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

by

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Certificate of authorship and originality

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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.

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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

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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.

Chapter 1:

Introduction

Chapter 1: Introduction

Summary

This chapter examines the diagnostic utility of cerebrospinal fluid with focus on the translational opportunities of detecting human diseases with neuroinflammatory mechanisms using cerebrospinal fluid metabolomics.

The chapter will first provide an overview of metabolomics, cerebrospinal fluid sample preparation and analysis, followed by an in depth literature investigation on the use of cerebrospinal fluid metabolomics to identify biomarkers for a broad spectrum of human diseases that have proven neuroinflammation and central nervous system diseases with emerging evidence of the role of neuroinflammation.

The bulk of this chapter is from a critical review that was submitted for publication to Clinical and Translational Immunology and under review.

Cerebrospinal fluid metabolomics: detection of neuroinflammation in human CNS disease

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1.1 Abstract

The high morbidity and mortality of neuroinflammatory diseases drives significant interest in understanding the underlying mechanisms involved in the innate and adaptive immune response of the central nervous system (CNS). Diagnostic biomarkers are important to define treatable and reversible neuroinflammation. Metabolomics is a rapidly evolving research area offering novel insights into metabolic pathways, and elucidation of reliable metabolites as biomarkers for diseases. We first review the technology surrounding metabolomics including the use of cerebrospinal fluid (CSF) as a biofluid and analytical methodology. We then focus on the emerging literature regarding the detection of neuroinflammation using CSF metabolomics in human cohort studies. Studies of classic neuroinflammatory disorders such as encephalitis, CNS infection and multiple sclerosis confirm the utility of CSF metabolomics. Additionally, studies in neurodegeneration and neuropsychiatry support the emerging potential of defining neuroinflammatory pathways in common CNS diseases such as Alzheimer's disease and depression. We demonstrate metabolites in the tryptophan-kynurenine pathway, nitric oxide pathway and neopterin show moderately consistent ability to differentiate patients with neuroinflammation from controls. We discuss the importance of methodological standardisation in the translation of CSF metabolomics into clinical practise.

Keywords: Cerebrospinal fluid, metabolomics, neuroinflammation, tryptophan-kynurenine, nitric oxide pathway, neopterin

1.2 Introduction

Neuroinflammation is inflammation of the CNS initiated in response to either infection, autoimmunity, traumatic brain injury, toxic metabolites or degeneration. In the case of acquired inflammation or infection, the inflammatory response is driven by invading immune cells such as infiltrating lymphocytes or monocytes. Alternatively, cellular and biochemical responses mediated by resident immune cells of the brain (such as microglia) contribute to repair or neuronal damage, and the disease expression of neurodevelopmental disorders, neurodegeneration and toxic injuries [1, 2].

The negative impact of neuroinflammation has directed research to identify the pathophysiological processes [3]. Encephalitis is inflammation of the brain commonly found in children as a result of viral infection or an autoimmune response. Meningitis is another dangerous inflammatory condition of the meninges surrounding the brain and is caused by invasive viruses and bacteria. The significant mortality and morbidity of encephalitis and meningitis have directed great attention to explore the pathophysiologic mechanisms and biomarkers for identification [4, 5]. Due to the lack of knowledge underlying the pathophysiological processes that contribute to the progression of human diseases with neuroinflammatory mechanisms, and the lack of accurate diagnostic markers and limited effective therapies, there is a great demand for novel approaches.

Metabolomics is a rapidly emerging discipline and increasingly involved in characterising neurological diseases. The key function of metabolomics is to describe the entire metabolome within a biological sample and correlate these metabolomic profiles with known physiological or pathological states [6]. CSF is mainly produced by the choroid plexus in the lateral and third ventricles, playing an important role in the maintenance of homeostasis, protection and nourishment of the brain. CSF is a useful biofluid for analysing brain metabolism, and provides an enhanced representation of neurochemical states and greater metabolite sources for neuroinflammation in human CNS diseases [7]. The information from CSF metabolomics can offer insights

into the correlations between the metabolomes and cellular processes, which can further provide a deeper understanding of the molecular disease mechanisms [8, 9].

This chapter will explore the diagnostic utility and developments in CSF metabolomics with focus on the use of metabolomics to identify biomarkers for a broad spectrum of conditions that have inflammation as a component of the disease including proven neuroinflammatory disorders such as encephalitis, meningitis, and multiple sclerosis. Moreover, CNS diseases with emerging evidence of the role of neuroinflammation such as common neurodegenerative and neuropsychiatric conditions [10, 11]. A background to neuroinflammation, metabolomics, CSF sample preparation and analysis methods will be provided first followed by highlighting potential metabolite biomarkers in the tryptophan-kynurenine pathway, nitric oxide pathway and neopterin for a range of studies detecting neuroinflammation in human diseases.

1.3 Neuroinflammation

Neuroinflammation is an inflammatory response of the brain or spinal cord initiated as a result of infection, traumatic brain injury, toxic metabolites, or autoimmunity. Inflammatory responses are coordinated through the interaction and movement of various signalling molecules and cells releasing mediators such as cytokines, chemokines, secondary messengers and reactive nitrogen and oxygen species. The production of inflammatory mediators is formed from peripherally derived immune cells, CNS glia and endothelial cells.

Peripheral inflammation initiates a neuroinflammatory response involving the blood-brain barrier (BBB), glia and neurons. The BBB is a highly specialised form of endothelial layer regarded as a key to the understanding of prolonged neuroinflammation due to peripheral injuries. As an active transport mechanism, BBB is not only permeable to pro-inflammatory mediators but can also facilitate the release and mobilisation of interleukin cytokines into the brain [12]. Microglial cells are resident macrophages of the CNS and are responsible for the release of pro-

inflammatory mediators in the neuroinflammation process [13]. In the state of chronic neuroinflammation microglia are activated for extended periods resulting in large quantities of neurotoxic molecules. Putative CSF microglial activation biomarkers have been identified, however, to date their capability of reflecting disease activity and progression cannot be determined. Astrocytes play an important role in synaptic regulation and stimulation of inflammatory molecules in the cortex and mid-brain [14]. Cytokines are signalling proteins that mediate the process of neuroinflammation such as tumour necrosis factor and interleukin are integral in the pathology of inflammation and acceleration of diseases. Chemokines are small cytokines with extremely low physiological concentrations in the CNS and take part in the chemotaxis and upregulation of microglia and astrocytes, causing damages in neuronal function [15].

Acute brain inflammation is a severe inflammatory disease and stands as an important global public health issue. The significant mortality and morbidity rates in both children and adults is estimated to affect approximately 5-10 per 100,000 individuals per year [16]. There are over 100 causes of acute brain inflammation as a sequelae of direct infection, post-infection or autoimmune process. Over the past decade, advances and improved understanding of neuroinflammatory mechanisms have uncovered new infectious and autoimmune causes of acute brain inflammation diseases [16]. However, 37 to 63% of acute brain inflammation cases remain without a known etiology [17, 18].

The development and diagnosis of acute brain inflammation remains a challenge to clinicians in its early stages given the non-specific and heterogeneity of clinical presentations. There are limited accurate diagnostic biomarkers to demonstrate and monitor inflammation and a lack of knowledge on the neuroinflammatory mechanisms underlying acute brain inflammation. Hence, there is a great demand for novel approaches to offer new insights into the pathophysiological processes, monitor neuroinflammation and responses to treatment.

1.4 Principle of Metabolomics

Metabolomics is an emerging field and increasingly used as a characteristic fingerprint in clinical sciences. Metabolomics depicts the systematic analysis of metabolism and metabolite responses to physiological stimuli including disease states or therapeutic interventions [19]. Metabolites are crucial for cellular functions whereby biological disturbances can cause a series of metabolic changes in the body. Small molecule metabolites such as amines, amino acids, neurotransmitters, fatty acids and products of cellular activities are a result of biological and environmental factors which hold potential in reflecting the metabolic functions and bridging knowledge of genotype and phenotype.

This novel technique is used to derive unique information on the downstream effects caused by diverse metabolite activity and regulations with different physical and chemical properties [6]. The strength of metabolomics is the discovery of correlations between the metabolome and cellular processes which identifies changes in metabolite profiles that are distinctive specific disease states. This aims to generate biomarkers for early diagnosis and monitoring patient responses to treatments [20].

The diversity of the CSF metabolome requires advanced analytical tools to identify changes and fluxes in the concentrations of endogenous metabolites [21]. The advancements in nuclear magnetic resonance (NMR), mass spectrometry (MS) based methods and chromatography has enabled new opportunities and enhanced metabolic profiling for biological fluids [22]. Advanced statistical tools such as chemometrics have become a dedicated and essential tool for the extraction of valuable metabolic signature information. Chemometrics can unravel information of significant metabolites for comparisons between disease patients and controls [23].

1.5 Cerebrospinal Fluid as a biofluid of diagnostic utility

Blood has been the most commonly used biological fluid for the diagnosis of clinical chemistry [24] due to its rich metabolome, less invasive nature (compared to CSF and brain biopsy) and ability to reflect metabolic states. CSF is a less commonly used biofluid compared to blood [24]. However, CSF is the closest biological matrix to the brain and directly reflects the pathophysiological alterations of the CNS [8]. CSF is a colourless filtrated product of blood plasma located in the subarachnoid spaces and ventricles of the brain. The production of CSF occurs mainly in the choroid plexus at a rate of four hundred to six hundred millilitres per day [25]. This is driven by a combination of processes including active transport and diffusion. CSF is mainly composed of water and contains enzymes, metal ions or salts, neurotransmitters, carbohydrates and short chain fatty acids [26]. CSF is circulated within the cranial and spinal arachnoid villi sites and absorbed through the arachnoid villi and into the venous outflow system. CSF is reabsorbed into the blood through arachnoid granulations. The analysis of CSF metabolites, interpretation of metabolite data and subsequent biochemical changes are fundamental to understand neuroinflammatory mechanisms, identify biomarkers, enable prognosis of disease developments and treatment strategies.

The workflow for CSF metabolomics analysis involves three major steps, pre-analytical work, analytical work and data processing [27]. The pre-analytical stages requires careful handling in the collection, pre-processing and storage steps of CSF to ensure the integrity of the samples before chemical analysis. In the analytical stage there are multiple steps involved in CSF metabolite extractions and data acquisition using analytical technologies. The data processing stage can comprise of feature extractions, metabolite identification and statistical analysis.

Standardised CSF sample handling procedures are imperative in the search for reliable biomarkers. It has been reported that delayed storage and blood contamination of CSF result in changes of prostaglandin D-synthase peptides, amino

acids and metabolites [28]. CSF samples are recommended to be centrifuged immediately after collection and stored at −80 °C.

1.5.1 Sample Preparation and Metabolite Extraction

Sample preparation and metabolite extraction plays an important role in exploring the cellular metabolome. The development of efficient sample preparation procedures for CSF is further challenged by the limited sample volumes available for analysis. The common extraction methods (such as organic solvent based precipitation, ultrafiltration, dilution, solid phase extraction) for CSF metabolites have been extensively reviewed [29-32]. Deproteinisation is a requirement for sample pre-treatment in all methods. The presence of proteins is deemed to reduce accuracy, precision and instrument lifetime [33]. CSF samples are generally analysed with minimal sample preparation to maintain a wide coverage in metabolites. For this reason, non-selective sample preparation methods such as dilute-and-shoot or solvent-protein precipitation are more commonly used [34, 35].

In metabolomic studies the most frequently used extraction methods involve organic solvent based protein precipitation, and subsequently ultrafiltration or centrifugation [36]. Organic solvent-based protein precipitation can extract hydrophobic and hydrophilic compounds whereby the extent of metabolite recovery is dependent upon the nature of solvents used. For example, Bruce et al. reported acetonitrile enhances protein removal but methanol, methanol/ethanol or methanol/acetonitrile provides better metabolic coverage in human plasma [37]. In larger cohort experiments, automated solvent based protein precipitation with 96-well plate formats have become recently available [38]. This extraction approach was successfully demonstrated in a study conducted by Fuertig et al [39]. The researchers introduced the use of 96-well plates to perform single-step ultrafiltration for CSF, plasma and brain tissue samples. This significantly reduced the time and cost of sample preparation and allowed direct injection of the filtrate into LC-MS/MS systems. Turbulent flow chromatography is an alternate automated online procedure

used for removing proteins. The technique enables the crude biological sample to be directly injected into a turbo flow column at a high flow rate [40].

1.5.2 Analytical Tools for Metabolomics

The diversity of chemical and physical properties in the CSF metabolome requires advanced analytical tools to ensure the accuracy of metabolite identification and improve the coverage of metabolites. NMR [41-43] and mass spectrometry based methods (such as liquid chromatography and gas chromatography) [44-48] are principal technological platforms employed for metabolomics. The behaviour of small molecules is exploited on the ^{1}H NMR system through a magnetic field which distinguishes different nuclei based on their resonant frequency. In comparison, mass spectrometry identifies the composition of ions through the mass-to-charge ratio in charged molecules. The data generated by both analytical approaches are powerful means of understanding biochemical consequences of diseases whereby the spectrum of peaks establishes associations between cellular responses and the levels of metabolites.

1.5.2.1 Nuclear Magnetic Resonance Spectroscopy

NMR spectroscopy has significantly contributed to the field of metabolomics holding advantages in the scope of metabolite quantification, metabolite identification, good experimental reproducibility and non-destructive nature[41]. The capacity of NMR to resolve thousands of peaks in complex biological mixtures and unambiguous structural identification of new metabolites led to the initial choice of development in the field of metabolomics [49]. As a powerful approach for identification the main advantages of using NMR over MS analysis is its great reproducibility and highly quantitative nature where metabolite peaks are assigned an integral value which is directly in proportion to the concentration of the metabolite [50, 51]. NMR is capable of detecting uncharged and polar compounds including amines, sugars and volatile substances that are often undetectable by GC or LC-MS based methods. NMR offers

capabilities to identify active metabolic pathways, measure enzyme activities and metabolite fluxes by tracing nuclei flow using ^{1}H, ^{13}C and ^{15}N[52].

The large number of metabolites in biological samples requires the development of enhanced methods. With numerous types of NMR available, ^{1}H NMR spectroscopy has evolved as the standard technique for most NMR-based metabolomics research [53]. The data collection process is characterised as reproducible, simple, rapid and readily yields spectra of peaks. A major advantage of ^{1}H-NMR is the non-destructive nature and limited sample preparation which is beneficial for the CSF specimens. However, a major challenge in ^{1}H techniques is the reliable quantification of metabolites due to the generation of overlapping signals in the spectra. The overlap of signals is caused by limited spectral dispersion of proton chemical shifts leading to uncertainties in the assignment of metabolites.

1.5.2.2 Mass Spectrometry

With high demands on the development of analytical methods to manage and diagnose numerous human diseases, MS has undertaken significant advancements in experimental designs, software, instrumentations and databases [50]. Over the last decade, MS-based methods for metabolite analysis has been extensively used for clinical diagnoses [54, 55]. The high sensitivity, good mass accuracy, wide dynamic range of metabolite coverage and minimal sample volume required are extremely beneficial for the analysis of CSF metabolites.

The accuracy and sensitivity of MS detection relies on a number of experimental and instrumental conditions such as separation, ionisation and detection methods. The complex nature of biological samples often requires desired metabolites separated prior to the acquisition of MS. As a result, MS hyphenated with different chromatographic techniques such as liquid chromatography (LC) and gas chromatography (GC) play increased roles in metabolomics studies[45]. GC-MS is a versatile platform with a long-standing history of established protocols for analysing metabolites [48, 56]. GC-MS offers better separation of metabolites and its gaseous

phase avoids ion suppression. However, GC is mainly disadvantaged by the requirement of a chemical derivatisation for metabolic species prior to instrumental analysis [57]. LC-MS has undertaken tremendous growth in the last decade and greater interest due to its capability of detecting a larger pool of metabolites without the need of chemical modifications [58]. Conventional reverse phase chromatography is employed to separate non-polar to slightly polar metabolites. The advancements in hydrophilic interaction liquid chromatography are becoming increasingly popular for the analysis of polar metabolites [59].

One of the most crucial steps in MS detection and measurement of metabolites is ionisation [60]. Electron ionisation is commonly employed in GC-MS studies for high chromatographic resolution and rich fragmentation patterns of low molecular weight and volatile compounds. Electrospray ionisation is a soft ionisation technique more commonly used in LC-MS studies with the ability to adequately ionise small and large molecules.

Metabolite detection approaches acquiring high sensitivity and resolution are of great interest. The challenge remains for the achievement of both goals in any single MS detection as higher resolution leads to reduced sensitivity and vice versa. Global metabolomic profiling is predominantly conducted using high resolution mass spectrometry such as quadrupole-time-of-flight, orbitrap and fourier transform ion cyclotron resonance instrumentations due to their higher mass resolving power [60]. For targeted studies, triple-quadrupole ion trap and triple quadrupole mass analysers are deemed more suitable.

Given the diversity and size of the CSF metabolome there is no single platform able to identify and quantify all the metabolites. The unique strengths in NMR and mass spectrometry technologies have undoubtedly contributed to the rapid growth of metabolomics and shown to be highly complementary. There is increased recognition on the importance of combining both NMR and MS metabolomics and are demonstrated in several studies [61-63].

1.5.3 Statistics and Metabolite Annotation

The advancement of analytical technologies has led to the demand of different data-analysis tools required in the process of extracting relevant information. Chemometrics has developed into a well-established statistical tool in areas such as multivariate calibration, pattern recognition, multivariate statistical process control and quantitative structure modelling [23, 64-66]. The diverse disciplines of chemometrics offer reliable data measurement, enabling researchers to model data and generate visual representations. The most commonly used chemometrics methods are principal component analysis (PCA) and partial least squares – discriminant analysis (PLS-DA) [67].

As an unsupervised method, PCA explores the overall structure of a dataset in an unbiased view by discovering grouping and trends within a dataset. The objective of PCA is to preserve as much variance in the lower dimensionality output at a linear transformation [68]. The main benefit of PCA is due to the simple nature of the non-parametric method; it can project MS and NMR into lower dimensional space, providing characteristic data structure and reduced dimensional representation of the original data [69]. Despite the widespread use of PCA in metabolomics, there are limitations in this approach. First of all, PCA has no associated probabilistic model exhibiting difficulties in the assessment of the PCA fit to the data. This limits the potential of extending the scope and application of PCA. Secondly, PCA can fail to display underlying groups of subjects in a set of data resulting in false interpretations.

Supervised methods support prior known structures of data to recognise patterns and rules for the prediction of new data. Partial least squares – discriminant analysis is the most frequently employed supervised method for classification [70]. Discriminant analysis aims to search for a linear function to maximise the ratio of between-class variance and minimise the ratio of within class variance. Discriminant analysis is a rapid and powerful tool where the optimisation of parameters is not required. PLS-DA possesses the ability to provide great insights into the cause of discrimination and

handling highly collinear data. The advantages of PLS-DA reflect its usefulness for biomarker discovery [66].

The human metabolome database (http://www.hmdb.ca) is currently the most comprehensive and largest database supporting the interpretation of metabolomics data[71]. The CSF Metabolome data (https://www.csfmetabolome.ca), METLIN (http://metlin.scripps.edu), ChemSpider (http://www.chemspider.com), NIST (http://chemdata.nist.gov) mass spectral library and mzCloud (https://www.mzcloud.org) are also popular avenues to perform metabolite identifications. Over the last decade, data pre-processing softwares, metabolite databases and libraries available for NMR and MS metabolomics research have significantly expanded [72-75] with increased dependence on the usage of metabolome repositories and querying platforms [76].

1.6 Cerebrospinal Fluid Metabolomics: Biomarkers of Neuroinflammation

The identification of biomarkers is clinically useful for an accurate diagnosis, prognosis and disease management [77]. CSF metabolomics applications which focus on biomarker discovery offer the promise of earlier detection and improved outcomes. Current biochemical and neuroimaging techniques are *a priori*, and restricted to a small set of metabolites for metabolic profiling [78]. Neuroimaging is limited due to its insensitive and low specificity nature for early diagnosis. There has been increasing focus using the metabolomics approach to explore the processes underlying neuroinflammation to identify unique metabolite biomarkers and disease associated metabolic networks. There are three main metabolic pathways involved in human studies of CNS inflammation that we now discuss in detail, specifically tryptophan-kynurenine, nitric oxide and neopterin (Figure 1-1). However, there are a number of other metabolites and pathways associated with inflammatory processes, including biogenic amines, amino acids, neurotransmitters, carbohydrates and lipids.

The research in these areas are on a smaller scale, are less consistent, and have broader variation of metabolic network coverage across independent studies, and these are not discussed in this review.

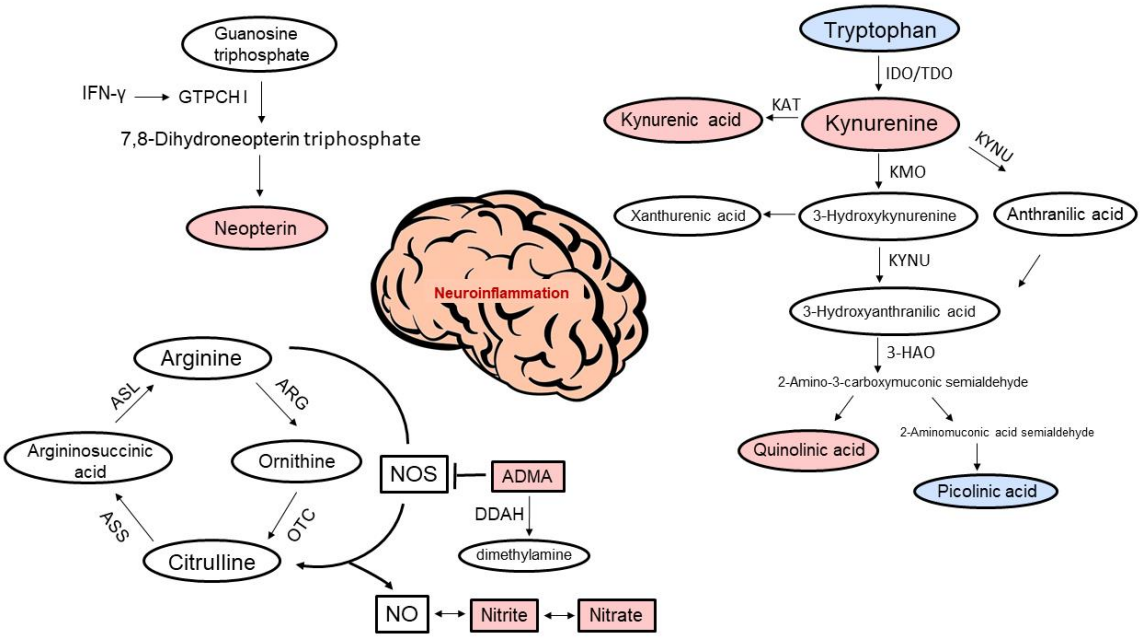

Figure 1-1 Major pathways involved in neurological diseases with confirmed or suspected neuroinflammation – tryptophan-kynurenine pathway (right), nitric oxide pathway (left bottom) and neopterin (left above). Trends are highlighted in red (representing statistically elevated in patients with neuroinflammation 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.

1.6.1 Tryptophan-Kynurenine Pathway

The tryptophan-kynurenine metabolic pathway commences with the conversion of tryptophan into kynurenine (Figure 1-1), stimulated by indoleamine 2,3-dioxygenase 1 (IDO-1) [79]. Kynurenine is further metabolised by three main enzymes, kynurenine aminotransferase, kynurenine 3-monooxygenase and kynurenase dividing into three arms generating its metabolites, kynurenic acid (KA), 3-hydoxykynurenine (3-HK) and anthranilic acid (AA) respectively. 3-HK and AA can be converted to 3-hydroxyanthranilic acid (3-HAA) and afterwards interacted with 3-hydroxyanthranilic acid oxygenase to produce quinolinic acid (QA) and picolinic acid (PIC).

The kynurenine pathway is involved in neuroinflammation due to activation of IDO and related enzymes, causing depletion of tryptophan and imbalanced formation of neuroprotective and neurotoxic metabolites (Figure 1-1). Table 1-1 summarises human cohort studies (with controls) of the tryptophan/kynurenine pathway as a biomarker of inflammation in CSF. As shown, the studies vary in the size of patient and control cohorts (Table 1-1). The disease states are separated into CNS infection such as encephalitis, meningitis or other infections known to affect the CNS (hepatitis C, HIV, malaria etc). Subsequently, studies on multiple sclerosis, a recognised neuroinflammatory disorder of proposed autoimmune aetiology, are reported. Furthermore, Table 1-1 shows studies of diseases where inflammation is increasingly described, such as in neurodegeneration and mental health, followed by other entities with possible inflammatory associations. As seen in Table 1-1, there are general trends that inflammation results in decreased tryptophan, elevated kynurenine or kynurenic acid, with elevated kynurenine/tryptophan ratio (or decreased tryptophan/ kynurenine ratio). Quinolinic acid was almost universally elevated and picolinic acid was generally reduced when measured. The analysis of CSF metabolites in the tryptophan-kynurenine pathway therefore holds promises as inflammatory biomarkers in the early diagnosis and prognosis of neurological

pathologies and provides insights into their pathophysiology. As recently reviewed, it should be highlighted that inflammation induced by activation of IDO and tryptophan 2,3-dioxygenase (TDO) is often inferred due to changes in metabolite ratios, rather than actual measurement of the IDO/TDO enzyme protein or activation status [80, 81]. The development of inflammatory mediated neuropathology is associated with the changes of quinolinic acid levels [82]. Quinolinic acid is an important metabolite inducing immunosuppression, and has been hypothesised to induce toxicity in brain cells [83] and associated with glutamate neurotoxicity [84]. Further studies in common neurological diseases with possible inflammatory mechanisms such as neurodegeneration, neuropsychiatry and neurodevelopmental disorders are therefore warranted.

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).

Disease Cohort	**Description of control group**	**Analytical Platform**	**Findings**					**Ref**
			TRP	KYN	KA	QA	Other	
Encephalitis, meningitis and infection								
Encephalitis (infectious, autoimmune, unknown, n=10); acute aseptic meningitis (n=25); acute bacterial meningitis (n=6)	NIND (n=42)	LC-MS/MS targeted	↓	↑	↑	↑	↑ PIC; ↑ AA; ↑ 3-HK; ↑ KYN/TRP; ↓ KA/(3HK +QA)	[85]

Neuroborreliosis (n=34); Bacterial meningitis (n=32); Multiple sclerosis (n=17); VZV meningoencephalitis (n=15); Enterovirus meningitis (n=10); HSV encephalitis (n=9); Anti-NMDA-R encephalitis (n=8)	NIND (n=66)	LC-MS/MS targeted	↓	↑			↑ KYN/TRP	[86]
Enterovirus Meningitis (n=10)	NIND (n=19)	LC-MS/MS untargeted		↑				[87]
Tuberculosis meningitis survivors (n=15); Tuberculosis meningitis non-survivors (n=17)	Controls with no infection (n=22)	LC-MS/MS untargeted	↓		↑	↑	↑ AA	[88]
Cerebral malaria (n=69)	Controls with no infection (n=8)	HPLC-UV targeted		↑	↑			[89]
Subacute sclerosing panencephalitis (n=32)	Epileptic and other encephalopathy controls (n=43)	GC-MS targeted				↑		[90]
Hepatitis C treated with IFN-α/ribavirin (n=16)	Hepatitis C - no treatment (n=20)	HPLC-FD, HPLC-UV targeted	↔	↑	↑	↑		[91]
Bacterial meningitis (n=13) Aseptic meningitis (n=7)	Controls no infection (n=8)	HPLC-UV targeted	↔	↑	↑		↑ AA; ↑ KYN/TRP	[92]
Inflammatory neurological disease (n=92)	NIND (n=201)	HPLC targeted	↓	↑	↑	↑	↑QA/KA	[93]
Tick-borne encephalitis (n=108)	Controls no infection (n=52)				↑			[94]
P. falciparum malaria (n=261)	Controls no infection (n=20)	HPLC-FD targeted			↔	↑	↑ PIC; ↑ QA/KA	[95]

Herpes Simplex Virus 1 Encephalitis (n=25)	Controls no infection (n=25)	HPLC targeted			↑			[96]
HIV positive with virologic control on cART (n=43)	HIV-negative (n=23)	UHPLC & GC-MS targeted	↓	↔		↑	↑ KYN/TRP; ↓ PIC	[97]
HIV positive (n=134)	HIV-negative controls (n=79)	HPLC targeted					↑ KYN/TRP	[98]
HIV positive with Depression and Cognitive Impairment (n=91)	HIV-negative (n=66)	HPLC targeted	↓	↔			↑NEO; ↔ KYN/TRP	[99]
HIV-1 positive (n = 22)	Healthy controls (n=22)	HPLC targeted			↑			[100]
Multiple Sclerosis (MS)								
MS (n=37)	NIND (n=22)	LC-MS/MS targeted	↓	↑	↔	↑		[101]
Relapsing Remitting MS (n=30), secondary progressive MS (n=16)	NIND (n=10)	LC-HRMS untargeted		↑	↑			[102]
Relapsing–Remitting MS (n=20)	NIND (n=14)	UHPLC-MS targeted			↓	↑	↑ QA/KA; ↓PIC; ↓ PIC/QA; ↓ KA/KYN;	[103]
Untreated MS (n = 38); RRMS (n=48)	NIND (n=20); Other inflammatory neurology (n=13)	LC-MS/MS targeted	↓	↔	↔	↑	↑ QA/KA; ↑ KYN/TRP; ↔ KA/KYN	[104]
MS (n=26); CNS infectious disease (n=16)	NIND (n=23)	HPLC-FD targeted			↓		KA lower in MS compared to CNS infection	[105]
Neurodegeneration								

AD (n=20)	Controls without AD (n=18)	UHPLC, HPLC and GC/MS targeted	↓	↔	↑	↓	↑ KYN/TRP; ↑ 3-HK/KYN; ↓ QA/KA; ↓ 3-HAA	[106]
AD (n=40)	Controls without AD (n=34)	LC-MS/MS untargeted	↓		↑	↑		[107]
Probable mild AD (n=41); AD mild (n=24); Moderate-severe AD (n=20); Frontotemporal dementia (n=8); Amyotrophic lateral sclerosis (n=8); Progressive supranuclear palsy (n=8)	Controls non-demented (n=23)	ELISA kit targeted	↔		↑		↑ KA/TRP	[108]
Amyotrophic lateral sclerosis (n=140)	Suspected meningitis (n=35)	HPLC-UV, GC-MS targeted	↑	↑		↑	↓ PIC	[109]
Mental Health/Neuropsychiatry								
Bipolar disorder (n=163)	Healthy controls (n=114)	HPLC-UV targeted			↑			[110]
Depression and suicidality (n=64)	Healthy controls (n=35)	HPLC-UV, GC-MS targeted				↑	↑ KYN/TRP; ↓ PIC; ↓ PIC/QUIN	[111]
Schizophrenia (n=22)	Controls (n=26)	LC-MS/MS targeted		↑	↑	↔	↓ QA/KA	[112]
Schizophrenia on olanzapine treatment (n = 16)	Controls (n=29)	HPLC-UV targeted	↔	↑	↑			[113]
Chronic Schizophrenia (n=23)	Controls (n=37)	HPLC targeted	↔	↑	↑		↓ TRP/KYN; ↓ TRP/KYNA	[114]
Other								
Trisomy 21 (Down syndrome, n=50)	Controls (n=50)	UHPLC-HRMS untargeted		↑		↑	↑ formyl-kynurenine; ↑ KYN/TRP	[115]

Severe traumatic brain injury (n=28)	Controls (n=11)	HPLC and GC-MS targeted	↔	↑	↑	↑	↑ QA/KYN; ↑ QA/KA ; ↔ AA; ↔ 3HAA	[116]

Abbreviations: kynurenine (KYN), tryptophan (TRP), kynurenic acid (KA), quinolinic acid (QA), picolinic acid (PIC), anthranilic acid (AA), 3-hydroxyanthranilic acid (3-HAA), 3-ydroxykynurenine (3-HK), Non-inflammatory neurology disease (NIND), Human Immunodeficiency virus (HIV), Alzheimer's disease (AD), Multiple Sclerosis (MS), combination antiretroviral therapy (cART).

1.6.2 Nitric Oxide Pathway

The conversion of arginine to nitric oxide and citrulline is stimulated by nitric oxide synthase (NOS). In the body there are three isoforms of NOS, whereby inducible NOS (iNOS) is extensively involved in the pathophysiology of inflammation and responsible for the production of nitric oxide [117, 118]. The inhibition of iNOS occurs by the endogenous production of asymmetric dimethylarginine (ADMA). Nitric oxide is further metabolised to reactive nitrogen species, including nitrate and nitrite. Citrulline is recycled to form arginine by argininosuccinate and argininosuccinate lyase, known as the citrulline-nitric oxide cycle. Conversely, arginine can be hydrolysed to produce orthinine via arginase and subsequently converted to citrulline by ornithine transcarbamylase.

