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

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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.

What this paper adds

- The kynurenine/tryptophan and anthranilic acid/3-hydroxyanthranilic acid ratios hold great potential as biomarkers of neuroinflammation.
- The elevation of the ADMA/arginine ratio in acute brain inflammation shows dysregulation of the nitric oxide pathway.

Abbreviations

3-HAO 3-hydroxyanthranilic acid oxidase

ADMA asymmetric dimethylarginine

BBB blood brain barrier

CNS central nervous system

CSF cerebrospinal fluid

IDO indoleamine-2,3-dioxygenase

LC-HRMS liquid chromatography coupled - high resolution mass spectrometry

OPLS-DA orthogonal partial least squares discriminant analysis

PCA = Principal component analysis

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.

Participants, Materials and Method

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). The MRI brain 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.

Local 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.

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.

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.

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 500. 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 $[M+Na]^+$ and a mass tolerance of 5 ppm. To reduce false positives and negative parameters eliminating 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 S1).

Results

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 1). This indicated good reproducibility and robustness of the metabolomics analysis method.

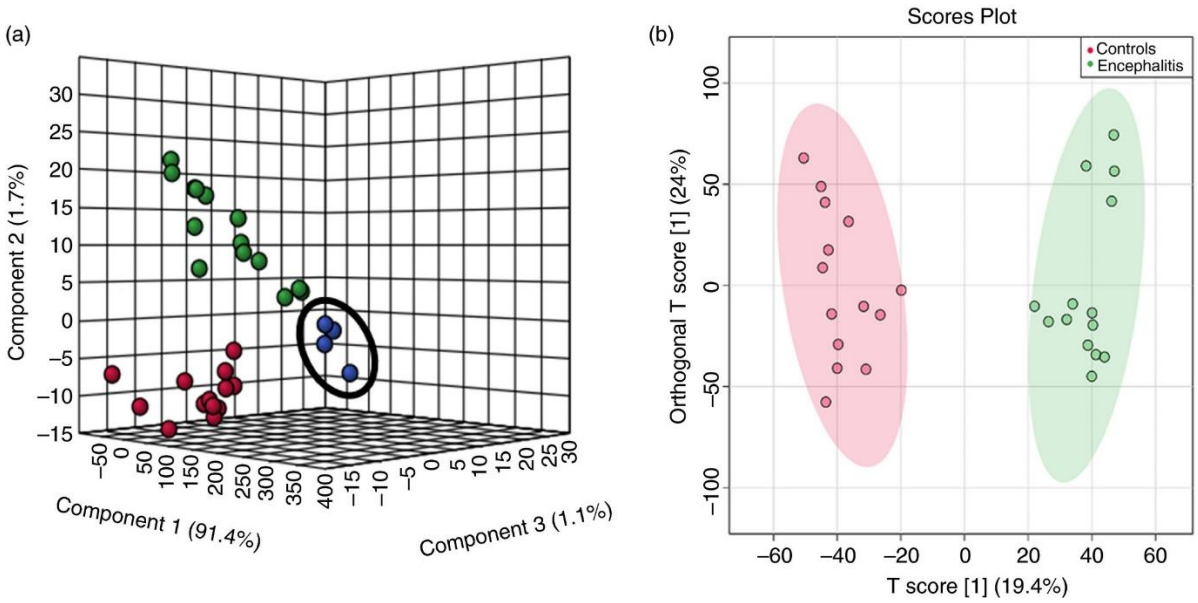

Figure 1: Statistical analysis of blinded CSF study using UPLC-HRMS. [A] PCA score plot on 14 control patients (red dots), 14 encephalitis patients (green dots) and 4 QC samples (blue dots) showing clustering of the QC samples. [B] OPLS-DA score plot presenting a clear separation of 14 control patients (red dots) from 14 encephalitis patients (green dots).

The differences in CSF levels between encephalitis and controls were explored using OPLS-DA (Figure 1). 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.

Among the thirty-five CSF metabolites, nine compounds were involved in the tryptophan-kynurenine pathway (Figure 2). 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 3A). The anthranilic acid/3-hydroxyanthranilic acid ratio has also been proposed in neuroinflammation [16-18] and was increased ($p < 0.001$) (Figure 3B), inferring 3-hydroxyanthranilic acid oxidase enzymatic activation during neuroinflammation.

