and Spill Management, http://www.usyd.edu.au/ohs/policies/ohs/deconguidelines.shtml#3, accessed 16th Feb 2010.

Formalin-fixed and paraffin-embedded tissues, particularly of the brain, remain infectious for long periods, if not indefinitely. They should be assumed to remain infectious through the processes of embedding, sectioning, staining and mounting on slides. The most effective chemical treatment for decontaminating formalin-fixed tissue is 96% formic acid for 1 hour.

Current recommendations for the sterilisation of articles or specimens that could be contaminated by prions are 18 minutes at 134°C in a pre-vacuum pressure steam steriliser or 1 hour at 132°C in a downward displacement pressure steam steriliser. The recommended chemical disinfectant for effective decontamination of articles or specimens that could be contaminated by prions is 20 000 ppm (2% sodium hypochlorite) available chlorine for 1 hour.

Infectivity is strongly stabilised by drying or fixing, so contaminated material should be kept wet between the time of use and disinfection.

Following decontamination, prion waste should then be treated as cytotoxic waste to ensure the material will be incinerated. Solid waste that has been pressure steam sterilised should be placed into a Cytotoxic Clinical Waste Bin and the autoclave bag labelled with the words PRION WASTE – INCINERATE AT 1100 CELSIUS. Prion material that has been chemically decontaminated should be treated as chemical waste and labelled as PRION WASTE – INCINERATE AT 1100 CELSIUS

5.6 Disposal

5.6.1 Consumables

All consumable items such as disposable equipment, tissues and gloves must be disposed of as contaminated waste. Non-sharp items must be placed in yellow contaminated waste bags which will be transferred to commercial contaminated waste disposal bins.

5.6.2 Sharp Instruments

Sharp instruments such as scalpel blades, needles, syringes and pipettes, must be placed in rigid-walled, puncture-proof sharps containers. Needles must be discarded unsheathed immediately after use. Sharps containers must never be overfilled and lids must be secured before containers are collected as part of the contaminated waste disposal service.

5.6.3 Waste Specimens

Disposable items that have been in contact with blood must be discarded into yellow contaminated waste bags or sharps containers.

Blood or human tissue must be decontaminated by autoclaving (for large volumes). Where this is impractical, material should be chemically disinfected, and sent out for incineration as part of the contaminated waste disposal service.

5.7 Accidents

Any accidents involving materials of human origin, in particular skin punctures or splashes to the face, must be reported immediately to the School Safety Officer who will inform the Biosafety Officer if necessary.

Care must be taken to prevent injuries when:

- using needles, scalpels, and other sharp instruments or devices;
- handling sharp instruments after procedures;
- cleaning used instruments; and
- disposing of used needles.

5.7.1 Blood Accidents

In the event of contact with human blood, the individual should wash the contaminated body area and seek immediate medical advice.

When cleaning contaminated laboratory surfaces, gloves must be worn throughout the clean up procedure. Spills and surfaces can be decontaminated with 5000ppm (0.5%) sodium hypochlorite. After clean up, all waste should be placed in yellow contaminated waste bags and hands thoroughly washed.

5.7.2 Needle Stick Injuries

All needle stick or similar injuries involving human blood or body fluid must be reported. Medical assistance must be sought immediately.

6. MAINTENANCE OF SOP

If any part of this SOP changes, refer it to the ASAM Authorisation signatory for updating as soon as possible.

7. PAPERWORK REQUIREMENTS

If you were required to read this SOP, sign the Induction Record Sheet.

A4 Biobank Decontamination Protocol

4. Biobank Decontamination Protocol

Safety Considerations

- Wear Gloves, glasses and a labcoat (put in wash bin after processing)
- If a large spill occurs use 'Accidental spill kit' Red or Green components. These products are not disinfectants, so follow by wiping down area with bleach & 80% ethanol.

Waste Disposal

- When working with blood, place bag inside red stand in the processing hood.

Pipette Tips

- Dispose of waste tips in the above waste bag
- When or full or at the end of each day, tape close the waste bag securely and place inside GMO

Blood Tubes/Urine Containers

- After using blood tubes and urine containers recap tightly and place inside waste bag
- When or full or at the end of each day, tape close the waste bag securely and place inside GMO

Liquid Waste

- Pour liquid waste into 250ml, 1L or 2L containers supplied in the store
- Secure lid when ¾ full or at end of the day and place inside GMO bin

GMO bin

- When ¾ or before too heavy to easily manoeuvre, place in designated waste collection area

Decontamination

Biosafety cabinet and benches

- Thoroughly wipe down benches, the biosafety cabinet, pipettes and tip boxes with Decon QUAT (directions for making up on side of the bottle)
- Followed by 80% ethanol
- Turn on UV inside biosafety cabinet for 30 minutes
- Thorough clean benches and biosafety cabinet 2-3 times per year by wiping with neat Povidone-Iodine Surgical Scrub, leave for 10 mins and rinse with 80% ethanol

Centrifuge

- After centrifuging blood tubes remove the lids and buckets and place in the sink
- Make 1:10 dilution of 10% stock of betadine in spray bottle.
- Soak items in diluted betadine solution and leave for 10mins

- Spray inside centrifuge with diluted betadine solution and leave for 10 mins
- Thoroughly rinse centrifuge and buckets with 80% ethanol
- If an spill or break occurs; Autoclave sealed bucket & contents (121C/15 min). Ensure bucket lid loose to allow steam to enter.

QIAsymphony

- Liquid waste as per above
- Removal items as outlined in DNA and RNA extraction protocols; soak in DECON-QAT solution for 15 minutes
- Qiaymsphony outside and inside surfaces wiped down with DECON-QUAT

QIAgility

- To be filled in – no UV capability

PCR Workstation

- Wipe down surfaces, pipettes and tip boxes with 80% ethanol after use
- If using for RNA follow by wipe down with RNAse Zap
- Turn on UV for 15 minutes after use with tip boxes and pipettes inside (UV before also for RNA work)

Note:

Decontamination process to be tested out once before full implementation. Potentially replace diluted betadine solution with Decon QUAT solution and clean as per Qiasymphony.

A5 Project Request Form

Macquarie University
Neurodegenerative Disease
Biobank

Project Request Form

Faculty of Medicine & Health Sciences
F10A Building
2 Technology Place
Macquarie University,
NSW 2109,
Australia

Date of Request:

Project Title:

A. Investigator Information

Principle Investigator

Name	Email

MQ Lab Based Researcher

Name	Email

Additional Investigator

Name	Affiliation	Will samples be sent externally to investigator?

Add extra lines as required

B. Research Project

1. Project Approval

Biobank Project Number *(Include if amending existing application, otherwise for committee use)*	
Biobank HREC Number	5201600387
External Affiliation Ethics Details	
Grant Information and Number	

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Project Request Form

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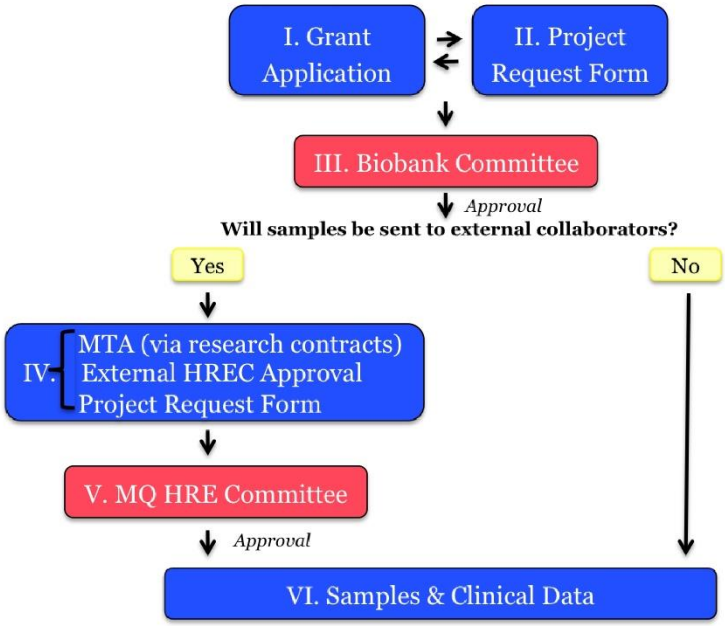
NSW 2109,
Australia

2. Application Process



I. ***Grant Applications:*** *Prior to submitting a grant application for a project that will utilise biobank samples and clinical data researchers must speak to the biobank manager to ensure feasibility of the project in terms of available samples and clinical data. The grant application and project request form (step 2) should be completed in parallel. Pilot studies without related grant applications are also welcomed in which case the application will occur after the pilot provides strong data to support the hypothesis.*

II. ***Project Request Form:*** *The form must be sent to the biobank manager for review and editing. Once the form is completed to a satisfactory standard, the request will be added to the agenda of the next scheduled biobank meeting.*

III. ***Biobank Committee****: The principle investigator and/or main contact on the project form will be invited to the meeting to discuss the project and answer any questions. Following which the committee will come to a formal agreement. Projects are reviewed on scientific merit, and sample and clinical data availability. Researchers should provide power calculations so the biobank can determine availability of samples to allow the project to have adequate statistical power. The review outcome will be either; approval, revision, request for more supporting data or rejection.*

IV. ***Documents for External Collaborations:*** *If the project involves sending samples to external collaborators, additional documents must be provided. The researcher must attain an MTA and a HREC approval letter for each external institution receiving samples. Relevant documents can be provided by biobank manager*

V. ***MQ HREC:*** *The biobank manager will send the project request form, MTA and external site HREC approval letter to MQ HREC for review and approval.*

VI. ***Obtaining Samples and Clinical Data:*** *Once the project is fully approved, biobank samples and clinical data will be given to the main contact on the request form.*

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Neurodegenerative Disease
Biobank
Project Request Form

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NSW 2109,
Australia

3. Project Summary

Please provide a summary with the aims, hypothesis and research plan of the project in which the samples will be used. Include justification for the amount/number/type of samples requested and how they will be used. Please also include proposed dates or whether this is an on-going project. If this is a pilot project please outline requirements for both pilot and full project.

4. Project keywords

Please provide up to four (4) keywords to describe your project (e.g. biomarker, SOD1, exome)

C. Sample Request

1. Criteria for *case* sample selection

Clinical Diagnosis (ALS, PLS etc.)	
Familial or Sporadic	
Gender	
Age (give range or > or <)	
Age of onset (give range or > or <)	
Other	

2. Criteria for *control* sample selection

Gender	
Age	
Other	

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Australia

3. Typical Samples Available

Sample Type	Aliquots	Max no. Aliquots per Collection	Storage
DNA	Variable	1 x stock, 1 x dilution	-30°C
RNA*	Variable	1 x stock	-80°C
Plasma	750µl	10	-80°C
Serum	500µl	6	-80°C
Urine	900µl	5	-80°C
Hair	Strands	Variable	Room Temp.

**Quality Score can be provided – discuss with Biobank staff*

4. Sample type and total number requested

Sample Type	Amount (e.g. ng)	# Cases	# Controls
DNA			
RNA			
Plasma			
Serum			
Urine			
Hair			

5. Clinical Data Information Requested for each sample
Clinical & Demographics

Patient Detail	Require?	Clinical Detail	Require?	Clinical Detail	Require?
UID		Date Of Onset		Date of NIV	
MQID		Date Of Diagnosis		Date of PEG	
DOB		Patient Site Onset		ALSFRSR ID	
Age		Bulbar LMN		*ALS-FRS-Total Score	
DOD		Bulbar LMN		*ALS-FRS-Creation Date	
DOD		Bulbar UMN		*ALS-FRS-Creation Date	
Gender		Cervical LMN		*ALS-FRS-Created By	
Familial ID		Cervical UMN		*ALS-FRS-Notes	
Collection Date		Lumbar LMN		*ALS-FRS not available for all patients	
Patient Type		Lumbar UMN			

Please discuss with biobank staff before completing this section

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Project Request Form

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NSW 2109,
Australia

6. Project Conditions

Project request application conditions are required to ensure efficient running of biobank projects and to comply with conditions of the MQ HREC approval. These conditions include:

	Condition	Project Complies (Yes or No)
I.	A MQ MND group leader must be the principle investigator on the project request.	
II.	A MQ MND group leader must be an investigator on existing or future grant applications relating to this project.	
III.	A staff member of the MQ MND group leader must be named as an additional investigator and in section A of the form, to liaise between biobank staff and the external researcher.	
IV.	A fee will be charged as per current fee schedule available from biobank manager	

7. Acknowledgement

In any publication resulting from use of samples and/or data from the Macquarie Neurodegenerative Disease Biobank, please acknowledge the biobank with the following statement: 'Biospecimens and related clinical data used in this research were obtained from the Macquarie University Neurodegenerative Disease Biobank, Macquarie University, New South Wales, Australia. All publications resulting from samples and/or data should be reported to the Neurodegenerative Disease Biobank.

PI Signature: **Date:**

BIOBANK EXECUTIVE COMMITTEE USE ONLY

Approved by:

Signed: **Date:**

Contact Details
Sarah Furlong PhD
Neurodegenerative Disease Biobank Manager
Faculty of Medicine & Health Sciences |
F10A Building 2 Technology Place Macquarie University,
NSW 2109, Australia
T: | +61 2 9850 2722,
E: | sarah.furlong@mq.edu.au

Chapter 3

BMAA and Neurotoxicity

This section has resulted in the publication of two journal articles.

2017 **NEUROTOXICITY RESEARCH**: NEUROTOXICITY OF THE CYANOTOXIN BMAA THROUGH AXONAL DEGENERATION AND INTECELLULAR SPREADING (ACCEPTED 21 JULY 2017)
Vanessa X. Tan, Benjamin Lassus, Chai K. Lim, Philippe Tixador, Josquin Courte, Alban Bessede, Gilles J. Guillemin & Jean-Michel Peyrin (Accepted 21 July 2017)

2017 **NEUROTOXICITY RESEARCH**: SYNERGISTIC NEUROTOXIC EFFECTS OF THE CYANOTOXINS L-BMAA AND DAB ON PRIMARY CORTICAL NEURONS (ACCEPTED 21 JULY 2017; FOUND IN CHAPTER 4.3)
Vanessa X. Tan (Co-First) and Claire Mazzocco, Bianca Varney, Dominique Bodet, Tristan A. Guillemin, Alban Bessede and Gilles J. Guillemin

Chapter 3: BMAA & Neurotoxicity

3.1 Research gap: Bridging BMAA and Neurotoxicity

Although there have been several studies demonstrating toxicity of BMAA (Dunlop, Cox, Banack, & Rodgers, 2013; Karlsson, Lindquist, Brittebo, & Roman, 2009; Meneely et al., 2016; Rao, Banack, Cox, & Weiss, 2006; Zeevalk & Nicklas, 1989) there exist opposing opinions on the role of BMAA in neurodegenerative diseases, with the fundamental problem of non-physiological quantities of BMAA remains key in this contention. Duncan et al (1990) have shown that washing of cycad flours, a primary carrier of ingested BMAA, removes more than 87% of BMAA found in the flour, and BMAA representing a mere 0.005% in flours by weight, or 50 µg/g of flour tested. The dosage of BMAA in animal and *in vitro* models appear to be considerably larger than possible to ingest through flour, bioaccumulating BMAA in flying foxes, fish, or other marine life, or even direct ingestion of cyanobacteria (Lee & McGeer, 2012).

What is clear is that BMAA is bioavailable, and can cross the BBB (Mark W. Duncan et al., 1991; Xie, Basile, & Mash, 2013), which is crucial when understanding its role in neurodegeneration. Previous studies that prove the neurotoxicity of BMAA investigate end-point outcomes of neurotoxicity, being cellular death, disregarding the roles of synaptic and axonal degeneration. In our study, we present the first investigation on the effects of sub-lethal concentrations of BMAA on synaptotoxicity, somatic and axonal health, and the ability of BMAA to spread transcellularly.

Bibliography

Duncan, M. W., Steele, J. C., Kopin, I. J., & Markey, S. P. (1990). 2-Amino-3-(methylamino)-propanoic acid (BMAA) in cycad flour: an unlikely cause of amyotrophic lateral sclerosis and parkinsonism-dementia of Guam. *Neurology*, *40*(5), 767–72. https://doi.org/10.1212/WNL.40.5.767

Duncan, M. W., Villacreses, N. E., Pearson, O. G., Wyatt, L., Rapoport, S. I., Kopin, I. J., … Smith, Q. R. (1991). 2-Amino-3-(Methylamino)-Propanoic Acid (BMAA) Pharmacokinetics and Blood-Brain Barrier Permeability in the Rat. *The Journal of Pharmacology and Experimental Therapeutics*, *258*(1), 27–35.

Dunlop, R. A., Cox, P. A., Banack, S. A., & Rodgers, K. J. (2013). The Non-Protein Amino Acid BMAA Is Misincorporated into Human Proteins in Place of l-Serine Causing Protein Misfolding and Aggregation. *PLoS ONE*, *8*(9), e75376. https://doi.org/10.1371/journal.pone.0075376

Karlsson, O., Lindquist, N. G., Brittebo, E. B., & Roman, E. (2009). Selective brain uptake and behavioral effects of the cyanobacterial toxin BMAA (??-N-Methylamino-L-alanine) following neonatal administration to rodents. *Toxicological Sciences*, *109*(2), 286–295. https://doi.org/10.1093/toxsci/kfp062

Lee, M., & McGeer, P. L. (2012). Weak BMAA toxicity compares with that of the dietary supplement β-alanine. *Neurobiology of Aging*, *33*(7), 1440–7. https://doi.org/10.1016/j.neurobiolaging.2010.11.024

Meneely, J. P., Chevallier, O. P., Graham, S., Greer, B., Green, B. D., & Elliott, C. T. (2016). β-methylamino-L-alanine (BMAA) is not found in the brains of patients with confirmed Alzheimer's disease. *Scientific Reports*, *6*, 36363. https://doi.org/10.1038/srep36363

Rao, S. D., Banack, S. A., Cox, P. A., & Weiss, J. H. (2006). BMAA selectively injures motor neurons via AMPA/kainate receptor activation. *Experimental Neurology*, *201*(1), 244–252. https://doi.org/10.1016/j.expneurol.2006.04.017

Xie, X., Basile, M., & Mash, D. C. (2013). Cerebral uptake and protein incorporation of cyanobacterial toxin β-N-methylamino-L-alanine. *Neuroreport*, *24*(14), 779–84. https://doi.org/10.1097/WNR.0b013e328363fd89

Zeevalk, G. D., & Nicklas, W. J. (1989). Acute excitotoxicity in chick retina caused by the unusual amino acids BOAA and BMAA: effects of MK-801 and kynurenate. *Neurosci Lett*, *102*(2–3), 284–290.

3.2 Key Results & Implications

Using microfluidic chips, we physically and fluidically separated neuronal somas in the proximal compartment from axons in the distal compartment. This allowed us to investigate the somatic application of lower levels of BMAA, and its axonal toxicity. We established that BMAA at 50 µM did not result in somatic death, but significant axonal degeneration. Using 10 µM of BMAA, we show that BMAA is taken up by first degree neurons in the proximal compartment. Further, we add striatal neurons to the distal compartment, recreating cortico-striatal networks. We showed that in 48h, second degree striatal cells not directly exposed to BMAA show presence of BMAA, indicating a trans-cellular spread of BMAA. This finding is crucial as (1) we demonstrate for the first time, a trans-cellular spread of BMAA, (2) low concentrations of BMAA may not be neurotoxic, however can cause axonal degeneration that may cause functional disruption in neurons.

3.3 Original Research Article: Neurotoxicity of the Cyanobacteria BMAA through Axonal Degeneration and Intercellular Spreading (Under Review)

2017 **NEUROTOXICITY RESEARCH**: NEUROTOXICITY OF THE CYANOTOXIN BMAA THROUGH AXONAL DEGENERATION AND INTECELLULAR SPREADING

Vanessa X. Tan, Benjamin Lassus, Chai K. Lim, Philippe Tixador, Josquin Courte, Alban Bessede, Gilles J. Guillemin & Jean-Michel Peyrin

This paper has been accepted after peer review to Neurotoxicity Research on 21 July 2017.

PT wrote macro used for calculation of fragmentation index, VT, BL assessed synaptic density, JC contributed in part to quantification of neurons. EL assisted with statistical analysis. AB provided BMAA antibody and kit. GJG and JMP assisted with research ideas. All other work is my own.

Abbreviations

ALS	Amyotrophic Lateral Sclerosis
ALS/PDC	Amyotrophic Lateral Sclerosis/Parkinson's dementia complex
BMAA	β-methylamino-L-alanine
CM	Culture Media
CSF	cerebrospinal fluid
DIV	Days *in vitro*
FI	Fragmentation Index
GFAP	Glial Fibrillary Acidic Protein
MAP2	Microtubule associated protein 2
MND	Motor Neuron Disease
MPP	1-methyl-4-phenylpyridinium
NMDA	N-methyl-d-aspartate

Original Research

NEUROTOXICITY OF THE CYANOTOXIN BMAA THROUGH AXONAL DEGENERATION AND INTERCELLULAR SPREADING

List of Authors:

Vanessa X Tan[1,2], Benjamin Lassus[2], Chai K Lim[1], Philippe Tixador[2], Josquin Courte[2], Alban Bessede[3], Gilles J Guillemin[1*] & Jean-Michel Peyrin[2*]

Affiliations:

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Acknowledgements

VXT, JMP, GJG formulated research ideas. VXT conducted the experiments. VXT, BL, PT, and JC contributed to data analyses. Statistical analysis was undertaken by CKL and VXT. VXT, JMP, GJG, CKL, and AB contributed to writing of paper. VXT was funded by Macquarie University Research Excellence Scholarship and Post Graduate Research Funds, and Campus France Eiffel Excellence Scholarship. GJG is funded by the Australian Research Council, Deb Bailey Foundation, MND and Me Foundation, and Macquarie University. JMP is funded by ERANET Neuron 2012. The authors thank Dr Pauline Vaur and Mr Maxime Pennisson for their expert assistance in microdissection.

Competing interests:

AB is the founder and CEO of ImmuSmol, and provided the BMAA antibody and StainPerfect Kit.