Nitric oxide is a critical gaseous molecule involved in neurotransmission, defence mechanisms, acute and chronic inflammation [117]. The nitric oxide pathway plays a critical role in the regulation of immunoprotective activities defending the body against infectious organisms. However, failure of immune regulation and over-activation of inflammatory pathways can result in disease states. The altered concentrations of CSF metabolites in the nitric oxide pathway have been implicated in a wide range of human diseases associated with inflammation as summarised in Table 1-2. A variation of analytical platforms, untargeted or targeted approaches and study cohorts have been used (Table 1-2). The cohort studies are sub-grouped in the same

way as Table 1-1. As shown in Table 1-2, asymmetric dimethylarginine, orthinine, nitrite and nitrate levels in CSF are generally increased in diseases with confirmed or suspected CNS inflammation. However, it should be noted that the studies differ in methodology and differ in the measured or reported metabolites. Figure 1-1 depicts the metabolites that are generally elevated or decreased. As is the case for the tryptophan-kynurenine pathway, the activation of iNOS is generally inferred by measuring the pre and post metabolites, rather than actually measuring iNOS.

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).

Disease Cohort	Description of control group	Analytical Platform	Findings				Ref
			ADMA	NO_2^-	NO_3^-	Other	
Encephalitis, meningitis and infection							
Segmental zoster (n=14); Facial nerve zoster (n=16); VZV meningitis/ encephalitis (n=15)	Controls no infection (n=36)	LC-MS/MS targeted				↓ ARG	[119]
Tuberculosis meningitis (n=31)	Controls no infection (n=20)	ELISA targeted				↑ NO	[120]
Streptococcus pneumonia (n=14); Neisseria (n=22); Haemophilus influenza (n=9) meningitis	Control no infection (n=7)	Colorimetric assay targeted		↑	↑		[121]
HIV-infected patients with syphilis infection (n=33)	HIV-negative and no infection (n=7)	colorimetric assay targeted		↑			[122]
Multiple Sclerosis (MS)							

Secondary progressive MS (n=12)	Healthy controls (n = 12)	LC-HRMS targeted	↑				[123]
MS (n=14); Neuromyelitis optica (n=9); Other neurological disease(n=26)	Healthy controls (n=11)	GC–MS/MS targeted	↑			↔hArg/ADMA; ↔ SDMA	[124]
MS exacerbation (n=24); MS remission (n=17); MS progression (n=20)	Controls with tension headache (n=19)	CE targeted		↑	↑		[125]
Relapsing remitting MS (n=15)	Healthy controls (n=15)	absorption spectro-photometry targeted		↑	↑	↑ Peroxynitrite	[126]
Neurodegeneration							
Amyotrophic lateral sclerosis (n=52)	Controls (n=21)	LC-MS/MS targeted	↑				[127]
Amyotrophic lateral sclerosis (n=22); Parkinson's disease (n=22)	Controls without neurodegeneration (n=28)	NMR untargeted				↑ dimethylamine	[128]
Amyotrophic lateral sclerosis (n=22); Parkinson's disease (n=22)	Controls without neurodegeneration (n=28)	GC & LC-MS/MS untargeted				↑ ornithine; ↓ ammonia in ALS compared to PD	[129]
Trauma and acute blood							
Traumatic brain injury (n=19)	Control no infection (n=5)	LC-MS/MS targeted	↑				[130]
Acute hydrocephalus due to hypertension (n=5); SAH (n= 3)	Peripheral neuropathy, ophthalmologic disorders & inactive neurocysticercosis (n=7)	HPLC-FD, HPLC-UV targeted			↔	↑ citrulline; ↓ARG/citrulline; ↔ ARG; ↔citrulline/nitrate	[131]
SAH with cerebral	SAH no	LC-MS/MS	↑			↑ SDMA	[132]

ischemia (n=20	ischemia (n=14)	targeted					
SAH (n=40)	Controls no infection (n=6)	GC and LC-TOFMS untargeted				↑ ornithine; ↔ citrulline; ↔ ARG	[133]
Cerebral Vasospasm After SAH (n=24)	Controls with hydrocephalus (n=6)	ELISA targeted	↑				[134]
SAH after traumatic brain injury (n=10); SAH after a non-traumatic injury (n=5)	Healthy controls (n=9)	LC–MS/MS targeted	↔			↓ ARG/ADMA; ↑SDMA; ↔ ARG	[135]
Other							
Glioblastoma IDH-WT (n = 7); IDH-mutant (n = 4); Metastatic CNS disease with lung cancer (n = 7); Metastatic CNS disease with breast cancer (n = 5)	Controls no cancer (n=8)	LC-MS targeted				↑Arginino-succinic acid in metastatic lung cancer to the CNS; ↑ ornithine in metastatic breast cancer to the CNS	[136]
Overt hepatic encephalopathy (n=14)	No neurological disease (n=27)	LC-HRMS untargeted				↑ ammonia	[137]
Episodic cluster headache (n=14)	Healthy controls (n=11)	CE targeted		↑	↑		[138]
Ischemic Stroke (n=88)	Controls (n=24)	HPLC targeted	↑			↑ SDMA	[139]

Abbreviations: asymmetric dimethylarginine (ADMA), nitrite (NO_2^-), nitrate (NO_3^-), arginine (ARG), nitric oxide (NO), symmetric dimethylarginine (SDMA), homoarginine (hArg), subarachnoid haemorrhage (SAH), Human Immunodeficiency virus (HIV), Multiple sclerosis (MS) and isocitrate dehydrogenase (IDH)

1.6.3 Neopterin

Neopterin is regarded as a valuable early biochemical marker of the cellular immune response during inflammation [140], and is sometimes used in clinical settings [141]. Guanosine triphosphate (GTP) is converted to 7,8-dihydroneopterin triphosphate via the actions of GTP cyclohydrolase I (Figure 1-1). The enzymatic activity of GTP cyclohydrolase I is activated by pro-inflammatory cytokines such as γ-interferon, leading to the conversion of neopterin. Neopterin is a direct product generated in the immune activation of γ-interferon able to be detected at low concentrations and practical for clinical assays [142].

The reported human cohort studies of CSF neopterin as a biomarker of inflammation are outlined in Table 1-3. The disease states have been classified into CNS infections including HIV, encephalitis, meningitis or other infections affecting the brain (HTLV-1, HAT etc). Moreover, studies investigated in multiple sclerosis, neurodegeneration, CNS tumours and autism are reported. CSF neopterin was found to be predominantly elevated in neurological diseases with inflammatory mechanisms. Strong correlations of elevated neopterin with the kynurenine/tryptophan ratio have also been reported [85, 143]. Therefore CSF neopterin serves as a strong inflammatory biomarker for practitioners.

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

Disease Cohort	Description of controls	Analytical Platform	Findings		Ref
			NEO		
Encephalitis, meningitis and infection					
HIV patients on cART neurocognitive impaired (n=70)	HIV patients on cART neurocognitive normal (n=29)	ELISA targeted	↑		[144]

HIV-positive patients (n=67)	HIV negative no neurological disease (n=45)	ELISA targeted	↑		[145]
Acute HIV Fiebig stage I (n=9); Acute HIV Fiebig stage II (n=10); Acute HIV Fiebig stage III (n=32); Chronic HIV (n=53)	HIV negative (n=18)	ELISA targeted	↑		[146]
Untreated HIV-infected (n=382); Untreated AIDS with CNS infections (n=73); Treated HIV patients (n=233)	HIV-seronegative (n=53)	EIA, RIA targeted	↑		[147]
Encephalitis (n=10); acute aseptic meningitis (n=25); acute bacterial meningitis (n=6)	Controls with similar symptoms without pleocytosis (n=42)	LC-MS/MS targeted	↑	Strong correlation KYN/TRP	[85]
CNS Lyme Disease (n=5); WNV meningoencephalitis (n=5); Clinically Isolated Syndrome of MS (n = 4); Rabies (n=10); Histoplasma meningitis (n=3)	No encephalopathy or encephalitis (n=25)	NMR targeted	↑	Elevated NEO in rabies, Lyme disease, and other neuro-infections	[148]
Acute encephalitis (n= 30); Neurodegeneration (n= 17); Febrile seizures (n= 6)	NIND (n=105)	HPLC-FD targeted	↑		[141]
Nephropathia epidemica caused by acute Puumala Hantavirus Infection (n=23)	Controls (n=19)	ELISA targeted	↑		[149]
Tumours of CNS (n=23); Peripheral infections (n=18); Meningitis/encephalitis (n=6); MS/polyneuropathy (n=9)	NIND (n=8)	RIA targeted	↑	Elevated NEO order: Meningitis or encephalitis > tumours of CNS >peripheral infections	[150]
Human African trypanosomiasis Stage 1 (n=20); Human African trypanosomiasis Stage 2 (n=20)	No history of HAT treatment (n=16)	LC-MS/MS untargeted	↑		[151]
Human T-lymphotropic virus 1 associated myelopathy/tropical	Human T-lymphotropic virus 1 infected asymptomatic carriers	HPLC targeted	↑		[152]

spastic paraparesis (n=52)	(n=23)				
Multiple Sclerosis (MS)					
MS (n=61); Autoimmune encephalitis (n=24)	Healthy controls (n=19)	ELISA targeted	↑	NEO elevated significantly in autoimmune encephalitis	[153]
MS (n=37)	NIND (n=22)	LC-MS/MS targeted	↑		[101]
Clinically isolated syndrome (n=27); Relapsing-remitting MS (n=44); Primary progressive MS (n=15)	NIND (n=39)	ELISA targeted	↑	Elevated NEO order: RRMS > PPMS > CIS	[154]
Neurodegeneration					
Alzheimer's disease (n=20)	Controls without AD (n=18)	HPLC, and GC/MS targeted	↑		[106]
Parkinson's disease (n=22)	Healthy controls (n=11)	HPLC targeted	↑	Strong correlation of neopterin with KYN/TRP	[143]
Cognitive impairment (n=10); Delirium & cognitive impairment (n=40); Delirium (n=40)	Controls (n=56)	HPLC-FD targeted	↑		[155]
CNS tumours					
Primary central nervous system lymphoma (PCNSL, n=21)	Other brain tumors (n=44), CNS Inflammatory diseases (n=34)	ELISA targeted	↑	Higher neopterin in PCNSL patients with multiple lesions	[156]
Other Brain Tumor Types (n=54); Pseudotumoral Inflammatory Lesions (n=13); PCNSL (n=28)	Non-tumefactive Inflammatory CNS Disorders (n=29)	HPLC-FD targeted	↑	NEO elevated significantly in PCNSL patients	[157]
Other					
Autism (n=12)	Other neurologic disorders (n=27)	HPLC targeted	↓	↑ biopterin	[158]

Abbreviations: Neopterin (NEO), Human Immunodeficiency virus (HIV), Non-inflammatory neurology disease (NIND), combination antiretroviral therapy (cART)

Acute HIV Fiebig stage I: HIV present in blood samples and RNA positive

Acute HIV Fiebig stage II: positive in RNA and HIV-1 p24 antigen test

Acute HIV Fiebig stage III: positive in RNA, HIV-1 antigen and EIA

Human African trypanosomiasis stage 1 is defined by presence of parasites in the blood and lymphatics.

Human African trypanosomiasis stage 2 is defines by parasites located beyond the blood-brain barrier in the CSF.

1.7 Challenges in CSF Metabolomics

The ultimate method for developing metabolomics analysis would be to explore the metabolome with minimal platforms, however, to date there is no single platform able to cover the full metabolome [38]. Further challenges in global metabolomics lie in the identification of metabolites and biological variation in human biofluids [159]. A major bottleneck in metabolomics studies is accurate metabolite annotation to perform biological interpretations. However, owing to the size of the metabolome, metabolite annotations are limited by lack of commercial reference standards available, absence of recorded reference mass spectra in databases and unavailability of new metabolites in databases.

Furthermore, variation in sample collection, preparation, analytical instrumentation and data processing can influence the set of observed metabolic changes within a study [160]. The optimisation of the experimental design for metabolomics studies is key to ensure standardisation and improve reproducibility of CSF metabolic biomarkers across studies. Despite the discriminative power of the CSF matrix there are many challenges involved in the accessibility of samples and limited sample volumes due to its invasive nature. In addition, given the invasive nature of CSF, garnering age-related normative ranges is not straight forward. Finally, there is a

paucity of studies which measure multiple metabolites in unison, in order to see whether there is correlation or key differences in tryptophan-kynurenine, nitric oxide and neopterin metabolites in different disease states. Given the importance of defining potentially damaging and reversible inflammatory mechanisms in common disorders such as neurodegeneration, neuropsychiatry and neurodevelopment, such large studies are vital to provide diagnostic biomarkers *in vivo*.

1.8 Aims of the Project

Metabolomics is an emerging approach increasingly used as a powerful comparative tool for the study of global metabolite levels in biological samples. Metabolic profiling exhibits strong discriminative power and great potential in the study of disease-specific metabolites unique to individuals. The application of CSF metabolomics offers a novel system for accurate diagnosis, prognosis and prediction of neuroinflammatory disease outcomes. There has been minimal literature investigating the small metabolic changes in the central nervous system for the accurate diagnosis of acute brain inflammation in its early stages. This study intends to address a number of gaps in current knowledge regarding the alteration of CSF metabolite profiles following acute brain inflammation and identification of a potential panel of CSF biomarkers for the opportunity to integrate omics research into a clinical diagnostic service.

The aim of this project is to develop an untargeted metabolomics analysis method for a rapid and high throughput detection of CSF metabolites using high resolution mass spectrometry for the application to a clinical cohort study. The specific aims of this research project were to:

1. Optimise sample preparation methods, chromatographic separation, spectrometric conditions and data processing steps for the analysis of metabolites in human CSF.
2. Evaluate the feasibility of the developed analysis method on a CSF pilot study.
3. Apply the untargeted metabolomics approach to an acute brain inflammation clinical study to identify a potential panel of CSF metabolic biomarkers.

1.9 Conclusion

Metabolomics is rapidly moving in an exciting direction, demonstrating great potential in diagnostic and treatment knowledge of diseases affecting the CNS. There is increasing evidence that the changes in metabolites involved in the tryptophan-kynurenine pathway, nitric oxide pathway and neopterin are strongly associated in a wide range of human CNS diseases with neuroinflammation mechanisms. Such metabolic CSF neuroinflammation biomarkers should be integrated into clinical practice. The Metabolomics Standard Initiative and Metabolomics repository avenues actively monitor and review recommendations to address the challenges in reproducibility across independent studies.

References

1. Bazan NG, Halabi A, Ertel M, Petasis NA: **Chapter 34 - Neuroinflammation.** In *Basic Neurochemistry (Eighth Edition).* Edited by Brady ST, Siegel GJ, Albers RW, Price DL. New York: Academic Press; 2012: 610-620

2. Cunningham C: **Microglia and neurodegeneration: The role of systemic inflammation.** *Glia* 2013, **61:**71-90.

3. DiSabato DJ, Quan N, Godbout JP: **Neuroinflammation: the devil is in the details.** *Journal of neurochemistry* 2016, **139 Suppl 2:**136-153.

4. Aksoy F, Yilmaz G, Aydin NN, Kaya S, Karahan SC, Koksal I: **Are New Biomarkers Useful in the Diagnosis of Meningitis in Adults?** *Open Forum Infectious Diseases* 2017, **4:**S303-S303.

5. Griffiths MJ, McGill F, Solomon T: **Management of acute meningitis.** *Clinical medicine (London, England)* 2018, **18:**164-169.

6. Sinclair AJ, Viant MR, Ball AK, Burdon MA, Walker EA, Stewart PM, Rauz S, Young SP: **NMR-based metabolomic analysis of cerebrospinal fluid and serum in neurological diseases--a diagnostic tool?** *NMR Biomed* 2010, **23:**123-132.

7. Wishart DS, Lewis MJ, Morrissey JA, Flegel MD, Jeroncic K, Xiong Y, Cheng D, Eisner R, Gautam B, Tzur D, et al: **The human cerebrospinal fluid metabolome.** *J Chromatogr B Analyt Technol Biomed Life Sci* 2008, **871:**164-173.

8. Dumas ME, Davidovic L: **Metabolic Profiling and Phenotyping of Central Nervous System Diseases: Metabolites Bring Insights into Brain Dysfunctions.** *J Neuroimmune Pharmacol* 2015, **10:**402-424.

9. Blanchet L, Smolinska A, Attali A, Stoop MP, Ampt KA, van Aken H, Suidgeest E, Tuinstra T, Wijmenga SS, Luider T, Buydens LM: **Fusion of metabolomics and proteomics data for biomarkers discovery: case study on the experimental autoimmune encephalomyelitis.** *BMC Bioinformatics* 2011, **12:**254.

10. Nagana Gowda GA, Raftery D: **Biomarker Discovery and Translation in Metabolomics.** *Current Metabolomics* 2013, **1:**227-240.

11. Tumani H, Teunissen C, Sussmuth S, Otto M, Ludolph AC, Brettschneider J: **Cerebrospinal fluid biomarkers of neurodegeneration in chronic neurological diseases.** *Expert Rev Mol Diagn* 2008, **8:**479-494.

12. Terrando N, Eriksson LI, Ryu JK, Yang T, Monaco C, Feldmann M, Jonsson Fagerlund M, Charo IF, Akassoglou K, Maze M: **Resolving postoperative neuroinflammation and cognitive decline.** *Ann Neurol* 2011, **70:**986-995.

13. Fung A, Vizcaychipi M, Lloyd D, Wan Y, Ma D: **Central nervous system inflammation in disease related conditions: mechanistic prospects.** *Brain Res* 2012, **1446:**144-155.

14. Abbott NJ, Ronnback L, Hansson E: **Astrocyte-endothelial interactions at the blood-brain barrier.** *Nat Rev Neurosci* 2006, **7:**41-53.

15. Sokolova A, Hill MD, Rahimi F, Warden LA, Halliday GM, Shepherd CE: **Monocyte chemoattractant protein-1 plays a dominant role in the chronic inflammation observed in Alzheimer's disease.** *Brain Pathol* 2009, **19:**392-398.

16. Graus F, Titulaer MJ, Balu R, Benseler S, Bien CG, Cellucci T, Cortese I, Dale RC, Gelfand JM, Geschwind M, et al: **A clinical approach to diagnosis of autoimmune encephalitis.** *The Lancet Neurology* 2016, **15:**391-404.

17. George BP, Schneider EB, Venkatesan A: **Encephalitis hospitalization rates and inpatient mortality in the United States, 2000-2010.** *PloS one* 2014, **9:**e104169-e104169.

18. Pillai SC, Hacohen Y, Tantsis E, Prelog K, Merheb V, Kesson A, Barnes E, Gill D, Webster R, Menezes M, et al: **Infectious and Autoantibody-Associated Encephalitis: Clinical Features and Long-term Outcome.** *Pediatrics* 2015, **135:**e974-e984.

19. Collino S, Martin F-PJ, Rezzi S: **Clinical metabolomics paves the way towards future healthcare strategies.** *British Journal of Clinical Pharmacology* 2013, **75:**619-629.

20. Piras D, Locci E, Palmas F, Ferino G, Fanos V, Noto A, D'aloja E, Finco G: **Rare disease: a focus on metabolomics.** *Expert Opinion on Orphan Drugs* 2016, **4:**1229-1237.

21. Roberts LD, Souza AL, Gerszten RE, Clish CB: **Targeted metabolomics.** *Curr Protoc Mol Biol* 2012, **Chapter 30:**Unit 30.32.31-24.

22. van der Greef J, Smilde AK: **Symbiosis of chemometrics and metabolomics: past, present, and future.** *Journal of Chemometrics* 2005, **19:**376-386.

23. Pinto RC: **Chemometrics Methods and Strategies in Metabolomics.** In *Metabolomics: From Fundamentals to Clinical Applications.* Edited by Sussulini A. Cham: Springer International Publishing; 2017: 163-190

24. Wu H, Chen Y, Li Z, Liu X: **Untargeted metabolomics profiles delineate metabolic alterations in mouse plasma during lung carcinoma development using UPLC-QTOF/MS in MS(E) mode.** *Royal Society open science* 2018, **5:**181143-181143.

25. Sakka L, Coll G, Chazal J: **Anatomy and physiology of cerebrospinal fluid.** *European Annals of Otorhinolaryngology, Head and Neck Diseases* 2011, **128:**309-316.

26. Di Terlizzi R, Platt S: **The function, composition and analysis of cerebrospinal fluid in companion animals: Part I – Function and composition.** *The Veterinary Journal* 2006, **172:**422-431.

27. Wu Y, Li L: **Sample normalization methods in quantitative metabolomics.** *Journal of Chromatography A* 2016, **1430:**80-95.

28. Rosenling T, Slim CL, Christin C, Coulier L, Shi S, Stoop MP, Bosman J, Suits F, Horvatovich PL, Stockhofe-Zurwieden N, et al: **The Effect of Preanalytical Factors on Stability of the Proteome and Selected Metabolites in Cerebrospinal Fluid (CSF).** *Journal of Proteome Research* 2009, **8:**5511-5522.

29. Albrecht B, Voronina E, Schipke C, Peters O, Parr MK, Díaz-Hernández MD, Schlörer NE: **Pursuing Experimental Reproducibility: An Efficient Protocol for the Preparation of Cerebrospinal Fluid Samples for NMR-based Metabolomics and Analysis of Sample Degradation.** *Metabolites* 2020, **10**.

30. Chetwynd AJ, Dunn WB, Rodriguez-Blanco G: **Collection and Preparation of Clinical Samples for Metabolomics.** In *Metabolomics: From Fundamentals to Clinical Applications.* Edited by Sussulini A. Cham: Springer International Publishing; 2017: 19-44

31. Trezzi J-P, Jäger C, Galozzi S, Barkovits K, Marcus K, Mollenhauer B, Hiller K: **Metabolic profiling of body fluids and multivariate data analysis.** *MethodsX* 2017, **4:**95-103.

32. Vuckovic D: **Current trends and challenges in sample preparation for global metabolomics using liquid chromatography-mass spectrometry.** *Anal Bioanal Chem* 2012, **403:**1523-1548.

33. Raterink R-J, Lindenburg PW, Vreeken RJ, Ramautar R, Hankemeier T: **Recent developments in sample-pretreatment techniques for mass spectrometry-based metabolomics.** *TrAC Trends in Analytical Chemistry* 2014, **61:**157-167.

34. Broeckling CD, Heuberger AL, Prenni JE: **Large Scale Non-targeted Metabolomic Profiling of Serum by Ultra Performance Liquid Chromatography-Mass Spectrometry (UPLC-MS).** *Journal of Visualized Experiments : JoVE* 2013**:**50242.

35. Fei F, Bowdish DM, McCarry BE: **Comprehensive and simultaneous coverage of lipid and polar metabolites for endogenous cellular metabolomics using HILIC-TOF-MS.** *Anal Bioanal Chem* 2014, **406:**3723-3733.

36. Courant F, Antignac J-P, Dervilly-Pinel G, Le Bizec B: **Basics of mass spectrometry based metabolomics.** *PROTEOMICS* 2014, **14:**2369-2388.

37. Tulipani S, Llorach R, Urpi-Sarda M, Andres-Lacueva C: **Comparative Analysis of Sample Preparation Methods To Handle the Complexity of the Blood Fluid Metabolome: When Less Is More.** *Analytical Chemistry* 2013, **85:**341-348.

38. Cajka T, Fiehn O: **Toward Merging Untargeted and Targeted Methods in Mass Spectrometry-Based Metabolomics and Lipidomics.** *Analytical Chemistry* 2016, **88:**524-545.

39. Fuertig R, Ceci A, Camus SM, Bezard E, Luippold AH, Hengerer B: **LC-MS/MS-based quantification of kynurenine metabolites, tryptophan, monoamines and neopterin in plasma, cerebrospinal fluid and brain.** *Bioanalysis* 2016, **8:**1903-1917.

40. Couchman L: **Turbulent flow chromatography in bioanalysis: a review.** *Biomedical Chromatography* 2012, **26:**892-905.

41. Emwas A-H, Roy R, McKay RT, Tenori L, Saccenti E, Gowda GAN, Raftery D, Alahmari F, Jaremko L, Jaremko M, Wishart DS: **NMR Spectroscopy for Metabolomics Research.** *Metabolites* 2019, **9:**123.

42. Wishart DS: **NMR metabolomics: A look ahead.** *Journal of Magnetic Resonance* 2019, **306:**155-161.

43. Nagana Gowda GA, Raftery D: **Recent Advances in NMR-Based Metabolomics.** *Analytical Chemistry* 2017, **89:**490-510.

44. Ren J-L, Zhang A-H, Kong L, Wang X-J: **Advances in mass spectrometry-based metabolomics for investigation of metabolites.** *RSC Advances* 2018, **8:**22335-22350.

45. Liu X, Zhou L, Shi X, Xu G: **New advances in analytical methods for mass spectrometry-based large-scale metabolomics study.** *TrAC Trends in Analytical Chemistry* 2019, **121:**115665.

46. Crutchfield CA, Thomas SN, Sokoll LJ, Chan DW: **Advances in mass spectrometry-based clinical biomarker discovery.** *Clinical Proteomics* 2016, **13:**1.

47. Cui L, Lu H, Lee YH: **Challenges and emergent solutions for LC-MS/MS based untargeted metabolomics in diseases.** *Mass Spectrometry Reviews* 2018, **37:**772-792.

48. Beale DJ, Pinu FR, Kouremenos KA, Poojary MM, Narayana VK, Boughton BA, Kanojia K, Dayalan S, Jones OAH, Dias DA: **Review of recent developments in GC–MS approaches to metabolomics-based research.** *Metabolomics* 2018, **14:**152.

49. Emwas A-H, Luchinat C, Turano P, Tenori L, Roy R, Salek RM, Ryan D, Merzaban JS, Kaddurah-Daouk R, Zeri AC, et al: **Standardizing the experimental conditions for using urine in NMR-based metabolomic studies with a particular focus on diagnostic studies: a review.** *Metabolomics* 2015, **11:**872-894.

50. Mishur RJ, Rea SL: **Applications of mass spectrometry to metabolomics and metabonomics: detection of biomarkers of aging and of age-related diseases.** *Mass Spectrom Rev* 2012, **31:**70-95.

51. Monteiro MS, Carvalho M, Bastos ML, Guedes de Pinho P: **Metabolomics analysis for biomarker discovery: advances and challenges.** *Curr Med Chem* 2013, **20:**257-271.

52. Ravanbakhsh S, Liu P, Bjorndahl TC, Mandal R, Grant JR, Wilson M, Eisner R, Sinelnikov I, Hu X, Luchinat C, et al: **Accurate, fully-automated NMR spectral profiling for metabolomics.** *PLoS One* 2015, **10:**e0124219.

53. Schicho R, Shaykhutdinov R, Ngo J, Nazyrova A, Schneider C, Panaccione R, Kaplan GG, Vogel HJ, Storr M: **Quantitative metabolomic profiling of serum, plasma, and urine by (1)H NMR spectroscopy discriminates between patients with inflammatory bowel disease and healthy individuals.** *J Proteome Res* 2012, **11:**3344-3357.

54. Crews B, Wikoff WR, Patti GJ, Woo H-K, Kalisiak E, Heideker J, Siuzdak G: **Variability analysis of human plasma and cerebral spinal fluid reveals statistical significance of changes in mass spectrometry-based metabolomics data.** *Analytical chemistry* 2009, **81:**8538-8544.

55. Dettmer K, Aronov PA, Hammock BD: **Mass spectrometry-based metabolomics.** *Mass Spectrom Rev* 2007, **26:**51-78.

56. Fiehn O: **Metabolomics by Gas Chromatography-Mass Spectrometry: Combined Targeted and Untargeted Profiling.** *Current protocols in molecular biology* 2016, **114:**30.34.31-30.34.32.

57. Dunn WB, Broadhurst D, Ellis DI, Brown M, Halsall A, O'Hagan S, Spasic I, Tseng A, Kell DB: **A GC-TOF-MS study of the stability of serum and urine metabolomes during the UK Biobank sample collection and preparation protocols.** *Int J Epidemiol* 2008, **37 Suppl 1:**i23-30.

58. Gowda GA, Djukovic D: **Overview of mass spectrometry-based metabolomics: opportunities and challenges.** *Methods Mol Biol* 2014, **1198:**3-12.

59. Bajad SU, Lu W, Kimball EH, Yuan J, Peterson C, Rabinowitz JD: **Separation and quantitation of water soluble cellular metabolites by hydrophilic interaction chromatography-tandem mass spectrometry.** *J Chromatogr A* 2006, **1125:**76-88.

60. Theodoridis GA, Gika HG, Want EJ, Wilson ID: **Liquid chromatography–mass spectrometry based global metabolite profiling: A review.** *Analytica Chimica Acta* 2012, **711:**7-16.

61. Marshall DD, Powers R: **Beyond the paradigm: Combining mass spectrometry and nuclear magnetic resonance for metabolomics.** *Progress in Nuclear Magnetic Resonance Spectroscopy* 2017, **100:**1-16.

62. Bhinderwala F, Wase N, DiRusso C, Powers R: **Combining Mass Spectrometry and NMR Improves Metabolite Detection and Annotation.** *Journal of Proteome Research* 2018, **17:**4017-4022.

63. Bingol K: **Recent Advances in Targeted and Untargeted Metabolomics by NMR and MS/NMR Methods.** *High Throughput* 2018, **7**.

64. Madsen R, Lundstedt T, Trygg J: **Chemometrics in metabolomics—A review in human disease diagnosis.** *Analytica Chimica Acta* 2010, **659:**23-33.

65. Trygg J, Gullberg J, Johansson AI, Jonsson P, Moritz T: **Chemometrics in Metabolomics — An Introduction.** In *Plant Metabolomics.* Edited by Saito K, Dixon RA, Willmitzer L. Berlin, Heidelberg: Springer Berlin Heidelberg; 2006: 117-128

66. Yi L, Dong N, Yun Y, Deng B, Ren D, Liu S, Liang Y: **Chemometric methods in data processing of mass spectrometry-based metabolomics: A review.** *Analytica Chimica Acta* 2016, **914:**17-34.

67. Sussulini A: *Metabolomics: From Fundamentals to Clinical Applications.* Springer International Publishing; 2017.

68. Jolliffe IT: **Principal Components in Regression Analysis.** In *Principal Component Analysis.* New York, NY: Springer New York; 2002: 167-198

69. Nyamundanda G, Brennan L, Gormley IC: **Probabilistic principal component analysis for metabolomic data.** *BMC Bioinformatics* 2010, **11:**571.

70. Liu Y, Rayens W: **PLS and dimension reduction for classification.** *Computational Statistics* 2007, **22:**189-208.

71. Wishart DS: **Metabolomic Data Exploration and Analysis with the Human Metabolome Database.** In *Computational Methods and Data Analysis for Metabolomics.* Edited by Li S. New York, NY: Springer US; 2020: 165-184

72. Chaleckis R, Meister I, Zhang P, Wheelock CE: **Challenges, progress and promises of metabolite annotation for LC–MS-based metabolomics.** *Current Opinion in Biotechnology* 2019, **55:**44-50.

73. Dona AC, Kyriakides M, Scott F, Shephard EA, Varshavi D, Veselkov K, Everett JR: **A guide to the identification of metabolites in NMR-based metabonomics/metabolomics experiments.** *Computational and Structural Biotechnology Journal* 2016, **14:**135-153.

74. Garcia-Perez I, Posma JM, Serrano-Contreras JI, Boulangé CL, Chan Q, Frost G, Stamler J, Elliott P, Lindon JC, Holmes E, Nicholson JK: **Identifying unknown metabolites using NMR-based metabolic profiling techniques.** *Nature Protocols* 2020, **15:**2538-2567.

75. Nash WJ, Dunn WB: **From mass to metabolite in human untargeted metabolomics: Recent advances in annotation of metabolites applying liquid chromatography-mass spectrometry data.** *TrAC Trends in Analytical Chemistry* 2019, **120:**115324.

76. Bingol K, Bruschweiler-Li L, Li D, Zhang B, Xie M, Brüschweiler R: **Emerging new strategies for successful metabolite identification in metabolomics.** *Bioanalysis* 2016, **8:**557-573.

77. Villoslada P, Baranzini S: **Data integration and systems biology approaches for biomarker discovery: Challenges and opportunities for multiple sclerosis.** *Journal of Neuroimmunology* 2012, **248:**58-65.

