CSF neopterin, quinolinic acid and kynurenine/tryptophan ratio are biomarkers of active neuroinflammation

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Summary

Background Defining the presence of acute and chronic brain inflammation remains a challenge to clinicians due to the heterogeneity of clinical presentations and aetiologies. However, defining the presence of neuroinflammation, and monitoring the effects of therapy is important given its reversible and potentially damaging nature. We investigated the utility of CSF metabolites in the diagnosis of primary neuroinflammatory disorders such as encephalitis and explored the potential pathogenic role of inflammation in epilepsy.

Methods Cerebrospinal fluid (CSF) collected from 341 paediatric patients (169 males, median age 5.8 years, range 0.1–17.1) were examined. The patients were separated into a primary inflammatory disorder group (n = 90) and epilepsy group (n = 80), who were compared with three control groups including neurogenetic and structural (n = 76), neurodevelopmental disorders, psychiatric and functional neurological disorders (n = 63), and headache (n = 32).

Findings There were statistically significant increases of CSF neopterin, kynurenine, quinolinic acid and kynurenine/tryptophan ratio (KYN/TRP) in the inflammation group compared to all control groups (all $p < 0.0003$). As biomarkers, at thresholds with 95% specificity, CSF neopterin had the best sensitivity for defining neuroinflammation (82%, CI 73–89), then quinolinic acid (57%, CI 47–67), KYN/TRP ratio (47%, CI 36–56) and kynurenine (37%, CI 28–48). CSF pleocytosis had sensitivity of 53%, CI 42–64). The area under the receiver operating characteristic curve (ROC AUC) of CSF neopterin (94.4% CI 91.0–97.7%) was superior to that of CSF pleocytosis (84.9% CI 79.5–90.4%) ($p = 0.005$). CSF kynurenic acid/kynurenine ratio (KYNA/KYN) was statistically decreased in the epilepsy group compared to all control groups (all $p \leq 0.0003$), which was evident in most epilepsy subgroups.

Interpretation Here we show that CSF neopterin, kynurenine, quinolinic acid and KYN/TRP are useful diagnostic and monitoring biomarkers of neuroinflammation. These findings provide biological insights into the role of inflammatory metabolism in neurological disorders and provide diagnostic and therapeutic opportunities for improved management of neurological diseases.

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Research in context

Evidence before this study

Inflammatory and autoimmune disorders have significant morbidity and mortality rates worldwide with a diverse range of genetic, infectious, autoimmune or unknown causes. Several studies have reported elevation of neopterin and alterations of the tryptophan-kynurenine pathway in human diseases associated with neuroinflammation. Moreover, our previous untargeted CSF metabolomics study and targeted inflammation panel pilot study support the emerging role of neopterin and the tryptophan-kynurenine pathway in neuroinflammation.

Added value of this study

In this study, we present data showing alterations in CSF neopterin, quinolinic acid, kynurenine and kynurenine/tryptophan ratio are useful biomarkers of neuroinflammation. Specifically, neopterin presented improved ROC AUC compared to CSF pleocytosis in the detection of inflammation. Depletions of kynurenic acid and lower kynurenic acid/kynurenine ratio were common in most epilepsy syndromes.

Implications of all the available evidence

There are very few studies comparing alterations in CSF metabolite profiles between different neuroinflammatory disorders. The reproducible quantification of CSF biomarkers in this cohort of children with paediatric neurological disorders of broad aetiologies provides evidence for potential translation into clinical practice.