Abstract

The cyanotoxin β-methylamino-L-alanine (BMAA) is implicated in neurodegeneration and neurotoxicity. Studies on BMAA neurotoxicity have been using high concentrations that are not physiologically relevant. We use microfluidic chips to investigate the effects of low doses of BMAA applied on neuronal somas. Using a newly developed BMAA antibody, we found that BMAA treatment at 10 µM is taken up by first degree neurons directly exposed to BMAA. Somatic exposure with BMAA at 50 µM did not result in somatic death, but led to a significant axonal degeneration. Within 48h, we detected a trans-cellular spread of BMAA to unexposed second-degree neurons and glial cells, demonstrating the ability of BMAA to spread. These results suggest that even low concentrations of BMAA can mediate axonal degeneration; and the evidence of transcellular spreading indicate a role for BMAA in the dying forward spread of diseases such as amyotrophic lateral sclerosis.

KEYWORDS

BMAA, axonal degeneration, spread, neurotoxic, microfluidics,

Introduction

β-methylamino-L-alanine (BMAA) is a secondary metabolite produced by cyanobacteria, and has been associated with Amyotrophic Lateral Sclerosis/Parkinson's dementia complex (ALS/PDC), and possibly other neurodegenerative diseases such as Alzheimer's and Parkinson's diseases [1], [2]. Increasing evidence points towards BMAA, as a potential contributing factor for sporadic Motor Neuron Disease (MND), or specifically Amyotrophic Lateral Sclerosis (ALS). An association with ALS was first observed in local Chamorro people of Guam, subsequently correlated to increased incidences of ALS/PDC in the Kii peninsular of Japan, and the Irian Jaya of New Guinea [3]–[5]. Fundamentally, these correlations were linked to the use of cycads for nutrition and medicine, and the consumption of flying foxes, which feed on cycad seeds, bioaccumulating BMAA [6]. Studies have shown that BMAA can be adsorbed into the circulatory system in rats [7], and non-human primates [1], [8], and documented transplacental transmission of BMAA [9]. BMAA has also been found in the brains of ALS/PDC patients [6], and has been detected in human cerebrospinal fluid (CSF) samples from both ALS and controls [10]. Scientists and neurologists in USA, France, and Canada have observed a high incidence of sporadic ALS cases in specific areas called "hot spots" [11]–[13]. In Australia, the Riverina is known for its persistent seasonal cyanobacterial blooms in both recreational and drinking water, and appears to have a 15-fold higher incidence than the national average. (Personal communication from Prof Dominic Rowe, Director of the MND clinic at Macquarie University)[14].

In recent years, studies have presented consistent data showing the neurotoxic effects of BMAA in patient tissue, animal models, as well as in culture models studies [6], [10], [15]–[23]. BMAA is associated with various neurotoxic mechanisms such as glutamate excitotoxicity [16], [18], [24], endoplasmic reticulum (ER) stress [20], mitochondrial dysfunction [18], oxidative damage [20], [22], and toxic protein aggregation [25] resulting in motor deficits, neuronal degeneration, and somatic death [8], [23], [26], [27]. Furthermore, the selective toxicity of BMAA towards motor neurons and pyramidal cells both *in vitro* and *in vivo* highlights the potential causal role of BMAA in ALS [15], [22], [27]. However, toxicological studies have been inconsistent with variability depending on model used, with studies focusing on mechanisms and cellular death [15], [19]–[21]. While apoptosis is an

important mechanism for neuronal death in the late-onset of several brain diseases including ALS [28], [29], it is becoming increasingly clear that cellular death is an endpoint, and better understanding of neuronal sufferance is required to interfere with the degeneration process.

Axonal and synaptic degeneration are critical events in neuronal degeneration processes [30]–[33], and the molecular pathways involved remain elusive [34]. In particular, the long axonal projections of motor neurons render them more susceptible than other neuronal cells to local and repetitive injuries [35]. In progressive neurodegenerative diseases such as ALS [36], axonal degeneration often proceeds through a protracted dying-back pattern in which dysfunction of nerve terminals precedes neuronal cell body destruction [35], [37], [38], and axonal degeneration is a known clinical and pathological feature of ALS [39]. Indeed, various studies have shown that ALS progresses spatially, and involves sequences of axonal shrinkage [40], muscle denervation [39], the loss of motor units; which in SOD1 mice, are present months before clinical symptoms appear [41], and a gradient of axonal pathology increasing from proximal to the distal nerves [42]. Other studies however, support a dying forward hypothesis during which pre-synaptic dysfunction leads to trans-synaptic damage to connected neurons [43]. Interestingly, recent neuropathological evidence indicates that ALS neuropathological hallmarks spreads along neural pathways in the brain. In line with these observations, recent studies shows that proteins such as SOD1, TDP-43 or can behave as prion like elements spreading from neuron to neuron [44]–[46], thus raising the possibility that toxic proteins disseminating along neuronal networks may hamper their robustness. Regardless of the mechanism of dying backward or forward, the preservation of neuronal cell bodies has been shown not to improve the symptoms or the lifespan of patients [39], nor aid axonal heath [47]. Furthermore, both dying forward and dying back processes triggers neuronal network disconnection and progressive network collapse. The present study thus investigates the neurotoxicity of L-BMAA using a novel microfluidic device [48], that allows distinct observations of toxicity in neuronal subcompartments of somas, and axons and synapses.

Here, using rodent derived neuronal networks reconstructed in microfluidic environments [48], [49] we investigate whether low doses of BMAA triggers progressive neuronal networks collapse. We therefore characterize two effects of BMAA toxicity on primary culture of fully differentiated cortico-striatal networks. Firstly, the transneuronal spread of BMAA from

neuron to neuron; second, if BMAA has a role in causing Wallerian degeneration in neurons through assessing axonal degeneration of neuronal soma treated with BMAA.

Materials and Methods

Primary culture in microfluidic chips

Animal ethics was approved by the C2EA - 05 Comité d'éthique en expérimentation animale Charles Darwin. Microfluidic chips were fabricated as in [48]. Briefly, two cell culture chambers (proximal and distal) each connected to two reservoirs are separated by narrowing 15µm - 3µm tapered micro channels (**Fig. 1**). A middle micro channel connected to a separate pair of reservoirs perpendicularly transverses the separating microchannels, maintaining fluidic pressure on both chambers. E14 Swiss mice (Janvier, Le Genest-Saint-Isle, France) were sacrificed and dissected in PBS-Glucose (PBS without calcium or magnesium, supplemented with 0.1% Glucose). Cortices and ganglionic eminences were micro dissected and washed gently twice in fresh PBS-Glucose before digestion with papain or trypsin respectively in DMEM with DNase, and mechanically dissociated. Cortical cells were seeded at 5.1 x 10^4 cells in the proximal chambers, and striatal cells at 1.36 x 10^4 on the distal chambers to create cortico-striatal networks. Cells were cultured in DMEM supplemented with 5% FCS, 1% N2, 2% B27, 1% Pen/Strep. Culture media was renewed every 3-5 days.

Pharmacological treatment

Ten to 15 days post seeding, microfluidic chips were screened by phase contrast microscopy before treatment. Cultures were selected for somatic survivals of >90%, <80% of axons <600µm long with <20% blebbing. To maintain fluidic isolation, hydrostatic pressure was created by over-pressurizing the untreated distal chamber. BMAA was diluted to final concentrations in CM, and all media refreshed before treatment.

Immunostaining

Cells were washed twice with PBS and fixed in 4% PFA-sucrose. Cells were permeabilized with 0.2% TritonX-1% BSA in 1x PBS. Primary antibodies were added in 1% BSA in PBS and incubated overnight at 4°C. Cells were rinsed in PBS then incubated with corresponding secondary antibodies if required. Primary antibodies include βIII-Tubulin (mouse monoclonal 1:500, Sigma), Microtubule Associated Protein-2 (mouse MAP2, 1:500, Sigma; Rabbit MAP2, 1:500, Millipore), Glial associated fibrillary protein (mouse GFAP, 1:500, Sigma Aldrich).

Secondary antibodies (1:2000, ThermoFisher Scientific) coupled to Alexa 350, 488, or 555 were used to visualize primary antibodies, and DAPI, when Alexa 350 was not used. For BMAA staining, cells were fixed and stained according to StainPerfect Immunostaining Kit A (ImmuSmol, Pessac, France). BMAA antibody (ImmuSmol) was used at 1:1000, and detected with the corresponding secondary antibody. Images were acquired with an Axio-observer Z1 (Zeiss, Stuttgart, Germany) fitted with a cooled CCD camera (CoolsnapHQ2, Ropert Scientific). The microscope was controlled with MetaMorph software (Molecular Devices, CA, USA) and images were analysed using Fiji software (ImageJ, US National Institutes of Health, Bethesda, Maryland, USA).

BMAA uptake in cortical cell cultures in microfluidic platform

To determine if BMAA is taken into cortical cells, 100 µM BMAA was applied to mature cultures for 48h, before being fixed and stained for BMAA and DAPI. Results were derived from at least six images per chamber, and three independent experiments conducted in triplicates.

Quantifying axonal degeneration and neuronal survival

To determine the effect of somatic application of BMAA on somatic death and axonal health, cortical cells were seeded in only the proximal chamber, and axons were allowed to grow across into the distal chamber as previously described [50]. Ten to 12 days post seeding, cells were treated with various concentrations of BMAA (0-1500µM), and 1000nM N-methyl-d-aspartate (NMDA) as a positive control, in only the proximal chamber for 48h, and subsequently fixed and stained with βIII-tubulin (axons) and Hoechst (cell nuclei). As previously described [51], healthy neurons were characterized by low percentage of condensed nuclei in the proximal chamber, as well as homogenous and continuous axons in the distal chamber, while blebbed axons exhibit fragmentation. Briefly, axonal fragmentation was quantified by applying the Otsu Threshold algorithm, calculating the ratio of the area of circular, fragmented axons (Analyse Particle function), against the area of continuous tubular axons (Tubeness function). Results were derived from at least six images per chamber, and at least three independent experiments conducted in triplicates. Results were assessed using repeated measures, one-way ANOVA, GraphPad Prism (GraphPad Software, Inc., CA, USA).

Transcellular transmission of BMAA in reconstructed cortico-striatal networks

To show anterograde transmission of BMAA, proximal chambers of cortico-striatal cultures were exposed to BMAA for 48h, fixed and stained for BMAA, and MAP2 or GFAP. At least six images per distal chamber were taken of second-order recipient striatal cells, each treatment done in a minimum of triplicates, in at least three independent experiments. Images were similarly processed using ImageJ, by fixing the threshold intensity in control images, and applying to all images. Total number of MAP2 or GFAP positive cells were enumerated, and compared to the number of BMAA and MAP2, or GFAP positive cells as a percentage. Microfluidic devices were over-pressured to create a pressure gradient across the three chambers to ensure any possible fluid exchange would be unidirectional. Chambers were filled with increasing amounts of media in each corresponding reservoir: 30 µL, 40 µl, 50 µL (Proximal, middle, distal). Results were analysed using one way ANOVA, GraphPad Prism.

Results

BMAA is structurally related to amino acids and has been proposed to triggers progressive neuronal death at high (mM) doses both *in vivo* and *in vitro*. While most of the previously published studies focused on neuronal death in our study we focused on the effect of low (µM) doses of BMAA on network related dysfunctions that are known to occur before neuronal soma destruction.

Primary culture models in microfluidics

Microfluidic chips allow for the physical and fluidic separation of neuronal subcompartments (**Fig. 1**). Neuronal somas are contained in the proximal chamber through size exclusion **(Fig. 1 a, g**), and axons allowed to grow through microchannels into the middle chamber (Fig 1 c), through to the distal chamber (**Fig. 1 e**). Distal chambers are used to assess axonal integrity (**Fig. 1 iii, h**), or to reconstruct oriented cortico-striatal networks (**Fig. 1 iv, j**). Fluidic isolation is maintained by the blockage of microchannels by neuronal axons, and creation of a pressure gradient in each chamber reservoir (**Fig. 1 b, d, f**). we have previously shown that after 15 days-*in vitro*, these networks are fully differentiated, and synaptically connected [52].

BMAA uptake by cortical cells

First, using a newly developed anti BMAA antibody (ImmuSmol, France) we determined the uptake of BMAA by cortical neurons grown in compartmentalized microfluidic chambers. Cortical cells in the proximal chamber were qualitatively assessed for their ability to take up BMAA into the cell body. For this, cortical neurons were exposed to 100 µM of BMAA. 48 hours post exposure; the somato-dendritic uptake of BMAA was evidenced by BMAA staining of the cortical cell chamber. Results show that BMAA is present in a subpopulation of cortical cell bodies, suggesting selectivity in uptake of BMAA by neuronal subtypes (**Fig. 2**). **Fig. 3** show intact axons stained with β3 Tubulin in the control, while treatment with 10 or 100 µM BMAA results in low-level fragmentation and blebbing in cortical axons, suggesting a potential deleterious effect of BMAA.

Sub-lethal concentrations of BMAA are neurotoxic to axons but not cell somas

To elucidate the effects of BMAA on neuronal subcompartments, BMAA was administered only to the proximal (somatic) chamber of mouse cortical neurons compartmentalized in a microfluidic environment. This allows for somato-dendritic exposure of cortical cells, leaving axons in distal chambers untreated (**Fig. 1 iii**). Using similar approaches, we have previously shown that somatic application of mild pro-apoptotic insults and beta amyloids triggers early and specific axonal degeneration [49], [50]. The percentage of nuclear condensation in cortical neurons was assessed as a measure of somatic health, with cells displaying nuclear morphology of condensed chromatin and intense DAPI staining with small nuclear size in cells indicative of the initiation of cell death processes. Cell death as observed by nuclear condensation was an overall significant, taking into consideration concentrations tested from 1-1500 µM (Repeated Measures, one-way ANOVA $p<0.001$, $R^2=0.8594$). Average percentage of nuclei condensation for untreated cells was 13%, and in BMAA treated cells ranged from 12-30%, and NMDA treated cells were 41% condensed. However, it is not significant for treatments less than 500 µM, and has an increasing significant effect at 500, 1000, and 1500 µM (**Fig. 4 a-g, o; Suppl. Table 1**).

Axonal health was assessed via analysis of axonal fragmentation index (FI) that determines continuous (tubular) axons as healthy, as a ratio to fragmented (particles) injured axons (**Fig. 4**). Results show that untreated cells had an FI of 0.304 (**Fig. 4 h**), and 1 µM BMAA had an FI of 0.342 (**Fig. 4 i**). BMAA treatments at 50, 500, 1000, and 1500 µM resulted in significant changes in FI to 0.392, 0.473, 0.495, and 0.555 respectively (Repeated Measures, one-way ANOVA $p<0.0001$, $R=0.9722$) (**Fig. 4 j-m, p; Suppl. Table 2**). Positive fragmentation control of NMDA treatment resulted in a FI of 0.972 ($p<0.0001$) (**Fig. 4 n**). A dose-dependent response can be observed with increases in axonal fragmentation as the concentration of BMAA increases from 50 -1500 µM, and is assessed by the Post-test for linear trend to be linear ($p<0.001$).

Transcellular spread of BMAA to second-order cells

To determine if BMAA can be passed from one neuron to another, we used the cortico-striatal network platform (**Fig. 1 iv**). We expose first-order cortical neurons of a days-*in vitro* 15 cortico-striatal network to 10 and 100 µM BMAA, and observe BMAA signals in second-order striatal neurons and astrocytes in the distal chamber. Results were derived from at least six images per chamber, and at least three independent experiments conducted in triplicates.

In the distal chamber, neuronal cells were labelled with MAP2 and DAPI, neuronal uptake of BMAA was defined by the presence of BMAA and MAP2 in the same cell, expressed as a percentage of total number of MAP2 positive cells (**Fig. 5 a**). Treatment with BMAA at 10 and 100 µM resulted in 14 and 62% of neurons (respectively) positive for both MAP2 and DAPI. This has a significance of $p>0.0014$ for a positive presence of BMAA in the striatal cell population of the BMAA treated cortical cells, with multiplicity adjusted p values of >0.163 and 0.001 for 10 and 100 µM respectively (**Fig. 5 b, Suppl. Table 3**).

Similarly, astrocytic uptake of BMAA was measured by the presence of GFAP and BMAA positive cells, expressed as a percentage of total GFAP positive cells. In GFAP expressing cells, we observed less BMAA infected cells with 12 and 23% of the cells positive for BMAA ($p<0.0589$), and multiplicity adjusted p values of 0.233 and 0.041 for 10 and 100 µM respectively (**Fig. 6, Suppl. Table 4**).

To confirm the fluidic isolation capabilities of the chambers, distal and middle chambers were over-pressurised with larger volumes of media in media reservoirs (**Fig. 1 d, f**), creating hydraulic resistance. Additionally, axons obstruct the microchannels, further limiting molecular diffusion between the proximal and distal chambers [51], [53]. With over-pressurizing, any possible fluid exchange would flow from distal towards the proximal chamber, thereby preventing flow of BMAA containing media in the proximal chamber towards the distal and middle chambers. Notwithstanding hydraulic resistance, BMAA is consistently observed to be present in the distal chambers (**Suppl. Fig. 1**), resulting in the conclusion that BMAA can indeed be passed from cell to cell, and is not an artefact of pressure driven movement. These results indicate that low doses of BMAA can be efficiently spread

from neuron to neuron and to astroglial cells, and that increasing amounts of BMAA leads to neuronal network collapse.

Discussion

This is the first study that 1) use sub-lethal doses of BMAA to observe changes in neuronal subcompartments; 2) investigates the effects of BMAA before cells start to die; and 3) investigate intercellular transmission of BMAA (**Fig. 8**). Our results are in line with notions showing that ALS related disorders spreads along neuronal pathways and support the notion that BMAA may initiates neuronal networks collapse. The outcomes of this study provide an intermediate between the conflicting views on the role of BMAA in neurodegeneration and neurodegenerative diseases.

Although BMAA has been suspected to be a causal agent for ALS for the last 40 years (Spencer, et al.), it has not been concretely linked to ALS. There are several rationales for the rejection of BMAA having a role in ALS. First, the ubiquitous distribution of BMAA producing cyanobacteria [54]–[56]. Secondly, the washing processes before consumption of cycad seeds results in the removal of a large amounts of BMAA [55]. Lastly, the pathophysiological concentrations of BMAA crossing the BBB, or in the body required to cause damage *in vitro* or in *in vivo* models [26], [57]–[59]. The neurotoxicity of BMAA has been demonstrated repeatedly on neuronal cell lines, primary cultures of rodent cells, and *in vivo* studies [6], [15]–[22].

Use of microfluidics in investigating BMAA uptake in cortical cells

Microfluidic devices represent a unique tool to study and characterise the cellular mechanisms induced by neurotoxins between cellular sub-compartments (**Fig. 1**). They can be used to assess therapeutic strategies and allow discovery and application of multipronged drugs that target not only cell death, but also axonal degeneration to repair or reduce neurotoxic damages [60]. We show here the application of a new BMAA antibody (ImmuSmol, France) in detecting intracellular BMAA on the microfluidic platform (**Fig. 2**). Murine cortical cells stain positive for BMAA when treated with 100µM of BMAA for 48h. In untreated cells, little axonal blebbing or degeneration is observed, axons are tubular and intact, indicating good health. However, in treated cells, we observe increased axonal blebbing coupled with nuclear condensation, signs of cellular stress (**Fig. 3**).

BMAA causes Wallerian-like degeneration

To further investigate the degeneration observed in cortical cells, cortical cultures (**Fig 1 iii**) was used to determine the effects of somatic application of BMAA on axons. We found that low-dose BMAA (50 µM) can cause a Wallerian-like degeneration process; damages that do not result in extensive somatic death, but cause axonal fragmentation (**Fig. 4**). We also observed a dose-dependent effect on the somatic health of neurons, with a significant increase in cell death starting at 500 µM of BMAA (**Fig. 4 a-g, o**). NMDA treatment was used as positive control (**Fig 4 g**). Concordant with our results, Lobner et al [16] found doses less than 1 mM of BMAA cause only slight neuronal death in mixed murine cortical cells, and showed that BMAA at 10 µM can potentiate β-Amyloid or 1-methyl-4-phenylpyridinium (MPP+) mediated neuronal damages. Similarly, Weiss and Choi reported that concentrations of BMAA of less than 300 µM induces limited cortical neuronal loss [26]. Furthermore, two studies reported that BMAA at 30µm cause specifically motor neuron death, and only limited injury to CA1 pyramidal neurons [1], [15], supporting the potential roles of BMAA in ALS neuropathology. Additionally, this study was conducted without adding bicarbonate, where studies show addition of bicarbonate during treatment results in increased cellular toxicity of [24], [61].

Axonal degeneration is evident in BMAA treated cells, as demonstrated by observations of increased blebbing and axonal fragmentation (**Fig. 3, 4 h-m**). Axonal fragmentation significantly increases with concentrations of BMAA from as low as 50 µM (**Fig. 4 p**). During the neurodegenerative processes, neuronal cell bodies are clearly affected; however, there is increasing evidence that axonal damage are more severe, and are crucial for the development and progression of the diseases. By demonstrating the preservation of cell body but not restoration of function, several studies have shown that rescuing only cell bodies is not sufficient to alter the course of diseases [31], [35], [47]. Additionally, considering the long distances in which motor neuron axons span, these cells are far more vulnerable to axonal insults and degeneration that would result in the characteristic dying back of distal regions of the axons in Wallerian-like degeneration. In ALS patients, this may explain the early asymptomatic changes occur before clinical symptoms [41], [62], [63], which is also consistent with results obtained with animal models [31], [37].

Although the treatment of BMAA was short and with sub-lethal doses, we observed a clear and significant changes in axonal integrity. This is conceivably more in line with the amounts

of BMAA that may be ingested via cycads or through the consumption of flying foxes that have biomagnified BMAA levels, but is not in the scope of this study [3], [64], [65]. Evidently, further studies are required to clarify the bioavailability and concentrations of BMAA ingested via consumption of contained food with in either the free or bound forms of BMAA [6]. Cumulatively, our results showing limited cell death and a clear decline in axonal integrity provide evidence that low-dose BMAA is toxic for primary neurons, contributing to axonal degeneration and withdrawal of support from a diseased cell body, recapitulating a dying-back pattern as observed in Wallerian-like and compartmentalized degeneration reminiscent of network disconnection [66], [67].

Transcellular spreading of BMAA

To understand the effect of BMAA at a network level, we use the cortico-striatal networks to observe if BMAA can be transmitted from cell-to-cell. We believe this is the first study that investigates BMAA spreading. The presence of BMAA in second order neurons and astrocytes (**Fig. 5 a, 6 a**) indicate anterograde axonal transport and transmission of BMAA in a dose-dependent manner, which has been shown in prion-like proteins such as α-synuclein and SOD-1, associated with neurodegenerative diseases [68], [69]. This is anticipated to occur in cells linked through synapses, or nanotubes, or via vesicles such as exosomes or microparticles [70]–[72]. In neurons, this transmission of BMAA is of particular interest as this could represent a mechanism for the focal neuroanatomical spread of neurodegenerative pathologies such as in ALS [73], [74].