78. Ivanisevic J, Siuzdak G: **The Role of Metabolomics in Brain Metabolism Research.** *Journal of neuroimmune pharmacology : the official journal of the Society on NeuroImmune Pharmacology* 2015, **10:**391-395.

79. Takikawa O: **Biochemical and medical aspects of the indoleamine 2,3-dioxygenase-initiated L-tryptophan metabolism.** *Biochem Biophys Res Commun* 2005, **338:**12-19.

80. Badawy AAB, Guillemin G: **The Plasma [Kynurenine]/[Tryptophan] Ratio and Indoleamine 2,3-Dioxygenase: Time for Appraisal.** *International journal of tryptophan research : IJTR* 2019, **12:**1178646919868978-1178646919868978.

81. Sorgdrager FJH, Naudé PJW, Kema IP, Nollen EA, Deyn PPD: **Tryptophan Metabolism in Inflammaging: From Biomarker to Therapeutic Target.** *Frontiers in Immunology* 2019, **10**.

82. Braidy N, Grant R: **Kynurenine pathway metabolism and neuroinflammatory disease.** *Neural Regeneration Research* 2017, **12:**39-42.

83. Badawy AAB: **Hypothesis kynurenic and quinolinic acids: The main players of the kynurenine pathway and opponents in inflammatory disease.** *Medical Hypotheses* 2018, **118:**129-138.

84. Lugo-Huitron R, Ugalde Muniz P, Pineda B, Pedraza-Chaverri J, Rios C, Perez-de la Cruz V: **Quinolinic acid: an endogenous neurotoxin with multiple targets.** *Oxid Med Cell Longev* 2013, **2013:**104024.

85. Quist-Paulsen E, Aukrust P, Kran A-MB, Dunlop O, Ormaasen V, Stiksrud B, Midttun Ø, Ueland T, Ueland PM, Mollnes TE, Dyrhol-Riise AM: **High neopterin and IP-10 levels in cerebrospinal fluid are associated with neurotoxic tryptophan metabolites in acute central nervous system infections.** *Journal of Neuroinflammation* 2018, **15:**327.

86. Suhs KW, Novoselova N, Kuhn M, Seegers L, Kaever V, Muller-Vahl K, Trebst C, Skripuletz T, Stangel M, Pessler F: **Kynurenine Is a Cerebrospinal Fluid Biomarker for Bacterial and Viral Central Nervous System Infections.** *J Infect Dis* 2019, **220:**127-138.

87. Ratuszny D, Suhs KW, Novoselova N, Kuhn M, Kaever V, Skripuletz T, Pessler F, Stangel M: **Identification of Cerebrospinal Fluid Metabolites as Biomarkers for Enterovirus Meningitis.** *Int J Mol Sci* 2019, **20**.

88. van Laarhoven A, Dian S, Aguirre-Gamboa R, Avila-Pacheco J, Ricaño-Ponce I, Ruesen C, Annisa J, Koeken VACM, Chaidir L, Li Y, et al: **Cerebral tryptophan metabolism and outcome of tuberculous meningitis: an observational cohort study.** *The Lancet Infectious Diseases* 2018, **18:**526-535.

89. Holmberg D, Franzén-Röhl E, Idro R, Opoka RO, Bangirana P, Sellgren CM, Wickström R, Färnert A, Schwieler L, Engberg G, John CC: **Cerebrospinal fluid kynurenine and kynurenic acid concentrations are associated with coma duration and long-term neurocognitive impairment in Ugandan children with cerebral malaria.** *Malar J* 2017, **16:**303.

90. Inoue H, Matsushige T, Ichiyama T, Okuno A, Takikawa O, Tomonaga S, Anlar B, Yüksel D, Otsuka Y, Kohno F, et al: **Elevated quinolinic acid levels in cerebrospinal fluid in subacute sclerosing panencephalitis.** *Journal of Neuroimmunology* 2020, **339:**577088.

91. Raison CL, Dantzer R, Kelley KW, Lawson MA, Woolwine BJ, Vogt G, Spivey JR, Saito K, Miller AH: **CSF concentrations of brain tryptophan and kynurenines**

during immune stimulation with IFN-α: relationship to CNS immune responses and depression. *Molecular Psychiatry* 2010, **15:**393-403.

92. Coutinho LG, Christen S, Bellac CL, Fontes FL, de Souza FRS, Grandgirard D, Leib SL, Agnez-Lima LF: **The kynurenine pathway is involved in bacterial meningitis.** *Journal of Neuroinflammation* 2014, **11:**169.

93. Heyes MP, Saito K, Crowley JS, Davis LE, Demitrack MA, Der M, Dilling LA, Elia J, Kruesi MJ, Lackner A, et al.: **Quinolinic acid and kynurenine pathway metabolism in inflammatory and non-inflammatory neurological disease.** *Brain* 1992, **115 (Pt 5):**1249-1273.

94. Holtze M, Mickiené A, Atlas A, Lindquist L, Schwieler L: **Elevated cerebrospinal fluid kynurenic acid levels in patients with tick-borne encephalitis.** *Journal of Internal Medicine* 2012, **272:**394-401.

95. Medana IM, Hien TT, Day NP, Nguyen HP, Nguyen THM, Chu'ong LV, Tran THC, Taylor A, Salahifar H, Stocker R, et al: **The Clinical Significance of Cerebrospinal Fluid Levels of Kynurenine Pathway Metabolites and Lactate in Severe Malaria.** *The Journal of Infectious Diseases* 2002, **185:**650-656.

96. Atlas A, Franzen-Röhl E, Söderlund J, Jönsson EG, Samuelsson M, Schwieler L, Sköldenberg B, Engberg G: **Sustained elevation of kynurenic Acid in the cerebrospinal fluid of patients with herpes simplex virus type 1 encephalitis.** *Int J Tryptophan Res* 2013, **6:**89-96.

97. Anderson AM, Croteau D, Ellis RJ, Rosario D, Potter M, Guillemin GJ, Brew BJ, Woods SP, Letendre SL: **HIV, prospective memory, and cerebrospinal fluid concentrations of quinolinic acid and phosphorylated Tau.** *Journal of Neuroimmunology* 2018, **319:**13-18.

98. van Zoest RA, Underwood J, De Francesco D, Sabin CA, Cole JH, Wit FW, Caan MWA, Kootstra NA, Fuchs D, Zetterberg H, et al: **Structural Brain Abnormalities in Successfully Treated HIV Infection: Associations With Disease and Cerebrospinal Fluid Biomarkers.** *The Journal of Infectious Diseases* 2017, **217:**69-81.

99. Keegan MR, Chittiprol S, Letendre SL, Winston A, Fuchs D, Boasso A, Iudicello J, Ellis RJ: **Tryptophan Metabolism and Its Relationship with Depression and Cognitive Impairment Among HIV-infected Individuals.** *Int J Tryptophan Res* 2016, **9:**79-88.

100. Atlas A, Gisslén M, Nordin C, Lindström L, Schwieler L: **Acute psychotic symptoms in HIV-1 infected patients are associated with increased levels of kynurenic acid in cerebrospinal fluid.** *Brain, Behavior, and Immunity* 2007, **21:**86-91.

101. Rajda C, Galla Z, Polyák H, Maróti Z, Babarczy K, Pukoli D, Vécsei L: **Cerebrospinal Fluid Neurofilament Light Chain Is Associated with Kynurenine Pathway Metabolite Changes in Multiple Sclerosis.** *Int J Mol Sci* 2020, **21**.

102. Herman S, Åkerfeldt T, Spjuth O, Burman J, Kultima K: **Biochemical Differences in Cerebrospinal Fluid between Secondary Progressive and Relapsing¯Remitting Multiple Sclerosis.** *Cells* 2019, **8**.

103. Tömösi F, Kecskeméti G, Cseh EK, Szabó E, Rajda C, Kormány R, Szabó Z, Vécsei L, Janáky T: **A validated UHPLC-MS method for tryptophan metabolites: Application in the diagnosis of multiple sclerosis.** *J Pharm Biomed Anal* 2020, **185:**113246.

104. Aeinehband S, Brenner P, Ståhl S, Bhat M, Fidock MD, Khademi M, Olsson T, Engberg G, Jokinen J, Erhardt S, Piehl F: **Cerebrospinal fluid kynurenines in multiple sclerosis; relation to disease course and neurocognitive symptoms.** *Brain, Behavior, and Immunity* 2016, **51:**47-55.

105. Rejdak K, Bartosik-Psujek H, Dobosz B, Kocki T, Grieb P, Giovannoni G, Turski WA, Stelmasiak Z: **Decreased level of kynurenic acid in cerebrospinal fluid of relapsing-onset multiple sclerosis patients.** *Neuroscience Letters* 2002, **331:**63-65.

106. Jacobs KR, Lim CK, Blennow K, Zetterberg H, Chatterjee P, Martins RN, Brew BJ, Guillemin GJ, Lovejoy DB: **Correlation between plasma and CSF concentrations of kynurenine pathway metabolites in Alzheimer's disease and relationship to amyloid-β and tau.** *Neurobiol Aging* 2019, **80:**11-20.

107. van der Velpen V, Teav T, Gallart-Ayala H, Mehl F, Konz I, Clark C, Oikonomidi A, Peyratout G, Henry H, Delorenzi M, et al: **Systemic and central nervous system metabolic alterations in Alzheimer's disease.** *Alzheimers Res Ther* 2019, **11:**93.

108. González-Sánchez M, Jiménez J, Narváez A, Antequera D, Llamas-Velasco S, Martín AH, Arjona JAM, Munain AL, Bisa AL, Marco MP, et al: **Kynurenic Acid Levels are Increased in the CSF of Alzheimer's Disease Patients.** *Biomolecules* 2020, **10**.

109. Chen Y, Stankovic R, Cullen KM, Meininger V, Garner B, Coggan S, Grant R, Brew BJ, Guillemin GJ: **The Kynurenine Pathway and Inflammation in Amyotrophic Lateral Sclerosis.** *Neurotoxicity Research* 2010, **18:**132-142.

110. Sellgren CM, Gracias J, Jungholm O, Perlis RH, Engberg G, Schwieler L, Landen M, Erhardt S: **Peripheral and central levels of kynurenic acid in bipolar disorder subjects and healthy controls.** *Transl Psychiatry* 2019, **9:**37.

111. Brundin L, Sellgren CM, Lim CK, Grit J, Pålsson E, Landén M, Samuelsson M, Lundgren K, Brundin P, Fuchs D, et al: **An enzyme in the kynurenine pathway that governs vulnerability to suicidal behavior by regulating excitotoxicity and neuroinflammation.** *Translational psychiatry* 2016, **6:**e865-e865.

112. Kegel ME, Bhat M, Skogh E, Samuelsson M, Lundberg K, Dahl ML, Sellgren C, Schwieler L, Engberg G, Schuppe-Koistinen I, Erhardt S: **Imbalanced kynurenine pathway in schizophrenia.** *Int J Tryptophan Res* 2014, **7:**15-22.

113. Linderholm KR, Skogh E, Olsson SK, Dahl M-L, Holtze M, Engberg G, Samuelsson M, Erhardt S: **Increased Levels of Kynurenine and Kynurenic Acid in the CSF of Patients With Schizophrenia.** *Schizophrenia Bulletin* 2010, **38:**426-432.

114. Schwieler L, Larsson MK, Skogh E, Kegel ME, Orhan F, Abdelmoaty S, Finn A, Bhat M, Samuelsson M, Lundberg K, et al: **Increased levels of IL-6 in the cerebrospinal fluid of patients with chronic schizophrenia--significance for activation of the kynurenine pathway.** *Journal of psychiatry & neuroscience : JPN* 2015, **40:**126-133.

115. Powers RK, Culp-Hill R, Ludwig MP, Smith KP, Waugh KA, Minter R, Tuttle KD, Lewis HC, Rachubinski AL, Granrath RE, et al: **Trisomy 21 activates the kynurenine pathway via increased dosage of interferon receptors.** *Nat Commun* 2019, **10:**4766.

116. Yan EB, Frugier T, Lim CK, Heng B, Sundaram G, Tan M, Rosenfeld JV, Walker DW, Guillemin GJ, Morganti-Kossmann MC: **Activation of the kynurenine pathway and increased production of the excitotoxin quinolinic acid following traumatic brain injury in humans.** *Journal of Neuroinflammation* 2015, **12:**110.

117. Tripathi P, Tripathi P, Kashyap L, Singh V: **The role of nitric oxide in inflammatory reactions.** *FEMS Immunology & Medical Microbiology* 2007, **51:**443-452.

118. Lee J, Ryu H, Ferrante RJ, Morris SM, Jr., Ratan RR: **Translational control of inducible nitric oxide synthase expression by arginine can explain the arginine paradox.** *Proc Natl Acad Sci U S A* 2003, **100:**4843-4848.

119. Kuhn M, Sühs K-W, Akmatov MK, Klawonn F, Wang J, Skripuletz T, Kaever V, Stangel M, Pessler F: **Mass-spectrometric profiling of cerebrospinal fluid reveals metabolite biomarkers for CNS involvement in varicella zoster virus reactivation.** *Journal of neuroinflammation* 2018, **15:**20-20.

120. Nagesh Babu G, Kumar A, Kalita J, Misra UK: **Proinflammatory cytokine levels in the serum and cerebrospinal fluid of tuberculous meningitis patients.** *Neuroscience Letters* 2008, **436:**48-51.

121. Grandgirard D, Gäumann R, Coulibaly B, Dangy J-P, Sie A, Junghanss T, Schudel H, Pluschke G, Leib SL: **The Causative Pathogen Determines the Inflammatory Profile in Cerebrospinal Fluid and Outcome in Patients with Bacterial Meningitis.** *Mediators of Inflammation* 2013, **2013:**312476.

122. Cheng Y-J, Tsai H-C, Ye S-Y, Sy C-L, Wu K-S, Chen J-K, Lee SS-J, Chen Y-S: **Elevated cerebrospinal fluid nitrite level in human immunodeficiency virus-infected patients with neurosyphilis.** *Journal of Microbiology, Immunology and Infection* 2014, **47:**512-517.

123. Carlsson H, Abujrais S, Herman S, Khoonsari PE, Åkerfeldt T, Svenningsson A, Burman J, Kultima K: **Targeted metabolomics of CSF in healthy individuals and patients with secondary progressive multiple sclerosis using high-resolution mass spectrometry.** *Metabolomics* 2020, **16:**26.

124. Haghikia A, Kayacelebi AA, Beckmann B, Hanff E, Gold R, Haghikia A, Tsikas D: **Serum and cerebrospinal fluid concentrations of homoarginine, arginine, asymmetric and symmetric dimethylarginine, nitrite and nitrate in patients with multiple sclerosis and neuromyelitis optica.** *Amino Acids* 2015, **47:**1837-1845.

125. Danilov AI, Andersson M, Bavand N, Wiklund NP, Olsson T, Brundin L: **Nitric oxide metabolite determinations reveal continuous inflammation in multiple sclerosis.** *Journal of Neuroimmunology* 2003, **136:**112-118.

126. Calabrese V, Scapagnini G, Ravagna A, Bella R, Foresti R, Bates TE, Giuffrida Stella AM, Pennisi G: **Nitric oxide synthase is present in the cerebrospinal fluid of patients with active multiple sclerosis and is associated with**

increases in cerebrospinal fluid protein nitrotyrosine and S-nitrosothiols and with changes in glutathione levels. *J Neurosci Res* 2002, **70:**580-587.

127. Ikenaka K, Atsuta N, Maeda Y, Hotta Y, Nakamura R, Kawai K, Yokoi D, Hirakawa A, Taniguchi A, Morita M, et al: **Increase of arginine dimethylation correlates with the progression and prognosis of ALS.** *Neurology* 2019, **92:**e1868-e1877.

128. Wu J, Wuolikainen A, Trupp M, Jonsson P, Marklund SL, Andersen PM, Forsgren L, Öhman A: **NMR analysis of the CSF and plasma metabolome of rigorously matched amyotrophic lateral sclerosis, Parkinson's disease and control subjects.** *Metabolomics* 2016, **12:**101.

129. Wuolikainen A, Jonsson P, Ahnlund M, Antti H, Marklund SL, Moritz T, Forsgren L, Andersen PM, Trupp M: **Multi-platform mass spectrometry analysis of the CSF and plasma metabolomes of rigorously matched amyotrophic lateral sclerosis, Parkinson's disease and control subjects.** *Mol Biosyst* 2016, **12:**1287-1298.

130. Thampatty BP, Klamerus MM, Oberly PJ, Feldman KL, Bell MJ, Tyler-Kabara EC, Adelson PD, Clark RSB, Kochanek PM, Poloyac SM: **Hypothermia decreases cerebrospinal fluid asymmetric dimethylarginine levels in children with traumatic brain injury.** *Pediatric critical care medicine : a journal of the Society of Critical Care Medicine and the World Federation of Pediatric Intensive and Critical Care Societies* 2013, **14:**403-412.

131. Pérez-Neri I, Castro E, Montes S, Boll MC, Barges-Coll J, Soto-Hernández JL, Ríos C: **Arginine, citrulline and nitrate concentrations in the cerebrospinal fluid from patients with acute hydrocephalus.** *J Chromatogr B Analyt Technol Biomed Life Sci* 2007, **851:**250-256.

132. Appel D, Seeberger M, Schwedhelm E, Czorlich P, Goetz AE, Böger RH, Hannemann J: **Asymmetric and Symmetric Dimethylarginines are Markers of Delayed Cerebral Ischemia and Neurological Outcome in Patients with Subarachnoid Hemorrhage.** *Neurocrit Care* 2018, **29:**84-93.

133. Li Y-C, Wang R, Xu M-M, Jing X-R, A J-Y, Sun R-B, Na S-J, Liu T, Ding X-S, Sun C-Y, Ge W-H: **Aneurysmal Subarachnoid Hemorrhage Onset Alters Pyruvate Metabolism in Poor-Grade Patients and Clinical Outcome Depends on More: A Cerebrospinal Fluid Metabolomic Study.** *ACS Chemical Neuroscience* 2019, **10:**1660-1667.

134. Jung CS, Lange B, Zimmermann M, Seifert V: **The CSF concentration of ADMA, but not of ET-1, is correlated with the occurrence and severity of cerebral vasospasm after subarachnoid hemorrhage.** *Neurosci Lett* 2012, **524:**20-24.

135. Martens-Lobenhoffer J, Sulyok E, Czeiter E, Büki A, Kohl J, Firsching R, Tröger U, Bode-Böger SM: **Determination of cerebrospinal fluid concentrations of arginine and dimethylarginines in patients with subarachnoid haemorrhage.** *J Neurosci Methods* 2007, **164:**155-160.

136. Ballester LY, Lu G, Zorofchian S, Vantaku V, Putluri V, Yan Y, Arevalo O, Zhu P, Riascos RF, Sreekumar A, et al: **Analysis of cerebrospinal fluid metabolites in patients with primary or metastatic central nervous system tumors.** *Acta Neuropathol Commun* 2018, **6:**85.

137. Weiss N, Barbier Saint Hilaire P, Colsch B, Isnard F, Attala S, Schaefer A, Amador MD, Rudler M, Lamari F, Sedel F, et al: **Cerebrospinal fluid metabolomics highlights dysregulation of energy metabolism in overt hepatic encephalopathy.** *J Hepatol* 2016, **65:**1120-1130.

138. Steinberg A, Wiklund NP, Brundin L, Remahl AI: **Levels of nitric oxide metabolites in cerebrospinal fluid in cluster headache.** *Cephalalgia* 2010, **30:**696-702.

139. Brouns R, Marescau B, Possemiers I, Sheorajpanday R, De Deyn PP: **Dimethylarginine Levels in Cerebrospinal Fluid of Hyperacute Ischemic Stroke Patients are Associated with Stroke Severity.** *Neurochemical Research* 2009, **34:**1642-1649.

140. Pingle SK, Tumane RG, Jawade AA: **Neopterin: Biomarker of cell-mediated immunity and potent usage as biomarker in silicosis and other occupational diseases.** *Indian Journal of Occupational and Environmental Medicine* 2008, **12:**107-111.

141. Dale RC, Brilot F, Fagan E, Earl J: **Cerebrospinal fluid neopterin in paediatric neurology: a marker of active central nervous system inflammation.** *Dev Med Child Neurol* 2009, **51:**317-323.

142. Krause D, Suh H-S, Tarassishin L, Cui QL, Durafourt BA, Choi N, Bauman A, Cosenza-Nashat M, Antel JP, Zhao M-L, Lee SC: **The Tryptophan Metabolite 3-Hydroxyanthranilic Acid Plays Anti-Inflammatory and Neuroprotective Roles During Inflammation: Role of Hemeoxygenase-1.** *The American Journal of Pathology* 2011, **179:**1360-1372.

143. Widner B, Leblhuber F, Fuchs D: **Increased neopterin production and tryptophan degradation in advanced Parkinson's disease.** *Journal of Neural Transmission* 2002, **109:**181-189.

144. Edén A, Marcotte TD, Heaton RK, Nilsson S, Zetterberg H, Fuchs D, Franklin D, Price RW, Grant I, Letendre SL, Gisslén M: **Increased Intrathecal Immune Activation in Virally Suppressed HIV-1 Infected Patients with Neurocognitive Impairment.** *PLoS One* 2016, **11:**e0157160.

145. Guha D, Mukerji SS, Chettimada S, Misra V, Lorenz DR, Morgello S, Gabuzda D: **Cerebrospinal fluid extracellular vesicles and neurofilament light protein as biomarkers of central nervous system injury in HIV-infected patients on antiretroviral therapy.** *AIDS (London, England)* 2019, **33:**615-625.

146. D'Antoni ML, Byron MM, Chan P, Sailasuta N, Sacdalan C, Sithinamsuwan P, Tipsuk S, Pinyakorn S, Kroon E, Slike BM, et al: **Normalization of Soluble CD163 Levels After Institution of Antiretroviral Therapy During Acute HIV Infection Tracks with Fewer Neurological Abnormalities.** *J Infect Dis* 2018, **218:**1453-1463.

147. Hagberg L, Cinque P, Gisslen M, Brew BJ, Spudich S, Bestetti A, Price RW, Fuchs D: **Cerebrospinal fluid neopterin: an informative biomarker of central nervous system immune activation in HIV-1 infection.** *AIDS Research and Therapy* 2010, **7:**15.

148. French CD, Willoughby RE, Pan A, Wong SJ, Foley JF, Wheat LJ, Fernandez J, Encarnacion R, Ondrush JM, Fatteh N, et al: **NMR metabolomics of cerebrospinal fluid differentiates inflammatory diseases of the central nervous system.** *PLoS Negl Trop Dis* 2018, **12:**e0007045.

149. Hautala T, Partanen T, Sironen T, Rajaniemi SM, Hautala N, Vainio O, Vapalahti O, Kauma H, Vaheri A: **Elevated cerebrospinal fluid neopterin concentration is associated with disease severity in acute Puumala hantavirus infection.** *Clin Dev Immunol* 2013, **2013:**634632.

150. Rehák S, Malirová E, Cermanová M, Suba P, Cerman J, Plisek S, Melichar B: **Cerebrospinal Fluid Neopterin in Patients with Tumors and other Disorders.** 2008, **19:**86.

151. Vincent IM, Daly R, Courtioux B, Cattanach AM, Biéler S, Ndung'u JM, Bisser S, Barrett MP: **Metabolomics Identifies Multiple Candidate Biomarkers to**

Diagnose and Stage Human African Trypanosomiasis. *PLoS neglected tropical diseases* 2016, **10:**e0005140-e0005140.

152. Sato T, Coler-Reilly A, Utsunomiya A, Araya N, Yagishita N, Ando H, Yamauchi J, Inoue E, Ueno T, Hasegawa Y, et al: **CSF CXCL10, CXCL9, and neopterin as candidate prognostic biomarkers for HTLV-1-associated myelopathy/tropical spastic paraparesis.** *PLoS neglected tropical diseases* 2013, **7:**e2479-e2479.

153. Fominykh V, Brylev L, Gaskin V, Luzin R, Yakovlev A, Komoltsev I, Belousova I, Rosliakova A, Guekht A, Gulyaeva N: **Neuronal damage and neuroinflammation markers in patients with autoimmune encephalitis and multiple sclerosis.** *Metab Brain Dis* 2019, **34:**1473-1485.

154. Stilund M, Gjelstrup MC, Petersen T, Møller HJ, Rasmussen PV, Christensen T: **Biomarkers of inflammation and axonal degeneration/damage in patients with newly diagnosed multiple sclerosis: contributions of the soluble CD163 CSF/serum ratio to a biomarker panel.** *PloS one* 2015, **10:**e0119681-e0119681.

155. Hall RJ, Watne LO, Idland AV, Raeder J, Frihagen F, MacLullich AM, Staff AC, Wyller TB, Fekkes D: **Cerebrospinal fluid levels of neopterin are elevated in delirium after hip fracture.** *J Neuroinflammation* 2016, **13:**170.

156. Geng M, Xiao H, Liu J, Song Y, Fu P, Cheng X, Zhang J, Wang G: **The diagnostic role and dynamic changes in cerebrospinal fluid neopterin during treatment of patients with primary central nervous system lymphoma.** *Cancer Med* 2018, **7:**3889-3898.

157. Viaccoz A, Ducray F, Tholance Y, Barcelos GK, Thomas-Maisonneuve L, Ghesquières H, Meyronet D, Quadrio I, Cartalat-Carel S, Louis-Tisserand G, et al: **CSF neopterin level as a diagnostic marker in primary central nervous system lymphoma.** *Neuro Oncol* 2015, **17:**1497-1503.

158. Zimmerman AW, Jyonouchi H, Comi AM, Connors SL, Milstien S, Varsou A, Heyes MP: **Cerebrospinal Fluid and Serum Markers of Inflammation in Autism.** *Pediatric Neurology* 2005, **33:**195-201.

159. Mamas M, Dunn WB, Neyses L, Goodacre R: **The role of metabolites and metabolomics in clinically applicable biomarkers of disease.** *Arch Toxicol* 2011, **85:**5-17.

160. Villas-Bôas SG, Koulman A, Lane GA: **Analytical methods from the perspective of method standardization.** In *Metabolomics: A Powerful Tool in Systems Biology.* Edited by Nielsen J, Jewett MC. Berlin, Heidelberg: Springer Berlin Heidelberg; 2007: 11-52

Chapter 2: Method Development and Optimisation

Chapter 2: Method Development and Optimisation

2.1 Abstract

Metabolites are crucial for cellular functions whereby biological disturbances can cause a series of metabolic changes in the body. Metabolomics is a rapidly growing field providing new opportunities in profiling biological matrices to facilitate biomarker discovery and unravelling the pathophysiologic mechanisms underlying health and diseases. An important goal of untargeted metabolomics is to cover a comprehensive spectrum of metabolites as well as achieving reproducibility and sensitivity. The great advancements in metabolomics technologies from experimental design through to data curation and chemometrics tools have significantly contributed to our understanding of the human physiology. The detection of cerebrospinal fluid (CSF) metabolites were determined based on a simple and rapid methanol precipitation sample preparation method. In this work, three liquid chromatography columns and two different liquid chromatography coupled to high resolution mass spectrometry (LC-HRMS) systems are thoroughly compared using a metabolite standard mixture and pooled CSF samples. The chromatographic separation of metabolites was achieved within a twenty minute gradient elution. The Waters HSS T3 and Agilent HILIC columns presented reproducibility of retention times, highest peak resolution and intensity. A total of 203 metabolites were annotated from molecular features detected in pooled human CSF samples. The rapid sample preparation, high throughput of the UPLC separation and strong resolving power of the orbitrap mass spectrometer holds promise for untargeted metabolomics analysis.

2.2 Introduction

Small molecules metabolites such as amines, amino acids, neurotransmitters, fatty acids and products of cellular activities reflect metabolic functions in the brain and disturbances to basal conditions. The dysregulation of metabolites in the central nervous system have been implicated to a range of diseases and gained increasing attention over the past decade [1-4]. As the composition of cerebrospinal fluid (CSF) is dependent on the rates of metabolite production in the brain, the analysis of CSF specimens holds promise in the diagnosis of neurological pathologies and insights into their molecular background [5].

The emergence of metabolomics is a novel approach which endeavours to provide insights into the effects of cellular functions caused by diverse metabolite regulations and activities [6]. Metabolomics strategies are generally categorised into targeted and untargeted analysis [7]. Targeted analysis is based on the measurement of defined metabolite groups. In contrast, untargeted metabolomics focuses on all detectable metabolites and chemical unknowns in a sample.

Untargeted metabolomics is a hypothesis generating comparative analysis of changes in detectable metabolites between different phenotypes in a biological system. The application of untargeted metabolomics requires rapid and reproducible sample preparation methods as well as covering a wide range of molecules. The size and diversity of the metabolome requires careful optimisation of various common steps such as experimental design, biological sample preparation, instrumentation parameters, data processing and statistical data analysis, as these can influence the set of metabolites detected [8, 9]. As no single platform can cover the entire metabolome, the ultimate purpose for method development is to cover relevant pathways and molecules to a great extent with minimal analytical platforms whilst maintaining accuracy and precision [10].

The advancements of metabolomics methods and continuous development of new capabilities in analytical instrumentations has become extremely beneficial in the analysis of CSF metabolites and the screening of biomarkers [11, 12]. LC-MS based metabolomic technology has moved to the forefront, becoming extensively used for clinical research due to its high sensitivity and minimal sample volume required [13, 14]. The innovation of technologies to investigate the changes in metabolites within sample matrices has led to the demand for reliable data mining and statistical tools required in the process of extracting relevant information. As large quantities of data are generated, the use of chemometrics and multivariate data analysis techniques are strategies commonly employed especially in regards to disease diagnosis [15, 16].

The aim of this chapter is to develop an untargeted metabolomics analysis method exhibiting reproducibility, high resolution and sensitivity for the analysis of metabolites in human CSF. A series of optimisations and comparisons were conducted for sample preparation, chromatographic, spectrometric and data processing steps.

2.3 Materials and method

2.3.1 Chemicals and Reagents

A total of twenty-two reference standards were purchased representing metabolites of different classes and pathways to optimise the untargeted metabolomics analysis method.

Anthranilic acid, dopamine hydrochloride, epinephrine hydrochloride, L-glutamic acid, GABA, dopamine hydrochloride, metanephrine, kynurenic acid, L-kynurenine, and L-tryptophan, quinolinic acid, picolinic acid, 3-hydroxykynurenine, 3-hydroxyanthranilic acid, xanthurenic acid and ammonium formate were purchased as powders from Sigma Aldrich (Sydney, Australia). Homovanillic acid, 3,4-

dihydroxyphenylacetic acid, 5-hydroxyindoleacetic acid, neopterin, biopterin, sepiapterin, histamine and norepinephrine were purchased from Novachem (Victoria, Australia). D_3-Dopamine and D_3-Kynurenic acid were supplied by CDN Isotopes (Quebec, Canada). HPLC grade acetonitrile and methanol were purchased from Honeywell (Sydney, Australia). Formic acid and were supplied by RCI Labscan (Bangkok, Thailand).

2.3.2 Preparation of Standard Solutions

Standard stock solutions (2 µg/mL) of twenty-two metabolites were individually prepared in water and 0.1 % formic acid. A 500 ng/mL mixed standard solution containing the analytes was prepared and stored in -20 ^{o}C prior to usage.

Two internal standard solutions (D_3-Dopamine and D_3-Kynurenic acid) were used to monitor the consistency of instrument signals and retention time stability and normalisation. The internal standards were used for the optimised sample preparation method and quality control assessment. A concentration of 100 ng/mL of the internal standard solutions was added to the methanol solution used for the deproteinisation step.

2.3.3 Sample preparation methods for cerebrospinal fluid samples

Method 1: An acidified mobile phase was composed of 0.2 % formic acid, 0.05 % trifluoroacetic acid and 1:5 acetonitrile in water. 100 µL of the acidified mobile phase was added to the 100 µL of pooled CSF. Subsequently 1000 µL of methanol was added and allowed to rest for 30 minutes at -20 ^{o}C to assist in protein precipitation. The sample mixture was centrifuged for 15 minutes at 5 ^{o}C and at a velocity of 3000 *g*. The supernatant was collected and evaporated to dryness under nitrogen. The residue was reconstituted in 100 µL of the acidified mobile phase.

Method 2: 100 µL of CSF and 300 µL of the methanol was thoroughly mixed for 3 minutes on a vortex and ultra-sonicated for 10 minutes. For protein precipitation, the mixture was left on ice for 90 minutes and subsequent centrifugation for 15 minutes at 5 ^{o}C and velocity of 4000 *g*. The supernatant was collected and evaporated to dryness under gentle stream of nitrogen. 100 µL of 40:60 water/acetonitrile was added to the residue.