Introduction

Neuroinflammation is an inflammatory response of the brain or spinal cord initiated secondary to infection, traumatic brain injury, toxic metabolites, or autoimmunity. The inflammatory response is coordinated through the interaction of cellular and molecular signalling molecules released by mediators such as cytokines, chemokines, microglia, secondary messengers and reactive nitrogen and oxygen species.[1,2] For example, microglial cells are responsible for the release of pro-inflammatory mediators and contribute to neuronal repair or damage, and disease expression of neurodevelopmental disorders,[3] neurodegeneration[4] and toxic injuries.[5] Pro-inflammatory cytokines and chemokines are key intercellular mediators in acute and chronic inflammation, and are elevated in a variety of neuroinflammatory disorders.[6–8]

Inflammatory and autoimmune disorders of the central nervous system (CNS) can present with acute or chronic neurological symptoms, and early diagnosis is important due to their potentially treatable and reversible nature.[9,10] The acute and chronic presentations and the differential diagnoses of neuroinflammatory disorders can be challenging. Acute neurological disturbance can arise from inflammatory aetiologies such as encephalitis,[11] or alternatively other aetiologies such as functional neurological disease[12] or acute behavioural change (such as acute obsessive-compulsive disorder or psychosis),[13] all of which have different therapeutic pathways. Likewise, recognition of neuroinflammation in chronic disorders such as genetic interferonopathies and lymphocytic haemophagocytosis are important due to specific therapeutic pathways for these conditions.[14,15] Although there are already specific biomarkers of some neuroinflammatory disorders (such as anti-N-methyl-D-aspartate receptor (NMDAR) and myelin oligodendrocyte glycoprotein (MOG) autoantibodies),[16–19] many disorders lack specific diagnostic biomarkers, and therefore biomarkers to provide evidence of neuroinflammation are needed. In addition, there is increasing evidence supporting the role of neuroinflammation in the pathogenesis of epilepsy.[20–22] The impaired regulation of inflammatory cells and processes are major players in the development of epilepsy, however, the underlying mechanistic link to how inflammation contributes to epilepsy remains unclear.

Collection of cerebrospinal fluid (CSF) is an invasive but essential investigation to understand the CNS environment.[23] Although CSF pleocytosis is present in some patients with neuroinflammation, its absence does not exclude the presence of neuroinflammation.[24,25] The measurement of CSF cytokines is a common way to demonstrate neuroinflammation,[26] however, the assays are designed for research purposes only, and typically require batching, imposing impracticalities of translation into clinical diagnostic care with rapid turnaround times. There has been growing focus to explore the processes underlying neuroinflammation, with mounting evidence reporting that the neopterin[27,28] and tryptophan-kynurenine[29–31] metabolic pathways are associated with CNS inflammation (including in acute infection, multiple sclerosis, neurodegeneration and neuropsychiatry). CSF metabolomics also provides an opportunity to explore cellular function that influences neuroplasticity and neurodegeneration.[32,33]

To this end, we have developed a mass spectrometry method to measure inflammatory metabolites, with internal controls and accredited validation in a diagnostic laboratory, suitable for performing individualised testing and generating results within hours.[34] Building upon a significant emerging literature, we have shown that CSF neopterin and kynurenine pathway (KP) metabolites are useful biomarkers of inflammation.[28,35] In the present study, we aimed to explore the diagnostic utility and associations of these CSF metabolites in a large cohort of children with inflammatory and non-inflammatory neurological diseases. In addition, there is an increasing interest in the role of neuroinflammation in epilepsy, so we also explored the utility of these biomarkers in epilepsy compared to three control groups.

Methods

Study design and patient characteristics

341 individuals with available stored CSF were included. Patients presented to the Sydney Children's Hospitals Network (Westmead and Randwick), with acute or chronic neurological conditions between 2016–2022. As part of their routine investigation, CSF was taken for infective, inflammatory, and metabolic investigations including CSF for neurotransmitters, which was stored within an hour of arrival at the laboratory. Residual CSF that was not used for routine purposes was stored at –80 °C. The families of patients with residual CSF suitable for inclusion were written to gather consent, according to ethics protocol (2019/ETH06182). The total group of 341 patients included 169 males, with a median age of 5.8 years, and range of 0.1–17.1 years.