The role of astroglial cells in neurodegenerative disease is well known [75]–[78], and the presence of BMAA in astrocytes may be attributed to its role in regulation of amino acids and neurotransmitters, thus, explaining the presence of BMAA in astrocytes in the untreated chamber (**Fig. 6**) [75], [76], [79]. Furthermore, a study showed that astrocytes infected with prions have increased neurotoxicity in co-culture with neurons [80].

There are two possible explanations for the partial spreading of BMAA to the receiving second-order neurons in the distal chamber. First, BMAA may only contaminate second-order cells that are functionally connected to a neuron containing BMAA. Trans-neuronal spreading could thus rely on efficient synaptic connections, as proposed for Tau protein, or unknown limiting mechanisms. Importantly, BMAA triggering early axonal degeneration thus may

modify BMAA trans-neuronal spreading. Second, specific cell susceptibility, or a specific receptor regulating the uptake of BMAA into the cell. This is supported by earlier studies showing that BMAA is selectively taken up by specific neuronal cell subtypes such as motor and NADPH-diaphorase neurons, whereas higher degree cortical neurons display only a limited BMAA uptake and loss [9], [22], [26], [81]. In terms of cytotoxicity, it is important to highlight that BMAA was detected mostly in cell somas, however, exerted a stronger effect on neuronal axons rather than the cell body (**Fig. 4, 5 a**). BMAA is present in some axons, but appear to be absent in others as they may be below detectable limits of the technique.

Proposed mechanism of BMAA-mediated neurodegeneration and spreading

The excitotoxic mechanism triggered by BMAA is well described [21], [22], [26], [61], [82], and is believed to be initiated through the activation of both NMDA and non-NMDA receptors, leading to calcium increases, cell depolarisation and also production of reactive oxygen species [16], [22], [24], [26]. As mentioned, another cytotoxic effect of BMAA was identified by Dunlop *et al* [25], observing BMAA misincorporation into proteins, an important and new mechanism associated with neurodegenerative diseases.

Although this was not formally linked to the later observation, another recent study showed that BMAA promotes abnormal Tau phosphorylation and aggregation in hippocampal neurons [1]. It is thus tempting to speculate that misintegration of BMAA may trigger the misfolding of structural proteins that disrupt the cellular transport, which is crucial for the routing of trophic factors and nutrients to the axons. This will affect neuronal homeostasis leading to the destabilization of the cytoskeleton and cell signalling resulting in progressive functional impairments. and ultimately axonal degeneration and somatic loss. This corresponds to a Wallerian-like degeneration model. Additionally, formation of aberrant proteins or structures may also result in nucleation, the initiating process required in prion-like propagation, thereby imposing its aberrant structure on other normal proteins. The observation of intercellular spreading of BMAA to other cells not only results in an amplification of cytotoxicity, but also clinically reflects the focal spread of neurodegeneration in ALS. However, more work needs to be done to understand the processes by which BMAA spreads transcellularly.

Is BMAA involved in the development of ALS?

The results of this study show that only a low amount of BMAA, not previously considered as cytotoxic, can induce alter neuronal integrity. BMAA triggers a Wallerian-like degeneration in neurons, and cellular transmission of BMAA between cells indicate a transcellular transmission of BMAA (**Fig. 8**). Together with other factors such as genetics, environmental triggers, lifestyle, physical trauma, and ageing [83], [84], our results indicate that BMAA contributes to a complex interplay of accumulating factors in the multiple hit hypothesis in ALS [83]. A delay or inhibition of axonal degeneration may alleviate clinical symptoms of neurodegeneration, and further research into the capability of BMAA to trigger a prion-like phenomenon, and its roles in the disturbance of axonal transportation and degeneration may pave the road for better understanding of ALS mechanisms.

References

[1] **Paul Alan Cox, David A. Davis, Deborah C. Mash, JS. Metcalf, and Sandra Anne Banack**, "Dietary exposure to the environmental toxin b - N -methylamino- L -alanine triggers neurofibrillary tangles and amyloid deposits in the brain," *Proc. Biol. Sci.*, vol. 283, no. 1823, pp. 979–980, Jan. 2016.

[2] **J. Pablo, S. A. Banack, P. A. Cox, T. E. Johnson, S. Papapetropoulos, W. G. Bradley, A. Buck, and D. C. Mash**, "Cyanobacterial neurotoxin BMAA in ALS and Alzheimer's disease," *Acta Neurol. Scand.*, vol. 120, no. 4, pp. 216–225, Oct. 2009.

[3] **PeterS. Spencer, ValerieS. Palmer, Adam Herman, and Ahmed Asmedi H**, "CYCAD USE AND MOTOR NEURONE DISEASE IN IRIAN JAYA," *The Lancet*, vol. 330, no. 8570. pp. 1273–1274, 1987.

[4] **Peter S. Spencer, Masayuki Ohta, and Valerie S. Palmer**, "CYCAD USE AND MOTOR NEURONE DISEASE IN KII PENINSULA OF JAPAN," *The Lancet*, vol. 330, no. 8573. Elsevier, pp. 1462–1463, 1987.

[5] **P. S. Spencer, P. B. Nunn, J. Hugon, a C. Ludolph, S. M. Ross, D. N. Roy, and R. C. Robertson**, "Guam amyotrophic lateral sclerosis-parkinsonism-dementia linked to a plant excitant neurotoxin.," *Science*, vol. 237, no. 4814, pp. 517–522, 1987.

[6] **Susan J. Murch, Paul Alan Cox, and Sandra Anne Banack**, "A mechanism for slow release of biomagnified cyanobacterial neurotoxins and neurodegenerative disease in Guam.," *Proc. Natl. Acad. Sci. U. S. A.*, vol. 101, no. 33, pp. 12228–31, Aug. 2004.

[7] **Mark W. Duncan, Nelly E. Villacreses, Ohilip G. Pearson, Lance Wyatt, Stanley I. Rapoport, Irwin J. Kopin, Sanford P. Markey, and Quentin R. Smith**, "2-Amino-3-(Methylamino)-Propanoic Acid (BMAA) Pharmacokinetics and Blood-Brain Barrier Permeability in the Rat," *J. Pharmacol. Exp. Ther.*, vol. 258, no. 1, pp. 27–35, 1991.

[8] **Mark W. Duncan, Sanford P. Markey, Barton G. Weick, Philip G. Pearson, Herman Ziffer, Yulin Hu, and Irwin J. Kopin**, "2-Amino-3-(methylamino) Propanoic acid (BMAA) Bioavailability in the Primate," *Neurobiol. Aging*, vol. 13, no. 2, pp. 333–337, 1992.

[9] **Oskar Karlsson, Nils Gunnar Lindquist, Eva B. Brittebo, and Erika Roman**, "Selective brain uptake and behavioral effects of the cyanobacterial toxin BMAA (??-N-Methylamino-L-alanine) following neonatal administration to rodents," *Toxicol. Sci.*, vol. 109, no. 2, pp. 286–295, Jun. 2009.

[10] **L. Berntzon, L. O. Ronnevi, B. Bergman, and J. Eriksson**, *Detection of BMAA in the human central nervous system*, vol. 292. 2015.

[11] **Nathan Torbick, Sarah Hession, Elijah Stommel, and Tracie Caller**, "Mapping amyotrophic lateral sclerosis lake risk factors across northern New England," *Int J Heal. Geogr*, vol. 13, p. 1, Jan. 2014.

[12] **Estelle Masseret, Sandra Banack, Farid Boumédiène, Eric Abadie, Luc Brient, Fabrice Pernet 6¤, Raoul Juntas-Morales, Nicholas Nicolas Pageot, James Metcalf, Paul Cox, William Camu, A. Vaquer, Raul Morales, Nicholas Nicolas Pageot, James Metcalf, Paul Cox, William Camu, Fabrice Pernet, Raoul Juntas-Morales, Nicholas Nicolas Pageot, James Metcalf, Paul Cox,**

and William Camu, "Dietary BMAA Exposure in an Amyotrophic Lateral Sclerosis Cluster from Southern France," *PLoS One*, vol. 8, no. 12, p. e83406, Dec. 2013.

[13] **Richard H. Becker, Mohamed I. Sultan, Gregory L. Boyer, Michael R. Twiss, and Elizabeth Konopko**, "Mapping cyanobacterial blooms in the Great Lakes using MODIS," *J. Great Lakes Res.*, vol. 35, no. 3, pp. 447–453, Sep. 2009.

[14] **Sean Parnell**, "Algal bloom linked to motor neurone disease," *The Australian*, 2015.

[15] **Eric J. Buenz, and Charles L. Howe**, "Beta-methylamino-alanine (BMAA) injures hippocampal neurons in vivo," *Neurotoxicology*, vol. 28, no. 3, pp. 702–704, 2007.

[16] **Doug Lobner, Peachy Mae T. Piana, Abed K. Salous, and Robert W. Peoples**, "Beta-N-methylamino-L-alanine enhances neurotoxicity through multiple mechanisms.," *Neurobiol. Dis.*, vol. 25, no. 2, pp. 360–6, 2007.

[17] **Isaac M. Chiu, Emiko T. A. Morimoto, Hani Goodarzi, Jennifer T. Liao, Sean O'Keeffe, Hemali P. Phatnani, Michael Muratet, Michael C. Carroll, Shawn Levy, Saeed Tavazoie, Richard M. Myers, and Tom Maniatis**, "A Neurodegeneration-Specific Gene-Expression Signature of Acutely Isolated Microglia from an Amyotrophic Lateral Sclerosis Mouse Model," *Cell Rep.*, vol. 4, no. 2, pp. 385–401, 2013.

[18] **Alexander S. Chiu, Michelle M. Gehringer, Nady Braidy, Gilles J. Guillemin, Jeffrey H. Welch, and Brett a. Neilan**, "Excitotoxic potential of the cyanotoxin β-methyl-amino-l-alanine (BMAA) in primary human neurons," *Toxicon*, vol. 60, no. 6, pp. 1159–1165, 2012.

[19] **Oskar Karlsson, Erika Roman, Anna-Lena Berg, and Eva B. Brittebo**, "Early hippocampal cell death, and late learning and memory deficits in rats exposed to the environmental toxin BMAA (β-N-methylamino-l-alanine) during the neonatal period," *Behav. Brain Res.*, vol. 219, no. 2, pp. 310–320, 2011.

[20] **Oliver Okle, Kerstin Stemmer, Ulrich Deschl, and Daniel R. Dietrich**, "L-BMAA induced ER stress and enhanced caspase 12 cleavage in human neuroblastoma SH-SY5Y cells at low nonexcitotoxic concentrations.," *Toxicol. Sci.*, vol. 131, no. 1, pp. 217–24, Jan. 2013.

[21] **J. H. Weiss, and D. W. Choi**, "Beta-N-methylamino-L-alanine neurotoxicity: requirement for bicarbonate as a cofactor.," *Science*, vol. 241, no. 4868, pp. 973–5, Aug. 1988.

[22] **Shyam D. Rao, Sandra Anne Banack, Paul Alan Cox, and John H. Weiss**, "BMAA selectively injures motor neurons via AMPA/kainate receptor activation," *Exp. Neurol.*, vol. 201, no. 1, pp. 244–252, 2006.

[23] **M. D. Lewis, I. N. McQueen, and M. F. Scanlon**, "Motor neurone disease serum and beta-N-methylamino-L-alanine stimulate thyrotrophin-releasing hormone production by cultured brain cells.," *Brain Res.*, vol. 537, no. 1–2, pp. 251–255, 1990.

[24] **Delia M. Brownson, Tom J. Mabry, and Steven W. Leslie**, "The cycad neurotoxic amino acid, ß-N-methylamino-l-alanine (BMAA), elevates intracellular calcium levels in dissociated rat brain cells," *J. Ethnopharmacol.*, vol. 82, no. 2, pp. 159–167, 2002.

[25] **Rachael Anne Dunlop, Paul Alan Cox, Sandra Anne Banack, and Kenneth John Rodgers**, "The Non-Protein Amino Acid BMAA Is Misincorporated into Human Proteins in Place of l-Serine Causing Protein Misfolding and Aggregation," *PLoS One*, vol. 8, no. 9, p. e75376, 2013.

[26] **J. H. Weiss, J. Y. Koh, and D. W. Choi**, "Neurotoxicity of beta-N-methylamino-L-alanine (BMAA) and beta-N-oxalylamino-L-alanine (BOAA) on cultured cortical neurons.," *Brain Res.*, vol. 497, no. 1, pp. 64–71, Sep. 1989.

[27] **Oskar Karlsson, Anna-Lena Berg, Jörg Hanrieder, Gunnel Arnerup, Anna-Karin Lindström, and Eva B. Brittebo**, "Intracellular fibril formation, calcification, and enrichment of chaperones, cytoskeletal, and intermediate filament proteins in the adult hippocampus CA1 following neonatal exposure to the nonprotein amino acid BMAA," *Arch. Toxicol.*, vol. 89, no. 3, pp. 423–436, 2015.

[28] **Gregorio Valdez, Juan C. Tapia, Jeff W. Lichtman, Michael a Fox, and Joshua R. Sanes**, "Shared resistance to aging and ALS in neuromuscular junctions of specific muscles.," *PLoS One*, vol. 7, no. 4, p. e34640, Jan. 2012.

[29] **Katie Moisse, Jennifer Mepham, Kathryn Volkening, Ian Welch, Tracy Hill, and Michael J. Strong**, "Cytosolic TDP-43 expression following axotomy is associated with caspase 3 activation in NFL-/- mice: support for a role for TDP-43 in the physiological response to neuronal injury.," *Brain Res.*, vol. 1296, pp. 176–86, Nov. 2009.

[30] **Dick Jaarsma, Elize D. Haasdijk, J. a. C. Grashorn, Richard Hawkins, Wim van Duijn, Hein W. Verspaget, Jacqueline London, and Jan C. Holstege**, "Human Cu/Zn Superoxide Dismutase (SOD1) Overexpression in Mice Causes Mitochondrial Vacuolization, Axonal Degeneration, and Premature Motoneuron Death and Accelerates Motoneuron Disease in Mice Expressing a Familial Amyotrophic Lateral Sclerosis Mutant SO," *Neurobiol. Dis.*, vol. 7, no. 6, pp. 623–643, 2000.

[31] **Lindsey R. Fischer, Deborah G. Culver, Philip Tennant, Albert a. Davis, Minsheng Wang, Amilcar Castellano-Sanchez, Jaffar Khan, Meraida a. Polak, and Jonathan D. Glass**, "Amyotrophic lateral sclerosis is a distal axonopathy: evidence in mice and man," *Exp. Neurol.*, vol. 185, no. 2, pp. 232–240, Feb. 2004.

[32] **Michael Coleman**, "Axon degeneration mechanisms: commonality amid diversity," *Nat. Rev. Neurosci.*, vol. 6, no. 11, pp. 889–898, 2005.

[33] **Martin C. Raff, Alan V Whitmore, and John T. Finn**, "Axonal self-destruction and neurodegeneration.," *Science*, vol. 296, no. 5569, pp. 868–71, May 2002.

[34] **Vinay K. Godena, Giulia Romano, Maurizio Romano, Chiara Appocher, Raffaella Klima, Emanuele Buratti, Francisco E. Baralle, and Fabian Feiguin**, "TDP-43 regulates Drosophila neuromuscular junctions growth by modulating Futsch/MAP1B levels and synaptic microtubules organization.," *PLoS One*, vol. 6, no. 3, p. e17808, Jan. 2011.

[35] **Michal Dadon-Nachum, Eldad Melamed, and Daniel Offen**, "The 'dying-back' phenomenon of motor neurons in ALS.," *J. Mol. Neurosci.*, vol. 43, no. 3, pp. 470–7, Mar. 2011.

[36] **Luc Dupuis, and Jean-Philippe Loeffler**, "Neuromuscular junction destruction during amyotrophic lateral sclerosis: insights from transgenic models.," *Curr. Opin. Pharmacol.*, vol. 9, no. 3, pp. 341–6, Jun. 2009.

[37] **Hisashi Narai, Yasuhiro Manabe, Makiko Nagai, Isao Nagano, Yasuyuki Ohta, Tetsuro Murakami, Yasushi Takehisa, Tatsushi Kamiya, and Koji Abe**, "Early detachment of neuromuscular junction proteins in ALS mice with SODG93A mutation.," *Neurol. Int.*, vol. 1, no. 1, p. e16, Jan. 2009.

[38] **Mauro C. Dal Canto, and Mark E. Gurney**, "Neuropathological changes in two lines of mice carrying a transgene for mutant human Cu,Zn SOD, and in mice overexpressing wild type human SOD: a model of familial amyotrophic lateral sclerosis (FALS)," *Brain Res.*, vol. 676, no. 1, pp. 25–40, 1995.

[39] **Lindsey R. Fischer, and Jonathan D. Glass**, "Axonal degeneration in motor neuron disease.," *Neurodegener. Dis.*, vol. 4, no. 6, pp. 431–42, 2007.

[40] **Merete Karlsborg, Sverre Rosenbaum, Mette Wiegell, Helle Simonsen, Henrik Larsson, Lene Werdelin, and Ole Gredal**, "Corticospinal tract degeneration and possible pathogenesis in ALS evaluated by MR diffusion tensor imaging.," *Amyotroph. Lateral Scler. Other Motor Neuron Disord.*, vol. 5, no. 3, pp. 136–140, 2004.

[41] **A. Aggarwal, and G. Nicholson**, "Detection of preclinical motor neurone loss in SOD1 mutation carriers using motor unit number estimation.," *J. Neurol. Neurosurg. Psychiatry*, vol. 73, no. 2, pp. 199–201, Aug. 2002.

[42] **Robert B. Daroff, Gerald M. Fenichel, J. Jankovic, and John C. Mazziotta**, *Neuro-ophthalmology: Ocular Motor System | Bradley's Neurology in…*. 2012.

[43] **Ana C. Calvo, Raquel Manzano, Deise M. F. Mendonça, María J. Muñoz, Pilar Zaragoza, and Rosario Osta**, "Amyotrophic lateral sclerosis: a focus on disease progression.," *Biomed Res. Int.*, vol. 2014, p. 925101, Jan. 2014.

[44] **Edward Pokrishevsky, Leslie I. Grad, and Neil R. Cashman**, "TDP-43 or FUS-induced misfolded human wild-type SOD1 can propagate intercellularly in a prion-like fashion," *Sci. Rep.*, vol. 6, no. November 2015, p. 22155, 2016.

[45] **Oliver D. King, Aaron D. Gitler, and James Shorter**, "The tip of the iceberg: RNA-binding proteins with prion-like domains in neurodegnerative disease," *Brain Res.*, pp. 61–80, 2013.

[46] **Magdalini Polymenidou, and Don W. W. Cleveland**, "The seeds of neurodegeneration: Prion-like spreading in ALS," *Cell*, vol. 147, no. 3, pp. 498–508, 2011.

[47] **Y. Sagot, R. Vejsada, and A. C. Kato**, "Clinical and molecular aspects of motoneurone diseases: animal models, neurotrophic factors and Bcl-2 oncoprotein," *Trends Pharmacol. Sci.*, vol. 18, no. 9, pp. 330–337, 1997.

[48] **Jean-Michel Peyrin, Bérangère Deleglise, Laure Saias, Maéva Vignes, Paul Gougis, Sebastien Magnifico, Sandrine Betuing, Mathéa Pietri, Jocelyne Caboche, Peter Vanhoutte, Jean-Louis Viovy, and Bernard Brugg**, "Axon diodes for the reconstruction of oriented neuronal networks in microfluidic chambers.," *Lab Chip*, vol. 11, no. 21, pp. 3663–73, Nov. 2011.

[49] **Bérangère Deleglise, Sebastien Magnifico, Eric Duplus, Pauline Vaur, Vanessa Soubeyre, Morgane Belle, Maeva Vignes, Jean-Louis Viovy, Etienne Jacotot, Jean-Michel Peyrin, and Bernard Brugg**, "β-amyloid induces a dying-back process and remote trans-synaptic alterations in a microfluidic-based reconstructed neuronal network.," *Acta Neuropathol. Commun.*, vol. 2, p. 145, Jan. 2014.

[50] **Sébastien Magnifico, Laure Saias, Bérangère Deleglise, Eric Duplus, Devrim Kilinc, Marie-Christine Christine Miquel, Jean-Louis Louis Viovy, Bernard Brugg, and Jean-Michel Michel Peyrin**, "NAD acts on mitochondrial SirT3 to prevent axonal caspase activation and axonal degeneration," *FASEB J.*, vol. 27, no. 12, pp. 4712–22, Dec. 2013.

[51] **Devrim Kilinc, Jean-Michel Peyrin, Vanessa Soubeyre, @bullet Sébastien, Magnifico @bullet, Laure Saias, Jean-Louis Viovy, Bernard Brugg, Sébastien Magnifico, Laure Saias, Jean-Louis Viovy, and Bernard Brugg**, "Wallerian-like degeneration of central neurons after synchronized and geometrically registered mass axotomy in a three-compartmental microfluidic chip.," *Neurotox. Res.*, vol. 19, no. 1, pp. 149–61, Jan. 2011.

[52] **Bérangère Deleglise, Benjamin Lassus, Vaneyssa Soubeyre, Aurélie Alleaume-Butaux, Johannes J. Hjorth, Maéva Vignes, Benoit Schneider, Bernard Brugg, Jean Louis Viovy, and Jean Michel Peyrin**, "Synapto-Protective Drugs Evaluation in Reconstructed Neuronal Network," *PLoS One*, vol. 8, 2013.

[53] **Debjani Paul, Laure Saias, Jean-Cedric Pedinotti, Max Chabert, Sebastien Magnifico, Antoine Pallandre, Bertrand De Lambert, Claude Houdayer, Bernard Brugg, Jean-Michel Peyrin, and Jean-Louis Viovy**, "A 'dry and wet hybrid' lithography technique for multilevel replication templates: Applications to microfluidic neuron culture and two-phase global mixing.," *Biomicrofluidics*, vol. 5, no. 2, p. 24102, 2011.

[54] **Walter G. Bradley, and Deborah C. Mash**, "Beyond Guam: The cyanobacteria/BMAA hypothesis of the cause of ALS and other neurodegenerative diseases," *Amyotroph. Lateral Scler.*, no. sup2, pp. 7–20, Jan. 2009.

