Metabolomics of cerebrospinal fluids to identify novel biomarkers as a predictive tool for brain inflammatory conditions

by

Jingya Yan

A thesis submitted for the Degree of Doctor of Philosophy (Science)

University of Technology Sydney

October 2020

Certificate of authorship and originality

I certify that the work in this thesis has not previously been submitted for a degree nor has it been submitted as part of the requirements for a degree except as fully acknowledged within the text.

I also certify that the thesis has been written by me. Any help that I have received in my research work and the preparation of the thesis itself has been acknowledged. In addition, I certify that all the information sources and literature used are indicated in the thesis. This research is supported by an Australian Government Research Training Program Scholarship.

Jingya Yan

27/09/2020

Acknowledgements

First and foremost, I'd like to express my deepest gratitude to my primary supervisor, Professor Shanlin Fu. Your guidance, support and a wealth of knowledge has made this journey rewarding and memorable. Thank you for being my mentor for research and the many opportunities to present at conferences you have encouraged. I will always be grateful of your advice on the honours and PhD projects I chose.

To my co-supervisor, Dr Unnikrishnan Kuzhiumparambil, the success of my research projects could not have been possible without your support in the lab and wisdom on omics research. We certainly encountered some hurdles along the way but you made sure things would be silky smooth on my end. I am thankful for your encouragement and confidence in me from my honours through to PhD degrees.

A special thank you to my external supervisors, Dr Sushil Bandodkar and Professor Russell Dale from the Children's Hospital at Westmead. To Dr Sushil Bandodkar, for accessing clinical samples throughout the project, always finding time to check on my progress and project direction. To Professor Russell Dale, for sharing your clinical knowledge, designing the cohorts for the clinical study and your timely feedback. I would also like to thank them for the financial support throughout this project from supplying various reference standards to supporting my experimental work at the Sydney Mass Spectrometry services.

Thank you to all academic and professional staff at UTS for the technical support and assistance throughout my candidature. To Dr Ronald Shimmon, Dr Verena Taudte, Dr Dayanne Bordin and Dr Linda Xiao for the instrumental trainings and lab support. I wish to acknowledge Shanlin's toxicology research group for your support and feedback throughout my PhD.

I would also like to thank Professor Claude Roux, Centre for Forensic Science and the Forensic and Clinical Toxicology Association for supporting and funding my attendance at conferences.

Finally, I would like to extend my gratitude to family and friends for being by my side and supporting me throughout this chapter of my life and beyond.