Method 3: A mixture of 100 µL CSF was divided into two 50 µL portions. 50 µL of CSF was treated with 200 µL acidified methanol using formic acid. The other 50 µL of CSF was treated with 200 µL of methanol. Both mixtures were vortexed for 90 seconds, precipitated at -20 ^{o}C for 30 minutes and centrifuged for 10 minutes at 5 ^{o}C and velocity of 3500 *g*. 200 µL of the supernatant in both mixtures were transferred into a vial and evaporated to dryness under nitrogen. The dried extracts were reconstituted in 50 µL of 1:1 mixture of water/acetonitrile. The reconstituted volumes were combined into one sample volume for LC-MS/MS analysis.

Method 4: A mixture of 100 µL of CSF and 250 µL of methanol was vortexed for 5 minutes. The sample mixture was centrifuged for 10 minutes at 5 ^{o}C and velocity of 3500 *g*. The supernatant was filtered using Whatman Mini-UniPrep (0.2 µm pore size) syringeless filter vials for LC-MS/MS analysis.

For the final optimised sample preparation method, quality control samples were prepared by spiking the mixed standard solution to the pooled CSF. The concentrations of low, medium and high quality controls were 50, 350 and 800 ng/mL. Equal volumes from the three spiked CSF concentrations were obtained to form the QC sample.

2.3.4 Liquid Chromatography

The chromatographic separation of CSF metabolites was assessed on different columns (Table 2-1) using the Agilent 1200 Series LC Binary System coupled to an Agilent Quadrupole Time-of-Flight (QTOF) Mass Spectrometer (Agilent Technologies,

Santa Clara, CA, USA) and Thermo Scientific Vanquish system coupled to a Q Exactive HF-X Hybrid Quadrupole Orbitrap Mass Spectrometer (Thermo Fisher Scientific Inc., Massachusetts, CA, USA).

Table 2-1 Characteristics of chromatographic columns

Column	Supplier	Phase type	Dimensions (mm)	Particle size (um)
C18 RP	Agilent	Reverse	2.1 x 100	2.7
HILIC	Agilent	Normal	2.1 x 100	2.7
HSS T3	Waters	Reverse	2.1 x 100	1.8

The column oven temperature of 30 ^{o}C, flow rate of 0.3 mL/min and injection volume of 10 µL was suitable for all columns. Several experiments were conducted using different columns and mobile phase conditions to determine the optimal conditions for good peak separations.

2.3.5 Mass Spectrometry

The initial LC-MS/MS system used for the project was the Agilent Quadrupole Time-of-Flight (QTOF) Mass Spectrometer fitted with an electrospray (ESI) source in both positive and negative ion modes. The ESI source used for both modes included a spray voltage of 3500 V, capillary temperature of 300 ^{o}C, sheath gas flow of 10 and auxiliary gas flow of 40. The full scan acquisition ranged from 50 to 600 m/z.

The Q Exactive HF-X Hybrid Quadrupole Orbitrap Mass Spectrometer (Thermo Fisher Scientific Inc., Massachusetts, CA, USA) was acquired in the all ion fragmentation mode. The positive and negative ESI modes were assessed under a high mass resolution of 120 000, automatic gain control target at 3e6 and maximum injection time of 200 ms. The detection full scan ranged from m/z 50 to 600 in both ionisation

modes. The MS was operated with capillary voltage at 4 kV in the positive ionisation mode and capillary temperature of 300 ^{o}C.

The software interfaces for the two instruments were Agilent MassHunter and Thermo Xcalibur. Calibration of the mass spectrometer in the positive and negative modes was conducted prior to analysis using calibration mixtures provided by the manufacturer. A comparison of the two instruments were evaluated using the metabolite standard mixture and pooled CSF samples before a final decision was made to complete the method development work using HRMS.

2.3.6 Data Processing

The Agilent MassHunter Qualitative Analysis software package was used to process data acquired from the LC-QTOF. An initial screen of the number of molecular features detected in the raw data was conducted. The databases employed were the Human Metabolome Database, METLIN, UTS in-house CSF metabolite database and in-house metabolite personal compound database and library (PCDL). The MassHunter Profinder software was subsequently used for rapid batch processing feature extractions.

All MS raw data acquired from the Thermo LC-HRMS were preliminary processed using the Xcalibur software. During the initial stages of transferring to the HRMS instrument, SIEVE (Thermo Fisher Scientific) was employed for peak alignment and framing pooled CSF samples in one batch. This involved the generation of detailed information on features detected aligned by retention time, mass-to-charge ratio and peak area intensities. A low threshold cut off value was selected and each frame was visually inspected to confirm peak shapes. When subscriptions were obtained for the Compound Discoverer 3.0 software package, CSF raw data were interpreted using the in-built untargeted metabolomics processing workflow. Compound Discoverer 3.0 was employed for peak alignment and framing pooled CSF sample data in one batch. This involved the generation of information on features detected aligned by retention

time, mass-to-charge ratio and peak area intensities. Key parameters included a mass tolerance of 5 ppm, retention time window shift of 0.2 min, mass fragmentation ion species limited to $[M+H]^+$ and $[M+NH_4]^+$ and mass spectral features were set to consider features present in at least 75% of samples. Compound identification was performed using purchased commercial reference standards or putatively annotated using literature mass spectra. The databases used included, the Human Metabolome Database, ChemSpider, mzCloud and an in-house CSF database.

Multivariate analysis of quality control samples were analysed using an online metabolomics data analysis platform, MetaboAnalyst 4.0 [166]. Data on the retention time, molecular weight and peak intensities was scaled for unsupervised principal component analysis (PCA) to identify outliers and distinguish differences or similarities between sample groups.

2.4 Results and Discussion

2.4.1 Optimisation of Sample Preparation

The CSF biofluid has a less complex composition in comparison to plasma and blood resulting in a rapid sample preparation protocol [18]. However, concentrations for a majority of CSF metabolites are significantly lower. For this reason, highly sensitive analytical methods were selected to run the CSF samples. The preparation of samples is a critical step in acquiring high quality metabolomics data [19]. A number of important key factors were considered before conducting experiments. These included (i) rapid and minimal handling to reduce technical and analytical variability; (ii) costs of preparing samples for analysis to benefit larger sample sizes and (iii) experimental reproducibility. Sample preparation methods one [20] and two [21] were adopted from literature and fitted well with our criteria. Table 2-2 summarises the total number of molecular features detected in pooled CSF samples. The highest number of molecular features was identified using method 2 followed closely by

method 3. CSF samples prepared using methanol precipitation yielded a greater recovery of metabolites. In comparison, an acidified precipitation and combination of methanol precipitation and filtration approach obtained a lower number of identified molecular features.

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

Sample preparation method	No. of molecular features detected		
	Agilent RP	Agilent HILIC	Waters HSS T3
1	105	99	112
2	129	174	135
3	97	146	119
4	42	63	71

Further modifications on method 2 were trialled to reduce the time required for sample preparation. The duration of protein precipitation on ice was first experimented at 30, 45, 60 and 75 minutes. After obtaining a similar number of molecular features as ninety minutes, the protein precipitation time length was optimised to forty-five minutes. Subsequently, the vortex, ultra-sonication and centrifugation times were tested at a reduced time and optimised to 90 seconds, 5 minutes and 12 minutes respectively. Finally to increase the concentration of CSF metabolites in the samples the reconstitution volume was increased by two-fold to 50 µL which yielded a higher number of molecular features.

The optimised sample procedure protocol is as follows: 100 µL of CSF samples were deproteinised by 300 µL of the methanol mixture in microcentrifuge tubes. The samples were vortexed for 90 seconds, sonicated in ice-cold water for 5 minutes and precipitated in ice for 45 minutes. This was followed by centrifugations of the samples for 12 minutes at a temperature of 5 ^{o}C and velocity of 4000 *g*. The supernatant was

collected and evaporated to dryness under nitrogen. Subsequently, 50 µL of water/acetonitrile at a ratio of 40/60 was used to reconstitute the CSF residue for LC-HRMS analyses.

The reproducibility of the sample preparation method was assessed using QC samples (n=5). The PCA plot in Figure 2-1 shows clustering of the QC samples and the absence of outliers in the samples. This indicates the reproducibility, robustness and analytical stability of the method.

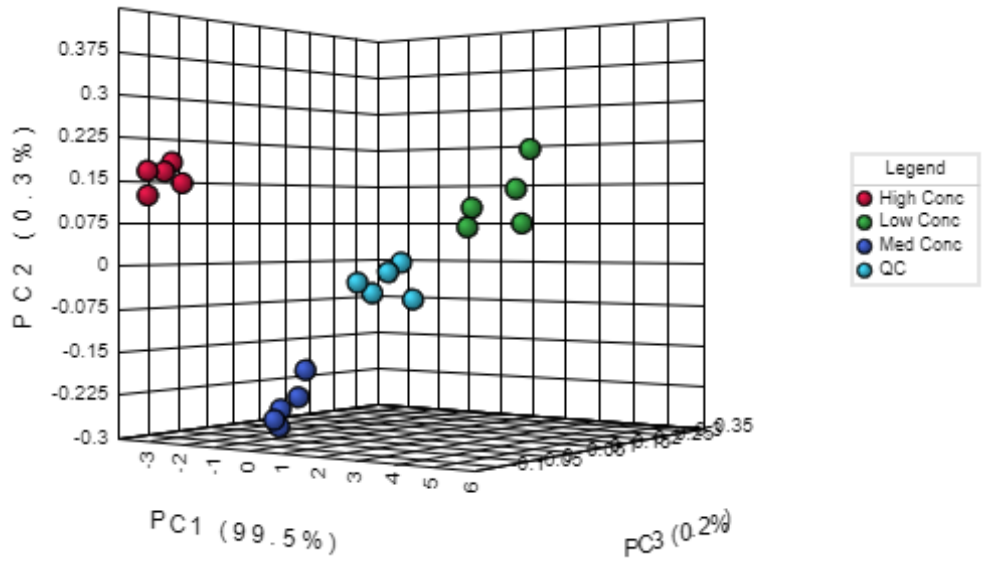

Figure 2-1 PCA 3D score plot of low concentration spiked samples (n=5, green dots), medium concentration spiked samples (n=5, dark blue dots), high concentration spiked samples (n=5, red dots) and QC samples (n=5, light blue dots).

2.4.2 Optimisation of chromatographic conditions

A literature search was conducted on publications that studied neuroinflammation particularly in the CSF biofluid, noting statistically significant metabolites previously reported or metabolites that hold great potential. From publications amino acid metabolites, tryptophan-kynurenine pathway metabolites, neopterin, histamine and biogenic amines were found to be major players during CNS inflammation. For this

reason reference standards of twenty-two metabolites representing different classes and pathways were used to optimise the chromatographic conditions of the metabolomics analysis method (Table 2-3).

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

Metabolite	**Formula**	**Expected m/z $[M+H]^+$**
Dopamine	$C_8H_{11}NO_2$	154.0863
Epinephrine	$C_9H_{13}NO_3$	184.0968
Metanephrine	$C_{10}H_{15}NO_3$	198.1125
Norepinephrine	$C_8H_{11}NO_3$	170.0812
Homovanillic acid	$C_9H_{10}O_4$	183.0652
3,4-Dihydroxyphenylacetic acid	$C_8H_8O_4$	169.0495
5-Hydroxyindoleacetic acid	$C_{10}H_9NO_3$	192.0655
Sepiapterin	$C_9H_{11}N_5O_3$	238.0935
Neopterin	$C_9H_{11}N_5O_4$	254.0884
Biopterin	$C_9H_{11}N_5O_3$	238.0935
Histamine	$C_5H_9N_3$	112.0869
Glutamic acid	$C_5H_9NO_4$	148.0604
GABA	$C_4H_9NO_2$	104.0706
Tryptophan	$C_{11}H_{12}N_2O_2$	205.0972
Kynurenine	$C_{10}H_{12}N_2O_3$	209.0921
Kynurenic acid	$C_{10}H_7NO_3$	190.0499
Anthranilic acid	$C_7H_7NO_2$	138.0555
3-hydroxyanthranilic acid	$C_7H_7NO_3$	154.0499
3-Hydroxykynurenine	$C_{10}H_{12}N_2O_4$	225.2133
Quinolinic acid	$C_7H_5NO_4$	168.0291

Picolinic acid	$C_6H_5NO_2$	124.0393
Xanthurenic acid	$C_{10}H_7NO_4$	206.0448

Systematic investigations were conducted on the effects of mobile phase compositions, buffer systems, pH and temperature using an Agilent 1200 Series LC Binary System. Several experiments were performed to obtain a good separation of the multi-class endogenous metabolite standards with high resolution and efficiency within an acceptable run time. This is of great importance for the identification of neuroactive metabolites as authentic CSF samples have low concentrations and presence of unknown additional peaks.

Several mobile phases were assessed for chromatographic separation of multi-class metabolites taking into consideration the hydrophilic and physicochemical properties of the analytes. A majority of the metabolite standards are mostly polar due to the various functional groups (amine, carboxy, hydroxyl). Experiments with different mobile phases were tested under acidic conditions using formic acid, ammonium formate and ammonium hydroxide buffer systems in water (aqueous mobile phase) and acetonitrile or methanol (organic mobile phase).

The choice of an organic modifier used in the mobile phase can have a strong impact on the chromatographic selectivity and in turn is an important tool in the development of a method. Methanol and acetonitrile are two commonly selected organic modifiers in liquid chromatography [22]. Acetonitrile is an example of a polar aprotic solvent with high dipole moment properties whereas methanol is a polar protic solvent. A major advantage of both solvents is their compatibility with common buffers and miscibility with water. Different organic modifiers can influence and alter the mechanism of retention (pressure, elution strength, separation selectivity and peak shape). A series of experiments using acetonitrile, methanol and 50:50 (v/v) acetonitrile/methanol were conducted on the HSS T3 column on the Agilent LC system. Acetonitrile showed greater affinity, better resolution and peak shape compared to methanol or combination of both solvents (Figure 2-2).

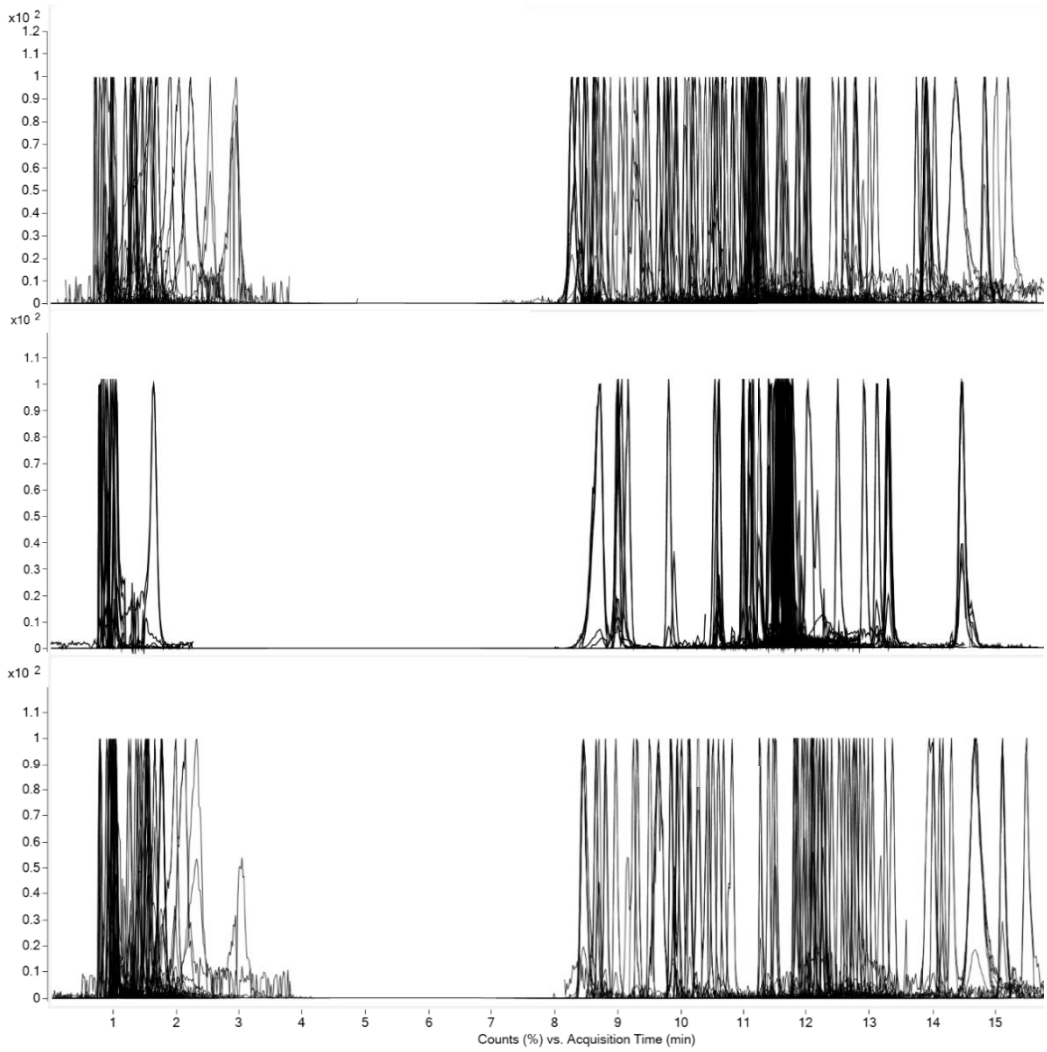

Figure 2-2 Representative total ion chromatograms of pooled CSF samples analysed in various organic solvents on the LC-QTOF/MS using the Waters HSS T3 column (from top to bottom): (1) acetonitrile, (2) methanol and (3) 50:50 (v/v) of acetonitrile/methanol.

Ammonium salts and formic acid are widely used additives for LC-MS/MS analysis and their electrospray ionisation efficacies on biogenic amines and amino acids have been preliminarily demonstrated in previous studies [20, 23, 24]. To improve the signals and peak separation of metabolite standards formic acid, ammonium formate and ammonium hydroxide buffers were tested. Formic acid and ammonium formate were found to improve peak shape and mass spectral signal intensity of the metabolite standards. The effects of ammonium formate concentrations (5, 10 and 20 mM) in the mobile phases were investigated. An improvement in peak shape and peak signal

intensity was evident with increasing the concentration of ammonium formate in the mobile phases. Hence, pH of the mobile phase serves as an important aspect for the retention of analytes.

Various commercial normal and reversed phase columns from two manufacturers (Agilent and Waters) were assessed (Table 2-1). To ensure the same column conditions were in place across all the sample runs an initial two minutes of equilibration time was applied before sample injections. The six, twelve and five minute re-equilibration time at the end of the gradient was established for RP, HILIC and HSS T3 methods respectively to avoid sample carryover. Subsequent analysis of blank samples confirmed that there was no carryover of analytes. In addition washing procedures of the autosampler prior and after sample injections were programmed.

The developed method showed good resolution and retention of the metabolite standards (Figure 2-3). The column oven temperature of 30 ^{o}C, flow rate of 0.3 mL/min and sample injection volume of 10 µL was deemed suitable for both methods. The optimised complementary methods were achieved under the following gradient elution conditions:

(i) The RP method had a run time of 30 minutes. The mobile phases consisted of 0.1% formic acid in water (aqueous) and 0.1% formic acid in acetonitrile (organic). The gradient program was: 0−3 min (0% B), 3-10 min (50% B), 11-15 min (70% B), 15-16 min (95% B), 16-21 (95% B), 21-23 (30% B), 23-24 min (0% B) and 24-30 min (0% B).

(ii) The HILIC method had a run time of 32 minutes. The mobile phases consisted of 20 mM ammonium formate in water (aqueous) and 20 mM ammonium formate in 90% acetonitrile (organic). The gradient program was: 0−1.5 min (90% B), 1.5−12 min (90−50% B), 12−16 min (50% B), 16−17 min (50−25% B), 17−20 min (25% B), 20−21 min (45−90% B) and 21−32 min (90% B).

(iii) The HSS T3 method had a run time of 22 minutes. The mobile phases consisted of 0.1% formic acid in water (aqueous) and 0.1% formic acid in acetonitrile (organic).

The gradient program was: 0−1 min (2% B), 1-6 min (2−40% B), 6−14 min (40−99% B), 14−16.5 min (99% B), 16.5−17 min (99−2% B) and 17−22 min (2% B).

Under these optimised conditions the amino acids and more polar metabolites were eluted at a higher ratio of the aqueous mobile phase and the more hydrophobic metabolites eluted at higher concentrations of the organic mobile phase.

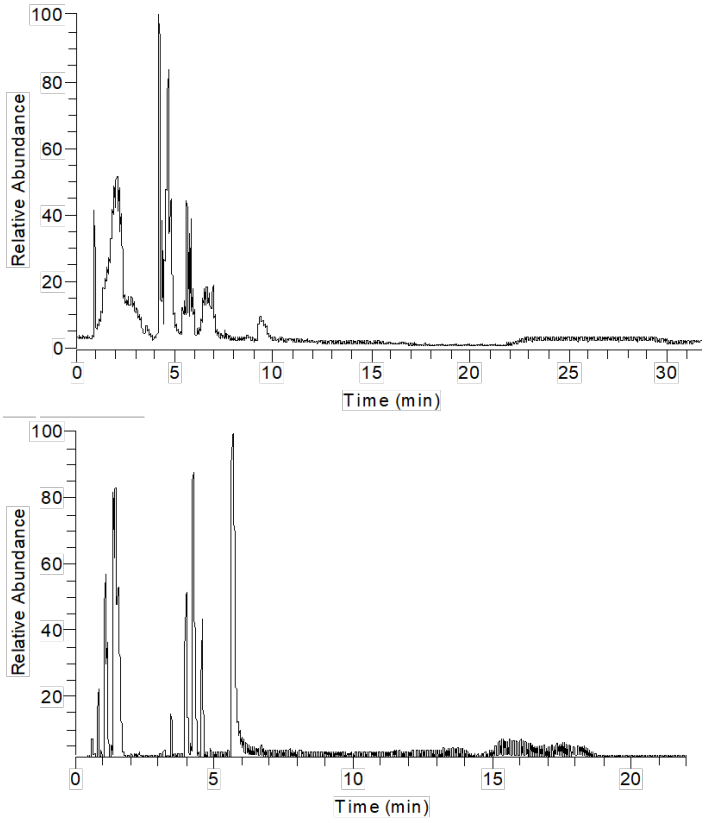

Figure 2-3 Representative total ion chromatograms of metabolite standards using the Agilent HILIC column (top) and Water HSS T3 Column (bottom) on the Thermo Q Exactive HF-X MS.

2.4.3 Optimisation of MS conditions

Initially, the Agilent QTOF/MS was operated in the full scan mode for the comparison of columns using the endogenous metabolite standards mixture listed in Table 2-3. The optimisation of mass spectrometry parameters (such as capillary voltage and temperature) plays an important role to achieve greater sensitivity of mass

spectrometry detection [25]. Several tune parameters were optimised for the most ideal operating conditions. The optimal gas temperatures were recognised between 290 to 350 ^{o}C. As there were no significant differences observed within this range, the instruments default setting of 300 ^{o}C was selected. The auxiliary and sheath gases performed satisfactory at 40 and 10 (arbitrary units) respectively, whereas decreased signal intensities were observed at values higher.

Due to issues experienced with the Agilent QTOF/MS, the Thermo Q Exactive HF-X Hybrid Quadrupole Orbitrap MS was trialled and an assessment of their performances were compared (Table 2-4). Using the previously optimised parameters the Q Exactive HF-X Hybrid Quadrupole Orbitrap MS showed higher sensitivity and reproducibility. A comparison of peak intensities of the metabolite standard mixture (Figure 2-5) demonstrated that the Q Exactive HF-X Hybrid Quadrupole Orbitrap MS is more suitable for the untargeted analysis of CSF metabolites.

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.

Metabolite	**Peak Area**	
	QTOF/MS	**Q Exactive HFX-MS**
Dopamine	8.82E+05	1.41E+10
Epinephrine	7.94E+04	3.39E+09
Metanephrine	9.31E+04	1.58E+09
Norepinephrine	1.65E+04	9.34E+09
Homovanillic acid	1.33E+04	3.37E+07
3,4-Dihydroxyphenylacetic acid		2.51E+06
5-Hydroxyindoleacetic acid		2.53E+07
Sepiapterin		4.28E+09

Neopterin	4.84E+04	1.64E+09
Biopterin		1.66E+09
Histamine		8.11E+08
Glutamic acid	2.73E+05	1.89E+10
γ-aminobutyric acid	8.94E+05	8.10E+07
Tryptophan	1.56E+06	1.11E+10
Kynurenine	9.28E+05	5.51E+08
Kynurenic acid	1.36E+05	2.57E+09
Anthranilic acid	6.62E+05	3.30E+08
3-hydroxyanthranilic acid	2.18E+05	1.80E+09
3-Hydroxykynurenine	5.39E+04	6.77E+09
Quinolinic acid	4.66E+05	5.67E+09
Picolinic acid	2.99E+05	2.07E+08
Xanthurenic acid		1.58E+08

The optimised MS conditions in the Agilent QTOF MS were applied as the starting settings to optimise the parameters of the Thermo Q Exactive HF-X MS system. A high mass resolution of 60,000 is essential to differentiate metabolites in complex biological matrices and identification of overlapping $[M+H]^+$ ion metabolites. For example, m/z 238.0925 and 238.0935 of two pterin species, biopterin and sepiapterin.

Full MS/all ion fragmentation and full MS/data dependent-MS^2 modes were explored and compared using pooled CSF samples. All ion fragmentation data was acquired using a data independent method under a high mass resolution of 120 000, automatic gain control target at 3e6 and maximum injection time of 200 ms. A significantly higher number of features and annotated metabolites in pooled CSF was determined in the MS/all ion fragmentation mode. MS/MS data mode acquired data using the data dependent top twenty compounds method had a mass resolution set to 30 000

for an improved MS/MS spectrum quality to accurately identify relevant individual metabolites of interest. This achieved a narrow precursor ion isolation width with minimal transmission losses of the targeted ions and confirmed metabolites of interest (listed in Table 2-3) were detectable and present in CSF. However, for an untargeted method the data dependent acquisition was limited in its sensitivity resulting in a lower number of features detected in the pooled CSF samples. A significantly higher number of features and annotated metabolites in pooled CSF was determined in the all ion fragmentation mode (Table 2-5). Overall, the full MS/all ion fragmentation mode was deemed more suitable for the untargeted metabolomics analysis method.

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.

Full MS fragmentation mode	**No. of molecular features**	**No. of annotations**
All Ion Fragmentation	296	98
Data Dependent-MS^2	167	53

A comparison of the positive and negative ion modes was also evaluated using the metabolite standard mixture and pooled CSF. For positive and negative ionisation modes, low frequency oscillations of the baseline were reduced with a higher spray voltage. A spray voltage of 4 kV for the positive mode and 3 kV for the negative ion mode were selected. Trialling at spray voltages higher than these selected values did not show further improvements. A large variation of metabolites (including all the metabolite standards) was detected in CSF using positive electrospray ionisation. In the negative mode, metabolite standards such as 3,4-dihydroxyphenylacetic acid, homovanillic acid, 5-hydroxyindoleacetic acid and GABA exhibited a higher signal intensity in the negative mode. However, other metabolites in the negative mode lacked sensitivity or could not be detected in pooled CSF samples. Detection using

positive electrospray ionisation was found to have a better overall performance in detecting the broad spectrum of multi-class metabolites in CSF.

2.4.4 Column comparison using pooled CSF samples and metabolite standards

Currently, there is no single platform able to cover the entire CSF metabolome given its size and diversity of metabolite properties. The metabolite standard solution contains a wide variation in hydrophobic and hydrophilic properties of metabolites. As a result, the physiochemical properties of such a broad spectrum of metabolites will influence their ability to bind onto different stationary phases in each column. The reverse phase nature of the RP column and HSS T3 column leads to the early elution of hydrophilic molecules. Conversely, hydrophilic molecules on the HILIC column eluted at a later stage and an earlier elution of hydrophobic compounds.

Preliminary evaluation of the different columns was performed using the metabolite standard mixture using the optimised gradient conditions in the positive ionisation mode. The columns listed in Table 2-1 displayed comparable retention time analysis for the mixed standard solutions. The elution of metabolite standards began from approximately 0.80 minutes after sample injection and the last metabolite standard eluted prior to 10 minutes. The C18 RP column presented the lowest retainment of the metabolite standard mixture.

The performance of the columns were evaluated based on retention time, peak area and peak width for each metabolite standard (Table 2-6 and Table 2-7). For the metabolite standards the HILIC and HSS T3 columns showed high retention time reproducibility with minimal shifts and low standard deviation values. The elution range of the metabolites ranged from 0.83 to 4.09, 0.97 to 9.37 and 0.82 to 5.89 minutes for the RP, HILIC and HSS T3 columns respectively. For the metabolite standards the HILIC and HSS T3 columns showed high retention time reproducibility with minimal shifts and low standard deviation values. The HSS T3 column yielded the

narrowest peaks from the three investigated columns. The HILIC column was the most sensitive, obtaining the highest peaks areas for the standard metabolites.

Following the transition to the Thermo UPLC-HRMS instrument the standards and pooled CSF were analysed on the HSS T3 and HILIC column. The HSS T3 and HILIC column showed good chromatographic separation of the standards and improved metabolite coverage in pooled CSF samples (Figure 2-4 and Figure 2-5).

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.

Metabolite	**RP Column**		**HILIC Column**		**HSS T3 column**	
	Mean	**SD**	**Mean**	**SD**	**Mean**	**SD**
Dopamine	2.57	0.01	5.23	0.00	1.58	0.02
Epinephrine	0.94	0.00	4.94	0.02	1.28	0.00
Metanephrine	1.03	0.02	2.16	0.01	1.44	0.02
Norepinephrine	2.89	0.06	3.17	0.00	1.12	0.01
Homovanillic acid			9.37	0.04	3.15	0.00
3,4-Dihydroxyphenylacetic acid			3.03	0.02	4.27	0.01
5-Hydroxyindoleacetic acid	1.11	0.04	1.51	0.02	4.90	0.00
Sepiapterin			1.73	0.01	3.79	0.00
Neopterin			2.71	0.02	1.26	0.03
Biopterin			4.04	0.00	2.12	0.02
Histamine			5.98	0.01		
Glutamic acid	0.83	0.01	7.85	0.03	0.82	0.01
γ-aminobutyric acid	1.52	0.02	5.8	0.02	0.98	0.00

Tryptophan	1.93	0.03	4.79	0.01	4.39	0.01
Kynurenine	4.09	0.03	4.38	0.02	3.77	0.01
Kynurenic acid	3.31	0.02	2.82	0.01	4.75	0.00
Anthranilic acid	1.37	0.01	0.97	0.01	5.89	0.03
3-hydroxyanthranilic acid	2.36	0.03	1.18	0.00	4.01	0.01
3-Hydroxykynurenine	2.15	0.01	3.33	0.00	3.44	0.02
Quinolinic acid	1.26	0.02	3.85	0.01	1.95	0.00
Picolinic acid	0.88	0.02	5.48	0.02	1.2	0.01
Xanthurenic acid	2.34	0.02	3.68	0.01	4.53	0.00

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.

Metabolite	**RP Column**		**HILIC Column**		**HSS T3 column**	
	Area	**Width**	**Area**	**Width**	**Area**	**Width**
Dopamine	2.57E+06	0.16	1.41E+10	0.07	4.16E+07	0.38
Epinephrine	7.44E+05	0.18	3.39E+09	0.25	1.58E+07	0.18
Metanephrine	1.98E+05	0.10	1.58E+09	0.42	1.61E+08	0.46
Norepinephrine	1.42E+04	0.51	9.34E+09	0.26	1.99E+06	0.18
Homovanillic acid			3.37E+07	0.55	9.04E+06	0.20
3,4-Dihydroxyphenylacetic acid			2.51E+06	0.44	1.26E+06	0.18
5-Hydroxyindoleacetic acid	2.84E+04	0.35	2.53E+07	0.14	4.29E+06	0.08
Sepiapterin			4.28E+09	0.18	1.64E+07	0.21
Neopterin			1.64E+09	0.38	4.03E+08	0.45
Biopterin			1.66E+09	0.28	2.01E+09	0.24

Histamine			8.11E+08	0.08		
Glutamic acid	4.38E+06	0.39	1.89E+10	0.26	3.51E+09	0.17
γ-aminobutyric acid	5.31E+04	0.29	8.10E+07	0.40	7.67E+07	0.20
Tryptophan	3.76E+07	0.26	1.11E+10	0.30	2.87E+10	0.22
Kynurenine	4.68E+06	0.35	5.51E+08	0.52	1.86E+07	0.24
Kynurenic acid	2.96E+05	0.47	2.57E+09	0.30	2.08E+10	0.16
Anthranilic acid	5.51E+05	0.22	3.30E+08	0.42	1.78E+07	0.41
3-hydroxyanthranilic acid	1.01E+06	0.18	1.80E+09	0.20	5.01E+08	0.26
3-hydroxykynurenine	2.18E+05	0.16	6.77E+09	0.24	5.98E+08	0.14
Quinolinic acid	1.62E+06	0.24	5.67E+09	0.22	4.05E+08	0.22
Picolinic acid	4.37E+04	0.43	2.07E+08	0.15	1.72E+07	0.20
Xanthurenic acid	6.37E+04	0.30	1.58E+08	0.14	2.07E+06	0.15

Initial experiments using the RP column exhibited poor retention of metabolites or difficulties of molecules to be retained on the column. This included histamine, homovanillic acid, pterins and a small number of amino acids. These metabolites have also been previously reported to be quite challenging to elute [26, 27]. Subsequent, trials were conducted using the Waters BEH amide column and a traditional normal stationary phase Agilent C18 column. The C18 column did not show improvements in the retention of metabolites. There were improvements observed in the BEH amide column for the elution of the metabolite standards, however, there was a lack of consistency and reproducibility of the peak shapes across different sequence runs.