[55] **Alexander S. Chiu, Michelle M. Gehringer, Jeffrey H. Welch, and Brett a. Neilan**, "Does α-Amino-β-methylaminopropionic Acid (BMAA) Play a Role in Neurodegeneration?," *Int. J. Environ. Res. Public Health*, vol. 8, no. 12, pp. 3728–3746, 2011.

[56] **Julie P. Meneely, Olivier P. Chevallier, Stewart Graham, Brett Greer, Brian D. Green, and Christopher T. Elliott**, "β-methylamino-L-alanine (BMAA) is not found in the brains of patients with confirmed Alzheimer's disease," *Sci. Rep.*, vol. 6, p. 36363, Nov. 2016.

[57] **Quentin R. Smith, Hiroshi Nagura, Yoshiaki Takada, and Mark W. Duncan**, "Facilitated Transport of the Neurotoxin, ?-N-Methylamino-l-Alanine, Across the Blood-Brain Barrier," *J. Neurochem.*, vol. 58, no. 4, pp. 1330–1337, Apr. 1992.

[58] **Oskar Karlsson, Cecilia Berg, Eva B. Brittebo, and Nils Gunnar Lindquist**, "Retention of the cyanobacterial neurotoxin *β - N* -methylamino-l-alanine in melanin and neuromelanin-containing cells - a possible link between Parkinson-dementia complex and pigmentary retinopathy," *Pigment Cell Melanoma Res.*, vol. 22, no. 1, pp. 120–130, Feb. 2009.

[59] **M. W. Duncan, J. C. Steele, I. J. Kopin, and S. P. Markey**, "2-Amino-3-(methylamino)-propanoic acid (BMAA) in cycad flour: an unlikely cause of amyotrophic lateral sclerosis and parkinsonism-dementia of Guam.," *Neurology*, vol. 40, no. 5, pp. 767–72, May 1990.

[60] **Marion Szelechowski, Alexandre Bétourné, Yann Monnet, Cécile A. Ferré, Anne Thouard, Charlotte Foret, Jean-Michel Peyrin, Stéphane Hunot, and Daniel Gonzalez-Dunia**, "A viral peptide that targets mitochondria protects against neuronal degeneration in models of Parkinson's disease," *Nat. Commun.*, vol. 5, p. 5181, Oct. 2014.

[61] **A. Copani, P. L. Canonico, M. V. Catania, E. Aronica, V. Bruno, E. Ratti, F. T. M. van Amsterdam, G. Gaviraghi, and F. Nicoletti**, "Interaction between ß-N-methylamino- l-alanine and excitatory amino acid receptors in brain slices and neuronal cultures," 1991.

[62] **Andrew Eisen, Seung Kim, and Bhanu Pant**, "Amyotrophic lateral sclerosis (ALS): A phylogenetic disease of the corticomotoneuron?," *Muscle Nerve*, vol. 15, no. 2, pp. 219–224,

Feb. 1992.

[63] **Brigitte van Zundert, Pamela Izaurieta, Elsa Fritz, and Francisco J. Alvarez**, "Early pathogenesis in the adult-onset neurodegenerative disease amyotrophic lateral sclerosis," *J. Cell. Biochem.*, vol. 113, no. 11, pp. 3301–3312, Nov. 2012.

[64] **Sandra Anne Banack, Susan J. Murch, and Paul Alan Cox**, "Neurotoxic flying foxes as dietary items for the Chamorro people, Marianas Islands," *J. Ethnopharmacol.*, vol. 106, no. 1, pp. 97–104, 2006.

[65] **Thomas L. Perry, Catherine Bergeron, Andrew J. Biro, and Shirley Hansen**, "β-N-Methylamino-l-alanine: Chronic oral administration is not neurotoxic to mice," *J. Neurol. Sci.*, vol. 94, no. 1, pp. 173–180, 1989.

[66] **Ikuo Tsunoda**, "Axonal degeneration as a self-destructive defense mechanism against neurotropic virus infection.," *Future Virol.*, vol. 3, no. 6, pp. 579–593, 2008.

[67] **Liqun Luo, and Dennis D. M. O'Leary**, "Axon retraction and degeneration in development and disease.," *Annu. Rev. Neurosci.*, vol. 28, pp. 127–56, Jan. 2005.

[68] **Eric C. Freundt, Nate Maynard, Eileen K. Clancy, Shyamali Roy, Luc Bousset, Yannick Sourigues, Markus Covert, Ronald Melki, Karla Kirkegaard, and Michel Brahic**, "Neuron-to-neuron transmission of α-synuclein fibrils through axonal transport," *Ann. Neurol.*, vol. 72, no. 4, pp. 517–524, Oct. 2012.

[69] **Christian Münch, and Anne Bertolotti**, "Self-propagation and transmission of misfolded mutant SOD1: Prion or prion-like phenomenon?," *Cell Cycle*, vol. 10, no. 11, p. 1711, 2011.

[70] **Gabor G. Kovacs, and Herbert Budka**, "Molecular pathology of human prion diseases.," *Int. J. Mol. Sci.*, vol. 10, no. 3, pp. 976–99, 2009.

[71] **Leslie I. Grad, and Neil R. Cashman**, "Prion-like activity of Cu/Zn superoxide dismutase," *Prion*, vol. 8, no. 1, pp. 33–41, 2014.

[72] **Satoshi Kuwabara, and Takanori Yokota**, "Propagation: prion-like mechanisms can explain spreading of motor neuronal death in amyotrophic lateral sclerosis?," *J. Neurol. Neurosurg. Psychiatry*, vol. 82, no. 11, pp. 1181–2, Nov. 2011.

[73] **Paul H. Gordon**, "Amyotrophic Lateral Sclerosis: An update for 2013 Clinical Features, Pathophysiology, Management and Therapeutic Trials.," *Aging Dis.*, vol. 4, no. 5, pp. 295–310, 2013.

[74] **H. Braak, and E. Braak**, "Neuropathological stageing of Alzheimer-related changes," *Acta Neuropathol*, vol. 82, pp. 239–259, 1991.

[75] **Nicholas J. Maragakis, and Jeffrey D. Rothstein**, "Mechanisms of Disease: astrocytes in neurodegenerative disease.," *Nat. Clin. Pract. Neurol.*, vol. 2, no. 12, pp. 679–689, 2006.

[76] **W. Liu, Y. Tang, and J. Feng**, "Cross talk between activation of microglia and astrocytes in pathological conditions in the central nervous system," *Life Sci.*, vol. 89, no. 5–6, pp. 141–146, 2011.

[77] **Daniela Rossi, and Andrea Volterra**, "Astrocytic dysfunction: Insights on the role in

neurodegeneration," *Brain Res. Bull.*, vol. 80, no. 4, pp. 224–232, 2009.

[78] **Makiko Nagai, Diane B. Re, Tetsuya Nagata, Alcmène Chalazonitis, Thomas M. Jessell, Hynek Wichterle, and Serge Przedborski**, "Astrocytes expressing ALS-linked mutated SOD1 release factors selectively toxic to motor neurons," *Nat. Neurosci.*, vol. 10, no. 5, pp. 615–622, May 2007.

[79] **K. Yamanaka, S. J. Chun, S. Boillee, N. Fujimori-Tonou, H. Yamashita, D. H. Gutmann, R. Takahashi, H. Misawa, and D. W. Cleveland**, "Astrocytes as determinants of disease progression in inherited amyotrophic lateral sclerosis," *Nat Neurosci*, vol. 11, no. 3, pp. 251–253, 2008.

[80] **S. Cronier, J. Carimalo, B. Schaeffer, E. Jaumain, V. Beringue, M. C. Miquel, H. Laude, and J. M. Peyrin**, "Endogenous prion protein conversion is required for prion-induced neuritic alterations and neuronal death," *FASEB J.*, vol. 26, no. 9, pp. 3854–3861, 2012.

[81] **J. Y. Koh, and D. W. Choi**, "Vulnerability of cultured cortical neurons to damage by excitotoxins: differential susceptibility of neurons containing NADPH-diaphorase.," *J. Neurosci.*, vol. 8, no. 6, pp. 2153–63, Jun. 1988.

[82] **Stephen M. Ross, Mary Seelig, and Peter S. Spencer**, "Specific antagonism of excitotoxic action of 'uncommon' amino acids assayed in organotypic mouse cortical cultures," *Brain Res.*, vol. 425, no. 1, pp. 120–127, 1987.

[83] **Ammar Al-Chalabi, Andrea Calvo, Adriano Chio, Shuna Colville, Cathy M. Ellis, Orla Hardiman, Mark Heverin, Robin S. Howard, Mark H. B. Huisman, Noa Keren, P.Nigel Leigh, Letizia Mazzini, Gabriele Mora, Richard W. Orrell, James Rooney, Kirsten M. Scott, William J. Scotton, Meinie Seelen, Christopher E. Shaw, Katie S. Sidle, Robert Swingler, Miho Tsuda, Jan H. Veldink, Anne E. Visser, Leonard H. van den Berg, and Neil Pearce**, "Analysis of amyotrophic lateral sclerosis as a multistep process: a population-based modelling study.," *Lancet. Neurol.*, vol. 13, no. 11, pp. 1108–13, Nov. 2014.

[84] **J. D. Mitchell, and G. D. Borasio**, "Amyotrophic lateral sclerosis.," *Lancet (London, England)*, vol. 369, no. 9578, pp. 2031–41, Jun. 2007.

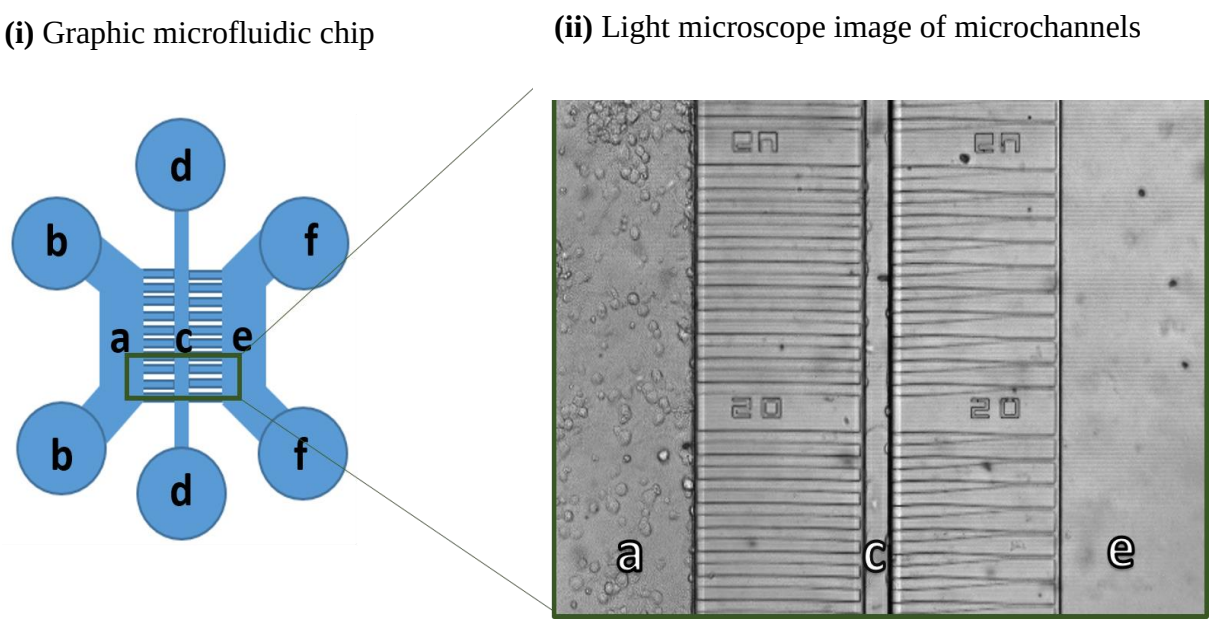


Fig. 1 Microfluidic Chips

(i) Graphical representation of microfluidic chip; **(ii)** photo of microfluidic chip showing middle microchannels **(a)** Proximal chamber, **(b)** proximal reservoirs, **(c)** middle microchannels of 15µM - 3µM tapered micro channels connecting the proximal to distal chambers, **(d)** middle reservoirs, **(e)** distal chamber, **(f)** distal reservoirs; **(iii)** cortical cultures, **(iv)** cortico-striatal networks; corresponding cultures images of **(g)** cortical somatic bodies in proximal compartment stained for axons (Green) and nuclei (Blue), and distal compartments, featuring **(h)** cortical axons stained for axons (Red), or **(j)** striatal neurons in the distal chamber to build a cortico-striatal network (MAP2, green; nuclei,

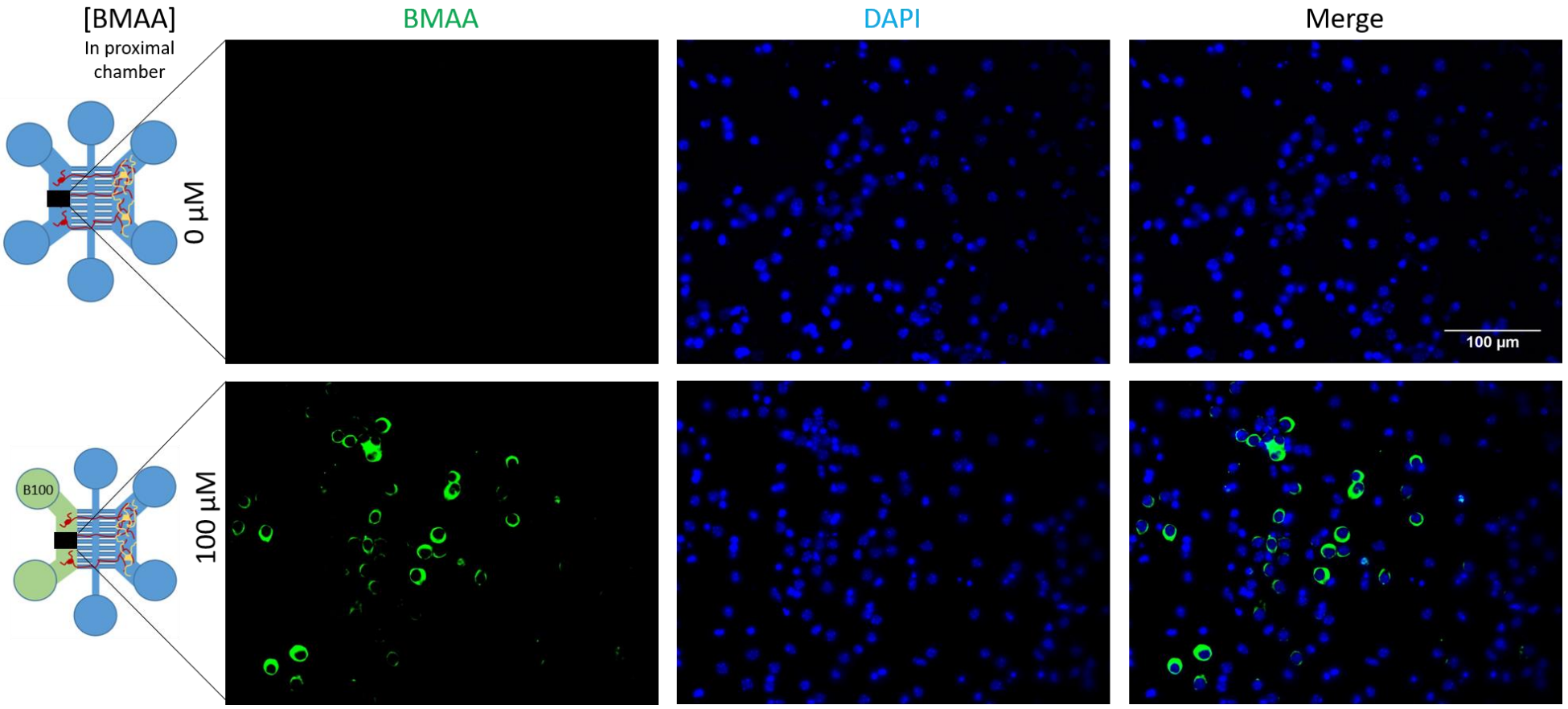


Fig. 2 Uptake of BMAA into treated somas in the proximal chamber

Proximal chamber of cortical cell bodies treated (100µM) or not treated (0) with BMAA showing uptake of BMAA into cell bodies. Cells not treated with BMAA do not show any BMAA staining, while cells treated with BMAA show a subpopulation of cells positive for BMAA. Microfluidic schematic represents treatments in fluidic isolation; Blue: complete media; Green: BMAA treated. Scale bar 100µm

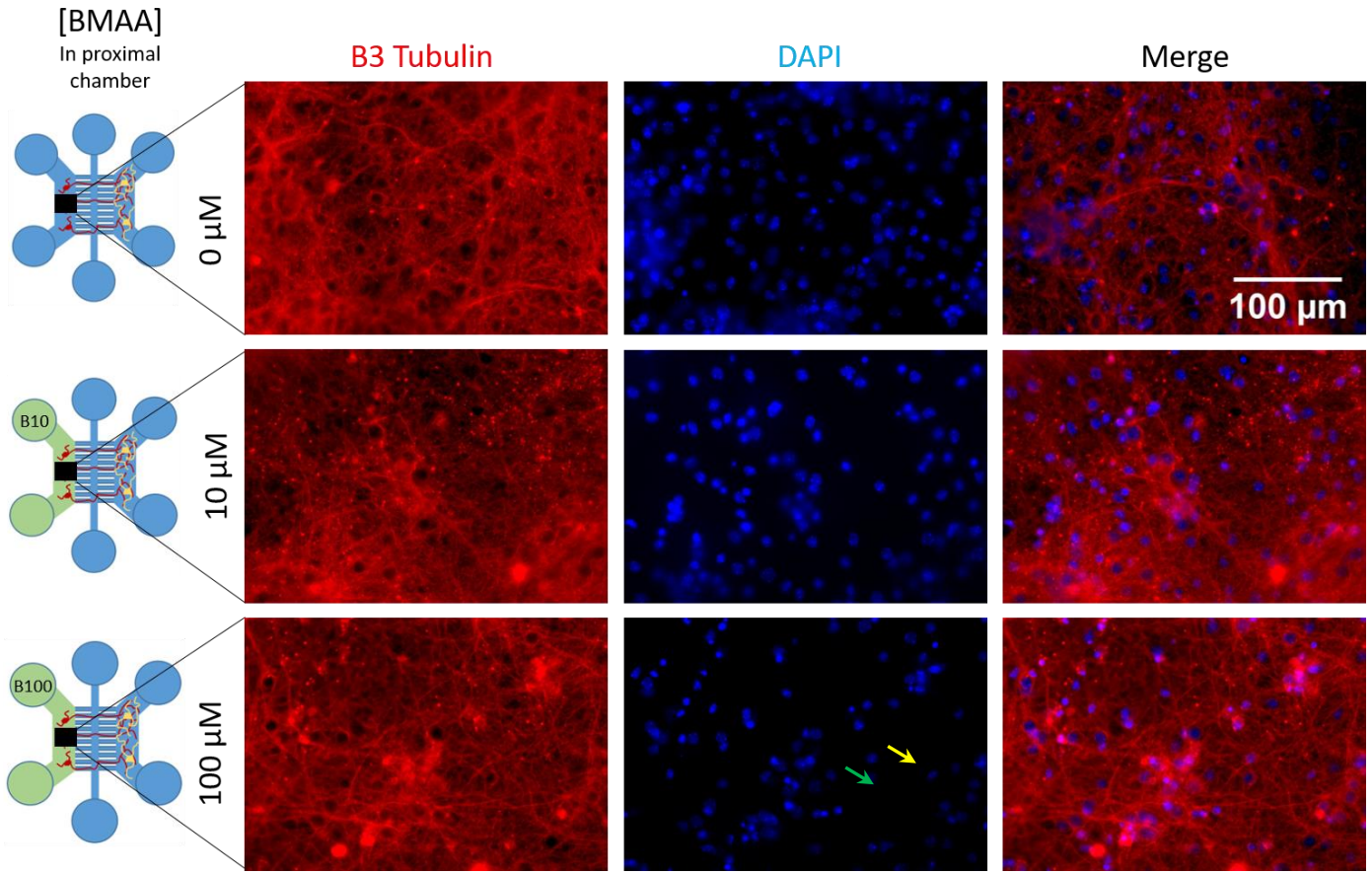
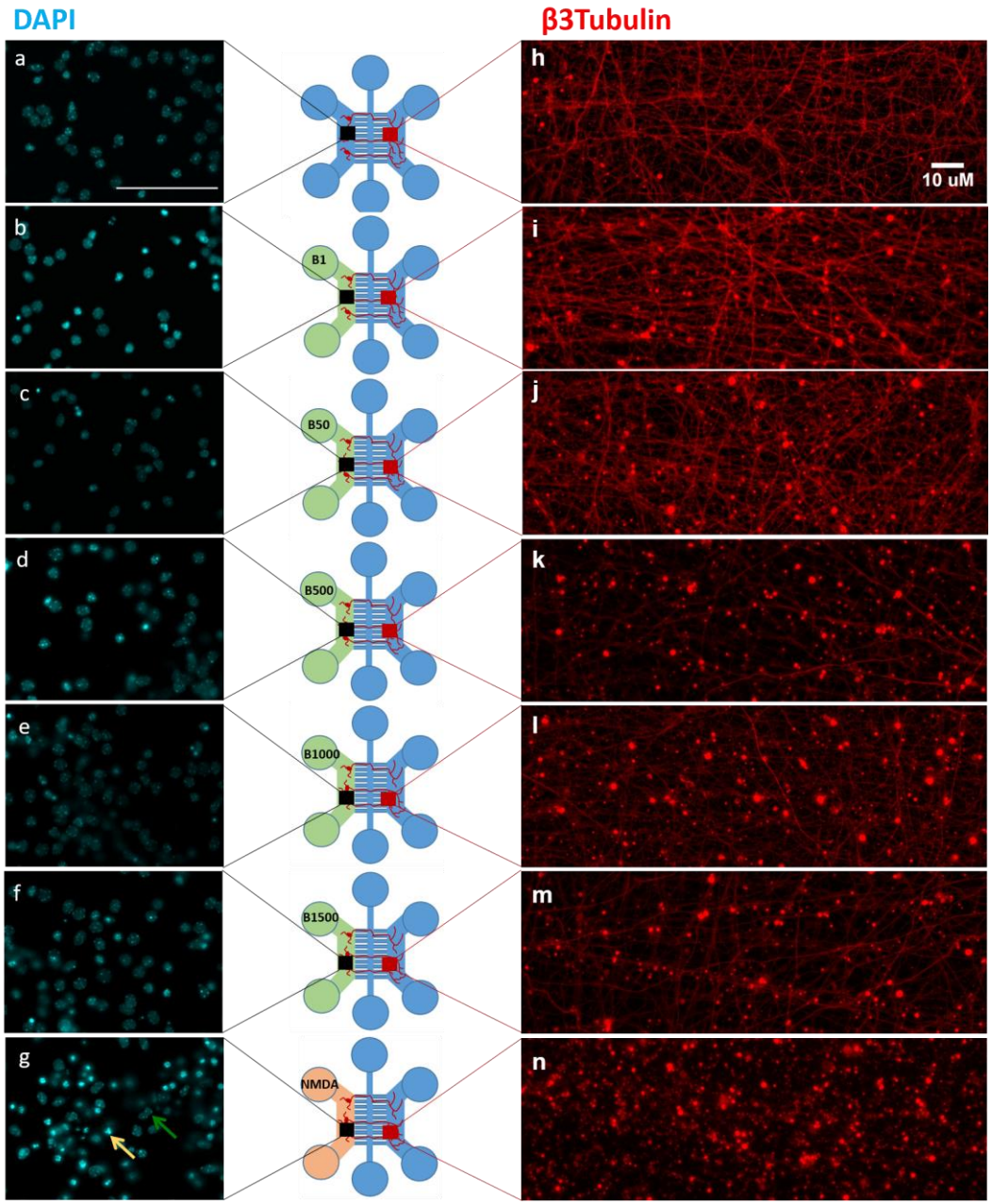