Table of contents

CERTIFICATE OF AUTHORSHIP AND ORIGINALITY II

ACKNOWLEDGEMENTS III

LIST OF FIGURES IX

LIST OF TABLES XII

ABBREVIATIONS XIV

PUBLICATIONS AND CONFERENCE PROCEEDINGS XVI

ABSTRACT XVIII

CHAPTER 1: INTRODUCTION 2

SUMMARY 2

1.1 ABSTRACT 4

1.2 INTRODUCTION 5

1.3 NEUROINFLAMMATION 6

1.4 PRINCIPLE OF METABOLOMICS 8

1.5 CEREBROSPINAL FLUID AS A BIOFLUID OF DIAGNOSTIC UTILITY 9

1.5.1 Sample Preparation and Metabolite Extraction 10

1.5.2 Analytical Tools for Metabolomics 11

1.5.2.1 Nuclear Magnetic Resonance Spectroscopy 11

1.5.2.2 Mass Spectrometry 12

1.5.3 Statistics and Metabolite Annotation 14

1.6 CEREBROSPINAL FLUID METABOLOMICS: BIOMARKERS OF NEUROINFLAMMATION 15

1.6.1 Tryptophan-Kynurenine Pathway 17

1.6.2 Nitric Oxide Pathway 22

1.6.3 Neopterin 26

1.7 CHALLENGES IN CSF METABOLOMICS 29

1.8 AIM OF THE PROJECT 30

1.9 CONCLUSION 31

REFERENCES 32

CHAPTER 2: METHOD DEVELOPMENT AND OPTIMISATION 51

2.1 ABSTRACT 51

2.2 INTRODUCTION 52

2.3 MATERIALS AND METHOD 53

2.3.1 Chemicals and Reagents 53

2.3.2 Preparation of Standard Solutions 54

2.3.3 Sample preparation methods for cerebrospinal fluid samples 54

2.3.4 Liquid Chromatography 55

2.3.5 Mass Spectrometry 56

2.3.6 Data Processing 57

2.4 RESULTS AND DISCUSSION 58

2.4.1 Optimisation of Sample Preparation 58

2.4.2 Optimisation of chromatographic conditions 60

2.4.3 Optimisation of MS conditions 65

2.4.4 Column comparison using pooled CSF samples and metabolite standards 69

2.4.5 Profiling of Metabolites in CSF samples 75

2.5 CONCLUSION 78

REFERENCES 79

CHAPTER 3: A PILOT STUDY TO EVALUATE CSF METABOLOMICS IN ACUTE BRAIN INFLAMMATION 85

3.1 ABSTRACT 85

3.2 INTRODUCTION 86

3.3 MATERIALS AND METHOD 87

3.3.1 Study Design and Participants 87

3.3.2 Chemicals and Reagents 87

3.3.3 Preparation of Standard Solutions 88

3.3.4 Cerebrospinal Fluid Metabolite Extraction 88

3.3.5 UPLC-HRMS Analysis 88

3.3.6 Data Treatment and Handling 89

3.4 RESULTS AND DISCUSSION 90

3.4.1 Quality Control Analysis 92

3.4.2 Statistical Analyses 93

3.4.3 Identified Metabolites and Pathways Involved 95

3.4.4 Statistically Significant Unknown Metabolites 99

3.5 CONCLUSION 101

REFERENCES 102

CHAPTER 4: APPLICATION TO A CLINICAL STUDY 108

SUMMARY 108

4.1 ABSTRACT 110

4.2 INTRODUCTION 111

4.3 PARTICIPANTS, MATERIALS AND METHOD 112

4.3.1 Study Design and Participants 112

4.3.2 Chemicals and Reagents 113

4.3.3 Sample Preparation..... 113

4.3.4 Liquid Chromatography – High Resolution Mass Spectrometry 114

4.3.5 Data Processing and Statistical Methods..... 115

4.4 RESULTS..... 117

4.5 DISCUSSION..... 126

4.6 CONCLUSION 130

REFERENCES 131

CHAPTER 5: CONCLUSION AND FUTURE WORK..... 138

5.1 CONCLUDING REMARKS..... 138

5.2 FUTURE WORK..... 140

List of figures

Figure 1-1 Major pathways involved in neurological diseases with confirmed or suspected neuroinflammation – tryptophan-kynurenine pathway (right), nitric oxide pathway (left bottom) and neopterin (left above). Trends are highlighted in red (representing statistically elevated in patients with neuroinflammation compared to controls) and blue (representing statistically decreased in patients with neuroinflammation compared to controls). Neopterin is the most valuable inflammatory metabolite in the GTP- tetrahydrobiopterin metabolism and therefore the full pathway isn't shown. 16

Figure 2-1 PCA 3D score plot of low concentration spiked samples (n=5, green dots), medium concentration spiked samples (n=5, dark blue dots), high concentration spiked samples (n=5, red dots) and QC samples (n=5, light blue dots)..... 60

Figure 2-2 Representative total ion chromatograms of pooled CSF samples analysed in various organic solvents on the LC-QTOF/MS using the Waters HSS T3 column (from top to bottom): (1) acetonitrile, (2) methanol and (3) 50:50 (v/v) of acetonitrile/methanol..... 63

Figure 2-3 Representative total ion chromatograms of metabolite standards using the Agilent HILIC column (top) and Water HSS T3 Column (bottom) on the Thermo Q Exactive HF-X MS. 65

Figure 2-4 Extracted ion chromatograms of metabolites in pooled CSF using Waters HSS T3 Column on the Thermo Scientific Vanquish system coupled to a Q Exactive HF-X Hybrid Quadrupole Orbitrap Mass Spectrometer. Metabolites were confirmed using reference standards. 73

Figure 2-5 Extracted ion chromatograms of metabolites in pooled CSF using Agilent HILIC Column on the Thermo Scientific Vanquish system coupled to a Q Exactive HF-X Hybrid Quadrupole Orbitrap Mass Spectrometer. Metabolites were confirmed using reference standards. 74

Figure 2-6 Summary of categorised subgroups in 203 annotated compounds identified in pooled CSF. 77

Figure 3-1 [A] PCA score plot of 4 control patients (red dots), 4 encephalitis patients (green dots) and 3 QC samples (blue dots) showing clustering of the QC samples using the HILIC

column [B] PCA score plot on 3 control patients (red dots), 3 encephalitis patients (green dots) and 3 QC samples (blue dots) showing clustering of the QC samples using the HSS T3 column. 92