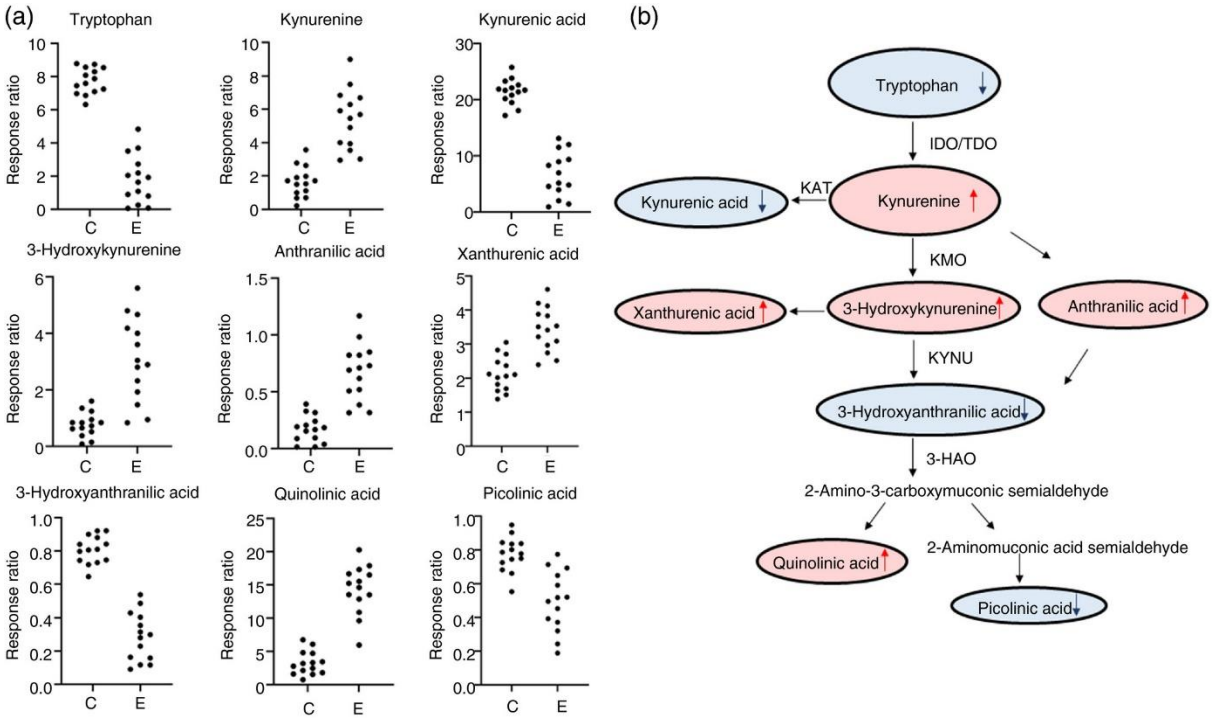

Figure 2: Tryptophan-kynurenine pathway metabolites [A] in encephalitis patients (E, n = 14) are compared to controls (C, n = 14). Significant differences were identified between the two groups in nine metabolites using ANOVA and Fishers LSD post-hoc analysis ($p < 0.001$). [B] Tryptophan-kynurenine metabolic pathway showing the increased metabolites (red) and decreased metabolites (blue) in encephalitis. IDO, indoleamine 2,3-dioxygenase; TDO, tryptophan 2,3-dioxygenase; KAT, kynurenine aminotransferase; KMO, kynurenine 3-monooxygenase; KYNU, kynureninase; 3-HAO, 3-hydroxyanthranilate oxidase.