Fig. 3 Somatic health of neurons treated or not treated with BMAA

Images of proximal chambers at **treated with 0, 10 or 100 µM** BMAA showing structure of axons **(β3 Tubulin; red)** and nuclear staining **(DAPI, blue**). Untreated cells have little blebbing in axonal structures, and modest nuclear condensation, while cells treated with BMAA show increased blebbing, and nuclear condensation. **Green arrow** shows intact nuclei, while **yellow arrow** shows condensed nuclei. Microfluidic schematic represents treatments in fluidic isolation; Blue: complete media; Green: BMAA treated. Scale bar 100 µm


DAPI
β3Tubulin
a
b
c
d
e
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i
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l
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B1
B50
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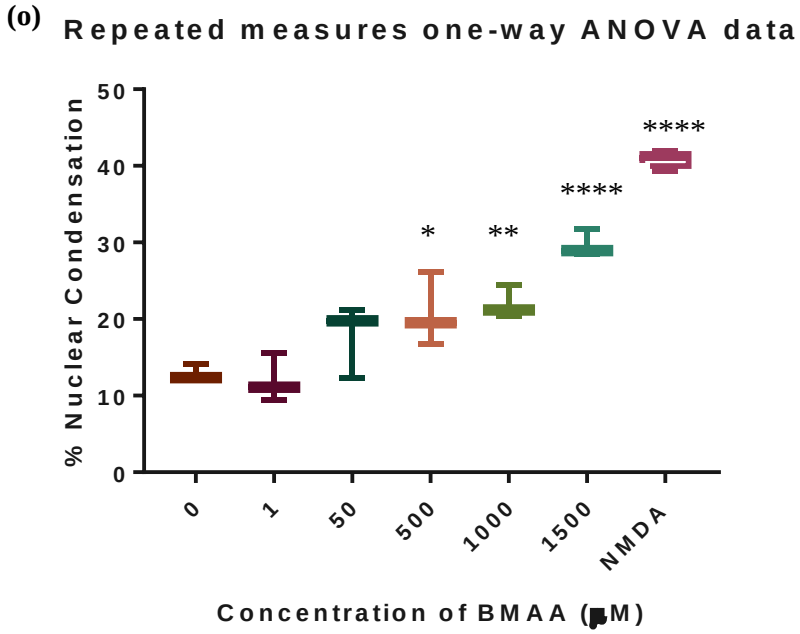
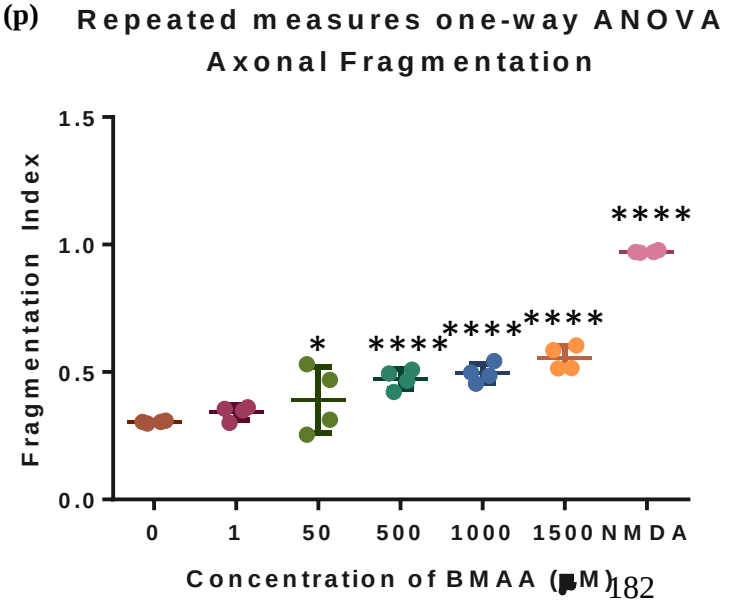
B1000
B1500
NMDA
10 uM


(o)
Repeated measures one-way ANOVA data
% Nuclear Condensation
0
10
20
30
40
50
*
**
****
****
0
1
50
500
1000
1500
NMDA
Concentration of BMAA (μM)


(p)
Repeated measures one-way ANOVA
Axonal Fragmentation
Fragmentation Index
0.0
0.5
1.0
1.5
*
****
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****
****
0
1
50
500
1000
1500
NMDA
Concentration of BMAA (μM)

Fig. 4 BMAA neurotoxicity in somatic and axonal subcompartments

BMAA treatment was applied on proximal chambers as represented by microfluidic schematic. Blue: complete media; Green: BMAA treated; Orange: NMDA.

Neurons are untreated (0), or treated with BMAA (1-1500µM), and 100µM NMDA as positive control.

Representative images of proximal chambers stained with DAPI (cyan): **(a)** untreated**, (b-f)** 1, 50, 500, 1000, and 1500 µM BMAA, **(g)** 100 µM NMDA. **Green arrow** shows intact nuclei, while **yellow arrow** shows condensed nuclei. Scale bar **100 µM**;

Representative high magnification images (63x) of cortical axons in distal chambers stained with β3-tubulin (red) and DAPI (cyan)): **(h)** untreated**, (i-m)** 1, 50, 500, 1000, and 1500 µM BMAA, **(n)** 100 µM NMDA Scale bar **10 µm**.

(o) The graph shows nuclear condensation as a result of treatment (0-1500µM BMAA, 100µM NMDA). Results are expressed as a percentage of total nuclei count, and significance evaluated by Repeated measures, one-way ANOVA test, overall P <0.0001**,** R^2=0.8594, multiplicity adjusted (to untreated control): *p-value<0.05, **p-value<0.01, ****p-value≤0.0001, Post test for linear trend: Linear, p≤0.0001 Results expressed as mean ±SD

(p) The graph represents the relative amount of fragmentation compared to the control, quantified by ratio of tubular and circular structures in axonal compartments. Analysed by Repeat measures, one-way ANOVA, overall p< 0.0001, R^2=0.9722, multiplicity adjusted (to untreated control): *p-value<0.05, ****p-value≤0.0001, Post test for linear trend: Linear, p≤0.0001) Results expressed as mean ±SD

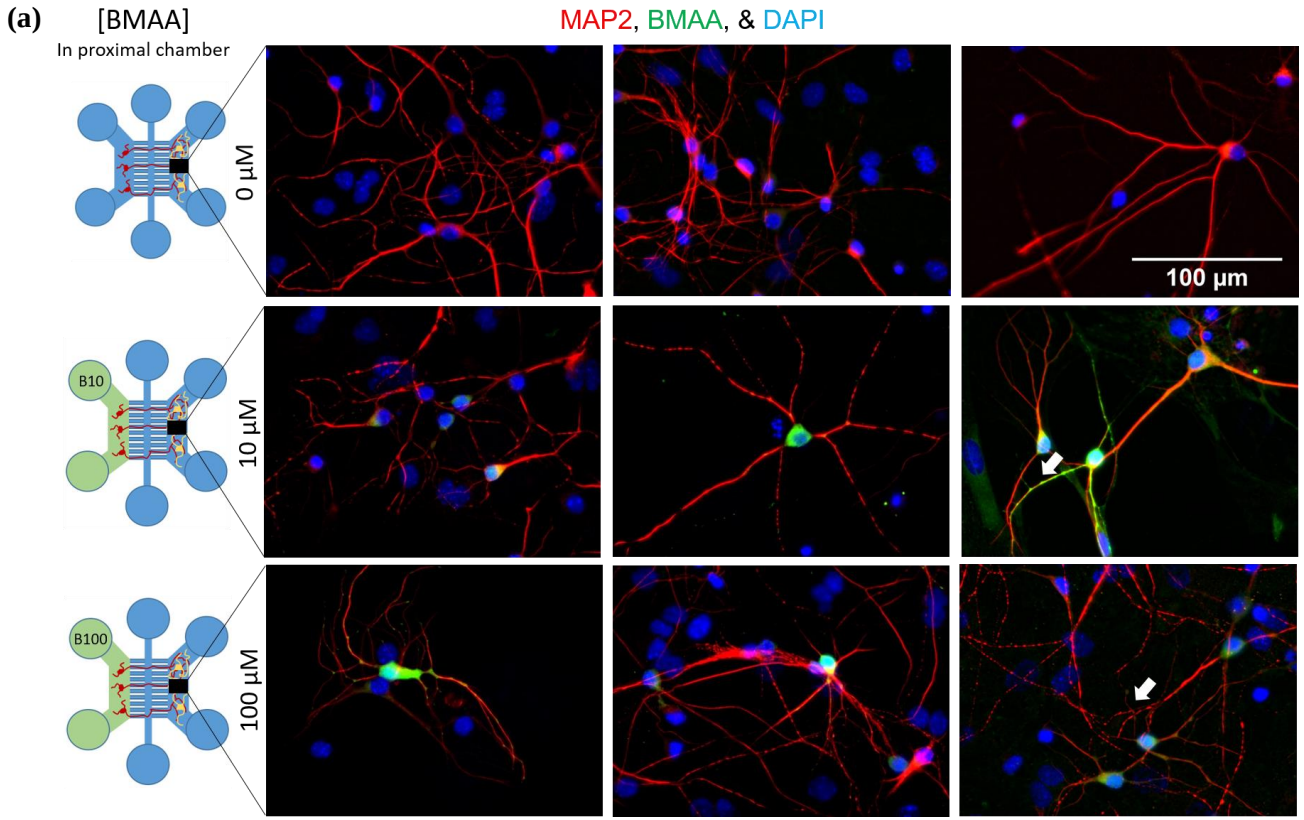
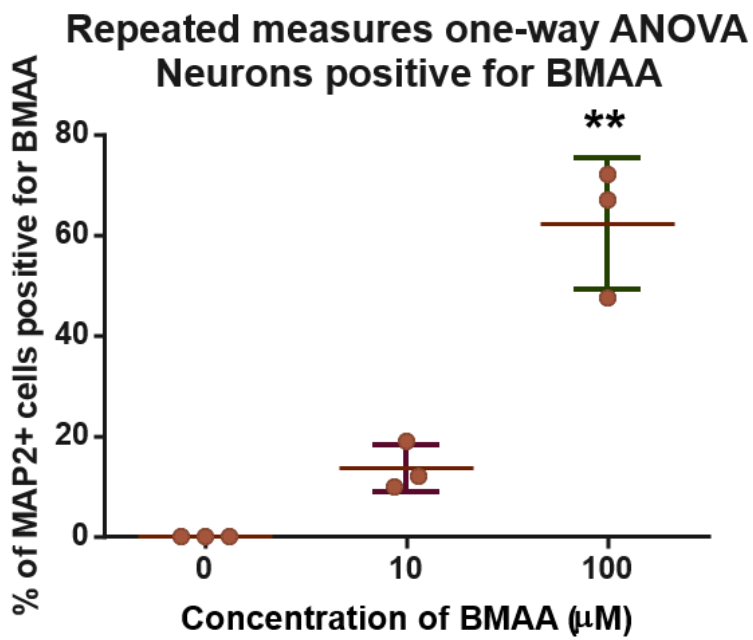


Fig. 5 Cellular uptake of BMAA in neurons of distal chambers

(a) Representative images of MAP2+ cells (Red) staining for BMAA (Green) in untreated, distal striatal chambers showing spread of BMAA from proximal chambers treated with 0, 10, and 100 µM of BMAA. White arrows indicate presence of blebbing. Microfluidic schematic represents treatments in fluidic isolation; Blue: complete media; Green: BMAA treated. Scale bar 100uM

(b)



(b) Quantification showing spread of BMAA into neurons of untreated distal chambers

Graph shows percentage quantification of second-order neurons positive for MAP2 and BMAA, of total MAP2 positive cells. Only proximal first-order neurons were treated with 0, 10, and 100 µM BMAA. Analysed by repeated measures, one way ANOVA with cumulative p value of 0.0014, and multiplicity adjusted values of 0.1634 and 0.0011 for 10 and 100 µm respectively. Results expressed as mean ±SD

(a)

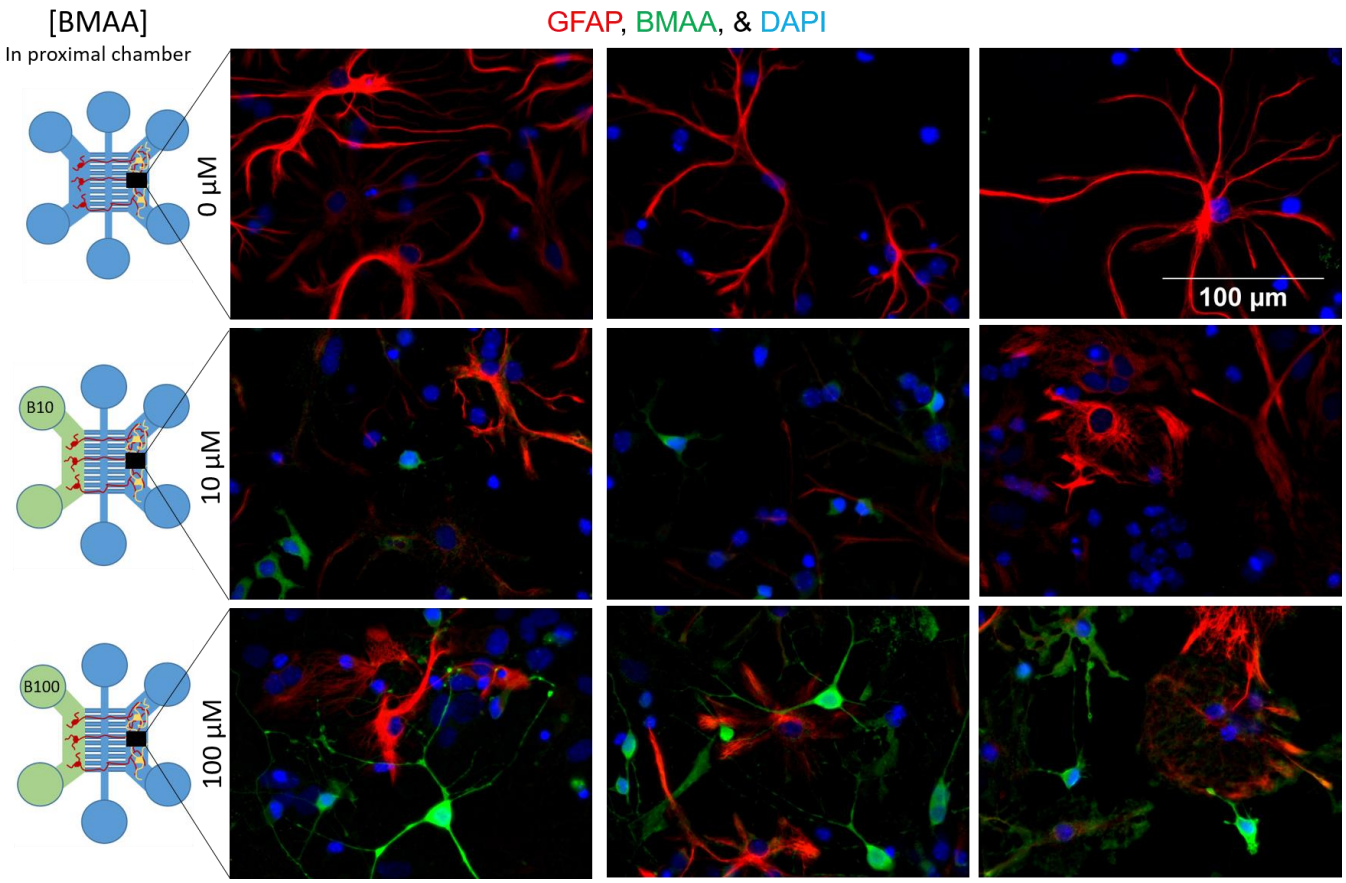


Fig. 6 (a) Cellular uptake of BMAA in astrocytes of distal chambers

Representative images of GFAP+ cells (Red) staining for BMAA (Green) in in untreated, distal striatal chambers showing spread of BMAA from proximal chambers treated with 0, 10, and 100 µM of BMAA. Microfluidic schematic represents treatments in fluidic isolation; Blue: complete media; Green: BMAA treated. Scale bar 100µm

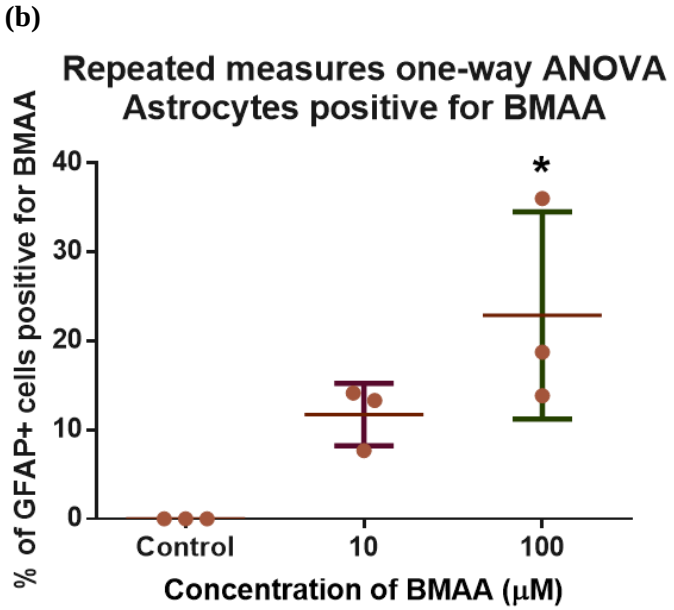


Fig. 6 (b) Quantification showing spread of BMAA into astrocytes of untreated distal chambers

Graph shows percentage quantification of second-order astrocytes positive for GFAP and BMAA, of total GFAP positive cells. Only proximal first-order neurons were treated with 0, 10, and 100 µM BMAA. Significant presence of BMAA was observed in GFAP-expressing astrocytic cells. Analysed by: repeated measures, one-way ANOVA with cumulative p value of 0.0589, and multiplicity adjusted values of 0.2332 and 0.0409 for 10 and 100 µm respectively. Results expressed as mean ±SD

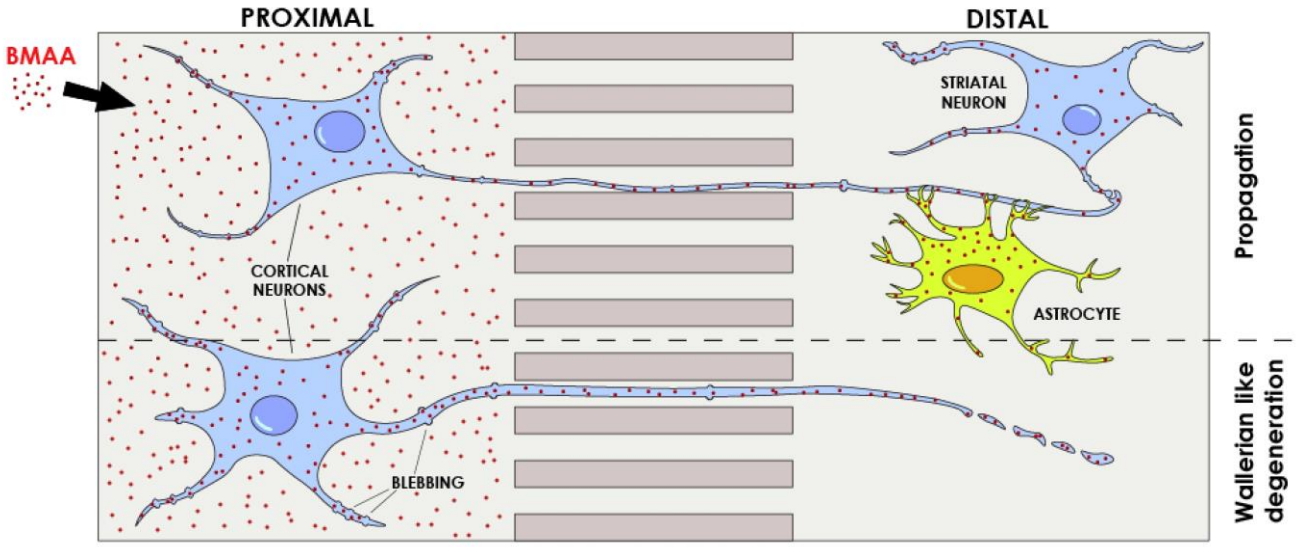


Fig. 7 Summary of BMAA toxicity Two modes of BMAA toxicity: Wallerian like degeneration, and propagation of BMAA from cell-to -cell

Chapter 4

Other Publications arising from this Candidature

Original Research Article: Defects in Optineurin and Myosin VI Mediated Cellular Trafficking in ALS, Human Molecular Genetics

Vinod Sundaramoorthy, Adam K. Walker, **Vanessa Tan**, Jennifer A. Fifita, Emily P. McCann, Kelly L. Williams, Ian P. Blair, Gilles J Guillemin, Manal A. Farg, Julie D. Atkin

I contributed to the culture of primary human motor neurons used in the article, and writing, editing and review of manuscript.