Figure 3-2 [A] OPLS-DA score plot presenting a clear separation of 4 control patients (red dots) and 4 encephalitis patients (green dots) using the HILIC column [B] OPLS-DA score plot presenting a clear separation score plot on 3 control patients (red dots) and 3 encephalitis patients (green dots) using the HSS T3 column. 93

Figure 3-3 Statistical differences were identified between encephalitis patients (E, n = 4) compared to controls (C, n = 4) in CSF metabolites using ANOVA and Fishers LSD post-hoc analysis [A] Six metabolites were identified from the tryptophan-kynurenine pathway [B] increased neopterin in encephalitis patients. 95

Figure 3-4 Statistical differences were identified between encephalitis patients (E, n = 4) compared to controls (C, n = 4) in CSF metabolites using ANOVA and Fishers LSD post-hoc analysis [A] Eight statistically significant amino acids [B] two lipid molecules showed an increase in encephalitis [C] increased epinephrine, choline and formyl isogluatmine and decreased adenine and acetyl-N-formyl-5-methoxykynurenamine in the encephalitis group.96

Figure 3-5 Schematic representation of the tryptophan-kynurenine pathway..... 97

Figure 3-6 Box plots (molecular weight/retention time in minutes) of metabolites with statistical difference using ANOVA and Fishers LSD post-hoc analysis ($p < 0.005$) between encephalitis patients and controls. 100

Figure 4-1 Representative extracted ion chromatograms of CSF metabolites in human cerebrospinal fluids..... 117

Figure 4-2 Statistical analysis of blinded CSF study using UPLC-HRMS. [A] PCA score plot on 14 control patients (red dots), 14 encephalitis patients (green dots) and 4 QC samples (blue dots) showing clustering of the QC samples. [B] OPLS-DA score plot presenting a clear separation of 14 control patients (red dots) from 14 encephalitis patients (green dots). 118

Figure 4-3 Tryptophan-kynurenine pathway metabolites [A] in encephalitis patients (E, n = 14) are compared to controls (C, n = 14). Significant differences were identified between the two groups in nine metabolites using ANOVA and Fishers LSD post-hoc analysis ($p < 0.001$).

[B] Tryptophan-kynurenine metabolic pathway showing the increased metabolites (red) and decreased metabolites (blue) in encephalitis. 120

Figure 4-4 Statistical analysis of ratios and neopterin in encephalitis patients (E, n = 14) compared with controls (C, n = 14). [A] kynurenine/tryptophan ratio ($p < 0.001$). [B] anthranilic acid/3-hydroxyanthranilic acid ratio ($p < 0.001$). [C] ADMA/arginine ratio ($p < 0.001$). [D] Neopterin ($p < 0.001$). 121

Figure 4-5 [A] Nitric Oxide pathway metabolites in encephalitis patients (E, n = 14) compared to controls (C, n = 14). Significant differences were identified between the two groups in four metabolites using ANOVA and Fishers LSD post-hoc analysis ($p < 0.001$). [B] Nitric oxide metabolic pathway showing the increased metabolites (red) and decreased metabolites (blue) in encephalitis. 122

Figure 4-6 [A] Statistical differences in six amino acid metabolites ($p < 0.001$) were identified In encephalitis patients (E, n = 14) compared to controls (C, n = 14) [B] histamine and its metabolite, imidazoleacetic acid were increased in encephalitis patients ($p < 0.001$) [C] histamine metabolic pathway showing the increased metabolites (red) in encephalitis [D] decreased levels of adenine and inosine and increased choline in encephalitis patients ($p < 0.001$). 124

Figure 4-7 [A] Catecholamine pathway metabolites in encephalitis patients (E, n = 14) in comparison with controls (C, n = 14). Statistical differences in six metabolites were identified ($p < 0.001$). [B] Catecholamine metabolic pathway showing the increased metabolites (red) and decreased metabolites (blue) in encephalitis [C] Four lipid molecules showed increase in encephalitis patients ($p < 0.001$). 125