The patients were grouped according to aetiology, and clinical phenotyping was done separately and blinded to the CSF metabolomic analysis. Patient phenotyping was done by KK, HJ, VH and RCD, using the electronic hospital records of the treating clinicians (KK, SM, RW, SAH, JA, MPM, ET, DG, SG, TK, HS, MAF, CT, PIA, SCP, RCD). The selected CSF was before immunological therapy, but not always before symptomatic treatment (e.g. anti-seizure medications). The cohort was then separated into 5 groups according to final diagnosis/syndrome (Table 1):

1. Inflammation group (n = 90) were diagnosed by the treating clinicians based on accumulated multifaceted clinical and radiological data, and included patients with encephalitis,[36] demyelination,[37] and other acute or chronic inflammatory or autoimmune disorders (Table 1).
2. Neurogenetic and structural disorders (n = 76) were included as a control group, and included patients with structural problems (cerebral palsy, tumour etc), or neurogenetic disorders (Table 1).
3. Neurodevelopmental disorders, psychiatric and functional neurological disorders (n = 63) were grouped together in a further control group.
4. Epilepsy was a large and heterogeneous group (n = 80) consisting of developmental and epileptic encephalopathy, status epilepticus (CSF taken as part of investigation of event), generalised epilepsy, and focal epilepsy.
5. Headache, the final control group (n-32), consisted of patients with diagnoses/investigations of intracranial hypertension, or atypical migraine/headache syndromes.

Quantification of neopterin and tryptophan-kynurenine pathway metabolites

The collection of CSF samples was conducted using an aseptic technique and frozen within 1 h of sampling and stored at –80 °C. The CSF tube analysed for this study was not used for routine testing and was thawed only once after sampling. The increasing role of the KP in inflammation, immune responses and neurodegeneration has received great attention.[28,38] Moreover, the alterations in KP are associated with several neurological disorders where inflammation plays a primary role or is secondary to other stimuli.[39] The selected metabolites of interest in the panel are associated with neuroinflammation and were reported as statistically significant in an untargeted metabolomics study.[35] We subsequently used a targeted metabolomics approach, which is regarded as the gold standard for the accurate and reproducible quantification of metabolites.[40,41] The measurement of neopterin and tryptophan-kynurenine pathway metabolites (TRP, KYN, KYNA, 3-HK, XAN, AA, 3-HAA, QUIN, and picolinic acid) was in accordance to the method of Yan et al.[34] The pathway is presented in Fig. 1. Briefly, the sample preparation for human CSF involved protein precipitation using metaphosphoric acid/ethylenediaminetetraacetic acid solution, followed by mixing and centrifugation in Nanosep 0.2 µM centrifugal devices. The collected supernatant was analysed on the Waters ACQUITY UPLC I-Class System UPLC system coupled to a Xevo TQ-XS triple quadrupole mass spectrometer. The chromatographic separation of metabolites was achieved using the Acquity UPLC BEH C18 column (2.1 mm × 150 mm 1.7 µm particle size) and a 12 min gradient program. The detection of metabolites on the mass spectrometer was carried in the multiple reaction monitoring mode (MRM) and positive electrospray ionisation.

Statistics

Statistical analyses were performed using GraphPad Prism 8 (GraphPad, San Diego, USA), R (The R Foundation for Statistical Computing, version 4.2.2) and SPSS version 26 (IBM Corp. Armonk, NY, USA). For the metabolomics data, pairwise non-parametric tests