The hydrophilic interaction chromatography (HILIC) has become a consolidated column for the analytical separation of polar based molecules and increasingly used as a complementary to conventional RP columns [28]. The highly orthogonal mechanism of HILIC is advantageous in the retainment and elution of metabolites (acidic, neutral and basic) and highly reproducible separations of strongly polar metabolites which elute in the void volume of reverse phase chromatography [29, 30]. The elution of compounds under high organic mobile phase compositions has

been found to improve signal intensity in MS analyses and efficiency of desolation processes [26]. The high specificity and selectivity of HILIC methods have been reported to provide a higher coverage of metabolites detected compared to RP chromatography [31].

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.

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.

In recent years, the HSS T3 column has gained attraction from its ability to retain both polar and non-polar molecules. HSS T3 columns contains a polar group bonded phase and treated silica particles forming a bonded sorbent beneficial for the retention of polar compounds under aqueous mobile phase conditions. The measurement of diverse metabolite properties in CSF using the HSS T3 column has reported to obtain satisfactory chromatographic resolution and retention in a number of studies [24, 32-34].

The method development process of HILIC methods can be challenging requiring careful optimisation and a prolonged column re-equilibration and conditioning duration [34, 35]. However, the reproducibility, metabolite intensity and coverage offered in HILIC methods outweigh these drawbacks. The use of complementary chromatographic methods is commonly employed in untargeted metabolomics analysis [34, 36, 37].

2.4.5 Profiling of Metabolites in CSF samples

The performances of the three columns were further evaluated by analysing metabolites extracted from pooled CSF. Injections of the metabolites in pooled CSF were initially performed on the Agilent LC-QTOF/MS using the RP and HILIC columns. Following the transition to the Thermo LC-HRMS, CSF metabolites were trialled using all three columns.

The reliability of metabolomics workflows to perform biological interpretations is facilitated by the advancements of metabolic libraries, deconvolution softwares and accessibility to commercial reference standards [38]. A major bottleneck in untargeted metabolomic studies is the accuracy of metabolite annotations. There has been a number of databases available to annotate CSF metabolites including the human metabolome database [39], CSF Metabolome [18], KEGG database (www.genome.jp/kegg/) and METLIN [40]. Several softwares have been developed for untargeted metabolomics analysis such as MassHunter profinder (Agilent

Technologies), Compound discoverer (Thermo Fisher Scientific), Metaboanalyst (www.metaboanalyst.ca) and Mass profiler professional (Agilent Technologies).

The LC-QTOF/MS data obtained from pooled CSF samples were processed using MassHunter Qualitative Analysis and MassHunter profinder. The total number of molecular features identified from pooled CSF samples (n = 5) are listed in Table 2-8. The Agilent HILIC column obtained the most number of molecular features identified in the positive ESI mode. However, the low number of molecular features able to be detected in the pooled CSF samples from both columns, further indicated the sensitivity challenges experienced in the analysis method.

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.

	LC-QTOF/MS		**UPLC -Q Exactive HFX/MS**	
	HSS T3	**HILIC**	**HSS T3**	**HILIC**
Elution range (min)	0.81 – 15.47	0.95 – 22.12	0.85 – 16.94	0.90 – 19.65
No. of molecular features	140	181	448	573
No. of annotations	47	55	129	167

Following the transition to the Thermo UPLC-HRMS, data was acquired on the optimised metabolomics workflow using Compound discoverer 3.0. A comparison of the pooled CSF results obtained from the columns (Table 2-9) showed the HILIC column yielded a greater number of molecular features identified and confirmed metabolite annotations, followed closely by the HSS T3 column.

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.

	RP	**HILIC**	**HSS T3**
Elution range (min)	0.83 – 15.50	0.90 – 19.65	0.85 – 16.94
No. of molecular features	239	573	448
No. of annotations	74	167	129

The HSS T3 and HILIC columns operated in the positive ionisation mode captured a wide variation of CSF metabolites. There were 93 annotated compounds shared between the two methods. As a result, a total of 203 compounds were annotated by reference standards or putatively through literature mass spectra. The annotated metabolites mainly consisted of endogenous neuroactive metabolites, organic acids and bases (Figure 2-6).

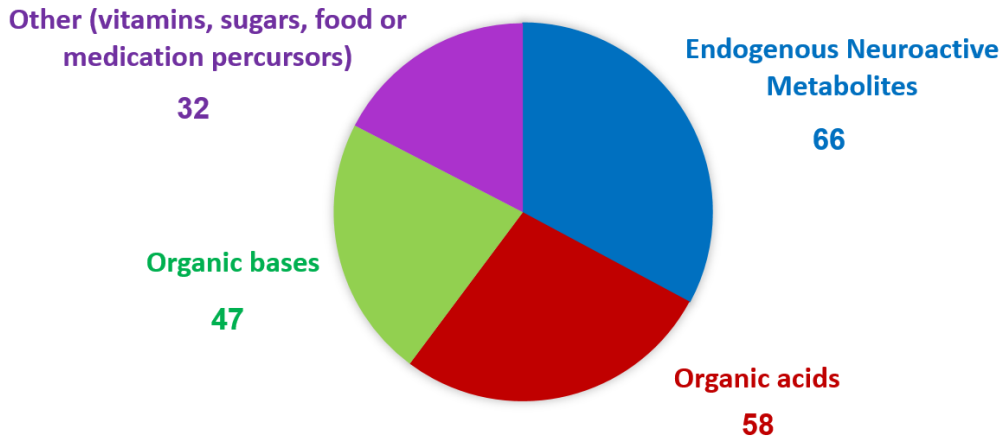

Figure 2-6 Summary of categorised subgroups in 203 annotated compounds identified in pooled CSF.

2.5 Conclusion

Metabolomics is a rapidly evolving research field providing new opportunities in facilitating biomarker discovery and unravelling the pathophysiologic mechanisms underlying health and diseases. An untargeted metabolomics analysis method using ultra-performance liquid chromatography coupled to high resolution mass spectrometry was developed for the screening of metabolites in human CSF. Sample preparation of CSF metabolites was achieved by a simple methanol precipitation method and demonstrated good reproducibility. The performance of three columns was assessed extensively using a metabolite standard mixture. The HSS T3 presented the narrowest peaks and the HILIC column obtained the highest peak area in the positive ionisation mode. Furthermore, pooled human CSF samples were evaluated and the HILIC column acquired the highest number of metabolites. The developed untargeted approach presents a useful addition and promising high throughput preliminary screening.

References

1. Botas A, Campbell HM, Han X, Maletic-Savatic M: **Metabolomics of neurodegenerative diseases.** *Int Rev Neurobiol* 2015, **122:**53-80.

2. Kasiappan R, Kamarajan P, Kapila YL: **5 - Metabolomics in head and neck cancer: A summary of findings.** In *Translational Systems Medicine and Oral Disease.* Edited by Sonis ST, Villa A: Academic Press; 2020: 119-135

3. Iida M, Harada S, Takebayashi T: **Application of Metabolomics to Epidemiological Studies of Atherosclerosis and Cardiovascular Disease.** *Journal of atherosclerosis and thrombosis* 2019, **26:**747-757.

4. Mandal R, Guo AC, Chaudhary KK, Liu P, Yallou FS, Dong E, Aziat F, Wishart DS: **Multi-platform characterization of the human cerebrospinal fluid metabolome: a comprehensive and quantitative update.** *Genome Medicine* 2012, **4:**38.

5. Wishart DS, Lewis MJ, Morrissey JA, Flegel MD, Jeroncic K, Xiong Y, Cheng D, Eisner R, Gautam B, Tzur D, et al: **The human cerebrospinal fluid metabolome.** *Journal of Chromatography B* 2008, **871:**164-173.

6. Fitzpatrick M, Young SP: **Metabolomics – A novel window into inflammatory disease.** *Swiss medical weekly* 2013, **143:**w13743-w13743.

7. Roberts LD, Souza AL, Gerszten RE, Clish CB: **Targeted metabolomics.** *Curr Protoc Mol Biol* 2012, **Chapter 30:**Unit 30.32.31-24.

8. Naz S, Vallejo M, García A, Barbas C: **Method validation strategies involved in non-targeted metabolomics.** *Journal of Chromatography A* 2014, **1353:**99-105.

9. Schrimpe-Rutledge AC, Codreanu SG, Sherrod SD, McLean JA: **Untargeted Metabolomics Strategies-Challenges and Emerging Directions.** *Journal of the American Society for Mass Spectrometry* 2016, **27:**1897-1905.

10. Cajka T, Fiehn O: **Toward Merging Untargeted and Targeted Methods in Mass Spectrometry-Based Metabolomics and Lipidomics.** *Analytical Chemistry* 2016, **88:**524-545.

11. Clish CB: **Metabolomics: an emerging but powerful tool for precision medicine.** *Cold Spring Harbor molecular case studies* 2015, **1:**a000588-a000588.

12. Zhang X-w, Li Q-h, Xu Z-d, Dou J-j: **Mass spectrometry-based metabolomics in health and medical science: a systematic review.** *RSC Advances* 2020, **10:**3092-3104.

13. Crews B, Wikoff WR, Patti GJ, Woo H-K, Kalisiak E, Heideker J, Siuzdak G: **Variability analysis of human plasma and cerebral spinal fluid reveals statistical significance of changes in mass spectrometry-based metabolomics data.** *Analytical chemistry* 2009, **81:**8538-8544.

14. Kennedy AD, Wittmann BM, Evans AM, Miller LAD, Toal DR, Lonergan S, Elsea SH, Pappan KL: **Metabolomics in the clinic: A review of the shared and unique features of untargeted metabolomics for clinical research and clinical testing.** *Journal of Mass Spectrometry* 2018, **53:**ii-ii.

15. Madsen R, Lundstedt T, Trygg J: **Chemometrics in metabolomics—A review in human disease diagnosis.** *Analytica Chimica Acta* 2010, **659:**23-33.

16. Pinto RC: **Chemometrics Methods and Strategies in Metabolomics.** In *Metabolomics: From Fundamentals to Clinical Applications.* Edited by Sussulini A. Cham: Springer International Publishing; 2017: 163-190

17. Chong J, Wishart DS, Xia J: **Using MetaboAnalyst 4.0 for Comprehensive and Integrative Metabolomics Data Analysis.** *Current Protocols in Bioinformatics* 2019, **68:**e86.

18. Wishart DS, Lewis MJ, Morrissey JA, Flegel MD, Jeroncic K, Xiong Y, Cheng D, Eisner R, Gautam B, Tzur D, et al: **The human cerebrospinal fluid metabolome.** *J Chromatogr B Analyt Technol Biomed Life Sci* 2008, **871:**164-173.

19. Want EJ, Masson P, Michopoulos F, Wilson ID, Theodoridis G, Plumb RS, Shockcor J, Loftus N, Holmes E, Nicholson JK: **Global metabolic profiling of animal and human tissues via UPLC-MS.** *Nat Protoc* 2013, **8:**17-32.

20. Fuertig R, Ceci A, Camus SM, Bezard E, Luippold AH, Hengerer B: **LC-MS/MS-based quantification of kynurenine metabolites, tryptophan, monoamines and neopterin in plasma, cerebrospinal fluid and brain.** *Bioanalysis* 2016, **8:**1903-1917.

21. Weiss N, Barbier Saint Hilaire P, Colsch B, Isnard F, Attala S, Schaefer A, Amador MD, Rudler M, Lamari F, Sedel F, et al: **Cerebrospinal fluid metabolomics highlights dysregulation of energy metabolism in overt hepatic encephalopathy.** *J Hepatol* 2016, **65:**1120-1130.

22. Rafferty JL, Siepmann JI, Schure MR: **Mobile phase effects in reversed-phase liquid chromatography: A comparison of acetonitrile/water and methanol/water solvents as studied by molecular simulation.** *Journal of Chromatography A* 2011, **1218:**2203-2213.

23. Yin S, Su M, Xie G, Li X, Wei R, Liu C, Lan K, Jia W: **Factors affecting separation and detection of bile acids by liquid chromatography coupled with mass spectrometry in negative mode.** *Analytical and bioanalytical chemistry* 2017, **409:**5533-5545.

24. Lo A, Guibal P, Doummar D, Rodriguez D, Hautem J-Y, Couderc R, Billette De Villemeur T, Roze E, Chaminade P, Moussa F: **Single-Step Rapid Diagnosis of Dopamine and Serotonin Metabolism Disorders.** *ACS Omega* 2017, **2:**5962-5972.

25. Pitt JJ: **Principles and applications of liquid chromatography-mass spectrometry in clinical biochemistry.** *The Clinical biochemist Reviews* 2009, **30:**19-34.

26. Danaceau JP, Chambers EE, Fountain KJ: **Hydrophilic interaction chromatography (HILIC) for LC–MS/MS analysis of monoamine neurotransmitters.** *Bioanalysis* 2012, **4:**783-794.

27. Noga S, Jandera P, Buszewski B: **Retention Mechanism Studies of Selected Amino Acids and Vitamin B6 on HILIC Columns with Evaporative Light Scattering Detection.** *Chromatographia* 2013, **76:**929-937.

28. Greco G, Letzel T: **Main Interactions and Influences of the Chromatographic Parameters in HILIC Separations.** *Journal of Chromatographic Science* 2013, **51:**684-693.

29. Gallart-Ayala H, Konz I, Mehl F, Teav T, Oikonomidi A, Peyratout G, van der Velpen V, Popp J, Ivanisevic J: **A global HILIC-MS approach to measure polar human cerebrospinal fluid metabolome: Exploring gender-associated variation in a cohort of elderly cognitively healthy subjects.** *Analytica Chimica Acta* 2018, **1037:**327-337.

30. Tang D-Q, Zou L, Yin X-X, Ong CN: **HILIC-MS for metabolomics: An attractive and complementary approach to RPLC-MS.** *Mass Spectrometry Reviews* 2016, **35:**574-600.

31. Wernisch S, Pennathur S: **Evaluation of coverage, retention patterns, and selectivity of seven liquid chromatographic methods for metabolomics.** *Analytical and bioanalytical chemistry* 2016, **408:**6079-6091.

32. Bergh MS-S, Bogen IL, Lundanes E, Øiestad ÅML: **Validated methods for determination of neurotransmitters and metabolites in rodent brain tissue and extracellular fluid by reversed phase UHPLC–MS/MS.** *Journal of Chromatography B* 2016, **1028:**120-129.

33. Jung-Klawitter S, Kuseyri Hübschmann O: **Analysis of Catecholamines and Pterins in Inborn Errors of Monoamine Neurotransmitter Metabolism-From Past to Future.** *Cells* 2019, **8**.

34. Ghosson H, Schwarzenberg A, Jamois F, Yvin J-C: **Simultaneous untargeted and targeted metabolomics profiling of underivatized primary metabolites in sulfur-deficient barley by ultra-high performance liquid chromatography-quadrupole/time-of-flight mass spectrometry.** *Plant methods* 2018, **14:**62-62.

35. King AM, Mullin LG, Wilson ID, Coen M, Rainville PD, Plumb RS, Gethings LA, Maker G, Trengove R: **Development of a rapid profiling method for the analysis of polar analytes in urine using HILIC–MS and ion mobility enabled HILIC–MS.** *Metabolomics* 2019, **15:**17.

36. Luo X, Zhang A, Wang X, Lu H: **UHPLC/MS based large-scale targeted metabolomics method for multiple-biological matrix assay.** *bioRxiv* 2019**:**642496.

37. Lee H-J, Kremer DM, Sajjakulnukit P, Zhang L, Lyssiotis CA: **A large-scale analysis of targeted metabolomics data from heterogeneous biological samples provides insights into metabolite dynamics.** *Metabolomics : Official journal of the Metabolomic Society* 2019, **15:**103-103.

38. Pinu FR, Goldansaz SA, Jaine J: **Translational Metabolomics: Current Challenges and Future Opportunities.** *Metabolites* 2019, **9:**108.

39. Wishart DS: **Metabolomic Data Exploration and Analysis with the Human Metabolome Database.** In *Computational Methods and Data Analysis for Metabolomics.* Edited by Li S. New York, NY: Springer US; 2020: 165-184

40. Smith CA, O'Maille G, Want EJ, Qin C, Trauger SA, Brandon TR, Custodio DE, Abagyan R, Siuzdak G: **METLIN: a metabolite mass spectral database.** *Ther Drug Monit* 2005, **27:**747-751.

Chapter 3: A pilot study to evaluate CSF metabolomics in acute brain inflammation

Chapter 3: A pilot study to evaluate CSF metabolomics in acute brain inflammation

3.1 Abstract

The response of the central nervous system to invading pathogens can be protective but also responsible for the harmful sequelae effects in neuroinflammatory diseases. The immune response upon inflammation induces the initiation and alteration of a wide range of neuroactive metabolites. A pilot study of four encephalitis patients and four age-matched controls were explored using the developed untargeted metabolomics analysis method. Chemometrics tools were performed to assess the statistical contribution of cerebrospinal fluid (CSF) metabolites to neuroinflammation. A statistical discrimination between encephalitis and control groups was achieved using orthogonal partial least squares discriminant (OPLS-DA) analysis. Univariate statistical methods annotated twenty two key metabolites as statistically discriminative between the encephalitis and control groups. Statistically discriminating CSF metabolites identified are mainly involved in the tryptophan-kynurenine pathway, neopterin and amino acid metabolism. The pilot study demonstrated that untargeted metabolomics using liquid chromatography coupled to high resolution mass spectrometry (LC-HRMS) is a robust procedure to generate CSF metabolic profiles and offers an important aid to the development of neuroinflammatory biomarkers.

3.2 Introduction

Inflammation of the brain is increasingly recognised as important in acute neuroinflammatory diseases (such as encephalitis), but also neurodevelopmental, neuropsychiatric and neurodegenerative processes. The inflammatory response of the central nervous system to invading cells can play a harmful role leading to neuronal damage and the progression of acute neuroinflammation [1]. Encephalitis is characterised by inflammation of the brain often due to infection (typically viral) or autoimmune reactivity [2]. Over 100 causes of encephalitis are described, and vary according to geographical region, but often the causes and neurological outcomes are unknown leading to significant challenges encountered by clinicians to provide a rapid diagnosis and treatment regimen.

Metabolomics has provided opportunities for identifying biomarkers with clinical significance and unravelling biochemical changes within a biological sample to offer a comprehensive understanding on a broad spectrum of metabolic profiles [3]. The improvements in technological platforms available for metabolomic workflows have directed great interest in the application of neuroinflammation research. CSF is regarded as the most useful biofluid in understanding the activities of the brain and a valuable source of potential clinical biomarkers [4].

The aim of this chapter is to assess the feasibility of the developed analysis method on human CSF cohort groups and determine whether the untargeted metabolomics approach can discriminate encephalitis patients from controls.

3.3 Materials and method

3.3.1 Study Design and Participants

Eight human CSF samples were obtained from the Department of Biochemistry at the Children's Hospital at Westmead (Sydney, Australia). CSF samples used for the study were withdrawn by lumbar puncture between the third or fifth lumbar vertebrae.

The encephalitis group (n=4, mean 6.25 years, median 6 years, 3 males) all fulfilled encephalitis criteria [5] (acute disseminated encephalomyelitis n=2, unknown infectious encephalitis n=1, enteroviral encephalitis n=1). The age matched control group (n=4, mean 6 years, median 5.5 years, 3 males) had non-inflammatory neurological diseases (cerebral palsy n=2, genetic dystonia n=1, genetic neuropathy n=1). CSF samples were frozen within 1 hour of sampling and stored at -40 ^{o}C until the time of processing. Prior to experiments, CSF samples were thawed, vortexed and aliquot into 2mL Eppendorf tubes.

Local Ethics committee approved this study, LNR/14/SCHN/275 (2019/ETH06182).

3.3.2 Chemicals and Reagents

Epinephrine hydrochloride, GABA, kynurenic acid, L-kynurenine, and L-tryptophan, quinolinic acid, 3-hydroxy-anthranilic acid, xanthurenic acid, neopterin and ammonium formate were purchased as powders from Sigma Aldrich (Sydney, Australia). D_3-Dopamine and D_3-metanephrine were supplied by CDN Isotopes (Quebec, Canada). HPLC grade acetonitrile was purchased from Honeywell (Chem Supply, Gillman, Australia). Formic acid was supplied by RCI Labscan (Bangkok, Thailand).

3.3.3 Preparation of Standard Solutions

A 500 ng/mL mixed solution of the metabolite standards were prepared and stored in -20 ^{o}C prior to usage. A mixed standard solution was injected with every batch run for confirmation of any analytes present in the CSF samples. A 50 ng/mL mixed internal standard solution containing D_3-Dopamine and D_3-Kynurenic acid was prepared and added to the methanol solution for the deproteinisation step.

3.3.4 Cerebrospinal Fluid Metabolite Extraction

100 µL of CSF samples and 300 µL of the methanol mixture were thoroughly mixed for 90 seconds on a vortex and sonicated for 5 minutes. For protein precipitation, the mixture was left in ice for 45 minutes. This was followed by centrifugations of the samples for 12 minutes at a temperature of 5 ^{o}C and velocity of 4000 g. The supernatant was collected and evaporated to dryness under nitrogen. Subsequently, 50 µL of water/acetonitrile at a ratio of 40/60 was used to reconstitute the CSF residue for UPLC-HRMS analyses.

Quality control (QC) samples were prepared from a pooled mixture of equal volumes of each original CSF sample. These were injected every three samples to evaluate the reproducibility of the method and analytical error for metabolites. The CSF samples were analysed independent of clinical status using a randomised injection sequence.

3.3.5 LC-HRMS Analysis

LC-HRMS analyses were performed using a Thermo Scientific Vanquish system coupled to a Q Exactive HF-X Hybrid Quadrupole Orbitrap Mass Spectrometer (Thermo Fisher Scientific Inc., Massachusetts, CA, USA) fitted with an electrospray source in both positive and negative ion modes. Calibration of the mass spectrometer

in the positive mode was conducted prior to analysis using a calibration mixture provided by the manufacturer.

The chromatographic separation of metabolites was achieved on an Agilent Infinity Lab HILIC column (2.1 x 100 mm, 2.7µm particle size) and the Waters Acquity UPLC HSS T3 column (2.1 x 10mm, 1.8µm particle size).

Mobile phases for the HILIC column consisted of an aqueous buffer of 20 mM ammonium formate in water and 90% acetonitrile with 20 mM ammonium formate as solvent B. For the HSS T3 column, 0.1% formic acid in A and acetonitrile containing 0.1% formic acid in B.

The gradient elution for both methods were performed at a flow rate of 0.30 mL/min and injection volume of 10 µL. For the HILIC column the gradient elution are as follows: 0−1.5 min (90% B), 1.5−12 min (90−50% B), 12−16 min (50% B), 16−17 min (50−25% B), 17−20 min (25% B), 20−21 min (45−90% B) and 21−32 min (90% B). The gradient program for the HSS T3 column are as follows: 0−1 min (2% B), 1-6 min (2−40% B), 6−14 min (40−99% B), 14−16.5 min (99% B), 16.5−17 min (99−2% B) and 17−22 min (2% B).

The MS was operated in the full scan/all ion fragmentation mode using positive electrospray ionisation. The detection scan range was achieved from m/z 50 to 600 in the positive ionisation mode. The MS was acquired under a high mass resolution of 120 000, capillary voltage at 4 kV and capillary temperature of 300 ^{o}C.

3.3.6 Data Treatment and Handling

Data processing was performed using Qualbrowser on the Xcalibur version 2.1 software and Compound Discoverer 3.0 software. (Thermo Fisher Scientific Inc.). Automated peak detection, integration, identification and determination of differences between samples sets were extracted using the metabolomics workflow in Compound Discoverer. Molecular features from the LC-HRMS data were extracted

using the following parameters: two positive ion species, $[M+H]^+$ and $[M+NH_4]^+$ and mass fragmentation tolerance of 5 ppm; aligned retention time window of 0.20 min and mass window of 5 ppm. The mass spectral features were set to consider only features present in at least 75% of samples and extracted peaks were manually inspected to reduce false positives and negative features.

The databases used for metabolite annotation and identification were the human metabolome database, m/z cloud, Chemspider and an in-house CSF database. For metabolites identified upon comparison with chemical reference standards ions had to meet the criteria proposed by the Metabolomics Standards Initiative [6] which includes a match in MS spectrum, retention time and accurate measure mass. In the absence of a reference chemical standard being available metabolites were putatively annotated upon comparison of mass spectra with literature spectra.

The pre-processed data sets extracted from compound discoverer were normalised against internal standards. An online platform, MetaboAnalyst 4.0 was employed for multivariate and univariate statistical analysis. The clustering and similarities or differences between sample sets (controls, encephalitis, QC) were evaluated using principal component analysis (PCA) and OPLS-DA. The score plots allowed visual inspections of the data distribution, trends and outliers in the data. Significant metabolites driving the discrimination between the control and encephalitis groups were analysed through ANOVA and Fishers LSD post-hoc analysis at a p-value cut off of 0.01.

3.4 Results and Discussion

CSF samples from 4 encephalitis patients and 4 controls were obtained for the purpose of this pilot study. In recent years, a few untargeted CSF metabolomics studies have been conducted on encephalitis employing NMR or LC-HRMS [7, 8]. The data treatment process from raw spectra to biological interpretations is an important component of the untargeted metabolomics workflow. Compound Discoverer is a

software containing workflows useful for the preliminary data pre-treatment of metabolomics data. Initially, a large number of peaks were extracted. This is commonly encountered in metabolomics studies where thousands of peaks can be initially detected [9]. Extensive filters such as mass fragmentation tolerances, retention time alignment tolerances and the exclusion of features corresponding to background noise and peaks that weren't present in more than 75% of samples were applied. This significantly reduced the manual data cleaning step resulting in 675 and 529 peaks detected for the HILIC and T3 methods respectively (Table 3-1). The CSF metabolites were eluted within a twenty minute time frame. The complementary method annotated a total of 234 different CSF metabolites. However, for the HSS T3 column method, one sample from both the encephalitis and control group was excluded due to significantly lower molecular features present and poor peak intensities.

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

Agilent HILIC	Number of molecular features	675
	Reference standard and database identified compounds	189
	Elution range	0.9 – 20 min
Water HSS T3	Number of molecular features	529
	Reference standard and database identified compounds	154
	Elution range	0.85 – 17.5 min

3.4.1 Quality Control Analysis

A total of three QC injections were inserted into the analytical run for this pilot study. The PCA for the two complementary methods showed a close cluster of the QC injections upon inspection of peak shape, peak areas and retention times (Figure 3-1). The 2D PCA plot indicated the absence of outliers and a good reproducibility of the developed analytical method. A comparison of the PCA score plots generated by the two methods showed the HILIC method to provide a greater statistical separation.

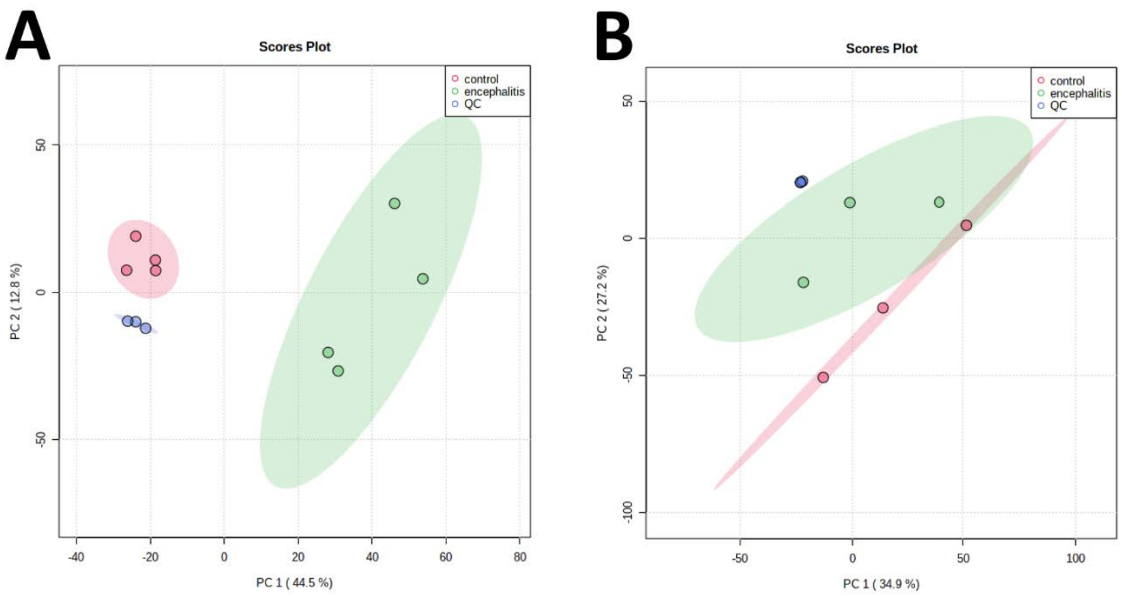

Figure 3-1 [A] PCA score plot of 4 control patients (red dots), 4 encephalitis patients (green dots) and 3 QC samples (blue dots) showing clustering of the QC samples using the HILIC column [B] PCA score plot on 3 control patients (red dots), 3 encephalitis patients (green dots) and 3 QC samples (blue dots) showing clustering of the QC samples using the HSS T3 column.

3.4.2 Statistical Analyses

The differences in CSF levels between encephalitis and non-inflamed patients were assessed using PLS-DA. The 2D OPLS-DA score plots showed that the controls and inflamed patients were well separated reflecting the metabolic differences between the two groups (Figure 3-2).

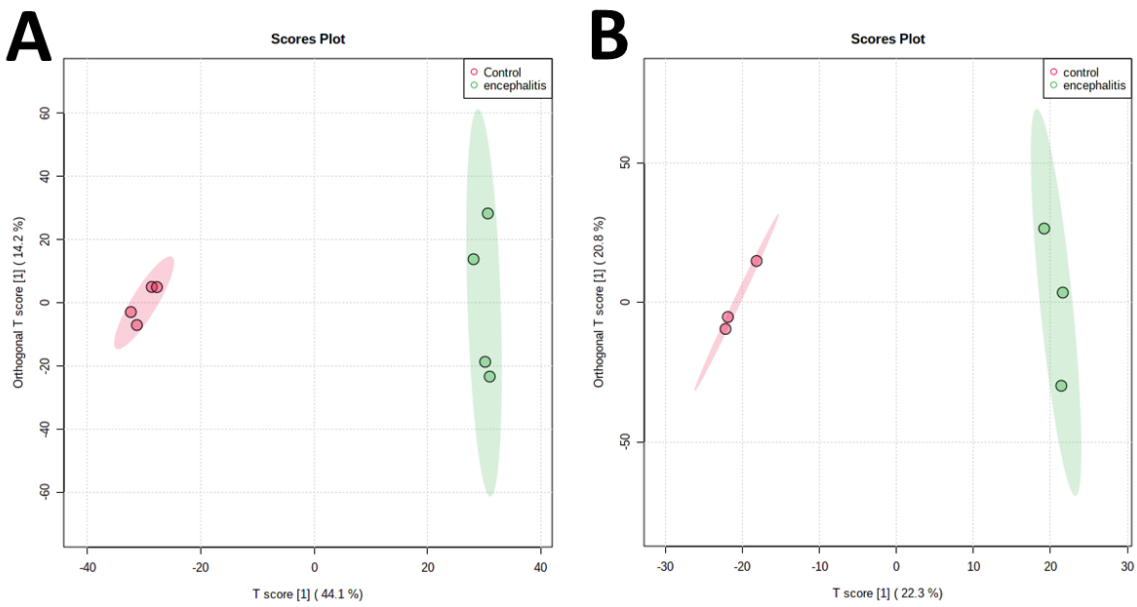

Figure 3-2 [A] OPLS-DA score plot presenting a clear separation of 4 control patients (red dots) and 4 encephalitis patients (green dots) using the HILIC column [B] OPLS-DA score plot presenting a clear separation score plot on 3 control patients (red dots) and 3 encephalitis patients (green dots) using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

ANOVA and post-hoc analysis at a low p-value cut off of 0.01 revealed a total of forty metabolites involved in driving the separation between encephalitis and control patients. The study was able to confidently annotate quinolinic acid, tryptophan, kynurenic acid, neopterin, GABA, kynurenine, 3-hydroxyanthranilic acid, xanthurenic acid and epinephrine as metabolites contributing to the clustering of inflamed patients using analytical reference standards. The remaining metabolites were putatively annotated using reported literature mass spectra from the Human Metabolome Database. As a result, thirteen metabolites were putatively confirmed and found to contribute to the discrimination. Although time consuming, the manual

integration provided a high degree of confidence for the twenty-two significant metabolites (Table 3-2).