Original Research Article: Kynurenine Pathway Metabolomics Predict and Provide Mechanistic Insight into Multiple Sclerosis Progression, Scientific Reports

Chai K. Lim, Ayse Bilfin, David B. Lovejoy, **Vanessa X. Tan**, Sonia Bustamante, Bruce V. Taylor, Alban Bessede, Bruce J. Brew, Gilles J. Guillemin

I contributed to the KP analysis and data collection, organization of clinical samples and data, data analysis and interpretation, and the writing and reviewing of manuscript.

Publications approved since submission of thesis

Original Research Article: Neurotoxicity of the Cyanotoxin BMAA Through Axonal Degeneration and Intercellular Spreading, Neurotoxicity Research

Vanessa X. Tan, Benjamin Lassus, Chai K. Lim, Philippe Tixador, Josquin Courte, Alban Bessede, Gilles J. Guillemin & Jean-Michel Peyrin

Original Research Article: Neurotoxicity of the Cyanotoxin BMAA Through Axonal Degeneration and Intercellular Spreading, Neurotoxicity Research

Vanessa X. Tan, Claire Mazzocco, Bianca Varney, Dominique Bodet, Tristan A. Guillemin, Alban Bessede, Gilles J. Guillemin

Articles are appended for reference.

Sundaramoorthy, V., al er, A. K., Tan, V., ifita, J. A., Mccann, .P., illiams, K. L., Blair, I. P., Guillemin, G. J., arg, M. A. & At in, J. D. (2015). Defects in optineurin- and myosin VI-mediated cellular traffic ing in amyotrophic lateral sclerosis. *u a o ecu ar e etics*, 24 13 , p. 3830-3846.

DOI: 10.1093/hmg dd 126

www.nature.com/scientificreports

4.2 Original Research Article: Kynurenine Pathway Metabolomics Predict and Provide Mechanistic Insight into Multiple Sclerosis Progression, Scientific Reports

SCIENTIFIC REPORTS

OPEN

Kynurenine pathway metabolomics predicts and provides mechanistic insight into multiple sclerosis progression

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Chai K. Lim[1,2], Ayse Bilgin[3], David B. Lovejoy[1], Vanessa Tan[1], Sonia Bustamante[4], Bruce V. Taylor[5], Alban Bessede[6], Bruce J. Brew[7,8,*] & Gilles J. Guillemin[1,7,*]

Activation of the kynurenine pathway (KP) of tryptophan metabolism results from chronic inflammation and is known to exacerbate progression of neurodegenerative disease. To gain insights into the links between inflammation, the KP and multiple sclerosis (MS) pathogenesis, we investigated the KP metabolomics profile of MS patients. Most significantly, we found aberrant levels of two key KP metabolites, kynurenic acid (KA) and quinolinic acid (QA). The balance between these metabolites is important as it determines overall excitotoxic activity at the N-methyl-D-Aspartate (NMDA) receptor. We also identified that serum KP metabolic signatures in patients can discriminate clinical MS subtypes with high sensitivity and specificity. A C5.0 Decision Tree classification model discriminated the clinical subtypes of MS with a sensitivity of 91%. After validation in another independent cohort, sensitivity was maintained at 85%. Collectively, our studies suggest that abnormalities in the KP may be associated with the switch from early-mild stage MS to debilitating progressive forms of MS and that analysis of KP metabolites in MS patient serum may have application as MS disease biomarkers.

Increasing evidence supports the hypothesis that inflammation contributes to neurodegeneration and is linked to multiple sclerosis (MS) progression[1]. Weiner HL (2009) described how the adaptive immunity mediated by auto-reactive T-cells, fuels the early stages of MS (i.e. relapsing-remitting multiple sclerosis; RRMS)[2], while monocytic cells of the innate immunity contribute to further neuronal degeneration that exacerbates disease progression leading to secondary progressive MS (SPMS)[2]. However, it is not well understood why approximately 50% of RRMS patients progress to secondary progressive MS, while 50% do not. We sought to better understand the mechanistic drivers of this switch. As the kynurenine pathway (KP) of the tryptophan metabolism is highly inducible in inflammatory environments, we hypothesized that changes in the KP may be associated with the progressive switch in MS.

The kynurenine pathway (KP) is the major route that breaks down tryptophan subsequently leading to the production of NAD^+. In the presence of pro-inflammatory cytokines[3,4], the KP is induced by activation of its first enzyme, indoleamine 2,3-dioxygenase (IDO-1). Metabolites produced along the KP can have neurotoxic or neuroprotective effects. Quinolinic acid (QA) is perhaps the most important, leading acutely to human neuronal death and chronically to dysfunction by at least 7 separate mechanisms, of which, N-methyl-D-Aspartate (NMDA) receptor excitotoxicity is the best characterised[5]. Within the brain and CNS, QA is produced by activated microglia and infiltrating macrophages but not in neurons or astrocytes[6]. Kynurenic acid (KA), produced by astrocytes[7], is an antagonist of ionotropic glutamate receptors and thus blocks the excitotoxic effects of QA, KA

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also has antioxidant activity, readily scavenging hydroxyl, superoxide anion and other free radicals[8]. Indeed, in disease states where excess QA is produced, it is thought that there is insufficient KA to block QA[9].

Accordingly, we examined the role of the KP in MS progression as it potentially links inflammation-induced activation of the KP[10], the production of the glutamatergic (NMDA)-modulatory metabolites, KA and QA, to excitotoxic neurodegeneration[11–13]. This metabolic shift may explain why the inflammatory milieu in RRMS changes to a neurodegenerative one in SPMS and may even constitute a unique metabolic biomarker of MS progression. Currently, there are no biomarkers that can identify this transition[14], and a suitable biomarker would be useful for assessing patient prognosis and potentially new therapeutics.

Materials and Methods

Sample Cohorts and Study design. All studies were carried out in accordance with the guidelines of the relevant institutional human research ethics committee and approved by St Vincent's Hospital Sydney (HREC–H03/037) and Macquarie University (HREC – 5201300333). Our study adheres to 'The code of ethics of the World Medical Association (Declaration of Helsinki)' for experiments involving human subjects. Written consent was obtained from respective sources that provided the samples. In order to thoroughly profile KP metabolism in MS, *Cohort 1* samples were obtained through the Accelerated Cure Project for Multiple Sclerosis (ACPMS), USA, and consisted of serum samples from patients with RRMS, SPMS, primary progressive (PPMS) and healthy controls (HC) subjects. Exclusion criteria included MS patients currently receiving disease modifying drugs or who had corticosteroid within the past 3 months, or presence of other medical conditions. Randomized samples were age and gender matched between the experimental groups (i.e. RRMS, SPMS, PPMS and HC) where possible. As repeated blood samples collected over two years were available, *Cohort 2* offered the ability to track changes in the KP longitudinally and was obtained through the Tasmanian MS Longitudinal Study conducted between 2003 and 2005. To further validate our study, we sourced another cohort of MS patients (*Cohort 3*) with matched same-patient serum and cerebrospinal fluid (CSF) from The Human Brain and Spinal Fluid Resource Center (HBSFRC), USA. All samples were unsorted and re-labelled for blinding prior to analysis.

Profiling of the kynurenine pathway metabolites. All reagents and KP metabolites were analytical reagent grade and were purchased from Sigma-Aldrich (St Louis, MO), unless otherwise stated. Deuterated internal standards were purchased from Medical Isotopes, Inc (Pelham, NH). KP metabolites were extracted using 10% (*w*/*v*) trichloroacetic acid (TCA) with equal volume of serum samples in accordance with methods previously described[15]. CSF samples were prepared similarly to serum samples except that deproteinization with TCA was not performed.

Concurrent analysis of tryptophan, kynurenine, 3-hydroxykynrenine (3-HK), 3-hydroxyanthranilic acid (3-HAA), and anthranilic acid (AA) was performed with UHPLC as described by Jones *et al.*[16], using an injection volume of 20 μL of the prepared extract from each samples. KA detection was performed using a gradient mobile phase comprise of 50 mM sodium acetate buffer supplement with 25 mM zinc acetate (dihydrate) to enhance fluorescence intensity and 2.25% acetonitrile as organic modifier (Solvent A), and 10% acetonitrile (Solvent B). Each sample (10 μL) was injected into a Poroshell RRHT C-18, 1.8 μm 2.1 × 100 mm column (Agilent Technologies, Inc, Santa Clara, CA) maintained at 38 °C for 12 min run time at a unison flowrate of 0.75 mL/min. The gradient elution consisted of 100% solvent A for 3 min and then 50% solvent A and 50% solvent B for 2 min, followed by 100% B for 2 min and 100% solvent A (run time 10 min). This gradient ensures sufficient time for KA retention while minimizing potential build-up of pressure due to precipitation of the high salt buffer. Detection of KA used fluorescence (excitation and emission wavelengths of 344 and 388 nm, respectively with a retention time of 1.5 min). Agilent OpenLAB CDS ChemStation (Edition C.01.04) was used to analyze the chromatograms (Supplementary Figure S1A and B).

For GCMS, 50 μL of the prepared extract were derivatized. Concurrent analysis of PA and QA were carried out as described by Smythe *et al.*[17] with slight modification using an Agilent 7890 A GC system coupled with Agilent 5975 C mass spectrometry detector and Agilent 7693 A autosampler (Agilent Technologies, Inc, Santa Clara, CA) with one microliter of derivatized mixture. Separation of PA and QA were achieved with a DB-5MS column, 0.25 μm film thickness, 0.25 mm × 30 m capillary column (Agilent Technologies, Inc, Santa Clara, CA) within 7 min but the assay run time was set for 12 min to prevent sample carryover. Concentrations of PA and QA were analyzed using Agilent GC/MSD ChemStation software (Edition 02.02.1431) and interpolated from the established six-point calibration curves based on the abundance count ratio of the metabolites to their corresponding deuterated internal standards within each standards and samples (Supplementary Figure S1C and D).

The intra- and inter-assay CV was within the acceptable range of 4–8% for UHPLC assays and 7–10% for GCMS assays calculated from the repeated measures of the metabolites standards incorporated during the sequence run. Nicotinamide adenine dinucleotide (NAD^+) was measured with 20 μL of neat serum using a previously described method[18].

Profiling of the inflammatory mediators. Quantification of multiplexing cytokines, chemokines and growth factors was performed using commercial 27-plex magnetic bead based immunoassay kits (Bio-Rad, Hercules, CA) on *Cohort 1*. Each assay was performed in accordance to manufacturer's instructions at the Australian Proteome Analysis Facility, as described by A. Khan[19]. Final readout of the sample concentration was expressed as picogram per millilitre (pg/mL) based on the standard curves integrated in the assay using the Bio-Plex Manager v5.0 software with reproducible intra- and inter-assay CV of 5–8%.

Statistical analysis and modelling. The normality of the variables, where required, was checked by Shapiro-Wilk and/or Kolmogorov Smirnov normality tests and equality of the variances by Levene's test for equality of variances. Comparisons between different MS subtypes and control group were performed by either

ANOVA or Kruskal Wallis test, depending on the normality and equal variances analyses. Correlations between variables in *Cohort 1* were assessed by Pearson's correlations; where required, log transformation of the variables was made before calculating correlations. A *p*-value of <0.05 was considered statistically significant. Paired t-tests or Wilcoxon Signed Ranks tests, depending on the distribution of the variables were applied to investigate the changes to the inflammatory mediators and KP variables from *Cohort 2* where the patients were observed between the years 2003 (baseline, cohort enrollment) and 2005. Four classification methods were used to identify the relative predictive value of each of the variables in each MS subtype in *Cohort 1* (Training set) and then validated against *Cohort 3* (Test set). These methods included the Classification and Regression Tree, Support Vector Machines, Discriminant Analysis and C5.0 Decision Tree[20,21]. A classification model was considered successful when it consistently returned high predictive accuracy rates across all three MS subtypes and control group. The model predictions were compared to random predictions (class specific lifts) to decide which model was best for future predictions. In addition, class-specific statistical powers were compared to identify the best classification model. All classification models were developed using IBM SPSS Modeler 14.2, and R[22] incorporated with various R packages such as Rcmdr, Lawstat, MASS[23], CAR and Lattice. To investigate whether changes to the CSF KP profiles mirrored serum KP profiles in the controls and MS subtypes of *Cohort 3*, we developed a series of models to identify the best relationships between the CSF KP variables and serum KP variables in the experimental groups. In the first model (model 1), we investigated whether it was possible to predict the CSF value for a KP variable based on its paired serum sample variable. In the second model (model 2), we added the experimental groups as covariates to model 1 to predict the CSF variables. In the last model (model 3), we used model 2 but adjusted for potential confounding factors from all other serum KP variables. We compared these models based on Akaike information criterion (AIC) and adjusted R^2, to identify the best model(s).

Results

Participants' characteristics. *Cohort 1* made up a total of 136 participants consisting of 50 RRMS, 20 SPMS, 17 PPMS and 49 HC. Age differences between the subtypes were adjusted accordingly in the subsequent analysis. Similarly a total of 59 participants consisting of 44 RRMS and 15 SPMS from *Cohort 2* were included in this study. Each *Cohort 2* participant provided a baseline and follow-up (1.72 years ± 0.27) serum samples for analysis. A total of 36 participants from *Cohort 3* met exclusion criteria and provided CSF and matching serum. Accordingly, *Cohort 3* consisted of 10 patients with RRMS, 20 patients with SPMS and 6 HC. Demographic and clinical characteristics of the subjects in all cohorts are summarized in Table 1.

Activation of the kynurenine pathway in MS. KP activation occurs when the activity of indoleamine 2,3-dioxygenase (IDO-1; the first enzyme in the KP) is significantly higher, leading to the consumption of kynurenine and higher kynurenine/tryptophan (K/T) ratio. In all the MS subtype groups the K/T ratio was significantly increased compared to the HC group ($p < 0.0001$, Fig. 1A–C; see Supplementary Table S2 for complete KP profile). These data also emphasize the importance of analyzing both TRP and KYN to determine KP activation, as relying on a single metabolite can lead to misinterpretation of data.

Abnormal downstream KP metabolites production in MS indicating excitotoxicity. As noted above, KA is capable of preventing glutamate-induced excitotoxicity induced by QA. Significantly, we found that KA levels were highest in the RRMS group relative to HC and progressive MS groups ($p < 0.0001$, Fig. 1D). However, KA levels were significantly lower in the progressive MS groups relative to controls. Picolinic acid, another known neuroprotective KP metabolite[24], showed a similar trend to KA, being highest in the RRMS but lowest in the PPMS, groups ($p < 0.0001$, Supplementary Table S2).

Production of QA increased uniformly in concert with disease severity and was particularly elevated in the PPMS group ($p < 0.0001$, Fig. 1E). We also found decreased NAD+ in all MS patient groups ($p < 0.0001$, Fig. 1F) which is also indicative of net QA elevation. The QA/KA ratio is indicative of excitotoxic potential with higher QA/KA ratio value favoring excitotoxicity. The QA/KA ratio was higher in both PPMS and SPMS groups compared to controls and the RRMS group ($p < 0.0001$, Fig. 1G). 3-HK, another potential neurotoxin, was found to be significantly higher in MS groups compared to healthy control ($p < 0.0001$, Supplementary Table S2). These data support our hypothesis that toxic KP metabolites fuel neurodegeneration in MS.

KP profile changes involving innate immunity are associated with MS disease severity and subtype. Pearson's correlation analysis showed that the KP variables correlated significantly to Expanded Disability Status Scale (EDSS) scores suggesting that perturbed KP metabolism may reflect the progression of the disease. Notably, the QA/KA ratio has the strongest correlation with EDSS ($r = 0.62$, $p < 0.0001$) underscoring the potential significance of these key KP parameters to the disability and severity of MS (Table 2). Using the Wilcoxon signed rank test for paired data analysis, we showed that the K/T ratio significantly increased over time ($p = 0.029$) in patients with RRMS demonstrating increased indoleamine 2,3-dioxygenase (IDO-1) activity (Table 3). We also found increased expression of the innate immunity signature proteins, macrophage inflammatory protein (MIP)-1α ($p = 0.004$) and MIP-1β ($p = 0.001$) over time in RRMS that are produced by infiltrating macrophages (Table 3). This is accompanied by decrease in adaptive immune respond evident by decreased IL-2 level. Although IL-7 was shown to be significantly increased over time, however, the degree of change is very small (from 13 to 18 pg/ml) and unlikely to be clinically relevant.

The KP and immune profiles predict the course of disease in MS. Currently, there are no validated biomarkers of MS[14] but our data suggests that certain metabolic KP signatures may discriminate MS subtype. To further explore the utility of KP metabolites as MS biomarkers, we applied predictive analytics. Initially, a predictive model was developed incorporating KP metabolites, inflammatory mediators and patient demographic information. The predictive model was then used to yield classification results, i.e., successful identification of MS

	RRMS	SPMS	PPMS	HC
Cohort 1, n	50	20	17	49
Female sex, n(%)	30 (60.0)	15 (75.0)	13 (76.5)	35 (71.4)
Age in years, mean (±SD)[A*]	43.4 (9.4)	53.45 (9.7)	52.24 (8.8)	45.29 (11.7)
Disease duration in year, mean (±SD)[B*]	6.92 (5.9)	16.5 (4.4)	16.18 (5.4)	N/A
Severity, EDSS, median (quartiles)[B*]	2.0 (1.5, 3.4)	6.0 (3.4, 6.5)	5.5 (2.5, 6.0)	N/A
Cohort 2, n	44	15		
Female sex, n(%)	32 (72.7)	7 (46.7)		
Age in years, mean (±SD)[A*] Baseline 2 years follow-up	47.8 (10.1) 49.4 (10.2)	59.4 (10.7) 61.5 (10.7)		
Disease duration in year, mean (±SD)[B*] Baseline Follow-up	8.73 (8.4) 10.3 (8.4)	18.2 (9.7) 20.3 (9.9)		
Severity, EDSS, mean (±SD)[B*] Baseline Follow-up	3.7 (1.9) 3.8 (2.0)	6.8 (1.5) 6.9 (1.5)		
Cohort 3, n	9	20		6
Female sex, n(%)	6 (66.7)	14 (70.0)		4 (66.7)
Age in years, mean (±SD)	49.2 (16.0)	49.7 (9.0)		43.67 (11.8)

Table 1. Demographic and clinical characteristics of Cohort 1, 2 and 3. Abbreviations: RRMS = Relapsing-remitting multiple sclerosis; SPMS = secondary progressive multiple sclerosis; PPMS = primary progressive multiple sclerosis; HC = healthy controls; EDSS = Expanded Disability Status Scale; N/A = not applicable. SD = Standard deviation. [A]ANOVA, Tukey HSD, [B]Kruskal Wallis non-parametric test, $*p < 0.001$, indicating significant difference between RRMS and SPMS groups. Adjustment had been made accordingly in subsequent analysis.

subtype. From a total of 37 potential predictors, we found six that were the most critical determinants successfully predicting MS subtype (Fig. 2A). These were KA, QA, tryptophan, PA, fibroblast growth factor-basic and tumour necrosis factor-α (in order of relevance). Using various classification models in Training Set, a C5.0 Decision Tree provided the best overall prediction achieving an accuracy of 91% shown in Fig. 2B. We further validated the model using a Test set (*Cohort 3*) and obtained a predictive accuracy of 83% (Fig. 2D). This confirmed that our panel of 6 predictors can be used as a MS subtype biomarker.

To confirm that a facile blood-based KP biomarker for MS is feasible, we correlated CSF KP variables to patient-paired serum profiles (Cohort 3). After adjusting for confounding factors (stratification of clinical grouping and presence of other KP parameters), our regression analysis indicated that serum KP metabolites were able to explain 62.9% ($p < 0.001$) of changes observed in patient-paired CSF KP variables (Table 4) with a moderately strong (65%) to strong (79.3%) correlation between CSF and serum KP metabolites.

Discussion

We evaluated the KP in MS in order to explore the links between inflammation, the KP and MS disease progression. Our data indicates that KP metabolism is aberrant in MS, as shown by elevated K/T ratio in MS patients compared to healthy controls, confirming previous studies[25,26]. Increased IDO-1 activity (as reflected by higher K/T ratios) is known to suppress the T-cell mediated response in MS[25,27] via activation of aryl hydrocarbon receptor (AhR)[10]. We recently showed that kynurenine, the by-product of IDO1, is an endogenous ligand of the AhR[10] that inhibits the inflammatory response in chronic experimental autoimmune encephalomyelitis (EAE) mice model of MS[28]. Indeed, inhibition of IDO1 in EAE mice leads to exacerbation of disease progression[29]. Initially, induction of the KP (i.e., up-regulation of IDO-1) may be beneficial as IDO-1 mediates an immunomodulatory effect in MS that partially explains the therapeutic effect exerted by interferons[4] and vitamin D[30] in early-mild stages of MS. This is also reflected in our longitudinal data (Cohort 2) with elevated K/T ratio (i.e., increased IDO1 expression) in RRMS but not SPMS to maintain a stable EDSS. However, chronic IDO-1 activation changes the excitotoxic balance due to increased QA production and may also disrupt the biosynthesis of serotonin and melatonin in the brain, as these neurotransmitters are produced in separate branches of the KP that are dependent on tryptophan (Fig. 1). Considering that lower serotonin and melatonin have been associated with depression in MS[31] and decreased melatonin is known to correlate to increased risk of MS relapse[32,33], restricted tryptophan availability caused by KP activation, may play a role in depression or relapse in MS.

Given that the KP is known to be induced by inflammation, we found surprisingly few correlations between inflammatory mediators and KP modulations. However, a positive correlation was found between interferon (IFN)-g-inducible protein (IP)-10 with K/T ratio ($r = 0.31$, $p < 0.001$) and QA ($r = 0.2$, $p < 0.001$) indicating an IFN-γ mediated response. Although we did not see significant up-regulation/correlation with IFN-γ and the KP, this may reflect the acute-phase nature of IFN-γ. Considering that the innate immunity markers (MIP-1α and MIP-1β) increased over time in the longitudinal RRMS patient samples (*Cohort 2*), this implies that even with stable EDSS, the innate immune activity is still constitutively active.

Activated innate cells such as macrophages or microglia are known to be the major source of pathophysiological concentrations of QA[34]. We previously showed that oligodendrocytes exposed to QA from activated microglia take up QA leading to oligodendrocyte death. This was reversed by targeting QA production, either by a KP inhibitor or a specific QA targeting antibody[35]. Collectively, our data provides evidence that, over time, the initially suppressive T-cell effect mediated by IDO-1 changes to a more chronic form of KP activation that leads to MS progression by the production of excitotoxic QA (and increased QA/KA ratio) by infiltrating macrophages.