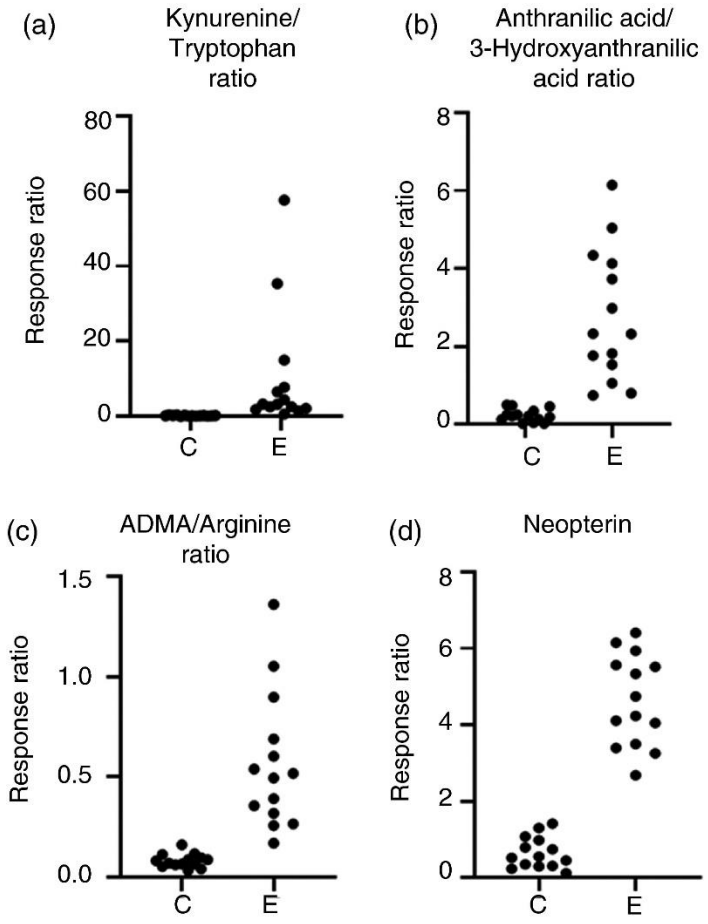

Figure 3: Statistical analysis of ratios and neopterin in encephalitis patients (E, n = 14) compared with controls (C, n = 14). [A] kynurenine/tryptophan ratio ($p < 0.001$). [B] anthranilic acid/3-hydroxyanthranilic acid ratio ($p < 0.001$). [C] ADMA/arginine ratio ($p < 0.001$). [D] Neopterin ($p 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

Four metabolites were altered in the nitric oxide pathway in encephalitis patients compared to controls (Figure 4). 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 3C).