Group	Subgroup	Number (males)	Median age (range)	Aetiology
Inflammation (n = 90, 42 males, median age 6.75 (0.1–17.1)	Encephalitis	33 (20)	7 (0.1–16.5)	Unknown (n = 9), suspected AE (n = 8), NMDAR ab (n = 3), enterovirus (n = 3), mycoplasma (n = 2), SSPE (n = 2), influenza, basal ganglia, brainstem, HHV6, HSV2, GAD-ab (all n = 1)
	Demyelination	33 (12)	9.6 (2.2–15.7)	MOG-ADEM (n = 11), multiple sclerosis (n = 7), ADEM (n = 3), MOG-myelitis (n = 3), MOG-ON (n = 3), relapsing demyelination nos (n = 2), ON nos (n = 2), myelitis nos (n = 1), AQP4 NMOSD (n = 1)
	Other	24 (10)	4.3 (0.4–17.1)	Post-infectious ataxia/cerebellitis (n = 8), genetic autoinflammation AGS and HLH (n = 5), opsoclonus myoclonus ataxia (n = 2), NPSLE (n = 2), pneumococcal meningitis (n = 2), viral meningitis (n = 2), listeria meningitis, Hashimoto encephalopathy, autoimmune chorea (all n = 1).
Neurogenetic and structural/CP (n = 76, 31 males, median age 3.35, range 0.1–16.2)	Structural	19 (9)	2.6 (0.5–15.5)	Cerebral palsy (n = 9), tumour (n = 5), hydrocephalus (n = 2), traumatic brain injury (n = 2), stroke (n = 1)
	Genetic[a]	57 (22)	4 (0.1–16.2)	Suspected genetic (n = 13), *GCH1* (n = 3), *NF1* (n = 2), *STXBP1* (n = 2), lissencephaly nos (n = 2), *MECP2* (n = 2), Trisomy 21 (n = 2), *SCN2A* (N = 2), all n = 1: *PTS*, *SPR*, *TITF1*, *TOR1A*, *TNPO2*, *FOXG1*, *MAP2K2*, duplication 1p, *ERCC8*, unbalanced translocation, *CMT1A*, *TCF4*, *SCN8A*, *SPG4*, *PNKP*, *SLC52A2*, *SLC9A6*, Prader Willi, *PUM1*, *KMT2B*, *ATP1A3*, *CACNA1A*, *EPM2A*, Marinesco Sjogren, Huntington, *NARS2*, Leigh disease nos, pontocerebellar hypoplasia type 9, *NUBOL*.
Neurodev., psychiatry, FND (n = 63, 39 males, median age 8, 0.3–15.8)	Neurodev.	46 (32)	5.3 (0.3–15.5)	Autistic regression (n = 15), ASD (n = 11), dev delay nos (n = 11), PANS (n = 4), ADHD/Tourette (n = 3), developmental motor syndrome (n = 2)
	Psychiatry	10 (3)	13.6 (11.9–15.8)	Psychosis (n = 10)
	Functional neurological disorder	7 (4)	11.4 (11.3–15.5)	Functional visual loss (n = 2), pseudo-seizures/attacks (n = 3), functional weakness (n = 2)
Epilepsy (n = 80, 46 males, median age 5.14, 0.2–15.5)	Developmental and epileptic encephalopathy (DEE)	27 (17)	4.2 (0.2–14.5)	Doose syndrome (n = 4), DEE nos (n = 4), *SCN1A* (n = 2), *CHD2* (n = 2), *SCN8A* (n = 2), Lennox Gastaut syndrome (n = 2), *CDKL5* (n = 2), GLUT1 (n = 2), all n = 1: *SYNGAP1*, 16p13del, Landau Kleffner syndrome, *GRIN2A*, *GRIN1*, *FOXG1*, Angelman syndrome, Aicardi syndrome.
	Status epilepticus	20 (9)	3.6 (0.3–7.3)	Afebrile status epilepticus (n = 10), Febrile status epilepticus (n = 10)
	Generalised epilepsy	9 (5)	4.1 (0.3–15.1)	Atypical absence epilepsy (n = 4), generalised tonic seizures (n = 4), drop myoclonic (n = 1)
	Focal epilepsy	24 (14)	4 (0.3–15.5)	Focal cortical dysplasia (n = 6), unknown (n = 6), benign focal epilepsy (n = 4), suspected genetic (n = 3), temporal lobe epilepsy (n = 2), frontal lobe epilepsy (n = 1), symptomatic post encephalitic (n = 1), focal febrile seizure (n = 1)
Headache (n = 32, 11 males, median age 11.8 (2.8–16)	Headache syndromes	32 (11)	11.8 (2.8–16)	Idiopathic intracranial hypertension (n = 16), headache nos (n = 5), common migraine (n = 4), confusional migraine (n = 3), chronic daily headache (n = 3), hemiplegic migraine (n = 1)