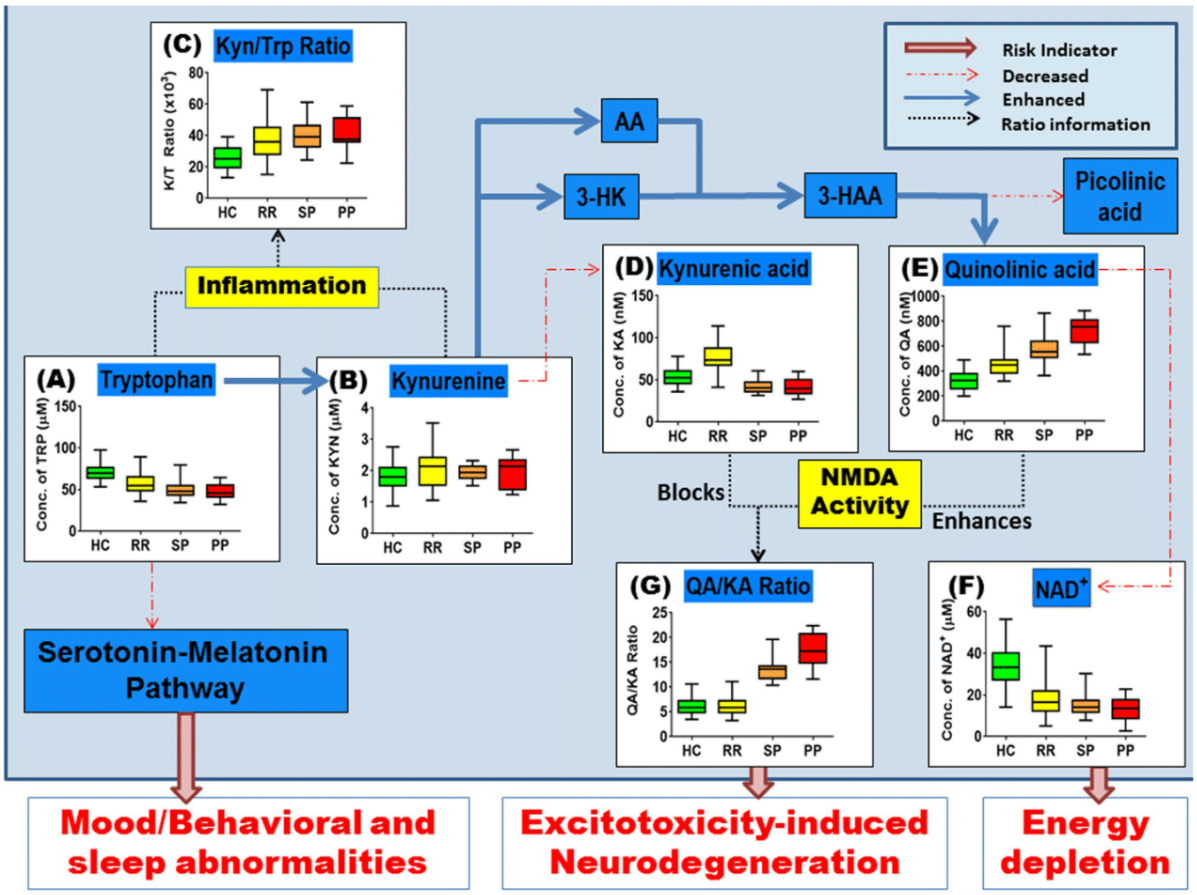

Figure 1. Overview of the kynurenine pathway with box plots of tryptophan (**A**), kynurenine (**B**), kynurenine/tryptophan (Kyn/Trp or K/T) ratio (**C**), kynurenic acid (**D**), quinolinic acid (**E**) NAD^+ (**F**) and quinolinic acid/kynurenic acid (QA/KA) ratio (**G**) in healthy control (HC), relapsing-remitting MS (RR), secondary progressive MS (SP) and primary progressive MS (PP) cohorts. The diagram illustrate how inflammation can influence the pathway leading to enhance (blue arrows) in some downstream metabolites such as 3-Hydroxykynurenine (3-HK), Anthranilic acid (AA) and 3-Hydroxyanthranilic acid (3-HAA) but not others (red dotted arrows), i.e. kynurenic acid, picolinic acid and nicotinamide adenine dinucleotide (NAD^+) based on patients with MS when compared to healthy control. The aberrant KP change in MS can potentially lead to mood/behavioural and sleep abnormalities, excitotoxicity-induced neurodegeneration and energy depletion related to cognitive fatigue in MS.

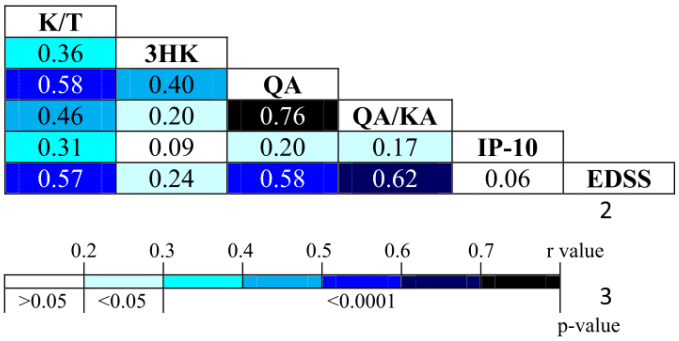

Table 2. Pearson correlation between KP variables, inflammatory mediators and MS severity (EDSS) scores in Cohort 1. Table is presented as a heat map, with darker colors depicting the strength of the correlation. All values are expressed as log transformation of the original concentration except K/T Ratio and EDSS.

Variables (n)	Baseline, median (quartiles)	Follow-up, median (quartiles)	Fold Change	*p*-value
K/T ratio (44)				
RRMS	54 (40, 65)	57 (47, 71)	0.06	0.029
SPMS	44 (39, 77)	56 (38, 95)	0.27	>0.05
IL-2 (24)				
RRMS	17 (3, 41)	4 (2, 11)	−0.76	0.011
SPMS	9 (2, 16)	3 (1, 7)	−0.67	>0.05
IL-7 (40)				
RRMS	13 (9, 19)	18 (13, 25)	0.38	0.024
SPMS	14 (11, 23)	20 (14, 24)	0.43	>0.05
MIP-1α (28)				
RRMS	5 (4, 10)	10 (5, 13)	1.00	0.004
SPMS	10 (4, 12)	9 (7, 12)	−0.10	>0.05
MIP-1β (41)				
RRMS	37 (21, 71)	69 (48, 90)	0.86	0.001
SPMS	42 (28, 94)	71 (48, 108)	0.69	>0.05

Table 3. KP and Immune profile changes between baseline and 2 years follow-up in Cohort 2 patients with RRMS. Wilcoxon signed ranks test was use and analyzed by disease group. Other variables measured that are not significant (i.e. $p > 0.05$) is not listed in the table. No significant changes were observed to any variables in the SPMS group over time. The EDSS was stable with little changes in both RRMS and SPMS shown in Table 1. Concentrations of all the inflammatory mediators are expressed in picogram per mililter (pg/ml).

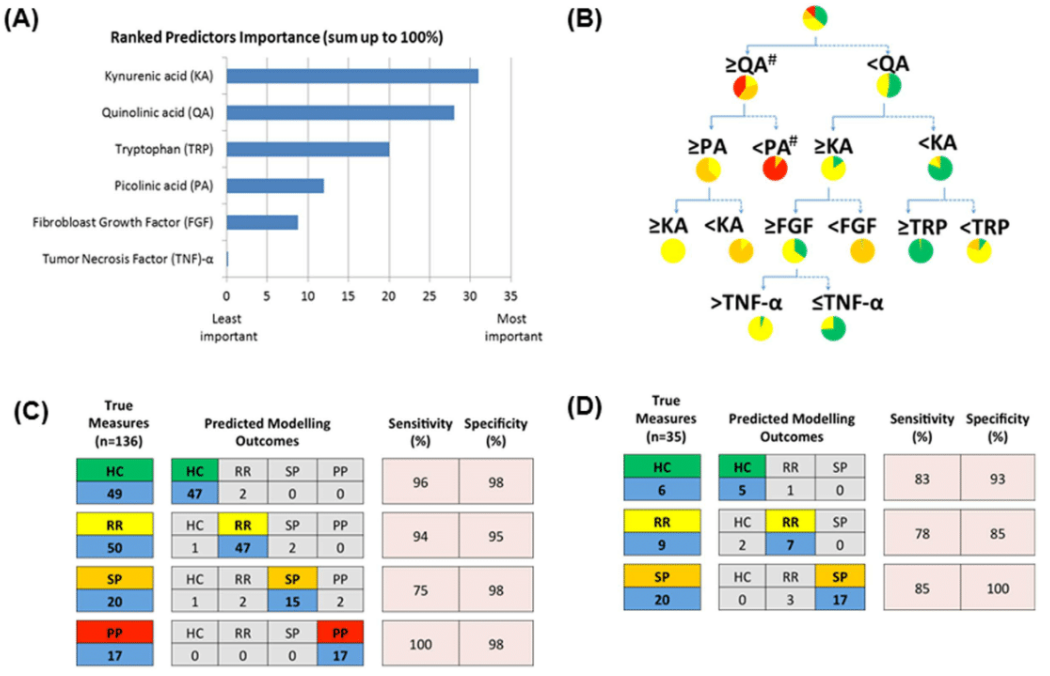

Figure 2. Biomarker for predicting MS severity using in silico approach. (**A**) Classification modelling was based on exploratory analysis on the variables in the dataset with the shortlisted six predictors, i.e., QA, PA, KA, FGF-basic, TRP and TNF-α and its ranked importance for the predictive analytics. (**B**) Training Set using a C5.0 Decision Tree comprised of pie chart proportions of healthy control or MS subtype after being split by the six predictors. To optimize the split, calculated cut-off concentrations for each predictor were determined by the analytic software. The aim is to define a set of predictors that results in a full circle for each experimental group. For example, a QA concentration ≥494 nM (#) results in isolation of the SP and PP MS subtypes, then applying a PA concentration of <313 nM (#), as the next predictor, results in 89.1% isolation of the PP MS subtype. The experimental groups are denoted: healthy control (HC; green), RRMS (RR; yellow), SPMS (SP; orange) and PPMS (PP; red). (**C**) The numbers of observed and correctly predicted HC and MS subtype in the Training Set are shown (blue boxes) along with proportions of true (sensitivity) and false (specificity) predictions. (**D**) A different Test Set was used to validate the predictive model built from the Training Set. The numbers of observed and correctly predicted HC and MS subtype in the Test Set are shown (blue boxes) along with proportions of true (sensitivity) and false (specificity) predictions.

CSF KP variables	Model (#)	Adjusted R^2	p value	AIC
TRP	1: TRP paired serum only	0.3027	0.0004	51
	2: TRP paired serum stratified by clinical groups	***0.4442***	***0.0003***	***44***
	3: TRP paired serum stratified by clinical groups and adjusted for other KP variables	0.3895	0.0073	52
KYN	1: KYN paired serum only	0.0938	0.0411	−202
	2: KYN paired serum stratified by clinical groups	***0.5051***	***<0.0001***	***−217***
	3: KYN paired serum stratified by clinical groups and adjusted for other KP variables	0.6142	<0.0001	−225
K/T Ratio	1: K/T Ratio paired serum only	0.2062	0.0036	243
	2: K/T Ratio paired serum stratified by clinical groups	***0.5592***	***<0.0001***	***201***
	3: K/T Ratio paired serum stratified by clinical groups and adjusted for other KP variables	0.4835	0.0008	233
KA	1: KA paired serum only	0.07	0.068	59
	2: KA paired serum stratified by clinical groups	***0.4234***	***0.0004***	***45***
	3: KA paired serum stratified by clinical groups and adjusted for other KP variables	0.3317	0.0178	54
PA	1: PA paired serum only	0.1593	0.01012	271
	2: PA paired serum stratified by clinical groups	***0.5172***	***<0.0001***	***229***
	3: PA paired serum stratified by clinical groups and adjusted for other KP variables	0.3548	0.1262	268
QA	1: QA paired serum only	0.334	0.0002	274.8
	2: QA paired serum stratified by clinical groups	***0.6293***	***<0.0001***	***215***
	3: QA paired serum stratified by clinical groups and adjusted for other KP variables	0.4592	0.0021	274

Table 4. Relationship of CSF and Matching Serum KP Profile. Abbreviations: AIC = Akaike information criterion. Note that selection of the best model among the proposed 3 models for each KP metabolites was based on the smallest AIC values. Overall, model 2 across all CSF KP variables has the smallest AIC value and the best model to predict CSF KP variables.

Our data support the concept that targeting innate cells may be a feasible immunotherapeutic approach to retard MS disease progression in MS which warrants further investigation.

Increased levels of neuroprotective metabolites, KA and PA, were only observed in RRMS but not in SPMS or PPMS, while toxic metabolites, 3-HK and QA level were progressively increased in both SPMS and PPMS. These observations may imply a role for neurotoxic KP metabolites in mediating neurodegeneration in MS. As described above, QA mediates potent excitotoxicity at the NMDA receptor[36], whereas KA plays a neuroprotective role as it antagonizes QA excitotoxicity at this receptor. Hence, the balance between QA and KA (expressed as the QA/KA ratio) defines the overall glutamatergic activity at the NMDA receptor and determines whether QA-mediated neurodegenerative excitotoxicity prevails[11–13]. The observed increased level of KA only in RRMS may be a compensatory mechanism in early-stage disease against QA-induced excitotoxicity, as suggested by the moderately strong correlation between QA/KA ratio and MS severity. It also indicates that the KP shunts towards production of KA, but not QA, during early disease course, whereas in later disease stages, the KP is shunted differently, favoring production of QA instead of KA. The mechanism(s) of this differential shunting remains unclear, but delineating these may suggest new therapeutic options. Additionally, as 3-HK is known to potentiate QA-induced excitotoxicity[37], the higher 3-HK levels in SPMS and PPMS patients observed in our study might also be relevant to the neurodegenerative process in MS. Our results are also consistent with the increased levels of 3-HK and QA found in tissues of the experimental autoimmune encephalomyelitis (EAE) rat model of MS[38]. Considered collectively, our results suggest that NMDA receptor mediated excitotoxicity is highly relevant in the neurodegeneration associated with progressive MS and may constitute a key threshold event in the switch from RRMS to SPMS.

To our knowledge, this is the first study using targeted KP metabolomics as a blood-based prognostic biomarker capable of distinguishing MS subtype. Previously, decreased tryptophan was found in MS patients and described as a potential biomarker[39]. However, this study only detected tryptophan and was not capable of detecting other downstream KP metabolites. This study also failed to distinguish between MS subtype. We showed that tryptophan and 3 other metabolites of the KP were important predictors of MS subtype and correlated to disease severity scores. Indeed, the four KP predictors accounted for approximately 90% of the predictive power of our built model with the two inflammatory mediators only adding 10% predictive power. This suggests that tryptophan metabolism is more relevant to MS pathology than general inflammation. The validity of model was confirmed when applied to an alternate and independent blinded set (Test set, *Cohort 3*) where we observed a reproducible accuracy of 83%.

One potential limitation of a blood based biomarker for neurological disease may be that blood parameters may not necessarily mirror those of the CNS. However, a previous study demonstrated that the blood KP profile followed closely with changes of the KP profile in CNS[40] and our analysis (which included 3 more KP variables, i.e. K/T ratio, KA and PA) showed generally good to-strong plasma-CNS KP metabolite correlations in the range

65–79.3%. Potential factors limiting these correlations may include the inherent differences in the magnitude of CSF parameters relative to serum values and the fact that the length of storage time (over 10 years) in some of these samples may have led to the partial degradation of some analytes. Although, the key metabolites kynurenine and QA have been reported as relatively stable even after many years of storage[41]. Notwithstanding these points, the moderately strong correlations between CSF and serum, confirms that the serum KP profile is a suitably sensitive blood-based predictor of disease progression in MS.

In conclusion, our results demonstrate that KP parameters have a strong association with MS subtype, correlating with disease severity scores. The changing levels of KP metabolites we observed also provides a mechanistic insight that may explain the transition from the milder RRMS form to the more debilitating SPMS disease form. KP profiling is likely to be relevant to the pathogenesis of other diseases characterized by inflammation and neurodegeneration, like Alzheimer's disease, Parkinson's disease and ALS, where aberrant KP metabolism has been reported[42]. Our results also suggest that strategies aimed rebalancing the KP, particularly in terms of QA/KA levels, could be useful therapeutic approaches in slowing neurodegeneration in MS.

References

1. Trapp, B. D. & Nave, K. A. Multiple sclerosis: an immune or neurodegenerative disorder? *Annu Rev Neurosci* **31,** 247–269 (2008).
2. Weiner, H. L. The challenge of multiple sclerosis: how do we cure a chronic heterogeneous disease? *Ann Neurol* **65,** 239–248 (2009).
3. O'Connor, J. C. *et al.* Interferon-γ and Tumor Necrosis Factor-α Mediate the Upregulation of Indoleamine 2,3-Dioxygenase and the Induction of Depressive-Like Behavior in Mice in Response to Bacillus Calmette-Guérin. *The Journal of Neuroscience* **29,** 4200–4209 (2009).
4. Guillemin, G. J. *et al.* IFN-beta1b induces kynurenine pathway metabolism in human macrophages: potential implications for multiple sclerosis treatment. *J Interferon Cytokine Res* **21,** 1097–1101 (2001).
5. Guillemin, G. J. Quinolinic acid, the inescapable neurotoxin. *FEBS J* **279,** 1356–1365 (2012).
6. Guillemin, G. J., Smythe, G., Takikawa, O. & Brew, B. J. Expression of indoleamine 2,3-dioxygenase and production of quinolinic acid by human microglia, astrocytes, and neurons. *Glia* **49** (2005).
7. Guillemin, G. J. *et al.* Kynurenine pathway metabolism in human astrocytes: a paradox for neuronal protection. *J Neurochem* **78,** 842–853 (2001).
8. Miranda, A. F., Boegman, R. J., Beninger, R. J. & Jhamandas, K. Protection against quinolinic acid-mediated excitotoxicity in nigrostriatal dopaminergic neurons by endogenous kynurenic acid. *Neuroscience* **78,** 967–975 (1997).
9. Foster, A. C., Vezzani, A., French, E. D. & Schwarcz, R. Kynurenic acid blocks neurotoxicity and seizures induced in rats by the related brain metabolite quinolinic acid. *Neurosci Lett* **48,** 273–278 (1984).
10. Bessede, A. *et al.* Aryl hydrocarbon receptor control of a disease tolerance defence pathway. *Nature* **511,** 184–190 (2014).
11. Pitt, D., Werner, P. & Raine, C. S. Glutamate excitotoxicity in a model of multiple sclerosis. *Nature medicine* **6,** 67–70 (2000).
12. Salter, M. G. & Fern, R. NMDA receptors are expressed in developing oligodendrocyte processes and mediate injury. *Nature* **438,** 1167–1171 (2005).
13. Lim, C. K., Bruce, B. J. & Guillemin, G. J. Understanding the roles of the kynurenine pathway in multiple sclerosis progression. *International Journal of Tryptophan Research* **3,** 157–167 (2010).
14. Teunissen, C. E., Malekzadeh, A., Leurs, C., Bridel, C. & Killestein, J. Body fluid biomarkers for multiple sclerosis[mdash]the long road to clinical application. *Nat Rev Neurol* **11,** 585–596 (2015).
15. Lim, C. K. *et al.* Altered kynurenine pathway metabolism in autism: Implication for immune-induced glutamatergic activity. *Autism research: official journal of the International Society for Autism Research* (2015).
16. Jones, S. P. *et al.* Expression of the Kynurenine Pathway in Human Peripheral Blood Mononuclear Cells: Implications for Inflammatory and Neurodegenerative Disease. *PLoS One* **10,** e0131389 (2015).
17. Smythe, G. A. *et al.* Concurrent Quantification of Quinolinic, Picolinic, and Nicotinic Acids Using Electron-Capture Negative-Ion Gas Chromatography-Mass Spectrometry. *Analytical Biochemistry* **301,** 21–26 (2002).
18. Braidy, N., Lim, C. K., Grant, R., Brew, B. J. & Guillemin, G. J. Serum nicotinamide adenine dinucleotide levels through disease course in multiple sclerosis. *Brain research* **1537,** 267–272 (2013).
19. Khan, A. Detection and quantitation of forty eight cytokines, chemokines, growth factors and nine acute phase proteins in healthy human plasma, saliva and urine. *Journal of proteomics* **75,** 4802–4819 (2012).
20. Hastie, T., Tibshirani, R., Friedman, J. & Franklin, J. The elements of statistical learning: data mining, inference and prediction. *The Mathematical Intelligencer* **27,** 83–85 (2005).
21. Han, J., Kamber, M. & Pei, J. *Data mining: concepts and techniques: concepts and techniques* (Elsevier, 2011).
22. Team, R.C. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria, 2012. (ISBN 3-900051-07-0, 2014).
23. Venables, W. & Ripley, B. Modern applied statistics with S. *Statistics and computing* (2002).
24. Grant, R. S., Coggan, S. E. & Smythe, G. A. The Physiological Action of Picolinic Acid in the Human Brain. *International Journal of Tryptophan Research: IJTR* **2,** 71–79 (2009).
25. Mancuso, R. *et al.* Indoleamine 2,3 Dioxygenase (IDO) Expression and Activity in Relapsing- Remitting Multiple Sclerosis. *PLoS One* **10,** e0130715 (2015).
26. Aeinehband, S. *et al.* Cerebrospinal fluid kynurenines in multiple sclerosis; relation to disease course and neurocognitive symptoms. *Brain, behavior, and immunity* **51,** 47–55 (2016).
27. Mellor, A. L. & Munn, D. H. IDO expression by dendritic cells: tolerance and tryptophan catabolism. *Nat Rev Immunol* **4,** 762–774 (2004).
28. Quintana, F. J. *et al.* An endogenous aryl hydrocarbon receptor ligand acts on dendritic cells and T cells to suppress experimental autoimmune encephalomyelitis. *Proceedings of the National Academy of Sciences of the United States of America* **107,** 20768–20773 (2010).
29. Kwidzinski, E. *et al.* Indolamine 2,3-dioxygenase is expressed in the CNS and down-regulates autoimmune inflammation. *The FASEB Journal* **19,** 1347–1349 (2005).
30. Correale, J., Ysrraelit, M. C. & Gaitan, M. I. Immunomodulatory effects of Vitamin D in multiple sclerosis. *Brain* **132,** 1146–1160 (2009).
31. Mohr, D. C., Goodkin, D. E., Islar, J., Hauser, S. L. & Genain, C. P. TReatment of depression is associated with suppression of nonspecific and antigen-specific th1 responses in multiple sclerosis. *Archives of Neurology* **58,** 1081–1086 (2001).
32. Farez Mauricio, F. *et al.* Melatonin Contributes to the Seasonality of Multiple Sclerosis Relapses. *Cell* **162,** 1338–1352 (2015).
33. Steiner, J. *et al.* Severe depression is associated with increased microglial quinolinic acid in subregions of the anterior cingulate gyrus: Evidence for an immune-modulated glutamatergic neurotransmission? *Journal of Neuroinflammation* **8,** 1–9 (2011).
34. Guillemin, G. J., Smythe, G., Takikawa, O. & Brew, B. J. Expression of indoleamine 2,3-dioxygenase and production of quinolinic acid by human microglia, astrocytes, and neurons. *Glia* **49,** 15–23 (2005).