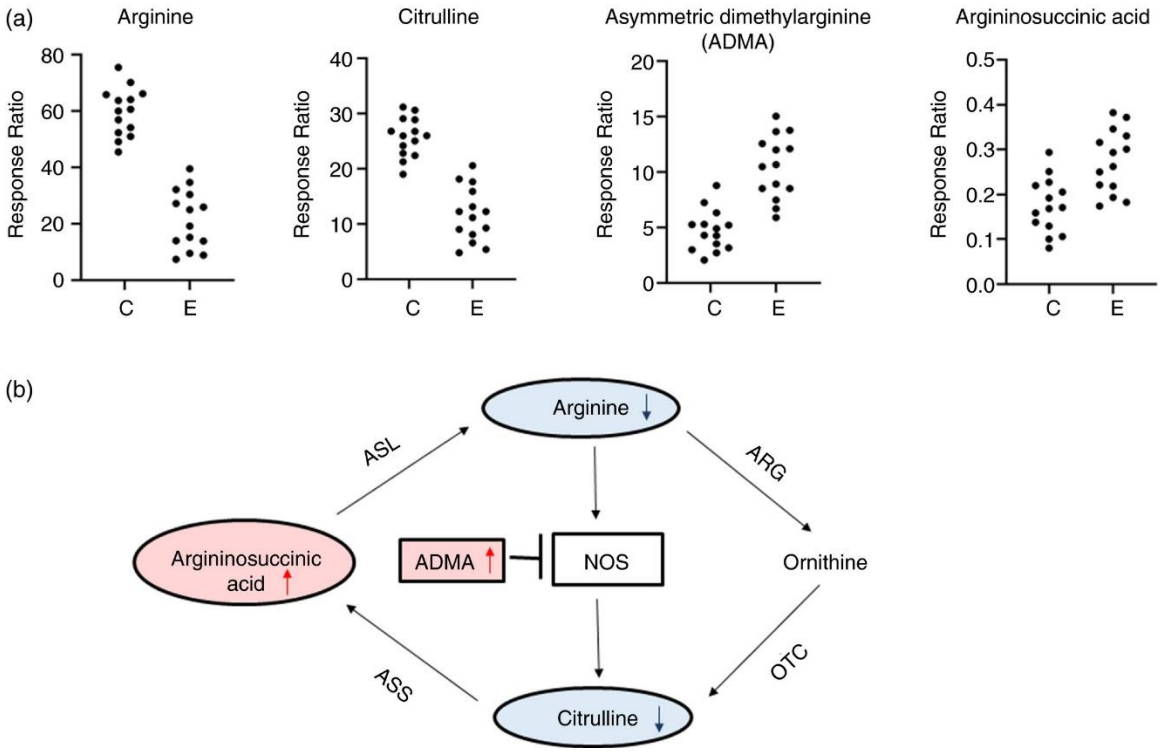

Figure 4: Nitric oxide pathway metabolites in patients with encephalitis (E, *n*=14) compared with controls (C, *n*=14). Significant differences were identified between the two groups in four metabolites using analysis of variance and Fisher's least significant difference post hoc analysis ($p<0.001$). (b) Nitric oxide metabolic pathway showing the increased metabolites (red) and decreased metabolites (blue) in encephalitis. ARG, arginine; ASL, argininosuccinate lyase; ASS, argininosuccinate synthase; NOS, nitric oxide synthase; OTC, ornithine transcarbamylase.

Neopterin was also significantly elevated in encephalitis groups compared to controls ($p < 0.001$) (Figure 3D). A statistical power analysis was conducted on the clinical study (n=28) comparing encephalitis and controls (Table S2). 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.

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

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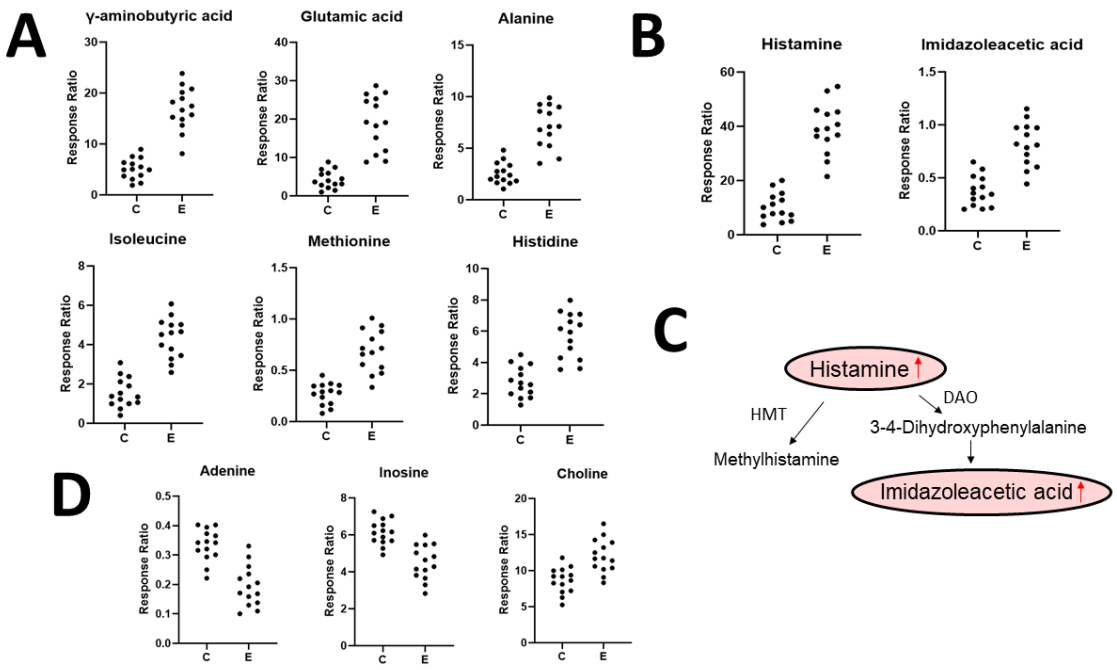

Figure S1). Likewise, metabolites in the catecholamine metabolism, lipids, histamine and its metabolite imidazoleacetic acid were also increased in encephalitis patients (Figure S2).

Finally, adenosine and inosine were decreased in the encephalitis group and choline was elevated