ADEM: acute disseminated encephalomyelitis, ADHD: attention deficit hyperactivity disorder, AE: autoimmune encephalitis, AGS: Aicardi Goutières syndrome, AQP4 NMOSD: aquaporin-4 neuromyelitis optica spectrum disorder, ASD: autistic spectrum disorder, GAD: glutamic acid decarboxylase, HHV6: Human herpes virus 6, HLH: hemophagocytic lymphocytic histiocytosis, HSV2: herpes simplex virus 2, MOG: myelin oligodendrocyte glycoprotein, nos: not otherwise specified, NPSLE: neuropsychiatric systemic lupus erythematosus, ON: optic neuritis, PANS: paediatric acute neuropsychiatric syndrome, SSPE: subacute sclerosing panencephalitis. Patients are separated into inflammation, neurogenetic/structural, neurodevelopment/psychiatry/functional neurological disorder, epilepsy and headache groups.
[a]Some of the patients with neurogenetic conditions had a previous seizure in the past, but epilepsy was not an ongoing management issue.

Table 1: **A total of n = 341 patients were tested (169 males, median age 5.8 (0.1–17.1)).**

(Mann–Whitney U test) were performed. GraphPad Prism was used for analyses of putative marker concentrations which, due to their substantially skewed distributions, are presented on the log2 scale. The two groups of interest were the inflammation group and the epilepsy group, and pairwise comparisons were conducted between these two groups of interest and the three control groups. The primary hypothesis was that patients in the inflammation and epilepsy groups have significantly different neopterin and KP metabolites compared to the three normative groups. Therefore, given the 7 pairwise comparisons and the 12 metabolites/ratios analysed, we used a Bonferroni correction for multiple testing (84 statistical tests, therefore $p < 0.0006$ as threshold). The pROC R package was used to generate ROC curves and areas under the curves (AUCs).[42] The 95% CI of the AUC was computed using the pROC *ci. auc* function and Delong method. Comparisons of two correlated (paired) ROC curves was computed using the pROC *roc. test* function and Delong method.[43]

Analysis of correlations between metabolites in the inflammation group and the epilepsy group was performed using Spearman's correlation coefficient (R) in SPSS. The correlations are presented in colour (red for positive correlations, and blue for negative correlations).

Ethics

The Sydney Children's Hospitals Network Ethics Committee approved this study (LNR/14/SCHN/275; 2019/ETH06182), including informed consent from parents and/or guardians, as per ethics protocol.