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

Metabolite	Relevance
3-Hydroxyanthranilic acid	kynurenine pathway metabolite
4-Amino-5-oxopentanoate	amino acid
10-Hydroxydecanoic acid	fatty acid
Acetyl-N-formyl-5-methoxykynurenamine	tryptophan pathway metabolite
Adenine	nucleobase
Arginine	amino acid
Choline	precursor to acetylcholine
Creatine	amino acid
Epinephrine	neurotransmitter, catecholamine
Formyl-isoglutamine	histidine metabolism
Gamma-aminobutyric acid	inhibitory neurotransmitter, amino acid
Hexanoylcarnitine	acylcarnitine, lipid
Histidine	amino acid, precursor to histamine
Homoarginine	endogenous non-proteinogenic amino acid
Isoglutamine	amino acid
Kynurenic acid	kynurenine pathway metabolite
Kynurenine	tryptophan pathway metabolite
Lysine	amino acid
Neopterin	catabolic product of guanosine triphosphate
Quinolinic acid	kynurenine pathway metabolite
Tryptophan	amino acid, precursor to kynurenine

Xanthurenic acid	kynurenine pathway metabolite

3.4.3 Identified Metabolites and Pathways Involved

The changes in twenty-two metabolites could discriminate between encephalitis and control groups. Twelve metabolite levels were decreased, and ten metabolites showed increased levels in encephalitis patients compared to controls (Figure 3-3 and Figure 3-4). The significant metabolites contributing to the discrimination between the two groups are involved in the tryptophan-kynurenine pathway, neopterin, amino acid metabolism (GABA, arginine, creatine, isoglutamine, lysine, histidine), lipid metabolism (carnitine, 10-hydroxydecanoic acid), catecholamine metabolism (epinephrine) and other endogenous molecules (choline, adenine). These findings are consistent with many previous studies on human diseases with neuroinflammatory mechanisms involved which reported alterations in neopterin [10-12], tryptophan metabolism [13-15] and amino acids [16, 17].

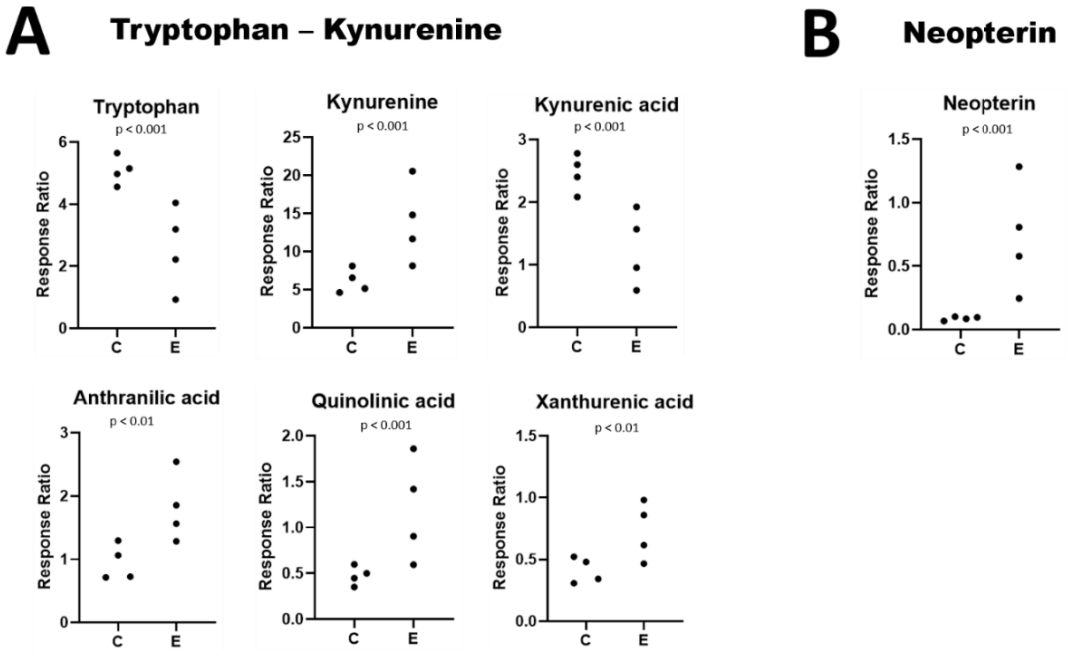

Figure 3-3 Statistical differences were identified between encephalitis patients (E, n = 4) compared to controls (C, n = 4) in CSF metabolites using ANOVA and Fishers LSD

post-hoc analysis [A] Six metabolites were identified from the tryptophan-kynurenine pathway [B] increased neopterin in encephalitis patients.

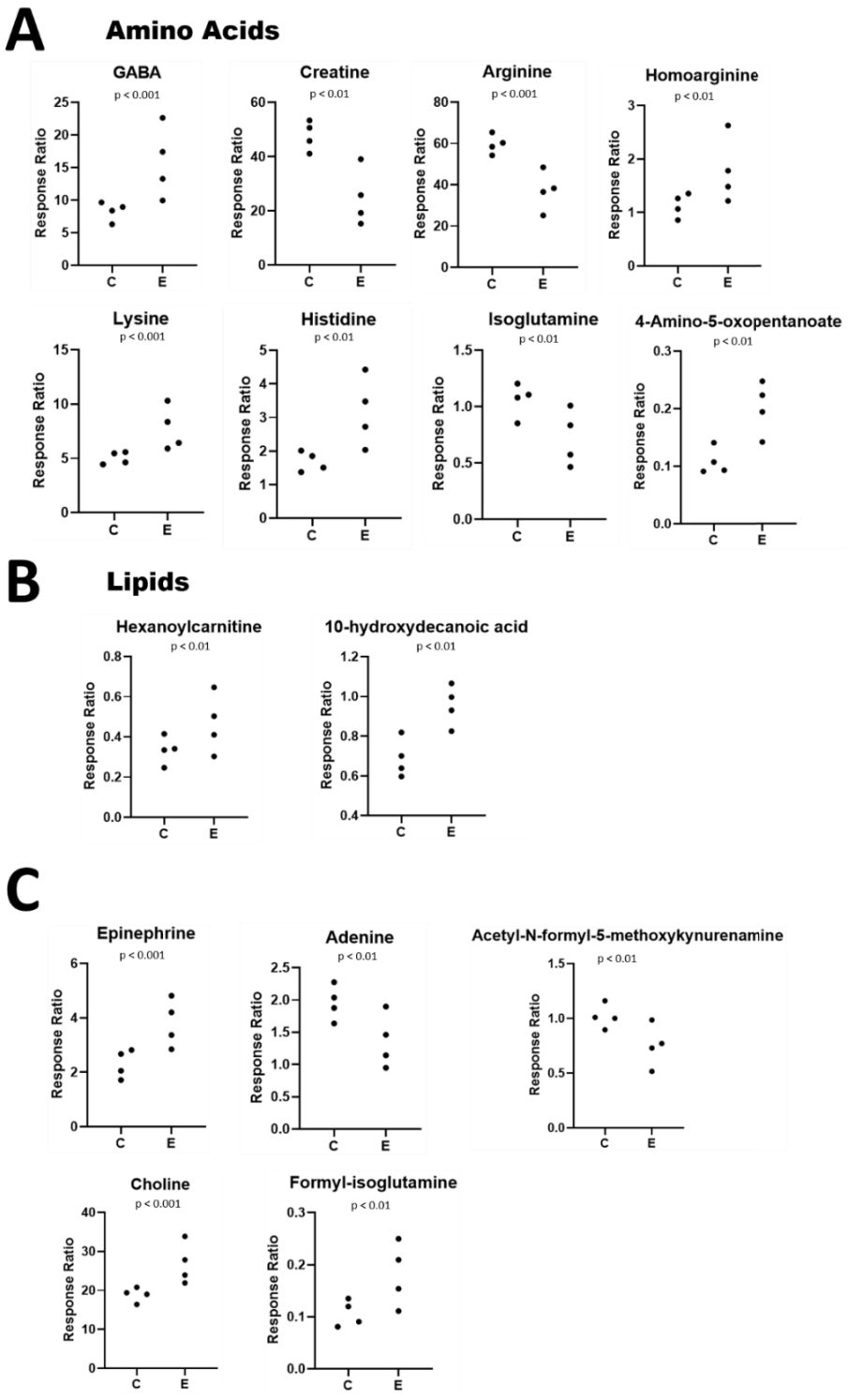

Figure 3-4 Statistical differences were identified between encephalitis patients (E, n = 4) compared to controls (C, n = 4) in CSF metabolites using ANOVA and Fishers LSD

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.

Among the twenty-two CSF metabolites, six metabolites were involved in the tryptophan-kynurenine pathway. The levels of quinolinic acid, kynurenine, anthranilic acid and xanthurenic acid were increased in CSF encephalitis patients, and levels of tryptophan and kynurenic acid were decreased. The kynurenine pathway is a major route for the metabolism of tryptophan and responsible for the regulation of immune activation [18, 19]. The initiation of the route for the conversion of tryptophan into kynurenine is metabolised by indoleamine 2,3-dioxygenase [20] forming its major metabolites illustrated in Figure 3-5.

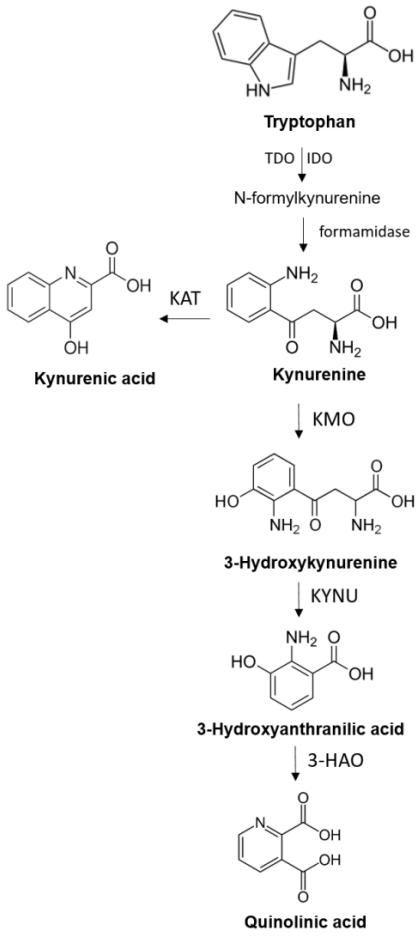

Figure 3-5 Schematic representation of the tryptophan-kynurenine pathway

The disruption of the tryptophan-kynurenine metabolism is strongly linked with neuroinflammation and immune activation. Under basal conditions, indoleamine 2,3-dioxygenase plays a minor role in the metabolism of tryptophan [21]. However, the enzyme is activated in response to cytokines, interferons and inflammatory signals released during inflammation [22]. The activation of indoleamine 2,3-dioxygenase stimulates the accelerated breakdown of tryptophan. This reduces the availability of tryptophan and imbalance formation of neuroprotective (e.g. kynurenic acid) and neurotoxic metabolites (e.g. 3-hydroxykynurenine, quinolinic acid).

A number of studies have reported the imbalances in the kynurenine metabolic pathway leads to alterations of two major metabolites, kynurenic acid and quinolinic acid. Kynurenic acid is a competitive N-methyl-D-aspartate (NMDA) antagonist and inhibits the functions of the α7 nicotinic acetylcholine receptor [23]. A majority of kynurenine is metabolised to kynurenic acid by astrocytes under basal conditions, however, following inflammation, there is increasing evidence that the metabolism of kynurenine favours the kynurenine 3-monooxygenase route producing quinolinic acid [24]. Quinolinic acid is a neurotoxin and NMDA agonist in the central nervous system with immune-regulatory and pro-inflammatory properties. A number of studies have reported the production of quinolinic acid to be generally elevated following inflammation [25-27]. During neuroinflammatory conditions the major sources contributing to the production of quinolinic acid include infiltrating macrophages and microglia [28]. The elevation of quinolinic acid will disruptively change the neuronal activity in the brain, as well as increasing the potential of glutamate excitotoxicity and pro-inflammation [29, 30].

Neopterin is metabolised from the guanosine triphosphate pathway and is a direct product generated by the stimulation of γ-interferon. Neopterin is a valuable biomarker of inflammation and infection in the CNS [10, 31]. Our findings show the elevation of neopterin in encephalitis patients are in line with published literature, and reported in a number of human diseases with neuroinflammatory mechanisms [10, 12, 32, 33]. However, CSF neopterin is a non-specific marker, unable to distinguish between different types of inflammatory diseases, and hence its

diagnostic value is interpreted within a given clinical context [34]. In addition, some studies simultaneously measure neopterin and tryptophan-kynurenine pathway metabolites [13, 35]. γ-interferon has been previously reported to influence the kynurenine pathway where an increase in the production of γ-interferon can result in the upregulation of the kynurenine pathway [13, 28, 36]. Increased CSF neopterin levels have showed a strong association with the changes in metabolites of the kynurenine pathway [13, 35, 37].

Amino acids and lipids are another category of metabolites associated with inflammatory processes [16, 38-40]. In this pilot study a total of eight amino acids were found to be statistically altered in encephalitis samples. An increase in lipid compounds, hexanoylcarnitine and 10-hydroxydecanoic acid was observed following inflammation. However, there is limited information available for the comparison of our findings to related studies. Research targeting amino acids and lipids in acute brain inflammation are scarce, less consistent and exhibit analytical challenges for the coverage of such a large variation of metabolic networks involved.

3.4.4 Statistically Significant Unknown Metabolites

LC-HRMS is an advantageous and comprehensive analytical tool with regards to the coverage of the CSF metabolome and provides important structural elucidation information for metabolites such as retention time, fragmentation patterns and accurate mass [41]. However, a large number of molecular features in metabolomics data sets remain unannotated [42, 43].

This pilot study identified ten statistically significant unknown metabolites represented by the box plots in Figure 3-6. Six metabolites were elevated and four metabolites decreased in encephalitis patients compared to controls. A comparison and search of the fragmentation data to existing mass spectra libraries and databases (such as METLIN, Human Metabolome database, mzCloud and ChemSpider) were unable to identify these metabolites.

The isolation of metabolites for NMR analysis is a possible solution for structural elucidation, however, this is a time-consuming process and may need larger volumes of CSF available. Alternatively, multi-stage tandem mass spectrometry in combination with in-silico fragmentation tools [44] can be useful for metabolite identification.

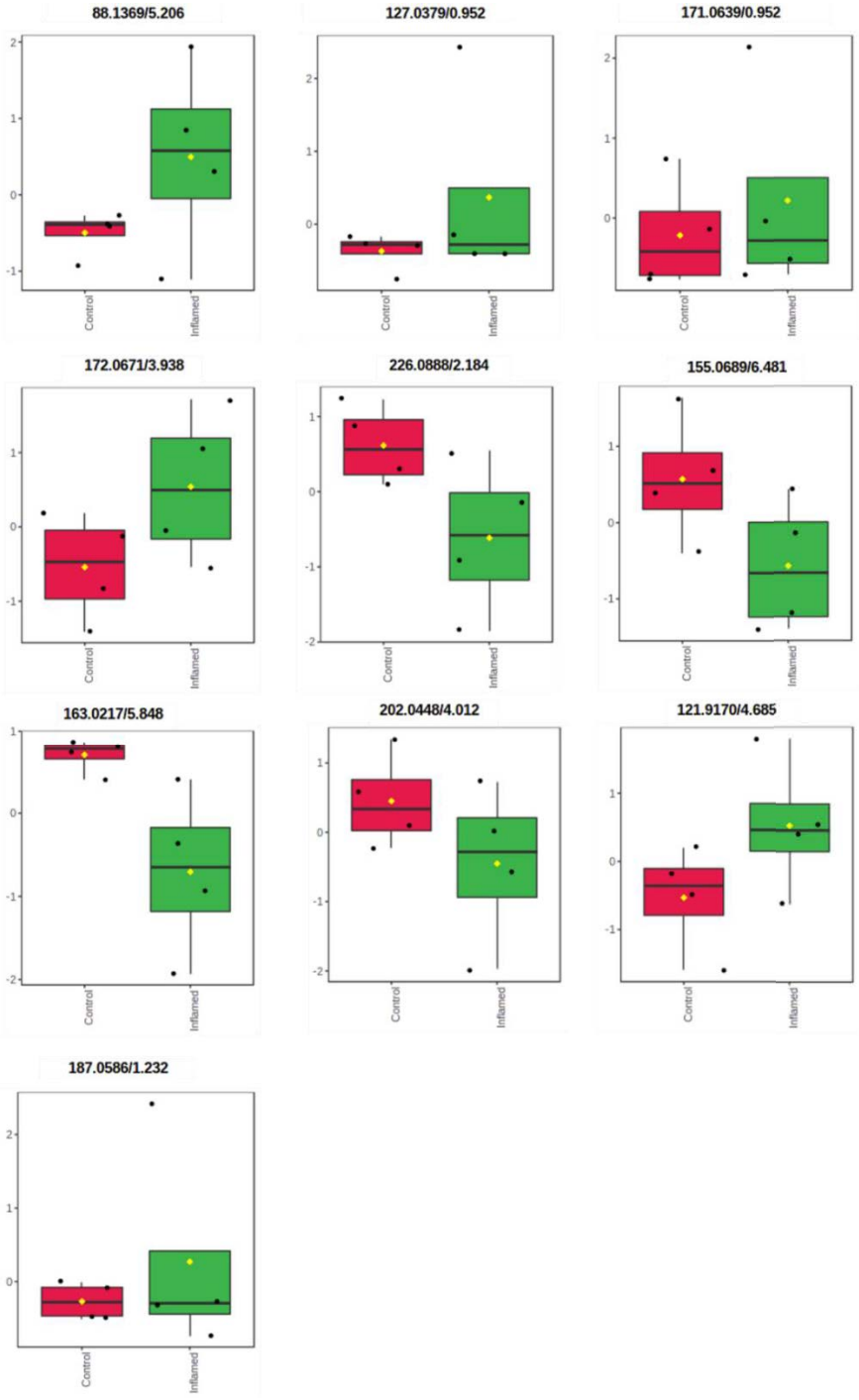

Figure 3-6 Box plots (molecular weight/retention time in minutes) of metabolites with statistical difference using ANOVA and Fishers LSD post-hoc analysis ($p < 0.005$) between encephalitis patients and controls.

3.5 Conclusion

The pilot study presents preliminary evidence that the developed untargeted analysis method is a robust strategy with great potential in facilitating the understanding of the CSF metabolome. The HILIC method was found to be more suitable for the analysis of CSF metabolites, obtaining excellent peak performance and a greater number of molecular features identified. The application of untargeted metabolomics on a small-scale CSF cohort showed discrimination between acute neuroinflammation patients from controls. The correlations observed require more mechanistic studies and a larger CSF sample cohort in order for a panel of biomarkers to be used in a clinical routine setting.

References

1. Sochocka M, Diniz BS, Leszek J: **Inflammatory Response in the CNS: Friend or Foe?** *Molecular neurobiology* 2017, **54:**8071-8089.

2. Ellul M, Solomon T: **Acute encephalitis - diagnosis and management.** *Clinical medicine (London, England)* 2018, **18:**155-159.

3. Nagana Gowda GA, Raftery D: **Biomarker Discovery and Translation in Metabolomics.** *Current Metabolomics* 2013, **1:**227-240.

4. Wishart DS, Lewis MJ, Morrissey JA, Flegel MD, Jeroncic K, Xiong Y, Cheng D, Eisner R, Gautam B, Tzur D, et al: **The human cerebrospinal fluid metabolome.** *J Chromatogr B Analyt Technol Biomed Life Sci* 2008, **871:**164-173.

5. Granerod J, Cunningham R, Zuckerman M, Mutton K, Davies NW, Walsh AL, Ward KN, Hilton DA, Ambrose HE, Clewley JP, et al: **Causality in acute encephalitis: defining aetiologies.** *Epidemiol Infect* 2010, **138:**783-800.

6. Sumner LW, Amberg A, Barrett D, Beale MH, Beger R, Daykin CA, Fan TWM, Fiehn O, Goodacre R, Griffin JL, et al: **Proposed minimum reporting standards for chemical analysis Chemical Analysis Working Group (CAWG) Metabolomics Standards Initiative (MSI).** *Metabolomics : Official journal of the Metabolomic Society* 2007, **3:**211-221.

7. Suhs KW, Novoselova N, Kuhn M, Seegers L, Kaever V, Muller-Vahl K, Trebst C, Skripuletz T, Stangel M, Pessler F: **Kynurenine Is a Cerebrospinal Fluid Biomarker for Bacterial and Viral Central Nervous System Infections.** *J Infect Dis* 2019, **220:**127-138.

8. French CD, Willoughby RE, Pan A, Wong SJ, Foley JF, Wheat LJ, Fernandez J, Encarnacion R, Ondrush JM, Fatteh N, et al: **NMR metabolomics of cerebrospinal fluid differentiates inflammatory diseases of the central nervous system.** *PLoS Negl Trop Dis* 2018, **12:**e0007045.

9. Blasco H, Corcia P, Pradat PF, Bocca C, Gordon PH, Veyrat-Durebex C, Mavel S, Nadal-Desbarats L, Moreau C, Devos D, et al: **Metabolomics in cerebrospinal fluid of patients with amyotrophic lateral sclerosis: an untargeted approach via high-resolution mass spectrometry.** *J Proteome Res* 2013, **12:**3746-3754.

10. Dale RC, Brilot F, Fagan E, Earl J: **Cerebrospinal fluid neopterin in paediatric neurology: a marker of active central nervous system inflammation.** *Dev Med Child Neurol* 2009, **51:**317-323.

11. Viaccoz A, Ducray F, Tholance Y, Barcelos GK, Thomas-Maisonneuve L, Ghesquières H, Meyronet D, Quadrio I, Cartalat-Carel S, Louis-Tisserand G, et al: **CSF neopterin level as a diagnostic marker in primary central nervous system lymphoma.** *Neuro Oncol* 2015, **17:**1497-1503.

12. Fominykh V, Brylev L, Gaskin V, Luzin R, Yakovlev A, Komoltsev I, Belousova I, Rosliakova A, Guekht A, Gulyaeva N: **Neuronal damage and neuroinflammation markers in patients with autoimmune encephalitis and multiple sclerosis.** *Metab Brain Dis* 2019, **34:**1473-1485.

13. Quist-Paulsen E, Aukrust P, Kran A-MB, Dunlop O, Ormaasen V, Stiksrud B, Midttun Ø, Ueland T, Ueland PM, Mollnes TE, Dyrhol-Riise AM: **High neopterin and IP-10 levels in cerebrospinal fluid are associated with neurotoxic tryptophan metabolites in acute central nervous system infections.** *Journal of Neuroinflammation* 2018, **15:**327.

14. Coutinho LG, Christen S, Bellac CL, Fontes FL, de Souza FRS, Grandgirard D, Leib SL, Agnez-Lima LF: **The kynurenine pathway is involved in bacterial meningitis.** *Journal of Neuroinflammation* 2014, **11:**169.

15. Jacobs KR, Lim CK, Blennow K, Zetterberg H, Chatterjee P, Martins RN, Brew BJ, Guillemin GJ, Lovejoy DB: **Correlation between plasma and CSF concentrations of kynurenine pathway metabolites in Alzheimer's disease and relationship to amyloid-β and tau.** *Neurobiol Aging* 2019, **80:**11-20.

16. Li Z, Du B, Li J, Zhang J, Zheng X, Jia H, Xing A, Sun Q, Liu F, Zhang Z: **Cerebrospinal fluid metabolomic profiling in tuberculous and viral meningitis: Screening potential markers for differential diagnosis.** *Clin Chim Acta* 2017, **466:**38-45.

17. Socha E, Koba M, Kośliński P: **Amino acid profiling as a method of discovering biomarkers for diagnosis of neurodegenerative diseases.** *Amino Acids* 2019, **51:**367-371.

18. Han Q, Cai T, Tagle DA, Li J: **Structure, expression, and function of kynurenine aminotransferases in human and rodent brains.** *Cellular and molecular life sciences : CMLS* 2010, **67:**353-368.

19. Campbell BM, Charych E, Lee AW, Möller T: **Kynurenines in CNS disease: regulation by inflammatory cytokines.** *Frontiers in neuroscience* 2014, **8:**12-12.

20. Takikawa O: **Biochemical and medical aspects of the indoleamine 2,3-dioxygenase-initiated L-tryptophan metabolism.** *Biochem Biophys Res Commun* 2005, **338:**12-19.

21. Yeung AW, Terentis AC, King NJ, Thomas SR: **Role of indoleamine 2,3-dioxygenase in health and disease.** *Clin Sci (Lond)* 2015, **129:**601-672.

22. Sorgdrager FJH, Naudé PJW, Kema IP, Nollen EA, Deyn PPD: **Tryptophan Metabolism in Inflammaging: From Biomarker to Therapeutic Target.** *Frontiers in Immunology* 2019, **10**.

23. Albuquerque EX, Schwarcz R: **Kynurenic acid as an antagonist of α7 nicotinic acetylcholine receptors in the brain: facts and challenges.** *Biochemical pharmacology* 2013, **85:**1027-1032.

24. Parrott JM, Redus L, Santana-Coelho D, Morales J, Gao X, O'Connor JC: **Neurotoxic kynurenine metabolism is increased in the dorsal hippocampus and drives distinct depressive behaviors during inflammation.** *Translational psychiatry* 2016, **6:**e918-e918.

25. Inoue H, Matsushige T, Ichiyama T, Okuno A, Takikawa O, Tomonaga S, Anlar B, Yüksel D, Otsuka Y, Kohno F, et al: **Elevated quinolinic acid levels in cerebrospinal fluid in subacute sclerosing panencephalitis.** *Journal of Neuroimmunology* 2020, **339:**577088.

26. Guillemin GJ, Smythe G, Takikawa O, Brew BJ: **Expression of indoleamine 2,3-dioxygenase and production of quinolinic acid by human microglia, astrocytes, and neurons.** *Glia* 2005, **49:**15-23.

27. Stone TW, Darlington LG: **The kynurenine pathway as a therapeutic target in cognitive and neurodegenerative disorders.** *British journal of pharmacology* 2013, **169:**1211-1227.

28. Chen Y, Guillemin GJ: **Kynurenine pathway metabolites in humans: disease and healthy States.** *International journal of tryptophan research : IJTR* 2009, **2:**1-19.

29. Lugo-Huitron R, Ugalde Muniz P, Pineda B, Pedraza-Chaverri J, Rios C, Perez-de la Cruz V: **Quinolinic acid: an endogenous neurotoxin with multiple targets.** *Oxid Med Cell Longev* 2013, **2013:**104024.

30. Stone TW: **Neuropharmacology of quinolinic and kynurenic acids.** *Pharmacol Rev* 1993, **45:**309-379.

31. Hoffmann G, Wirleitner B, Fuchs D: **Potential role of immune system activation-associated production of neopterin derivatives in humans.** *Inflamm Res* 2003, **52:**313-321.

32. Edén A, Marcotte TD, Heaton RK, Nilsson S, Zetterberg H, Fuchs D, Franklin D, Price RW, Grant I, Letendre SL, Gisslén M: **Increased Intrathecal Immune Activation in Virally Suppressed HIV-1 Infected Patients with Neurocognitive Impairment.** *PLoS One* 2016, **11:**e0157160.

33. Geng M, Xiao H, Liu J, Song Y, Fu P, Cheng X, Zhang J, Wang G: **The diagnostic role and dynamic changes in cerebrospinal fluid neopterin during treatment of patients with primary central nervous system lymphoma.** *Cancer Med* 2018, **7:**3889-3898.

34. Viaccoz A, Ducray F, Tholance Y, Barcelos GK, Thomas-Maisonneuve L, Ghesquières H, Meyronet D, Quadrio I, Cartalat-Carel S, Louis-Tisserand G, et al: **CSF neopterin level as a diagnostic marker in primary central nervous system lymphoma.** *Neuro-Oncology* 2015, **17:**1497-1503.

35. Widner B, Leblhuber F, Fuchs D: **Increased neopterin production and tryptophan degradation in advanced Parkinson's disease.** *Journal of Neural Transmission* 2002, **109:**181-189.

36. Yamada A, Akimoto H, Kagawa S, Guillemin GJ, Takikawa O: **Proinflammatory cytokine interferon-gamma increases induction of indoleamine 2,3-dioxygenase in monocytic cells primed with amyloid beta peptide 1-42: implications for the pathogenesis of Alzheimer's disease.** *J Neurochem* 2009, **110:**791-800.

37. Schroecksnadel K, Winkler C, Fuith LC, Fuchs D: **Tryptophan degradation in patients with gynecological cancer correlates with immune activation.** *Cancer Lett* 2005, **223:**323-329.

38. Bhandage AK, Jin Z, Korol SV, Shen Q, Pei Y, Deng Q, Espes D, Carlsson P-O, Kamali-Moghaddam M, Birnir B: **GABA Regulates Release of Inflammatory**

Cytokines From Peripheral Blood Mononuclear Cells and CD4(+) T Cells and Is Immunosuppressive in Type 1 Diabetes. *EBioMedicine* 2018, **30:**283-294.

39. Haghighatdoost F, Jabbari M, Hariri M: **The effect of L-carnitine on inflammatory mediators: a systematic review and meta-analysis of randomized clinical trials.** *European Journal of Clinical Pharmacology* 2019, **75:**1037-1046.

40. Shamim A, Mahmood T, Ahsan F, Kumar A, Bagga P: **Lipids: An insight into the neurodegenerative disorders.** *Clinical Nutrition Experimental* 2018, **20:**1-19.

41. Courant F, Royer AL, Chereau S, Morvan ML, Monteau F, Antignac JP, Le Bizec B: **Implementation of a semi-automated strategy for the annotation of metabolomic fingerprints generated by liquid chromatography-high resolution mass spectrometry from biological samples.** *Analyst* 2012, **137:**4958-4967.

42. da Silva RR, Dorrestein PC, Quinn RA: **Illuminating the dark matter in metabolomics.** *Proceedings of the National Academy of Sciences of the United States of America* 2015, **112:**12549-12550.

43. Blaženović I, Kind T, Ji J, Fiehn O: **Software Tools and Approaches for Compound Identification of LC-MS/MS Data in Metabolomics.** *Metabolites* 2018, **8**.

44. Ivanisevic J, Want EJ: **From Samples to Insights into Metabolism: Uncovering Biologically Relevant Information in LC-HRMS Metabolomics Data.** *Metabolites* 2019, **9:**308.

Chapter 4: Application to a Clinical Study

Chapter 4: Application to a Clinical Study

Summary

This chapter investigates the potential biomarkers to define neuroinflammation and explore mechanisms involved in the regulation of central nervous system immune responses. The application of the untargeted metabolomics approach to a clinical cerebrospinal fluid study consisting of encephalitis samples and controls is further evaluated.

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Cerebrospinal fluid metabolites in tryptophan-kynurenine and nitric oxide pathways are biomarkers of acute neuroinflammation

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4.1 Abstract

Objective To explore the cerebrospinal fluid (CSF) metabolite features in acute neuroinflammatory diseases and identify potential biomarkers to diagnose and monitor neuroinflammation.

Methods A cohort of 14 acute encephalitis patients (acute disseminated encephalomyelitis n=6, unknown suspected viral encephalitis n=3, enteroviral encephalitis n=2, seronegative autoimmune encephalitis n=2, herpes simplex encephalitis n=1; mean 7.73 years, median 9, 5 females) and age-matched non-inflammatory neurological disease controls (n=14) were investigated using an untargeted metabolomics approach. CSF metabolites were analysed with liquid chromatography coupled to high resolution mass spectrometry (LC-HRMS) followed by subsequent multivariate and univariate statistical methods.