List of tables

Table 1-1 CSF metabolomics studies reporting tryptophan-kynurenine pathway findings in neurological diseases with confirmed or suspected neuroinflammation. Cohorts separated into subgroups (e.g. encephalitis etc). ↑ represents statistically elevated metabolite in patients compared to controls, ↓ represents statistically decreased metabolite in patients compared to controls, ↔ reports no statistical difference between groups, and blank represents 'not reported or not measured'. Ratios are represented by x/y (e.g. KYN/TRP). .. 18

Table 1-2 CSF metabolomics studies reporting nitric oxide pathway findings in neurological diseases with confirmed or suspected inflammation. ↑ represents statistically elevated in patients compared to controls, ↓ represents statistically decreased in patients compared to controls, ↔ represents no statistical difference between groups, and blank represents 'not reported or not measured'. Ratios are represented by x/y (e.g. arginine/citrulline). 23

Table 1-3 CSF studies reporting neopterin findings in neurological diseases with confirmed or suspected neuroinflammation. ↑ represents statistically elevated in patients compared to controls. 26

Table 2-1 Characteristics of chromatographic columns 56

Table 2-2 Averaged number of molecular features determined in pooled CSF using the four sample preparation methods (n=5) using LC-QTOF/MS..... 59

Table 2-3 Metabolite standards used in the optimisation of methods 61

Table 2-4 Comparison of averaged peak area (n = 5) of metabolites standards using the QTOF/MS and Q Exactive HFX-MS using the Agilent HILIC column. The blank cells represents the metabolite was unable to be detected or poor chromatographic peak shape..... 66

Table 2-5 Number of molecular features and annotated compounds in pooled CSF samples (n=3) using the Agilent HILIC column on the Thermo Q Exactive HF-X MS system and data processed through SIEVE. 68

Table 2-6 Comparison of retention time in minutes with mean (n=5) and standard deviations (SD) across three columns in positive ionisation mode using the Thermo Scientific Vanquish

system coupled to a Q Exactive HF-X Hybrid Quadrupole Orbitrap Mass Spectrometer. The standard deviations show good retention time reproducibility. The blank cells represent the metabolite was unable to be detected or poor chromatographic peak shape. 70

Table 2-7 Comparison of peak area and peak width across three columns in the positive ionisation mode using the Thermo Scientific Vanquish system coupled to a Q Exactive HF-X Hybrid Quadrupole Orbitrap Mass Spectrometer. Blank cells represent the metabolite was unable to be detected. 71

Table 2-8 Summary of number of averaged molecular features and annotated features in pooled CSF samples (n = 5) using the HSS T3 and HILIC column on the Thermo Q Exactive HF-X MS system and data processed through Compounds Discoverer 3.0. 76

Table 2-9 Comparison of molecular annotated features in pooled CSF samples (n = 5) across three columns on the Thermo Scientific Vanquish system coupled to a Q Exactive HF-X Hybrid Quadrupole Orbitrap Mass Spectrometer. 77

Table 3-1 Summary of annotated peaks using the two complementary methods 91

Table 3-2 Summary of statistically significant metabolites identified and their relevance..... 94

Table 4-1 Summary of metabolites annotated from reference standards and putatively by literature mass spectra. 116

Table 4-2 Effect sizes of statistically significant tryptophan-kynurenine pathway metabolites, nitric oxide pathway metabolites, neopterin, kynurenine/tryptophan ratio, anthranilic acid/3-hydroxyanthranilic acid ratio and ADMA/arginine ratio. 123

Abbreviations

3-HK	3-hydoxy-kynurenine
3-HAA	3-hydroxyanthranilic acid
3-HAO	3-hydroxyanthranilic acid oxidase
BBB	blood brain barrier
AD	Alzheimer's disease
AA	anthranilic acid
ADMA	asymmetric dimethylarginine
CNS	central nervous system
CSF	cerebrospinal fluid
cART	combination antiretroviral therapy
GC	gas chromatography
GTP	guanosine triphosphate
HILIC	hydrophilic interaction chromatography
HIV	human Immunodeficiency virus
HRMS	high resolution mass spectrometry
IDO-1	indoleamine 2,3-dioxygenase 1
iNOS:	inducible nitric oxide synthase
IDH	isocitrate dehydrogenase
LC	liquid chromatography
KA	kynurenic acid
KYN	kynurenine
MS	mass spectrometry