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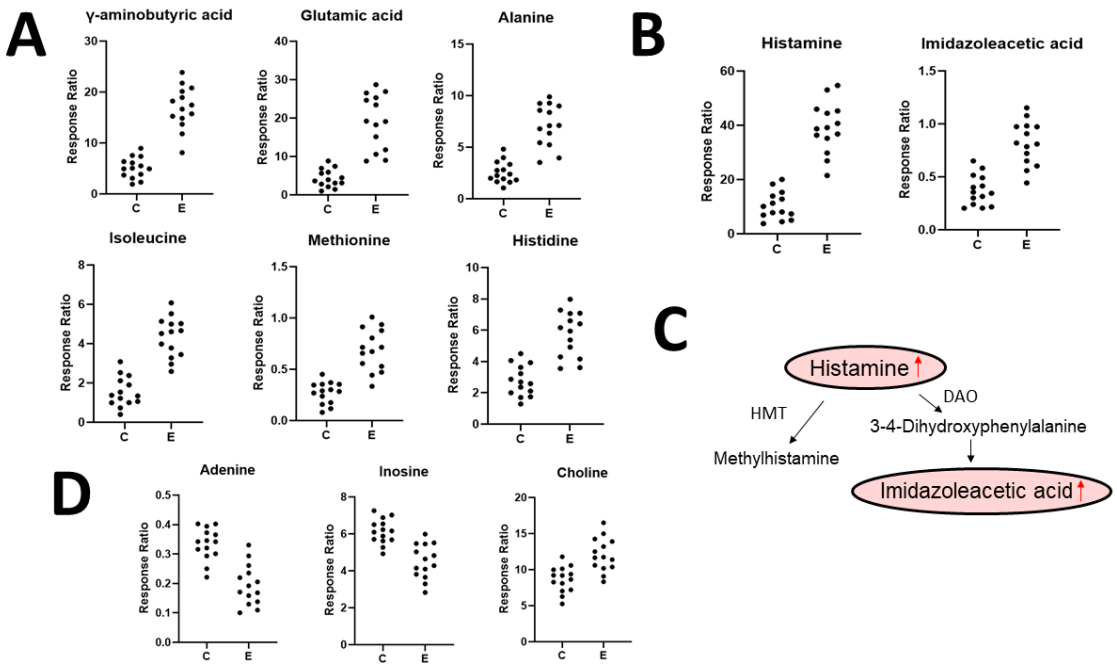

Figure S1).

Discussion

In recent years, a few untargeted CSF metabolomics studies have been conducted on encephalitis cohorts employing NMR or LC-HRMS [20,3,21]. 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]. However, it has been recently highlighted that only 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]. The anthranilic acid/3-

hydroxyanthranilic acid ratio was also a potentially useful biomarker of neuroinflammation, inferring 3-hydroxyanthranilic acid oxidase (3HAO) activation during inflammation. These findings are in agreement with previous targeted studies[17,20,18] 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[18,17]. 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[34,35,21]. 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[36]. 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[37] and rheumatoid arthritis[38]. 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[39].

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[40]. 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.

Limitations

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[41]. 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[42], neurodegeneration[43] and bipolar disorder[44]. 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 3. Further study in larger cohorts of encephalitis, and other syndromes with suspected or proven immune activation such as infection associated encephalopathy, acute seizure syndromes[45], 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).

Currently, there is no single platform able to cover the entire CSF metabolome given its size and diversity of metabolite properties. Taking into consideration the analytical and financial challenges of metabolomics, there is increasing evidence that LC-HRMS in combination with bioinformatics has the ability to curate large amounts of data successfully, as shown here.

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.

Acknowledgments

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No conflict of interest is reported by the authors.