Results A total of thirty-five metabolites were statistically discriminative between the groups using supervised orthogonal partial least squares discriminant analysis (OPLS-DA) and ANOVA. The tryptophan-kynurenine pathway contributed nine key metabolites. There was statistical increase of kynurenine, quinolinic acid and anthranilic acid in encephalitis patients, whereas tryptophan, 3-hydroxyanthrnailic acid and kynurenic acid were decreased. The nitric oxide pathway contributed four metabolites, with elevated asymmetric dimethylarginine (ADMA) and argininosuccinic acid and decreased arginine and citrulline in encephalitis patients. An increase in the CSF kynurenine/tryptophan ratio ($p < 0.001$), anthranilic acid/3-hydroxyanthranilic acid ratio ($p < 0.001$), ADMA/arginine ratio ($p < 0.001$), and neopterin ($p < 0.001$) strongly predicted neuroinflammation.

Interpretation The combination of alterations in tryptophan-kynurenine pathway, nitric oxide pathway and neopterin represent a useful potential panel for neuroinflammation and holds potential for clinical translation practice.

Keywords: cerebrospinal fluid, untargeted metabolomics, encephalitis, high resolution mass spectrometry

4.2 Introduction

Acute neuroinflammatory diseases such as encephalitis have significant mortality and morbidity rates worldwide [1]. Encephalitis is estimated to affect 500,000 people per year, with over 100 infectious or autoimmune causes. The inflammatory response of the central nervous system (CNS) to invading micro-organisms and cells plays a crucial role in the neuronal damage and progression of encephalitis. The detrimental impacts of encephalitis has directed great attention to understand the pathophysiologic mechanisms underlying the diseases and biomarker discovery research [2]. In some patients, clinical features, neuroimaging and routine CSF testing can define neuroinflammation, however, in some patients routine testing is negative. There is also emerging evidence of inflammation of the brain in neurodevelopmental disorders (such as autistic spectrum disorder), psychiatric disorders (including depression and schizophrenia) and neurodegeneration (such as Alzheimer's disease). Therefore translatable biomarkers of neuroinflammation are urgently required for the detection of inflammation in individual patients.

Metabolomics is a rapidly emerging approach increasingly used as a characteristic 'fingerprint' in CNS diseases. This powerful tool explores the changes of endogenous metabolites in biofluids and subsequent chemometrics data management to offer knowledge on underlying metabolic mechanisms [3, 4]. CSF is the most viable matrix for examining metabolic disturbances in the brain [5, 6]. The development and advancements in high throughput analytical instrumentations [7-10] has driven LC-HRMS to the forefront of accurately curating large amounts of data.

This study aimed to investigate the potential of CSF metabolic biomarkers covering a wide spectrum of endogenous pathways using an untargeted metabolomics approach to compare encephalitis patients and controls.

4.3 Participants, Materials and Method

4.3.1 Study Design and Participants

Twenty-eight human CSF were obtained from the Department of Biochemistry at the Children's Hospital at Westmead (Sydney, Australia). The encephalitis group (n=14, mean 7.73 years, median 9, 5 females) all fulfilled encephalitis criteria [11] (acute disseminated encephalomyelitis n=6, unknown suspected viral encephalitis n=3, enteroviral encephalitis n=2, seronegative autoimmune encephalitis n=2, herpes simplex encephalitis n=1). CSF was frozen within 1 hour of sampling and stored at -40 ^{o}C until the time of processing. 9 of the 14 patients had a CSF pleocytosis using >5cells/mm3 as a cut-off, and all patients tested had negative intrathecal oligoclonal bands (n=6 tested). Only 4 of 14 patients had a CSF protein above the upper reference range for children (0.4g/dl).

All CSF samples were acute, taken within 10 days (median 2, mean 3.3 days) of neurological symptom onset, and all samples were before starting immune modulatory therapy (corticosteroids or intravenous immunoglobulin), but not before treatment with anti-microbials (n=7) or anti-epileptics (n=10). Brain magnetic resonance imaging, performed at the same time as the CSF was abnormal in 10 of the 14 patients. Four of the patients had a short-lived admission to intensive care (usually related to seizures) and none needed inotropic support.

The age-matched control group (n=14, mean 7.86 years, median 7 years, 5 females) had non-inflammatory neurological disease (genetic episodic ataxia n=2, Hereditary Motor Sensory Neurology n=2, Cerebral Palsy n=2, Functional Neurological Disorder n=2, Neurodegeneration n=2, GLUT1 deficiency n=2, rapid onset Parkinsonism-Dystonia ATP1A3 positive n=1, Genetic epilepsy/developmental delay n=1). Four patients were taking medication at the time of CSF (SSRI n=1, anti-epileptic n=3). The CSF:serum albumin ratio in encephalitis patients was mean 0.01907 (range 0.00881-0.034), compared to controls mean 0.00373 (range 0.0026-0.0058), suggestive of blood brain barrier (BBB) disruption in encephalitis patients.

The ethnicity for both encephalitis patients and controls consisted of Australian Caucasians (n=10), Australian Indian subcontinent (n=2) and Australian Arab (n=2). The Sydney Children's Hospitals Network Ethics committee approved this study, LNR/14/SCHN/275 (2019/ETH06182). Written informed consent from parents and/or guardians and assent from children were obtained.

4.3.2 Chemicals and Reagents

3-hydroxyanthranilic acid, 3-hydroxykynurenine, anthranilic acid, kynurenic acid, quinolinic acid, L-tryptophan, L-kynurenine, picolinic acid, xanthurenic acid, dopamine hydrochloride, epinephrine hydrochloride, L-glutamic acid, GABA and ammonium formate were purchased as powders from Sigma Aldrich (Sydney, Australia). Homovanillic acid, 3,4-Dihydroxyphenylacetic acid, neopterin, histamine and norepinephrine were purchased from Novachem (Victoria, Australia). D_3-Dopamine and D_3-Kynurenic acid were supplied by CDN Isotopes (Quebec, Canada). HPLC grade acetonitrile and methanol were purchased from Honeywell (Sydney, Australia).

4.3.3 Sample Preparation

Prior to experiments, CSF samples were thawed, vortexed and aliquot into 2 mL Eppendorf tubes. One hundred microliters of CSF samples were deproteinised by 300 µL of the methanol mixture in microcentrifuge tubes. The samples were vortexed for 90 seconds, sonicated for 5 minutes and precipitated in ice for 45 minutes. This was followed by centrifugations of the samples for 12 minutes at a temperature of 5 ^{o}C and velocity of 4000 g. The supernatant was collected and evaporated to dryness under nitrogen. Subsequently, 50 µL of water/acetonitrile at a ratio of 40/60 was used to reconstitute the CSF residue for UPLC-HRMS analyses. Quality control (QC) samples were prepared from a pooled mixture of equal volumes from all CSF

samples. A blinded approached was undertaken where sample descriptions of all twenty-eight CSF samples were not revealed to the scientists prior to data analyses.

4.3.4 Liquid Chromatography – High Resolution Mass Spectrometry

UPLC-HRMS analyses were performed using a Thermo Scientific Vanquish system coupled to a Q Exactive HF-X Hybrid Quadrupole Orbitrap Mass Spectrometer (Thermo Fisher Scientific Inc., Massachusetts, CA, USA) fitted with an electrospray source in both positive and negative ion modes.

The chromatographic separation of metabolites was achieved on an Agilent Infinity Lab HILIC column (2.1 x 100 mm, 2.7µm particle size) at a flow rate of 0.30 mL/min. The mobile phases consisted of 20 mM ammonium formate in water (aqueous) and 20 mM ammonium formate in 90% acetonitrile (organic). The gradient program was: 0−1.5 min (90% B), 1.5−12 min (90−50% B), 12−16 min (50% B), 16−17 min (50−25% B), 17−20 min (25% B), 20−21 min (45−90% B) and 21−32 min (90% B). The UPLC autosampler was set to 4 ^{o}C and a sample injection volume of 10 µL. The solvents used for sample preparation and UPLC-HRMS analyses were high purity LC-MS grade, purchased from Honeywell Burdick and Jackson (Chem Supply, Gillman, Australia).

The MS was acquired in the all ion fragmentation mode using positive electrospray ionisation under a high mass resolution of 120 000, automatic gain control target at 3e6 and maximum injection time of 200 ms. The detection scan range was achieved from m/z 50 to 600 in the positive ionisation mode. The MS was operated with capillary voltage at 4 kV in the positive ionisation mode and gas temperature of 300 ^{o}C. Calibration of the MS in the positive mode was conducted prior to analysis using a calibration mixture provided by the manufacturer.

The injection sequence of CSF samples was randomised to ensure the order of samples analysed was independent of clinical condition. QC samples were injected after every seven samples to assess the reproducibility of the method and instrument.

4.3.5 Data Processing and Statistical Methods

Initial raw data were inspected in the Xcalibur version 2.1 software using the Qualbrowser. Automated peak detection, integration, identification and determination of differences between raw data sample sets were conducted using the Compound Discoverer 3.0 software. A number of workflow processing filters were applied to curate the large quantities of data. This included setting up a minimum absolute abundance threshold of 1500 counts at an m/z range of 70 to 600. For the alignment of retention times the tolerated shift window was set to 0.2 min and a mass tolerance of 5 ppm. For compound detection, the mass fragmentation were limited to two ion species $[M+H]^+$ and $[M+NH_4]^+$ and a mass tolerance of 5 ppm. To reduce false positives and negative parameters, eliminate mass spectral features were set to consider features present in at least 75% of samples. The databases used for metabolite annotation and identification were the human metabolome database, m/z cloud, Chemspider and an in-house CSF database.

The pre-processed data sets were extracted from compound discoverer and were normalised against internal standards and input for multivariate data analysis into Metaboanalyst 4.0 [12]. Principal component analysis (PCA) and OPLS-DA were performed across the sample sets (controls, encephalitis, QC). Significant metabolites statistically driving the separation between the groups were obtained through ANOVA and Fishers LSD post-hoc analysis at a p-value cut off of 0.001 to support the obtained differences are unlikely to occur due to random sampling. Several statistically significant metabolites identities were able to be confirmed by commercial reference standards obtained from Sigma Aldrich Australia. The remaining metabolites were putatively annotated and validated by conducting literature mass spectra searches (Table 4-1).

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

Metabolites confirmed using a reference standard	**Metabolites putatively annotated from literature mass spectra**
3-Hydroxyanthranilic acid	Citrulline
3-Hydroxykynurenine	Arginine
Anthranilic acid	Asymmetric dimethylarginine
Kynurenic acid	Argininosuccinic acid
Quinolinic acid	Tyrosine
Tryptophan	Imidazoleacetic acid
Kynurenine	Alanine
Picolinic acid	Histidine
Xanthurenic acid	Isoleucine
Neopterin	Methionine
Dopamine	Carnitine
Epinephrine	anandamide
Norepinephrine	Acetylcarnitine
Homovanillic acid	10-hydroxydecanoicacid
3,4-Dihydroxyphenylacetic acid	Choline
Histamine	Adenine
GABA	Inosine
Glutamic acid	

4.4 Results

The metabolites in the CSF samples were eluted within a twenty minute chromatographic window (Figure 4-1) showing good chromatographic separation.

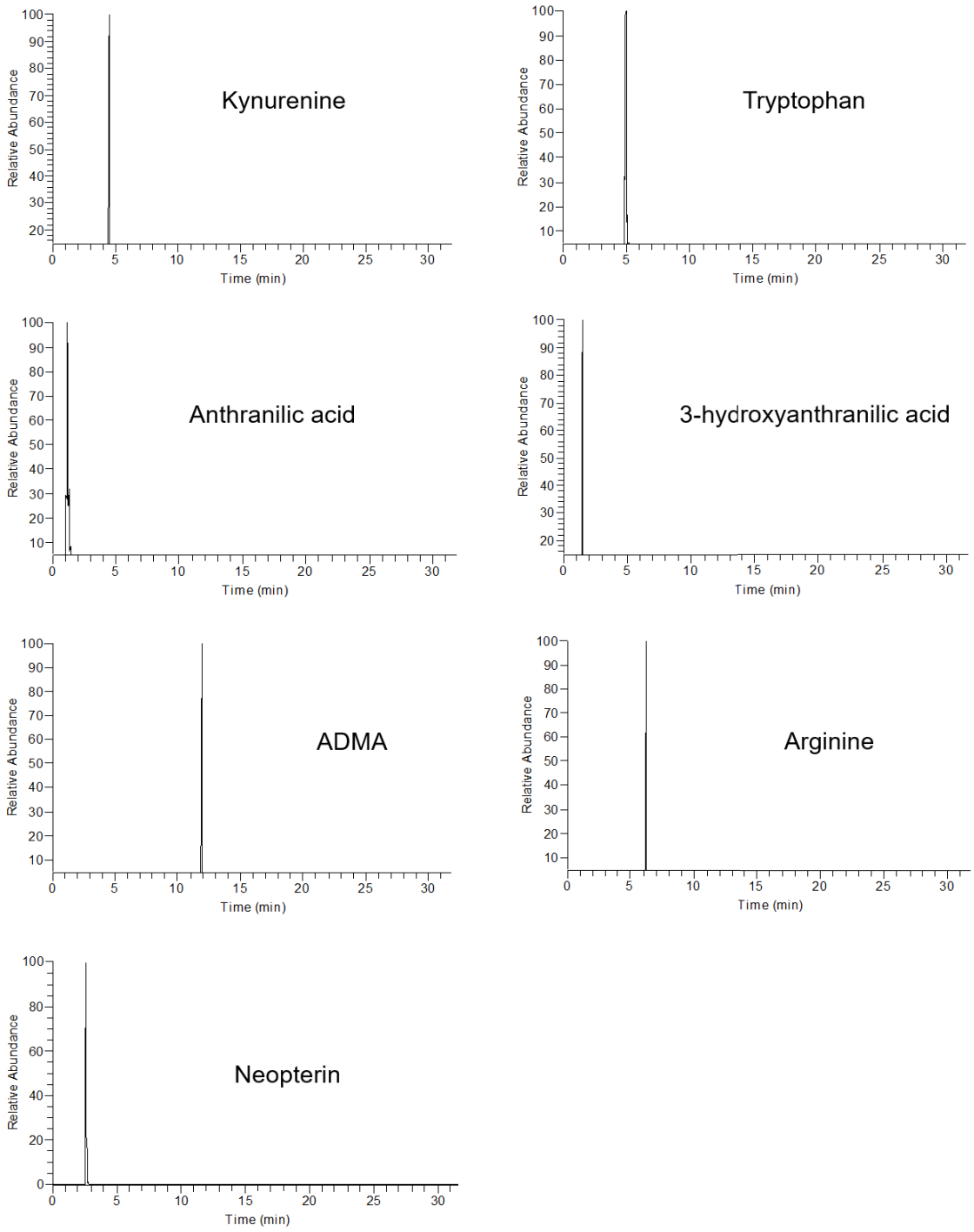

Figure 4-1 Representative extracted ion chromatograms of CSF metabolites in human cerebrospinal fluids.

Over a sequence run of twenty-eight CSF samples, a total of four QC injections were analysed. The PCA data presented a close cluster of the QC samples with the absence of outliers (Figure 4-2A). This indicated good reproducibility and robustness of the metabolomics analysis method.

The differences in CSF levels between encephalitis and controls were explored using OPLS-DA (Figure 4-2B). The OPLS-DA score plots showed distinct separation, reflecting the metabolic differences amongst the two groups. ANOVA and Fishers LSD post-hoc analysis at a p-value cut off of 0.001 revealed thirty-five metabolites were significantly involved in driving the discrimination between encephalitis patients and controls. Nine metabolites were present at lower levels in the encephalitis patients, and twenty-six metabolites showed increased levels in encephalitis patients, compared to controls.

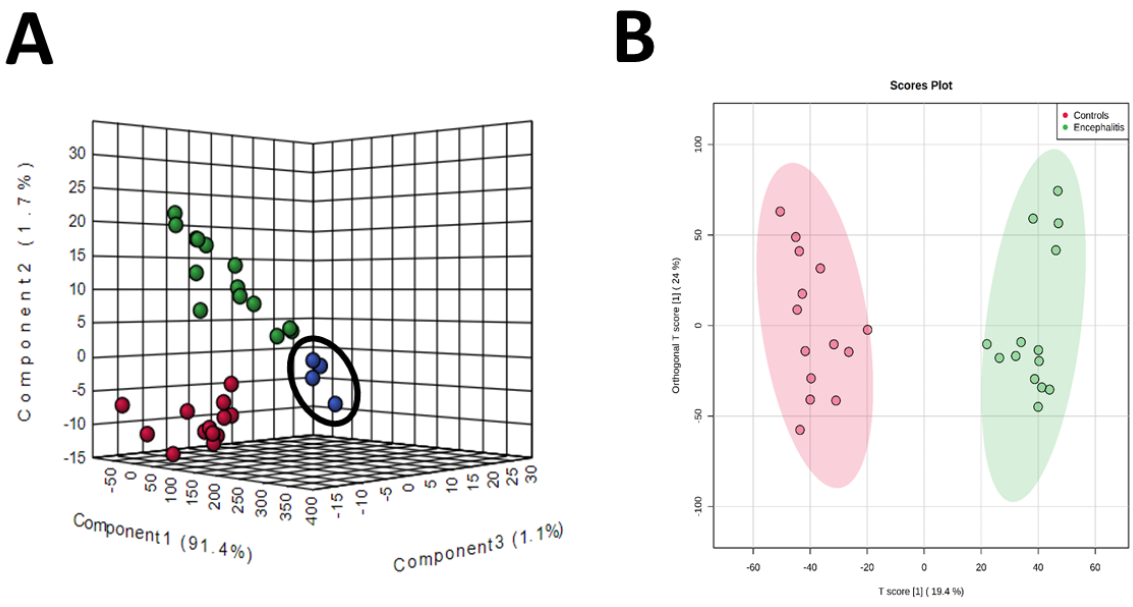

Figure 4-2 Statistical analysis of blinded CSF study using UPLC-HRMS. [A] PCA score plot on 14 control patients (red dots), 14 encephalitis patients (green dots) and 4 QC samples (blue dots) showing clustering of the QC samples. [B] OPLS-DA score plot presenting a clear separation of 14 control patients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

Among the thirty-five CSF metabolites, nine compounds were involved in the tryptophan-kynurenine pathway (Figure 4-3). The levels of kynurenine, 3-hydroxykynurenine, anthranilic acid, xanthurenic acid quinolinic acid were elevated in CSF of encephalitis patients. In encephalitis patients, there was a decrease in tryptophan, kynurenic acid, 3-hydroxyanthranilic acid and picolinic acid. The kynurenine/tryptophan ratio is used to infer the enzyme activity of indoleamine-2,3-dioxygenase (IDO) during inflammation [13-15]. Notably, in our analysis a marked decrease of tryptophan and increase of kynurenine levels and consequent elevated kynurenine/tryptophan ratio was observed in encephalitis samples compared to controls ($p < 0.001$) (Figure 4-4A). The anthranilic acid/3-hydroxyanthranilic acid ratio has also been proposed in neuroinflammation [16-18] and was increased ($p < 0.001$) (Figure 4-4B), inferring 3-hydroxyanthranilic acid oxidase enzymatic activation during neuroinflammation.

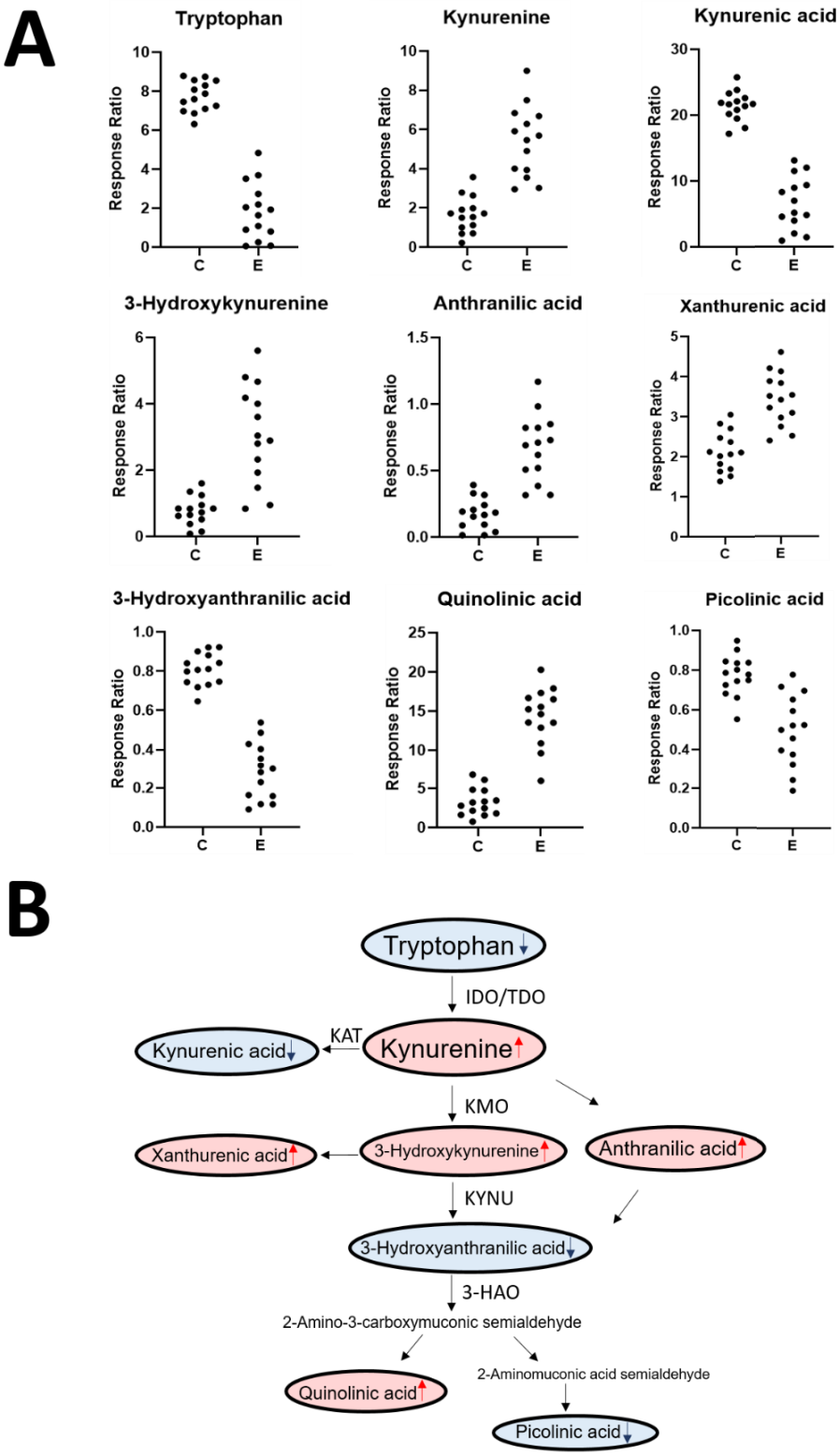

Figure 4-3 Tryptophan-kynurenine pathway metabolites [A] in encephalitis patients (E, n = 14) are compared to controls (C, n = 14). Significant differences were identified between the two groups in nine metabolites using ANOVA and Fishers LSD post-hoc analysis ($p < 0.001$). [B] Tryptophan-kynurenine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

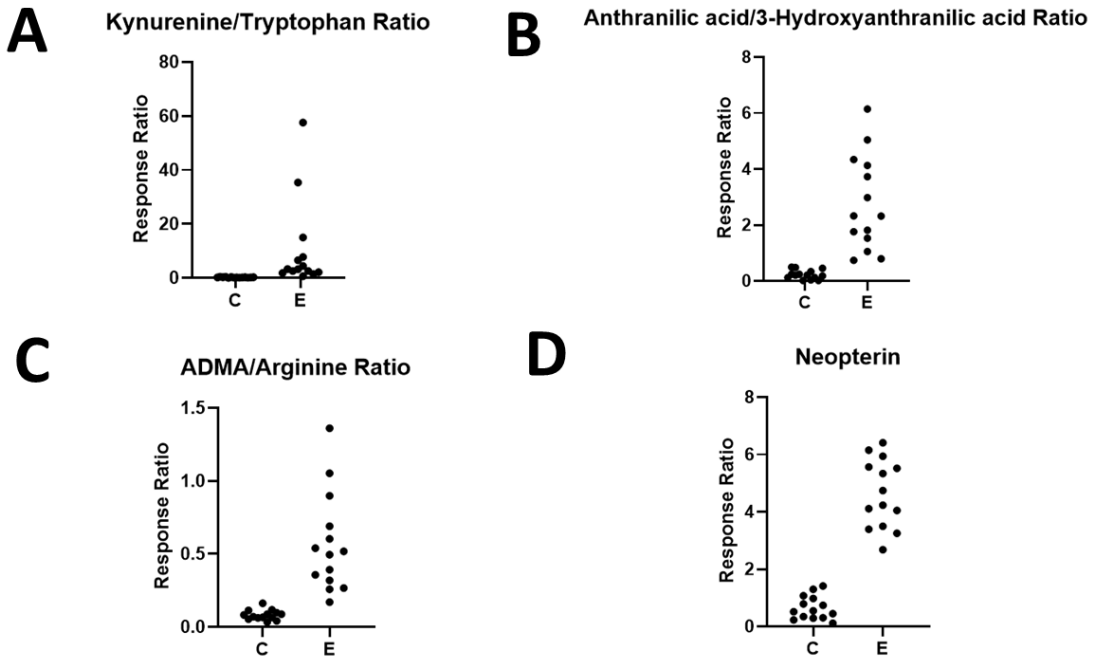

Figure 4-4 Statistical analysis of ratios and neopterin in encephalitis patients (E, n = 14) compared with controls (C, n = 14). [A] kynurenine/tryptophan ratio ($p < 0.001$). [B] anthranilic acid/3-hydroxyanthranilic acid ratio ($p < 0.001$). [C] ADMA/arginine ratio ($p < 0.001$). [D] Neopterin ($p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

Four metabolites were altered in the nitric oxide pathway in encephalitis patients compared to controls (Figure 4-5). The levels of arginine and citrulline were decreased, and asymmetric dimethylarginine (ADMA) and argininosuccinic acid were increased in encephalitis patients. ADMA serves as a competitive inhibitor to nitric oxide synthase which may limit arginine production. The ADMA/arginine ratio was elevated in the encephalitis group ($p < 0.001$) (Figure 4-4C).

Neopterin was also significantly elevated in encephalitis groups compared to controls ($p < 0.001$) (Figure 4-4D). A statistical power analysis was conducted on the clinical study (n=28) comparing encephalitis and controls (Table 4-2). The effect study showed a large difference using Cohen's criteria [19] for tryptophan-kynurenine metabolites, nitric oxide pathway metabolites, neopterin and adjacent ratios (kynurenine/tryptophan, anthranilic acid/3-hydroxyanthranilic acid and

ADMA/arginine). This supported the statistical significance obtained from the ANOVA results.

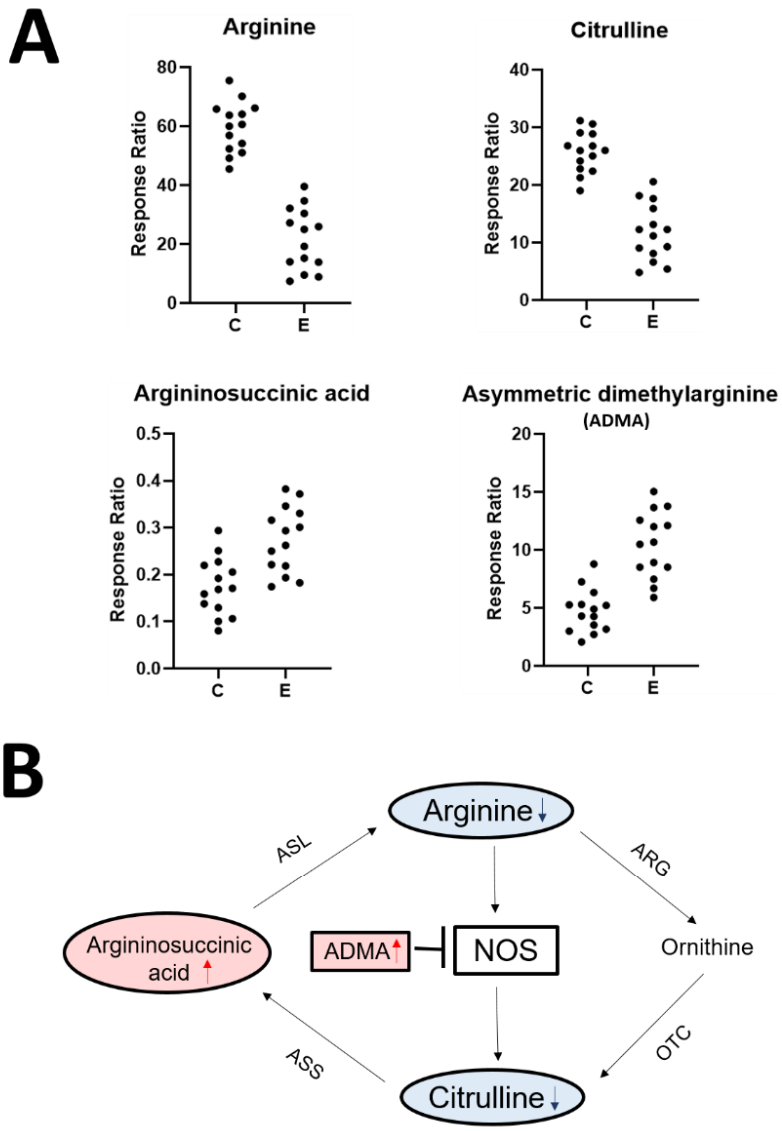

Figure 4-5 [A] Nitric Oxide pathway metabolites in encephalitis patients (E, n = 14) compared to controls (C, n = 14). Significant differences were identified between the two groups in four metabolites using ANOVA and Fishers LSD post-hoc analysis ($p < 0.001$). [B] Nitric oxide metabolic pathway showing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

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.

Metabolite	Effect Size
Tryptophan	1.90
Kynurenine	1.67
Kynurenic acid	1.89
3-Hydroxykynurenine	1.49
Anthranilic acid	1.77
Xanthurenic acid	1.26
3-Hydroxyanthranilic acid	1.89
Quinolinic acid	1.81
Picolinic acid	1.38
Arginine	1.83
Citrulline	1.60
ADMA	1.73
Argininosuccinic acid	1.68
NEO	1.59
kynurenine/tryptophan ratio	0.99
anthranilic acid/3-hydroxyanthranilic acid ratio	1.48
ADMA/arginine ratio	1.44

There was also a general increase in amino acid levels in the encephalitis group, in particular a significant difference between the two groups for major inhibitory neurotransmitter, γ-aminobutyric acid and major excitatory neurotransmitter, glutamic acid (Figure 4-6). Likewise, metabolites in the catecholamine metabolism, lipids, histamine and its metabolite imidazoleacetic acid were also increased in

encephalitis patients (Figure 4-7). Finally, adenosine and inosine were decreased in the encephalitis group and choline was elevated (Figure 4-6).