NEO	neopterin
NO	nitric oxide
NOS	nitric oxide synthase
NIND	non-inflammatory neurology disease
NMR	nuclear magnetic resonance
OPLS-DA	orthogonal partial least squares discriminant analysis
PLS-DA	partial least squares discriminant analysis
PIC	picolinic acid
PCA	principal component analysis
RP	reverse phase
QA	quinolinic acid
QC	quality control
SAH	subarachnoid hemorrhage
SDMA	symmetric dimethylarginine
TDO	tryptophan 2,3-dioxygenase

Publications and conference proceedings

Refereed journal publications directly related to this project

Yan, J., Kuzhiumparambil, U., Bandodkar, A., Bandodkar, S., Dale, R.C. and Fu, S. 2021, Cerebrospinal fluid metabolites in tryptophan-kynurenine and nitric oxide pathways: biomarkers for acute neuroinflammation. *Developmental Medicine and Child Neurology*. https://doi.org/10.1111/dmcn.14774

Refereed journal publications from other related research activities

Yan, J., Kuzhiumparambil, U., Bandodkar, S., Solowij, N., Fu, S. 2017, Development and validation of a simple, rapid and sensitive LC-MS/MS method for the measurement of urinary neurotransmitters and their metabolites, *Analytical and Bioanalytical Chemistry*. https://doi.org/10.1007/s00216-017-0681-3

Refereed conference proceedings (oral presentations)

Yan, J., Kuzhiumparambil, U., Bandodkar, S., Dale, R.C., Fu, S. Development of an untargeted metabolomics analysis of cerebrospinal fluid samples for clinical laboratories. The 57th Annual Meeting of the International Association of Forensic Toxicologists. Birmingham, United Kingdom, September 2-6, 2019.

Yan, J., Kuzhiumparambil, U., Bandodkar, S., Dale, R.C., Fu, S. The development of an untargeted metabolomics assay for cerebrospinal fluids using LC-MS/MS. Forensic and Clinical Toxicology Association Conference. Adelaide, Australia, June 16-19, 2019.

Yan, J., Kuzhiumparambil, U., Bandodkar, S., Fu, S. Development of a Targeted Metabolomics Assay for Forensic and Clinical Laboratories. Australian and New Zealand Forensic Science Society 24th International Symposium on Forensic Sciences. Perth, Australia, September 9-13, 2018.

Yan, J., Beale, C., Kuzhiumparambil, U., Solowij, N., Fu, S. Investigation of neurotransmitter level change in urine of chronic cannabis users following prolonged cannabidiol administration. Forensic and Clinical Toxicology Association Conference. Melbourne, Australia, November 19-22, 2017.

Abstract

Inflammation of the brain is increasingly recognised as important in encephalitis. The high mortality and morbidity rates of acute neuroinflammatory diseases has directed significant interest in the investigation of biomarkers to define neuroinflammation and explore mechanisms involved in the regulation of central nervous system immune responses. Metabolomics is a rapidly emerging research field increasingly recognised as a powerful approach for addressing the gaps in knowledge underlying the pathophysiologic mechanisms involved in neuroinflammation and accurate diagnostic biomarkers.

The advancements in analytical platforms followed by subsequent chemometrics tools have revolutionised untargeted metabolomics analyses. With liquid chromatography coupled to high resolution mass spectrometry moving to the forefront, an untargeted metabolomics analysis method was developed and optimised to identify multi-class metabolites in human cerebrospinal fluids. The detection of cerebrospinal fluid metabolites were determined based on a simple and rapid methanol precipitation sample preparation method. The chromatographic separation was achieved within a twenty minute gradient elution using hydrophilic interaction chromatography. The method exhibited good reproducibility, high efficiency chromatographic separation and strong mass resolving mass spectrometry analysis. The practicality and robustness of the developed method on a pilot study further demonstrated the potential of the untargeted metabolomics strategy to identify biomarkers and understand the biochemical pathways involved in neuroinflammation.

With metabolites as the downstream products of cellular function, the application of metabolomics data is to understand the pathogenesis of neuroinflammatory mechanisms involved in encephalitis. Preliminary evidence showed statistically

discriminative metabolites in the tryptophan-kynurenine pathway, nitric oxide pathway and elevation of neopterin. The use of the adjacent ratios such as kynurenine/tryptophan, anthranilic acid/3-hydroxyanthranilic acid and ADMA/arginine in combination with neopterin can serve as a potential cerebrospinal fluid biomarker panel to predict neuroinflammation, particularly when routine tests and neuroimaging return a negative result in encephalitis patients. The emergence of cerebrospinal metabolomics holds significant promise incorporating omics research into a clinical diagnostic service.