35. Sundaram, G. *et al.* Quinolinic acid toxicity on oligodendroglial cells: relevance for multiple sclerosis and therapeutic strategies. *Journal of Neuroinflammation* **11,** 204 (2014).
36. Stone, T. W. Neuropharmacology of quinolinic and kynurenic acids. *Pharmacol Rev* **45,** 309–379 (1993).
37. Guidetti, P. & Schwarcz, R. 3-Hydroxykynurenine potentiates quinolinate but not NMDA toxicity in the rat striatum. *Eur J Neurosci* **11,** 3857–3863 (1999).
38. Chiarugi, A., Cozzi, A., Ballerini, C., Massacesi, L. & Moroni, F. Kynurenine 3-mono-oxygenase activity and neurotoxic kynurenine metabolites increase in the spinal cord of rats with experimental allergic encephalomyelitis. *Neuroscience* **102,** 687–695 (2001).
39. Cocco, E. *et al.* 1H-NMR analysis provides a metabolomic profile of patients with multiple sclerosis. *Neurology - Neuroimmunology Neuroinflammation* **3** (2016).
40. Raison, C. L. *et al.* CSF concentrations of brain tryptophan and kynurenines during immune stimulation with IFN-[alpha]: relationship to CNS immune responses and depression. *Molecular psychiatry* **15,** 393–403 (2010).
41. Midttun, O., Hustad, S. & Ueland, P. M. Quantitative profiling of biomarkers related to B-vitamin status, tryptophan metabolism and inflammation in human plasma by liquid chromatography/tandem mass spectrometry. *Rapid communications in mass spectrometry: RCM* **23,** 1371–1379 (2009).
42. Chen, Y. & Guillemin, G. J. Kynurenine Pathway Metabolites in Humans: Disease and Healthy States. *International Journal of Tryptophan Research* **2,** 1–19 (2009).

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Author Contributions

C.K.L., G.J.G., and B.J.B. conceptualized and designed the study. C.K.L. and D.B.L. wrote the manuscript. C.K.L., V.T., S.B., Al.B. performed K.P. metabolomics analysis and data collection. G.J.G., V.T., B.V.T., collected and organized the clinical samples and data. C.K.L., D.B.L. performed the immunological profiling analysis and data collection. Ay.B. and C.K.L. completed the statistical analysis and classification modelling. All authors contributed to data interpretation, reviewed the manuscript and approved the final version.

Additional Information

Supplementary information accompanies this paper at http://www.nature.com/srep

Competing financial interests: Several authors (C.K.L., G.J.G. and B.J.B.) are named inventors on international patent specifications "Method and prognostic kit for monitoring multiple sclerosis (MS)" initially published in 2013 (WO/2015/008111) that contain partial information found in this manuscript. All other authors declare no competing financial interest.

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Kynurenine pathway metabolomics predicts and provide mechanistic insight into multiple sclerosis progression

Supplementary Information

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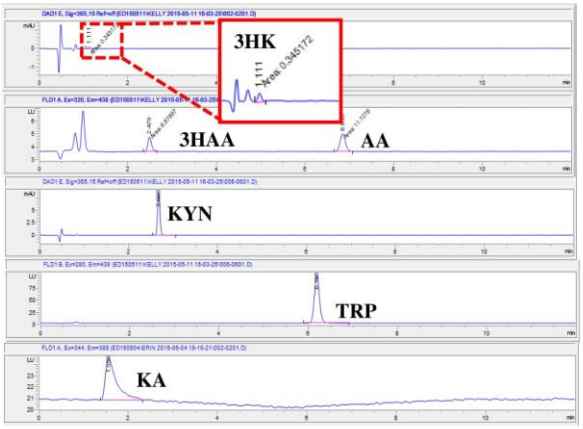
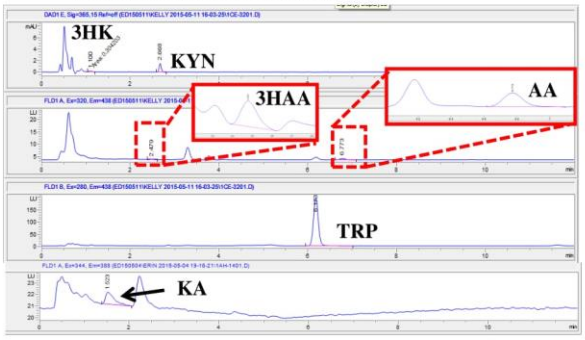
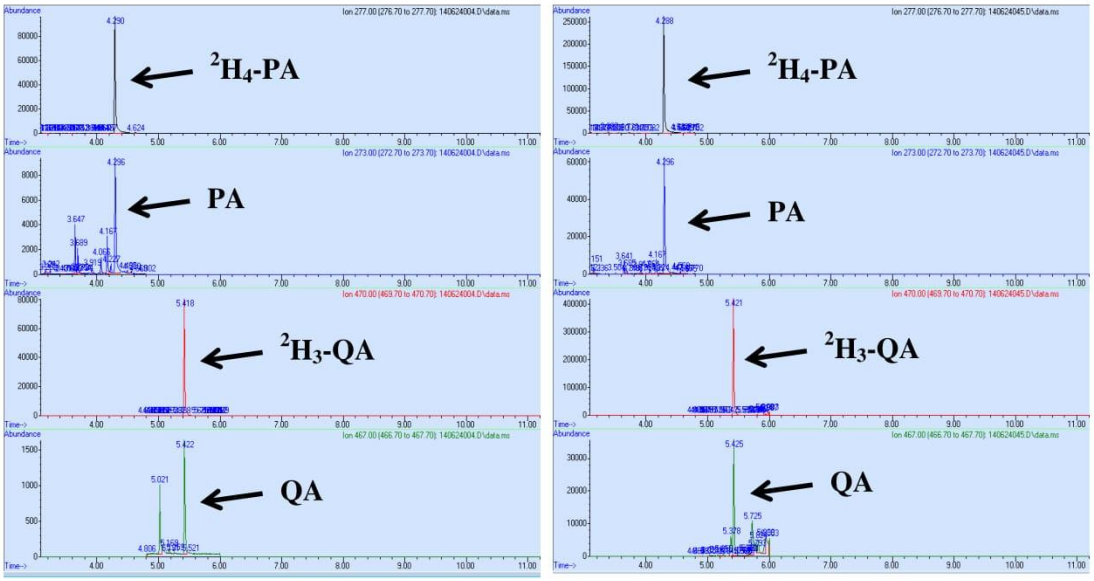
SUPPLEMENTARY FIGURE S1A



SUPPLEMENTARY FIGURE S1B



SUPPLEMENTARY FIGURE S1C **SUPPLEMENTARY FIGURE S1D**



Supplementary Figure 1. Snapshot of chromatograms of various KP metabolites standards (A) and serum sample (B) by UHPLC. Chromatogram of PA and QA standards (C) and serum samples (D) by GCMS. Although 3HAA was measured in our serum samples, the level was abnormally low. 3HAA is a relatively unstable metabolites having limited application as a biomarker and was not shown in our data.

SUPPLEMENTARY TABLES

Supplementary Table S2. Cohort 1 KP Metabolic Profiles

Metabolite	Mean or Median	Standard deviation or IQR	MS versus control Differences	*P value*
TRP[µM]				
Control (n=49)	71.11	± 10.20		
RRMS (n=50)	57.99	± 12.83	(D) -13.12	<0.0001[A]
SPMS (n=20)	50.44	± 11.63	(D) -20.67	<0.0001[A]
PPMS (n=17)	47.08	± 8.75	(D) -24.02	<0.0001[A]
KYN[µM]				
Control (n=49)	1.81	± 0.41		
RRMS (n=50)	2.05	± 0.51	(U) 0.25	0.03[A]
SPMS (n=20)	1.95	± 0.24		N.S[A]
PPMS (n=17)	1.91	± 0.50		N.S[A]
K/T Ratio				
Control (n=49)	26.02	± 7.29		
RRMS (n=50)	36.78	± 11.04	(U) 10.75	<0.0001[A]
SPMS (n=20)	40.12	± 9.09	(U) 14.09	<0.0001[A]
PPMS (n=17)	41.15	± 10.27	(U) 15.13	<0.0001[A]
KA[nM]				
Control (n=49)	52.77	46.16 – 60.65		
RRMS (n=50)	73.84	67.48 – 87.42	(U) 22.64	<0.0001[B]
SPMS (n=20)	40.46	36.52 – 46.25	(D) -11.33	0.01[B]
PPMS (n=17)	40.01	34.17 – 49.19	(D) -12.05	<0.01[B]
3-HK[nM]				
Control (n=49)	49.15	37.84 – 54.46		
RRMS (n=50)	63.67	48.87 – 81.68	(U) 25.06	<0.0001[B]
SPMS (n=20)	83.01	51.40 – 95.62	(U) 32.13	<0.0001[B]
PPMS (n=17)	83.61	69.46 – 88.92	(U) 33.74	<0.0001[B]
AA[nM]				
Control (n=49)	130.02	± 80.83		
RRMS (n=50)	116.73	± 54.35		N.S[A]
SPMS (n=20)	134.42	± 46.57		N.S[A]
PPMS (n=17)	145.54	± 55.36		N.S[A]
PA[nM]				
Control (n=49)	392.96	± 77.11		
RRMS (n=50)	459.13	± 91.74	(U) 66.17	<0.001[A]
SPMS (n=20)	388.10	± 66.94		N.S[A]
PPMS (n=17)	246.90	± 51.53	(D) -146.07	<0.0001[A]
QA[nM]				
Control (n=49)	322.13	262.26 – 377.54		
RRMS (n=50)	448.97	388.76 – 490.58	(U) 119.97	<0.0001[B]
SPMS (n=20)	552.94	511.34 – 617.71	(U) 240.07	<0.0001[B]
PPMS (n=17)	754.62	629.20 – 803.55	(U) 388.33	<0.0001[B]
QA/KA Ratio				
Control (n=49)	5.94	4.88 – 7.15		
RRMS (n=50)	5.88	4.91 – 7.08		N.S[B]
SPMS (n=20)	13.63	11.78 – 14.12	(U) 7.28	<0.0001[B]
PPMS (n=17)	17.23	14.95 – 20.56	(U) 11.41	<0.0001[B]
NAD^+				
Control (n=49)	33.54	27.28 – 39.92		

RRMS (n=50)	16.81	12.36 – 21.83	(D) -14.87	<0.0001[B]
SPMS (n=20)	14.20	11.83 – 16.61	(D) -18.32	<0.0001[B]
PPMS (n=17)	13.49	8.63 – 17.13	(D) -20.30	<0.0001[B]

Mean concentration of tryptophan (TRP), kynurenine (KYN), kynurenine/tryptophan (K/T) ratio, kynurenic acid (KA), 3-hydroxykynurenine (3-HK), picolinic acid (PA), quinolinic acid (QA) and QA/KA ratio in stratified MS groups and healthy controls. Metabolites that pass the Shapiro-Wilk normality test ($p \geq 0.05$; data not shown), ANOVA (denoted with A) was used for comparison and reported as mean with standard deviation, otherwise, Kruskal Wallis (denoted as B) was applied and reported as median with interquartile range (IQR) provided. A p value of ≤ 0.05 is considered significant for mean difference between the control and stratified MS subtypes with N.S. denotes as no significant. Further illustration of the mean differences is denote by up (U), and down (D).

Supplementary Table S3. Cohort 1 27-Plex Cytokines, Chemokine and Growth Factor Profiles

Cytokines, Chemokines and Growth Factors	**Median**	**IQR**	**MS versus control Differences[Φ]**	***P value***
IL-1β				
Control (n=49)	1.42	1.12-1.74		
RRMS (n=50)	1.15	0.92-1.59		N.S
SPMS (n=20)	1.34	0.95-1.53		N.S
PPMS (n=17)	1.29	1.01-1.69		N.S
IL-1ra				
Control (n=49)	69.53	49.65-107.31		
RRMS (n=50)	55.49	37.23-87.09	(D)	N.S
SPMS (n=20)	77.93	49.16-96.47	(U)	N.S
PPMS (n=17)	57.35	48.24-110.38	(D)	N.S
IL-2				
Control (n=49)	8.29	5.69-12.02		
RRMS (n=50)	5.54	3.64-9.94	(D)	N.S
SPMS (n=20)	10.21	5.71-12.11	(U)	N.S
PPMS (n=17)	8.99	5.96-12.65		N.S
IL-4				
Control (n=49)	1.27	0.97-1.52		
RRMS (n=50)	1.16	0.92-1.37		N.S
SPMS (n=20)	1.17	1.09-1.46		N.S
PPMS (n=17)	1.25	1.04-1.51		N.S
IL-5				
Control (n=49)	2.58	1.97-3.08		
RRMS (n=50)	2.14	1.68-2.84		N.S
SPMS (n=20)	2.34	1.90-3.16		N.S
PPMS (n=17)	2.30	2.06-3.12		N.S
IL-6				
Control (n=49)	4.68	3.33-6.21		
RRMS (n=50)	3.44	2.48-5.33	(D)	N.S
SPMS (n=20)	5.29	3.22-6.98	(U)	N.S
PPMS (n=17)	4.50	4.17-5.40		N.S
IL-7				
Control (n=49)	5.88	3.95-7.92		
RRMS (n=50)	5.26	4.27-8.85		N.S
SPMS (n=20)	6.80	5.57-7.75	(U)	N.S

PPMS (n=17)	6.78	5.65-7.98	(U)	N.S
IL-8				
Control (n=49)	13.13	8.73-14.72		
RRMS (n=50)	9.95	8.26-13.06	(D)	N.S
SPMS (n=20)	12.57	10.76-14.36		N.S
PPMS (n=17)	12.98	9.90-13.25		N.S
IL-9				
Control (n=49)	9.63	7.10-13.26		
RRMS (n=50)	7.82	6.01-9.78	(D)	N.S
SPMS (n=20)	8.48	6.71-10.84	(D)	N.S
PPMS (n=17)	8.49	7.36-8.49	(D)	N.S
IL-10				
Control (n=49)	9.71	6.75-14.76		
RRMS (n=50)	6.49	4.95-10.26	(D)	N.S
SPMS (n=20)	8.09	6.54-12.22	(D)	N.S
PPMS (n=17)	8.79	6.34-12.78	(D)	N.S
IL-12 (p70)				
Control (n=49)	20.76	12.13-29.81		
RRMS (n=50)	14.73	10.51-26.75	(D)	N.S
SPMS (n=20)	17.39	11.17-28.48	(D)	N.S
PPMS (n=17)	25.47	13.47-43.14	(U)	N.S
IL-13				
Control (n=49)	7.50	5.67-12.71		
RRMS (n=50)	5.55	4.07-10.72	(D)	N.S
SPMS (n=20)	8.68	5.95-12.23	(U)	N.S
PPMS (n=17)	12.02	8.32-15.51	(U)	N.S
IL-15				
Control (n=49)	9.77	7.05-16.48		
RRMS (n=50)	9.54	6.84-12.96		N.S
SPMS (n=20)	9.60	7.98-12.30		N.S
PPMS (n=17)	7.83	6.49-10.49	(D)	N.S
IL-17				
Control (n=49)	35.34	26.19-45.91		
RRMS (n=50)	28.34	22.72-35.88	(D)	N.S
SPMS (n=20)	32.37	25.60-37.97	(D)	N.S
PPMS (n=17)	30.49	23.52-36.96	(D)	N.S
Eotaxin				
Control (n=49)	69.92	42.24-105.27		
RRMS (n=50)	58.19	42.52-89.70	(D)	N.S
SPMS (n=20)	88.98	69.05-137.97	(U)	N.S
PPMS (n=17)	62.06	46.94-95.00	(D)	N.S
FGF-basic				
Control (n=49)	14.65	12.34-18.50		
RRMS (n=50)	12.24	9.53-15.33	(D)	N.S
SPMS (n=20)	13.55	9.53-17.89	(D)	N.S
PPMS (n=17)	13.85	10.23-16.96	(D)	N.S
G-CSF				
Control (n=49)	101.74	81.24-122.01		
RRMS (n=50)	83.17	70.24-103.33	(D)	N.S
SPMS (n=20)	97.59	80.27-128.77	(D)	N.S
PPMS (n=17)	97.91	85.10-116.96	(D)	N.S
GM-CSF				
Control (n=49)	7.64	5.87-12.21		

RRMS (n=50)	5.23	4.03-7.05	(D)	N.S
SPMS (n=20)	4.80	2.34-9.21	(D)	N.S
PPMS (n=17)	6.70	2.03-9.30	(D)	N.S
IFN-γ				
Control (n=49)	31.30	24.39-39.38		
RRMS (n=50)	24.83	17.90-32.90	(D)	N.S
SPMS (n=20)	30.12	25.58-37.85	(D)	N.S
PPMS (n=17)	28.72	20.22-33.44	(D)	N.S
IP-10				
Control (n=49)	269.58	224.74-465.89		
RRMS (n=50)	296.87	232.79-434.15	(U)	N.S
SPMS (n=20)	379.88	218.55-468.55	(U)	N.S
PPMS (n=17)	324.31	250.42-433.99	(U)	N.S
MCP-1				
Control (n=49)	45.35	36.44-65.58		
RRMS (n=50)	33.39	22.46-57.41	(D)	N.S
SPMS (n=20)	47.23	24.62-62.18	(U)	N.S
PPMS (n=17)	45.95	28.91-60-56		N.S
MIP-1α				
Control (n=49)	1.75	1.37-2.70		
RRMS (n=50)	1.37	1.00-1.78		N.S
SPMS (n=20)	1.71	1.27-2.03		N.S
PPMS (n=17)	1.69	1.41-2.84		N.S
MIP-1β				
Control (n=49)	34.47	26.16-47.07		
RRMS (n=50)	28.01	20.76-41.40	(D)	N.S
SPMS (n=20)	32.17	26.59-39.78	(D)	N.S
PPMS (n=17)	40.23	30.76-54.01	(U)	N.S
PDGF-bb				
Control (n=49)	1207.17	966.12-1577.31		
RRMS (n=50)	1193.95	919.28-1611.26		N.S
SPMS (n=20)	1203.98	977.71-1642.65		N.S
PPMS (n=17)	1201.54	1038.91-1380.43		N.S
RANTES				
Control (n=49)	1484.55	1269.85-2120.25		
RRMS (n=50)	1469.61	1158.78-1746.00		N.S
SPMS (n=20)	1602.06	1217.05-1602.06	(U)	N.S
PPMS (n=17)	1835.80	1620.58-2440.05	(U)	N.S
TNF-α				
Control (n=49)	19.68	14.68-25.83		
RRMS (n=50)	15.79	12.56-21.68	(D)	N.S
SPMS (n=20)	17.18	12.01-22.47	(D)	N.S
PPMS (n=17)	17.45	13.20-25.64	(D)	N.S
VEGF				
Control (n=49)	47.58	24.57-70.41		
RRMS (n=50)	33.27	20.52-53.41	(D)	N.S
SPMS (n=20)	32.43	21.74-47.87	(D)	N.S
PPMS (n=17)	44.53	35.57-83.25	(D)	N.S

Median concentration of interleukin-1β (IL-1β), interleukin-1 receptor antagonist (IL-1ra), interleukin-2 (IL-2), interleukin-4 (IL-4), interleukin-5 (IL-5), interleukin-6 (IL-6), interleukin-7 (IL-7), interleukin-8 (IL-8), interleukin-9 (IL-9), interleukin-10 (IL-10), interleukin-12 (p70) [IL-12 (p70)], interleukin-13 (IL-13), interleukin-15 (IL-15), interleukin-17 (IL-17), Eotaxin, Fibroblast growth factor-basic (FGF-basic), granulocyte

colony-stimulating factor (G-CSF), granulocyte-macrophages colony-stimulating factor (GM-CSF), interferon gamma (IFN-γ), IFN-γ-inducible protein 10 (IP-10), monocyte chemotactic protein-1 (MCP-1), macrophages inflammatory protein-1α (MIP-1 α), platelet-derived growth factor (PDGF-bb), macrophages inflammatory protein-1β (MIP-1β), regulated upon activation normal T-cell expressed and secreted (RANTES), tumour necrosis factor-alpha (TNF-α) and vascular endothelial growth factor (VEGF) in stratified MS groups and healthy controls. Kruskal Wallis nonparametric test was used and reported with interquartile range (IQR). We did not see any significance in mean difference between the control and stratified MS subtypes with N.S. denotes as no significant. Further illustration of noticeable mean differences is denote by up (U), and down (D). Concentrations of all the inflammatory mediators are expressed in picogram per mililter (pg/ml).

Tan, V. X., Lassus, B., Lim, C. K., Tixador, P., Courte, J., Bessede, A., Guillemin, G. J. & Peyrin J.M. (2018). Neurotoxicity of the cyanotoxin BMAA through axonal degeneration and intercellular spreading. *Neurotoxicity Research*, 33, p. 62-75.

DOI: 10.1007/s12640-017-9790-1

Tan, V. X., Mazzocco, C., Varney, B., Bodet, D., Guillemin, T. A., Bessede, A., & Guillemin, G. J. (2018). Detection of the cyanotoxins L-BMAA uptake and accumulation in primary neurons and astrocytes. *Neurotoxicity Research*, 33, p. 55-61.

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