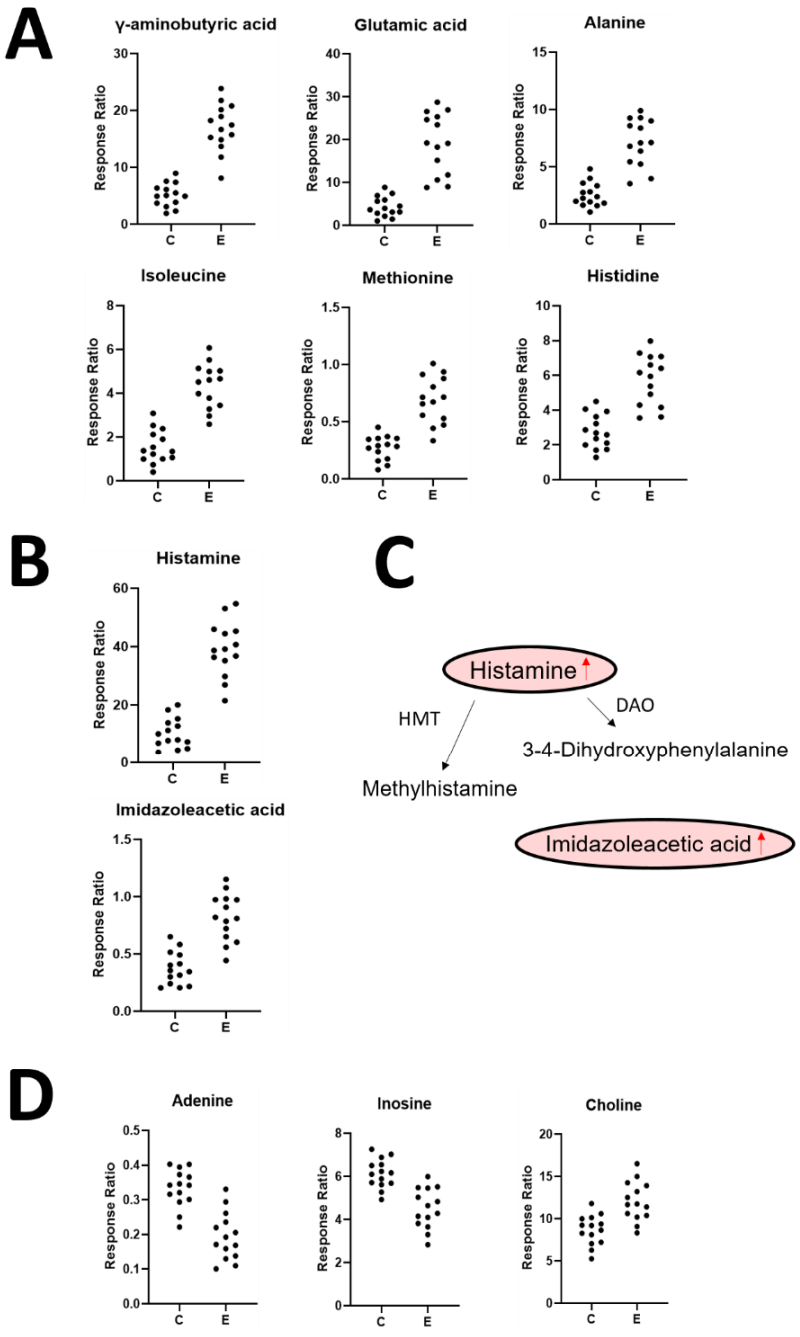

Figure 4-6 [A] Statistical differences in six amino acid metabolites ($p < 0.001$) were identified In encephalitis patients (E, n = 14) compared to controls (C, n = 14) [B] histamine and its metabolite, imidazoleacetic acid were increased in encephalitis patients ($p < 0.001$) [C] histamine metabolic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

metabolites (red) in encephalitis [D] decreased levels of adenine and inosine and increased choline in encephalitis patients ($p < 0.001$).

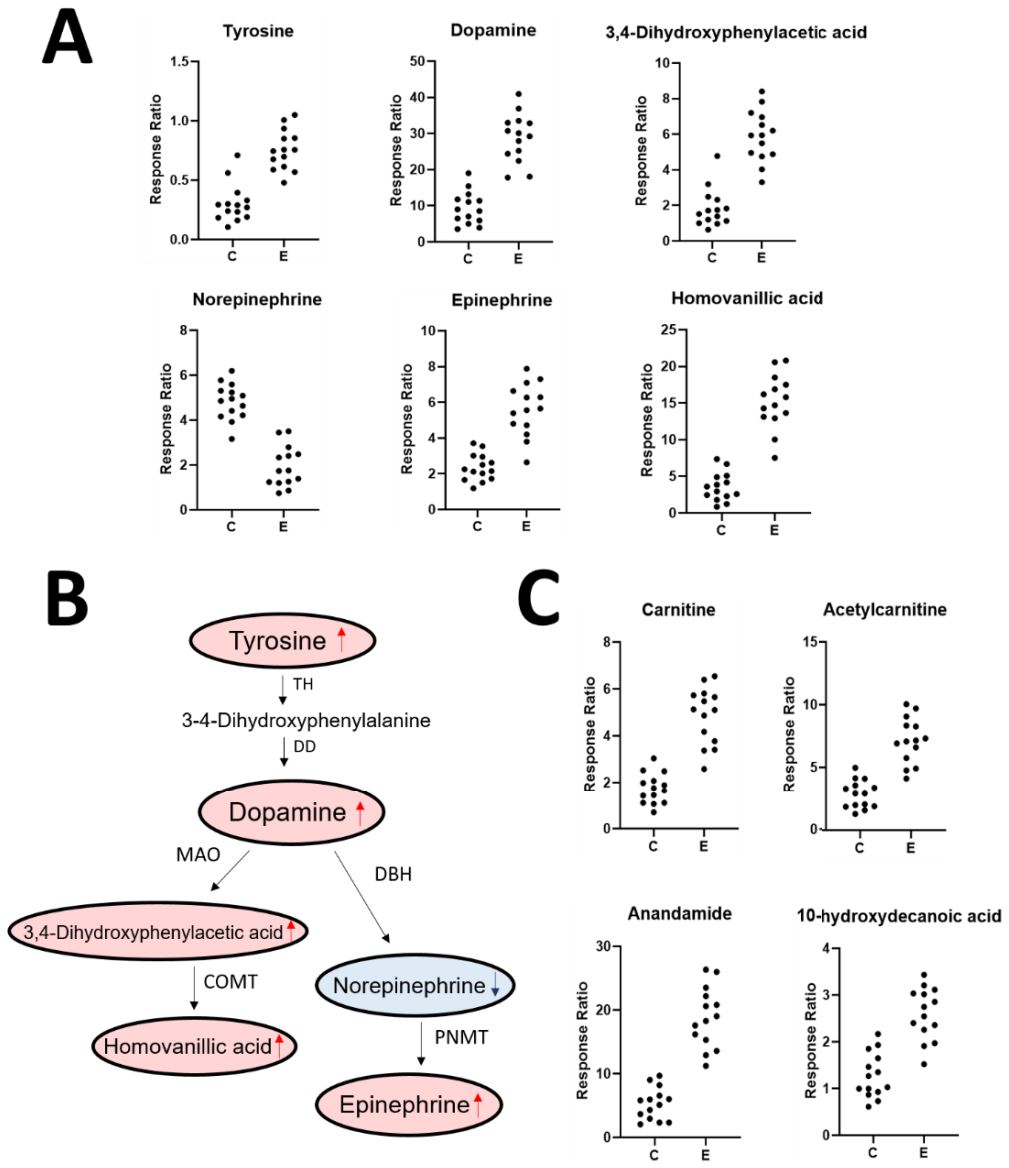

Figure 4-7 [A] Catecholamine pathway metabolites in encephalitis patients (E, n = 14) in comparison with controls (C, n = 14). Statistical differences in six metabolites were identified ($p < 0.001$). [B] Catecholamine metabolic pathway showing the increased metabolites (red) and decreased metabolites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

4.5 Discussion

In recent years, a few untargeted CSF metabolomics studies have been conducted on encephalitis cohorts employing NMR or LC-HRMS [3, 20, 21]. However, there is limited availability of definite diagnostic biomarkers to establish a standard protocol for routine work. This encephalitis study provides a comprehensive and blinded analysis of a broad spectrum of CNS neuroactive metabolites from patients with neuroinflammatory disorders using an untargeted metabolomics approach. Given the fact there is commonly blood brain barrier disruption in encephalitis, as evident in this cohort, we believe metabolic pathways that have both elevated and decreased metabolites are more likely to represent better neuroinflammatory biomarkers (such as metabolites in the tryptophan-kynurenine pathway and nitric oxide pathway), as opposed to metabolites that were generally elevated in encephalitis patients (such as other amino acids, catecholamines, and lipids). Utilising elevated and decreased adjacent metabolites (such as kynurenine/tryptophan ratio) has been previously proposed as a useful marker of neuroinflammation and is strongly supported by our study.

The tryptophan-kynurenine pathway has been predominantly evaluated in HIV [22-25] and neurodegenerative disorders [26-29]. In our study, an increased kynurenine/tryptophan ratio in encephalitis patients suggests activation of the indoleamine 2,3-dioxygenase (IDO) enzyme resulting in the catabolism of tryptophan forming imbalanced levels of neuroprotective and neurotoxic kynurenine pathway metabolites such as quinolinic acid and kynurenic acid [30-33]. IDO expression is generally low under basal conditions and upregulated following inflammation. The significance of the role IDO plays has led to several studies examining the clinical diagnostic utility of quantifying the enzyme as a biomarker for the implication of neuroinflammation [34-36]. However, it has been recently highlighted measuring IDO enzyme itself can definitely demonstrate activation of the enzyme, and measuring the kynurenine/tryptophan ratio alone can only infer activation of the enzyme [31].

There is growing evidence of the role 3-hydroxyanthranilic acid may impose on the cellular functions in the CNS. Moreover, clinical studies in a broad spectrum of human diseases with neuroinflammatory mechanisms have shown alterations in the levels of 3-hydroxyanthranilic acid and its association with changes in anthranilic acid [37-39]. The anthranilic acid/3-hydroxyanthranilic acid ratio is also a potentially useful biomarker of neuroinflammation, inferring 3-hydroxyanthranilic acid oxidase (3HAO) activation during inflammation. A marked increase in anthranilic acid and decrease in 3-hydroxyanthranilic acid in the encephalitis group are in line with findings reported by several studies. The reciprocal relationship of the two metabolites may suggest the existence of a consistent biochemical change following inflammation as opposed to random changes of metabolite levels. However, the exact biochemical mechanism resulting in the changes of anthranilic acid and 3-hydroxyanthranilic acid levels are unknown.

These findings are in agreement with previous targeted studies [17, 18, 20] reporting increased kynurenine/tryptophan ratio in CNS infections and decrease of 3-hydroxyanthranilic acid/anthranilic acid ratio in many neurological diseases, showing the clinical significance of the ratios as markers of neuroinflammatory diseases. Furthermore, the enzymes, IDO and 3HAO have been shown to be induced during inflammation [17, 18]. Although, it would be ideal to directly measure these enzymes in CSF, this is currently impractical in the CSF matrix and imposes an analytical constraint due to the extremely low or undetectable nature of these enzymes [13].

There have been limited studies reporting the association of nitric oxide pathway in encephalitis [21, 40, 41]. These studies are mainly performed in HSV-infected encephalitis or measure CSF nitric oxide concentrations alone in CNS infectious diseases. Nitric oxide plays a critical role in the regulation of neurotransmission, inflammatory cell growth and death, and defence mechanisms against intruding cells [42]. However, the short half-life and diffusion rate of nitric oxide remains a challenge for detection and quantification. For these reasons more stable metabolites in the nitric oxide pathway such as arginine, citrulline, ADMA and argininosuccinic acid hold more promise as biomarkers.

The higher ADMA/arginine ratio exhibited in encephalitis patients possesses potential as an inflammatory biomarker. In recent years, there is increasing attraction towards ADMA as a potential inflammatory marker in cardiovascular diseases [43] and rheumatoid arthritis [44]. ADMA is a competitive inhibitor of nitric oxide synthase and its balance with arginine act as an important indicator in the bioavailability of nitric oxide [45]. The increased levels of ADMA following inflammation has been inferred to be associated with the activation of protein arginine methyltransferases and consequently induces the inhibition of dimethylarginine dimethylaminohydrolase [46, 47].

Neopterin is a well-established clinical inflammatory marker and the increase of the metabolite in our encephalitis cohort is strongly supported by previous cohort studies [48]. Neopterin is a sensitive marker of inflammation but lacks specificity for any particular form of inflammation. Furthermore, with increasing recognition of brain inflammation in common neurological disorders such as autism, schizophrenia and dementia, there is urgent need for improved biomarkers to define and monitor neuroinflammation. We therefore propose that a CSF panel including tryptophan-kynurenine pathway metabolites, nitric oxide pathway metabolites and neopterin may provide strong discriminative power for neuroinflammatory conditions, particularly in encephalitis patients with negative neuroimaging and routine CSF findings.

A first limitation of the study is the cohorts and controls were of modest size (n=14), and the encephalitis syndromes were heterogeneous. Substantially larger cohorts are required to further examine the sensitivity and specificity of these biomarkers, and to test the translatability into clinical practise for individual patients.

A further limitation of the present study is the absence of blood samples to conduct simultaneous analysis with CSF, or urine samples to determine if less invasive tissues can aid in the diagnosis of neuroinflammation [49]. The BBB plays an important role in the maintenance of neurotransmitter levels in the brain, protection of the CNS against neurotoxins and regulation of molecules between the CNS and peripheral

blood. Evidence of BBB disruption has been reported to be associated with CNS diseases with neuroinflammation such as CNS infections [50], neurodegeneration [51] and bipolar disorder [52]. However, the pathogenesis resulting in BBB disruptions are unknown. With knowledge that the encephalitis patients used in the study have disrupted BBB, the elevated levels of metabolites in the amino acids, catecholamines, lipids and histamine metabolism may be simply secondary to BBB disruption. To combat the potential effects of BBB disruption on interpretation, we propose to use ratios of adjacent metabolites, as in Figure 4-4. Further study in larger cohorts of encephalitis, and other syndromes with suspected or proven immune activation such as infection associated encephalopathy, acute seizure syndromes [53], neurodevelopmental, neuropsychiatric and neurodegeneration disorders are warranted. Furthermore, combined CSF and plasma analysis may improve knowledge regarding the origins of the inflammatory process (peripheral and/or central).

The CSF matrix has a less complex composition in comparison to plasma and blood resulting in a rapid sample preparation protocols [5]. The rapid and simple metabolite extraction of CSF samples is beneficial for the analysis of neuroactive molecules in larger scale population studies. The concentrations for a majority of CSF metabolites are known to be significantly lower compared to other matrices [54]. Currently, there is no single platform able to cover the entire CSF metabolome given its size and diversity of metabolite properties. There is increasing evidence that LC-HRMS in combination with bioinformatics has the ability to curate large amounts of data successfully, as shown here. As the quest of integrating metabolomics into a clinical diagnostic service continues, the novelty of the findings in this CSF encephalitis study warrants further clinical validation.

4.6 Conclusion

The early diagnosis of encephalitis is pivotal for patients to receive immediate successful treatments and disease management. The emergence of CSF metabolomics holds promise incorporating clinical research experience into clinical translation practice. Elevated kynurenine/tryptophan, anthranilic acid/3-hydroxyanthranilic acid and ADMA/arginine ratios in encephalitis suggests the dysregulation of the tryptophan-kynurenine and nitric oxide pathways. Further confirmation and validation in a clinical setting with a substantial patient cohort is crucial for future research.

References

1. Venkatesan A, Geocadin RG: **Diagnosis and management of acute encephalitis: A practical approach.** *Neurology Clinical practice* 2014, **4:**206-215.

2. Ellul M, Solomon T: **Acute encephalitis - diagnosis and management.** *Clinical medicine (London, England)* 2018, **18:**155-159.

3. French CD, Willoughby RE, Pan A, Wong SJ, Foley JF, Wheat LJ, Fernandez J, Encarnacion R, Ondrush JM, Fatteh N, et al: **NMR metabolomics of cerebrospinal fluid differentiates inflammatory diseases of the central nervous system.** *PLoS Negl Trop Dis* 2018, **12:**e0007045.

4. Vasilopoulou CG, Margarity M, Klapa MI: **Metabolomic Analysis in Brain Research: Opportunities and Challenges.** *Frontiers in Physiology* 2016, **7:**183.

5. Wishart DS, Lewis MJ, Morrissey JA, Flegel MD, Jeroncic K, Xiong Y, Cheng D, Eisner R, Gautam B, Tzur D, et al: **The human cerebrospinal fluid metabolome.** *J Chromatogr B Analyt Technol Biomed Life Sci* 2008, **871:**164-173.

6. Wu J, Wuolikainen A, Trupp M, Jonsson P, Marklund SL, Andersen PM, Forsgren L, Öhman A: **NMR analysis of the CSF and plasma metabolome of rigorously matched amyotrophic lateral sclerosis, Parkinson's disease and control subjects.** *Metabolomics* 2016, **12:**101.

7. Beale DJ, Pinu FR, Kouremenos KA, Poojary MM, Narayana VK, Boughton BA, Kanojia K, Dayalan S, Jones OAH, Dias DA: **Review of recent developments in GC–MS approaches to metabolomics-based research.** *Metabolomics* 2018, **14:**152.

8. Liu X, Zhou L, Shi X, Xu G: **New advances in analytical methods for mass spectrometry-based large-scale metabolomics study.** *TrAC Trends in Analytical Chemistry* 2019, **121:**115665.

9. Smolinska A, Blanchet L, Buydens LM, Wijmenga SS: **NMR and pattern recognition methods in metabolomics: from data acquisition to biomarker discovery: a review.** *Anal Chim Acta* 2012, **750:**82-97.

10. Mehl F, Gallart-Ayala H, Konz I, Teav T, Oikonomidi A, Peyratout G, van der Velpen V, Popp J, Ivanisevic J: **LC-HRMS data as a result of untargeted**

metabolomic profiling of human cerebrospinal fluid. *Data in brief* 2018, **21:**1358-1362.

11. Granerod J, Cunningham R, Zuckerman M, Mutton K, Davies NW, Walsh AL, Ward KN, Hilton DA, Ambrose HE, Clewley JP, et al: **Causality in acute encephalitis: defining aetiologies.** *Epidemiol Infect* 2010, **138:**783-800.

12. Chong J, Wishart DS, Xia J: **Using MetaboAnalyst 4.0 for Comprehensive and Integrative Metabolomics Data Analysis.** *Current Protocols in Bioinformatics* 2019, **68:**e86.

13. Badawy AAB, Guillemin G: **The Plasma [Kynurenine]/[Tryptophan] Ratio and Indoleamine 2,3-Dioxygenase: Time for Appraisal.** *International journal of tryptophan research : IJTR* 2019, **12:**1178646919868978-1178646919868978.

14. Wang Q, Liu D, Song P, Zou MH: **Tryptophan-kynurenine pathway is dysregulated in inflammation, and immune activation.** *Front Biosci (Landmark Ed)* 2015, **20:**1116-1143.

15. Braidy N, Grant R: **Kynurenine pathway metabolism and neuroinflammatory disease.** *Neural Regeneration Research* 2017, **12:**39-42.

16. Kindler J, Lim CK, Weickert CS, Boerrigter D, Galletly C, Liu D, Jacobs KR, Balzan R, Bruggemann J, O'Donnell M, et al: **Dysregulation of kynurenine metabolism is related to proinflammatory cytokines, attention, and prefrontal cortex volume in schizophrenia.** *Molecular Psychiatry* 2019.

17. Quist-Paulsen E, Aukrust P, Kran A-MB, Dunlop O, Ormaasen V, Stiksrud B, Midttun Ø, Ueland T, Ueland PM, Mollnes TE, Dyrhol-Riise AM: **High neopterin and IP-10 levels in cerebrospinal fluid are associated with neurotoxic tryptophan metabolites in acute central nervous system infections.** *Journal of Neuroinflammation* 2018, **15:**327.

18. Darlington LG, Forrest CM, Mackay GM, Smith RA, Smith AJ, Stoy N, Stone TW: **On the Biological Importance of the 3-hydroxyanthranilic Acid: Anthranilic Acid Ratio.** *Int J Tryptophan Res* 2010, **3:**51-59.

19. Cohen J: *Statistical power analysis for the behavioral sciences.* Hillsdale, N.J.: L. Erlbaum Associates; 1988.

20. Suhs KW, Novoselova N, Kuhn M, Seegers L, Kaever V, Muller-Vahl K, Trebst C, Skripuletz T, Stangel M, Pessler F: **Kynurenine Is a Cerebrospinal Fluid**

Biomarker for Bacterial and Viral Central Nervous System Infections. *J Infect Dis* 2019, **220:**127-138.

21. Kuhn M, Sühs K-W, Akmatov MK, Klawonn F, Wang J, Skripuletz T, Kaever V, Stangel M, Pessler F: **Mass-spectrometric profiling of cerebrospinal fluid reveals metabolite biomarkers for CNS involvement in varicella zoster virus reactivation.** *Journal of neuroinflammation* 2018, **15:**20-20.

22. Yap SH, Abdullah NK, McStea M, Takayama K, Chong ML, Crisci E, Larsson M, Azwa I, Kamarulzaman A, Leong KH, et al: **HIV/Human herpesvirus co-infections: Impact on tryptophan-kynurenine pathway and immune reconstitution.** *PloS one* 2017, **12:**e0186000-e0186000.

23. Anderson AM, Croteau D, Ellis RJ, Rosario D, Potter M, Guillemin GJ, Brew BJ, Woods SP, Letendre SL: **HIV, prospective memory, and cerebrospinal fluid concentrations of quinolinic acid and phosphorylated Tau.** *Journal of Neuroimmunology* 2018, **319:**13-18.

24. van Zoest RA, Underwood J, De Francesco D, Sabin CA, Cole JH, Wit FW, Caan MWA, Kootstra NA, Fuchs D, Zetterberg H, et al: **Structural Brain Abnormalities in Successfully Treated HIV Infection: Associations With Disease and Cerebrospinal Fluid Biomarkers.** *The Journal of Infectious Diseases* 2017, **217:**69-81.

25. Keegan MR, Chittiprol S, Letendre SL, Winston A, Fuchs D, Boasso A, Iudicello J, Ellis RJ: **Tryptophan Metabolism and Its Relationship with Depression and Cognitive Impairment Among HIV-infected Individuals.** *Int J Tryptophan Res* 2016, **9:**79-88.

26. Davies NW, Guillemin G, Brew BJ: **Tryptophan, Neurodegeneration and HIV-Associated Neurocognitive Disorder.** *Int J Tryptophan Res* 2010, **3:**121-140.

27. Maddison DC, Giorgini F: **The kynurenine pathway and neurodegenerative disease.** *Seminars in Cell & Developmental Biology* 2015, **40:**134-141.

28. Herman S, Åkerfeldt T, Spjuth O, Burman J, Kultima K: **Biochemical Differences in Cerebrospinal Fluid between Secondary Progressive and Relapsing¯Remitting Multiple Sclerosis.** *Cells* 2019, **8**.

29. Jacobs KR, Lim CK, Blennow K, Zetterberg H, Chatterjee P, Martins RN, Brew BJ, Guillemin GJ, Lovejoy DB: **Correlation between plasma and CSF**

concentrations of kynurenine pathway metabolites in Alzheimer's disease and relationship to amyloid-β and tau. *Neurobiol Aging* 2019, **80:**11-20.

30. Inoue H, Matsushige T, Ichiyama T, Okuno A, Takikawa O, Tomonaga S, Anlar B, Yüksel D, Otsuka Y, Kohno F, et al: **Elevated quinolinic acid levels in cerebrospinal fluid in subacute sclerosing panencephalitis.** *Journal of Neuroimmunology* 2020, **339:**577088.

31. Guillemin GJ, Smythe G, Takikawa O, Brew BJ: **Expression of indoleamine 2,3-dioxygenase and production of quinolinic acid by human microglia, astrocytes, and neurons.** *Glia* 2005, **49:**15-23.

32. Stone TW, Darlington LG: **The kynurenine pathway as a therapeutic target in cognitive and neurodegenerative disorders.** *British journal of pharmacology* 2013, **169:**1211-1227.

33. Badawy AAB: **Hypothesis kynurenic and quinolinic acids: The main players of the kynurenine pathway and opponents in inflammatory disease.** *Medical Hypotheses* 2018, **118:**129-138.

34. Adu-Gyamfi CG, Snyman T, Hoffmann CJ, Martinson NA, Chaisson RE, George JA, Suchard MS: **Plasma Indoleamine 2, 3-Dioxygenase, a Biomarker for Tuberculosis in Human Immunodeficiency Virus-Infected Patients.** *Clin Infect Dis* 2017, **65:**1356-1358.

35. Mancuso R, Hernis A, Agostini S, Rovaris M, Caputo D, Fuchs D, Clerici M: **Indoleamine 2,3 Dioxygenase (IDO) Expression and Activity in Relapsing-Remitting Multiple Sclerosis.** *PLOS ONE* 2015, **10:**e0130715.

36. Mbongue JC, Nicholas DA, Torrez TW, Kim N-S, Firek AF, Langridge WHR: **The Role of Indoleamine 2, 3-Dioxygenase in Immune Suppression and Autoimmunity.** *Vaccines* 2015, **3:**703-729.

37. Mackay GM, Forrest CM, Stoy N, Christofides J, Egerton M, Stone TW, Darlington LG: **Tryptophan metabolism and oxidative stress in patients with chronic brain injury.** *Eur J Neurol* 2006, **13:**30-42.

38. Stoy N, Mackay GM, Forrest CM, Christofides J, Egerton M, Stone TW, Darlington LG: **Tryptophan metabolism and oxidative stress in patients with Huntington's disease.** *J Neurochem* 2005, **93:**611-623.

39. Darlington LG, Mackay GM, Forrest CM, Stoy N, George C, Stone TW: **Altered kynurenine metabolism correlates with infarct volume in stroke.** *Eur J Neurosci* 2007, **26:**2211-2221.

40. Marques CP, Cheeran MCJ, Palmquist JM, Hu S, Lokensgard JR: **Microglia are the major cellular source of inducible nitric oxide synthase during experimental herpes encephalitis.** *Journal of Neurovirology* 2008, **14:**229-238.

41. 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 and Development* 2003, **25:**200-202.

42. Tripathi P, Tripathi P, Kashyap L, Singh V: **The role of nitric oxide in inflammatory reactions.** *FEMS Immunology & Medical Microbiology* 2007, **51:**443-452.

43. Deidda M, Noto A, Bassareo PP, Cadeddu Dessalvi C, Mercuro G: **Metabolomic Approach to Redox and Nitrosative Reactions in Cardiovascular Diseases.** *Frontiers in physiology* 2018, **9:**672-672.

44. Di Franco M, Lucchino B, Conti F, Valesini G, Spinelli FR: **Asymmetric Dimethyl Arginine as a Biomarker of Atherosclerosis in Rheumatoid Arthritis.** *Mediators of inflammation* 2018, **2018:**3897295-3897295.

45. Visser M, Vermeulen MA, Richir MC, Teerlink T, Houdijk AP, Kostense PJ, Wisselink W, de Mol BA, van Leeuwen PA, Oudemans-van Straaten HM: **Imbalance of arginine and asymmetric dimethylarginine is associated with markers of circulatory failure, organ failure and mortality in shock patients.** *Br J Nutr* 2012, **107:**1458-1465.

46. Lee DY, Teyssier C, Strahl BD, Stallcup MR: **Role of Protein Methylation in Regulation of Transcription.** *Endocrine Reviews* 2005, **26:**147-170.

47. Sydow K, Münzel T: **ADMA and oxidative stress.** *Atherosclerosis Supplements* 2003, **4:**41-51.

48. Dale RC, Brilot F, Fagan E, Earl J: **Cerebrospinal fluid neopterin in paediatric neurology: a marker of active central nervous system inflammation.** *Dev Med Child Neurol* 2009, **51:**317-323.

49. Jurynczyk M, Probert F, Yeo T, Tackley G, Claridge TDW, Cavey A, Woodhall MR, Arora S, Winkler T, Schiffer E, et al: **Metabolomics reveals distinct, antibody-independent, molecular signatures of MS, AQP4-antibody and MOG-antibody disease.** *Acta Neuropathologica Communications* 2017, **5:**95.

50. Spindler KR, Hsu T-H: **Viral disruption of the blood-brain barrier.** *Trends in microbiology* 2012, **20:**282-290.

51. Sweeney MD, Sagare AP, Zlokovic BV: **Blood-brain barrier breakdown in Alzheimer disease and other neurodegenerative disorders.** *Nature reviews Neurology* 2018, **14:**133-150.

52. Patel JP, Frey BN: **Disruption in the Blood-Brain Barrier: The Missing Link between Brain and Body Inflammation in Bipolar Disorder?** *Neural Plasticity* 2015, **2015:**708306.

53. Kothur K, Bandodkar S, Wienholt L, Chu S, Pope A, Gill D, Dale RC: **Etiology is the key determinant of neuroinflammation in epilepsy: Elevation of cerebrospinal fluid cytokines and chemokines in febrile infection-related epilepsy syndrome and febrile status epilepticus.** *Epilepsia* 2019, **60:**1678-1688.

54. Batllori M, Casado M, Sierra C, Salgado MdC, Marti-Sanchez L, Maynou J, Fernandez G, Garcia-Cazorla A, Ormazabal A, Molero-Luis M, Artuch R: **Effect of blood contamination of cerebrospinal fluid on amino acids, biogenic amines, pterins and vitamins.** *Fluids and Barriers of the CNS* 2019, **16:**34.

Chapter 5: Conclusions and Future Work

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5.1 Concluding Remarks

Metabolomics is a rapidly growing field opening new opportunities in facilitating biomarker discovery and unravelling the pathophysiologic mechanisms underlying neuroinflammatory diseases. The employment of untargeted metabolomics using high resolution mass spectrometry is a powerful approach to examine the alterations in CSF metabolites following acute brain inflammation.

The development of an untargeted metabolomics analysis method was crucial to this research, in order to analyse a broad spectrum of metabolites in human CSF. Currently, there is no single platform able to detect all CSF metabolites due to the diversity, complexity and size of the metabolome. The advancements in LC-HRMS and subsequent chemometrics tools have provided researchers the ability to uncover metabolites and their association with neuroinflammation. The developed and validated untargeted metabolomics analysis method using hydrophilic interaction liquid chromatography and data independent acquisition exhibited high reproducibility, excellent chromatographic separation, and strong resolving power covering a wide range of multi-class CSF metabolites. The rapid and simple metabolite extraction of CSF samples is beneficial for the analysis of neuroactive molecules in larger scale population studies.

The feasibility of the developed analysis method was further evaluated through a human CSF pilot study which consisted of four encephalitis patients and four age-matched controls. Advanced statistical analysis showed a strong discrimination between the encephalitis cohort and controls. The tryptophan-kynurenine pathway presented a number of statistically significant metabolites with both increased and decreased metabolites. The accelerated breakdown of tryptophan leads to the imbalanced formation of kynurenine metabolites, which is known to play a key role in the immune response. Furthermore, the identified elevation of neopterin in acute

brain inflammatory diseases is similar to previous published cohort studies. The results of the pilot study demonstrated that the untargeted metabolomics analysis method is a robust strategy with the potential to facilitate the understanding of biochemical pathways involving neuroinflammatory mechanisms. Additionally, the greater chromatographic performance and reproducibility of hydrophilic interaction chromatography further highlighted its suitability in the analysis of CSF metabolites.

Early detection of encephalitis is crucial to ensure efficient treatments and improved clinical outcomes. Metabolites are important indicators of physiological states and their relationship with enzymes play a significant role in the regulation of biological processes. A larger CSF encephalitis cohort study conducted in this thesis showed alterations of metabolites in the tryptophan-kynurenine pathway inferring the activation of indoleamine 2,3-dioxygenase and 3-hydroxyanthranilic acid oxidase during inflammation.

The cost-effective nature of metabolomics studies has opened the window of opportunity for the identification of potential biomarkers and unique insights of metabolic pathways involved in acute brain inflammation. A notable finding in this study was the statistical significance of metabolites in the nitric oxide pathway. The nitric oxide pathway is recognised as an important mediator and regulator of the immune system, playing a critical role in the pathogenesis of inflammation. The participation of the nitric oxide pathway has been increasingly reported to be implicated with a number of human diseases with proven or suspected inflammatory mechanisms. With current analytical constraints measuring nitric oxide, the analysis of more stable metabolites in the nitric oxide pathway with both increased and decreased levels exhibits potential of nitric oxide pathway metabolites as neuroinflammatory biomarkers.

This research has demonstrated CSF metabolomics is a powerful platform for the identification of novel neuroinflammatory biomarkers to significantly improve early diagnosis. The preliminary finding of elevated neopterin and changes in the ratios of adjacent metabolites in the tryptophan-kynurenine pathway (kynurenine/tryptophan

and anthranilic acid/3-hydroxyanthranilic acid) and nitric oxide pathway (ADMA/arginine) holds potential as a CSF panel in the clinical setting. The findings in this research holds promise in the integration of metabolomics research experience to a clinical diagnostic service.

5.2 Future Work

Metabolism is an integral reflection of cell function and health status of the organism. The use and interpretation of high-quality metabolomics data is fundamental to understand neuroinflammatory mechanisms, identify biomarkers, enable prognosis of disease development and treatment strategies.

The alterations in metabolite profiles of human diseases often occur well before the signs of clinical symptoms. The preliminary identification of a potential panel of CSF metabolites shows promise in the discrimination of encephalitis from controls, and holds promise for translations into clinical settings. The development and validation of a targeted method to quantify CSF metabolites will enhance our knowledge of their diagnostic potential. The quest for potential biomarkers to be clinically approved will require the metabolite(s) to be tested using hundreds of specimens and demonstrate sensitivity, specificity and reproducibility.

Further confirmation and validation in larger cohorts of encephalitis in combination with other human central nervous system diseases with suspected or proven neuroinflammatory mechanisms is a revenue for future research. Larger population studies will determine if these biomarkers can demonstrate neuroinflammation, to improve treatments and reduced mortality. In addition, the simultaneous analysis of CSF and blood samples will be useful in addressing whether the origins of observed metabolite changes are purely from CSF or secondary to blood brain barrier disruptions.