Supporting Information

Table S1: 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	

Table S2: 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

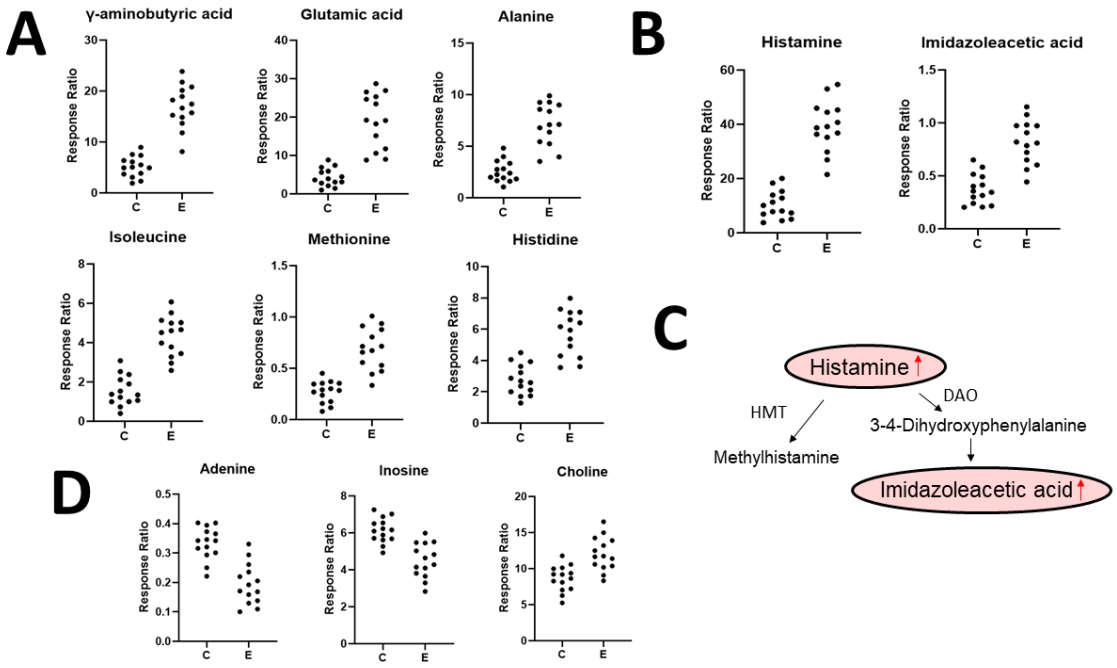

Figure S1: [A] Statistical differences in six amino acid metabolites ($p < 0.001$) were identified In encephalitis patients (E, n = 14) compared to controls (C, n = 14) [B] histamine and its metabolite, imidazoleacetic acid were increased in encephalitis patients ($p < 0.001$) [C] histamine metabolic pathway showing the increased metabolites (red) in encephalitis [D] decreased levels of adenine and inosine and increased choline in encephalitis patients ($p < 0.001$)

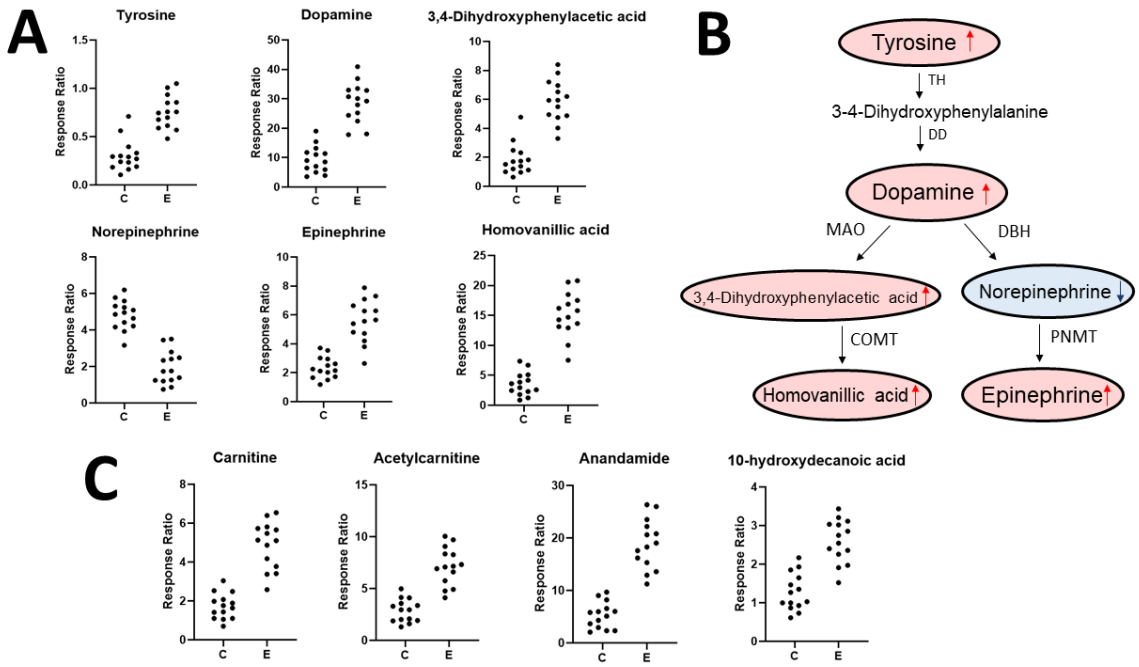

Figure S2: [A] Catecholamine pathway metabolites in encephalitis patients (E, n = 14) in comparison with controls (C, n = 14). Statistical differences in six metabolites were identified ($p < 0.001$). [B] Catecholamine metabolic pathway showing the increased metabolites (red) and decreased metabolites (blue) in encephalitis [C] Four lipid molecules showed increase in encephalitis patients ($p < 0.001$).

References

1 Venkatesan A, Geocadin RG. Diagnosis and management of acute encephalitis: A practical approach. *Neurol Clin Pract* 2014; **4**: 206-15.

2 Ellul M, Solomon T. Acute encephalitis - diagnosis and management. *Clin Med (Lond)* 2018; **18**: 155-9.

3 French CD, Willoughby RE, Pan A, Wong SJ, Foley JF, Wheat LJ, Fernandez J, Encarnacion R, Ondrush JM, Fatteh N, Paez A, David D, Javaid W, Amzuta IG, Neilan AM, Robbins GK, Brunner AM, Hu WT, Mishchuk DO, Slupsky CM. NMR metabolomics of cerebrospinal fluid differentiates inflammatory diseases of the central nervous system. *PLoS neglected tropical diseases* 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, Sawhney S, Bamforth F, Greiner R, Li L. The human cerebrospinal fluid metabolome. *Journal of chromatography. B, Analytical technologies in the biomedical and life sciences* 2008; **871**: 164-73.

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 Brief* 2018; **21**: 1358-62.

11 Granerod J, Cunningham R, Zuckerman M, Mutton K, Davies NW, Walsh AL, Ward KN, Hilton DA, Ambrose HE, Clewley JP, Morgan D, Lunn MP, Solomon T, Brown DW, Crowcroft NS. Causality in acute encephalitis: defining aetiologies. *Epidemiology and infection* 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-.

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**: 1116-43.

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, Lenroot R, Guillemin GJ, Weickert TW. 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. *J 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. *International journal of tryptophan research : IJTR* 2010; **3**: 51-9.

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. *The Journal of infectious diseases* 2019; **220**: 127-38.

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. *J Neuroinflammation* 2018; **15**: 20-.

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

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**: 13-8.

24 van Zoest RA, Underwood J, De Francesco D, Sabin CA, Cole JH, Wit FW, Caan MWA, Kootstra NA, Fuchs D, Zetterberg H, Majoie CBLM, Portegies P, Winston A, Sharp DJ, Gisslén M, Reiss P, Collaboration obotCiRtA. 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. *International journal of tryptophan research : IJTR* 2016; **9**: 79-88.

26 Davies NW, Guillemin G, Brew BJ. Tryptophan, Neurodegeneration and HIV-Associated Neurocognitive Disorder. *International journal of tryptophan research : IJTR* 2010; **3**: 121-40.

27 Maddison DC, Giorgini F. The kynurenine pathway and neurodegenerative disease. *Seminars in Cell & Developmental Biology* 2015; **40**: 134-41.

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, Hoshide M, Ohga S, Hasegawa S. 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. *Br J Pharmacol* 2013; **169**: 1211-27.

33 Badawy AAB. Hypothesis kynurenic and quinolinic acids: The main players of the kynurenine pathway and opponents in inflammatory disease. *Medical Hypotheses* 2018; **118**: 129-38.

34 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**: 229-38.

35 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**: 200-2.

36 Tripathi P, Tripathi P, Kashyap L, Singh V. The role of nitric oxide in inflammatory reactions. *FEMS Immunology & Medical Microbiology* 2007; **51**: 443-52.

37 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-.

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

39 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. *The British journal of nutrition* 2012; **107**: 1458-65.

40 Dale RC, Brilot F, Fagan E, Earl J. Cerebrospinal fluid neopterin in paediatric neurology: a marker of active central nervous system inflammation. *Developmental medicine and child neurology* 2009; **51**: 317-23.

41 Jurynczyk M, Probert F, Yeo T, Tackley G, Claridge TDW, Cavey A, Woodhall MR, Arora S, Winkler T, Schiffer E, Vincent A, DeLuca G, Sibson NR, Isabel Leite M, Waters P, Anthony DC, Palace J. Metabolomics reveals distinct, antibody-independent, molecular signatures of MS, AQP4-antibody and MOG-antibody disease. *Acta neuropathologica communications* 2017; **5**: 95.

42 Spindler KR, Hsu T-H. Viral disruption of the blood-brain barrier. *Trends Microbiol* 2012; **20**: 282-90.

43 Sweeney MD, Sagare AP, Zlokovic BV. Blood-brain barrier breakdown in Alzheimer disease and other neurodegenerative disorders. *Nature reviews. Neurology* 2018; **14**: 133-50.

44 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.

45 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**: 1678-88.