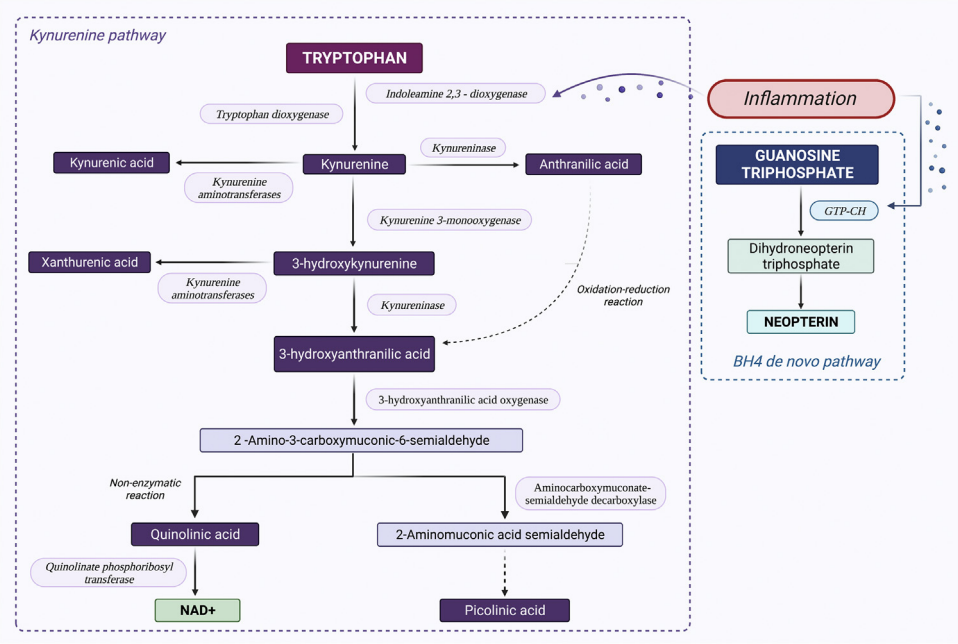


Fig. 1: **Neopterin and kynurenine pathways including enzymes.** GTP-CH: guanosine triphosphate cyclohydrolase, NAD+: Nicotinamide adenine dinucleotide+.

Role of funders

Financial support for the study was granted by Dale NHMRC Investigator grant APP1193648, University Sydney, Petre Foundation, and Cerebral Palsy Alliance. Funding sources had no role in the design of this study, and did not have any role during its execution, analyses, interpretation of the data, or decision to submit results.

Results

The inflammation group was the primary group of interest, and given the role of inflammation in epilepsy, the epilepsy group was the secondary group of interest. The other three groups (neurogenetic/structural, neurodevelopment/psychiatry/FND, headache) were considered the normative controls. The inflammation group was significantly older than the neurogenetic/structural ($p = 0.007$) (Mann Whitney U test) and epilepsy groups ($p = 0.002$) (Mann Whitney U test) and significantly younger than the headache group ($p = 0.02$) (Mann Whitney U test). The epilepsy group was significantly younger than the headache group ($p < 0.0001$) (Mann Whitney U test) and neurodevelopment group ($p < 0.002$) (Mann Whitney U test) (all ages in Table 1).

Cerebrospinal fluid metabolomics

We summarise in Table 2 the Mann–Whitney p-values of the seven pairwise between-group tests for each of the 12 metabolites/ratios, with statistically significant findings after Bonferroni multiple comparison correction boldfaced.

Inflammation group

CSF neopterin, kynurenine and quinolinic acid were significantly elevated in the inflammation group compared to all 3 control groups (Fig. 2A, B, C, Table 2). Kynurenic acid was significantly higher in the inflammation group compared to the epilepsy group (Fig. 2D, Table 2). 3-hydroxykynurenine and anthranilic acid were elevated in the inflammation group, and tryptophan was decreased in the inflammation group, but these differences were not statistically significant after Bonferroni correction, and are presented in supplementary Fig. S1, along with xanthurenic acid, 3-hydroxyanthranilic acid and picolinic acid (which were not differentiating between groups, Table 2).

Ratios are used to compare adjacent metabolites substrate which can infer enzymatic activity (which can be activated due to inflammation). The ratio KYN/TRP was significantly elevated in the inflammation group (Fig. 2E, Table 2). The ratio KYNA/KYN was significantly decreased in the inflammation group compared to the three control groups (Fig. 2F, Table 2).

A 95% centile threshold was created by combining the 3 control groups (dotted lines in Figures). The 95% centile appears to have validity throughout the paediatric age groups (supplementary Fig. S2). Further analysis separately comparing age and gender strata in each of the inflammation and combined control groups showed no statistically significant or otherwise substantial differences (one-way ANOVA) across age and gender strata, which suggests that the changes are driven by the

Comparison	Inflammation v neurogen + structural/CP	Inflammation v Dev + psych + FND	Inflammation v epilepsy	Inflammation v headache	Epilepsy v neurogen + structural/CP	Epilepsy v Dev + psych + FND	Epilepsy v headache
Neopterin	**p < 0.0001**	**p < 0.0001**	**p < 0.0001**	**p < 0.0001**	0.0018	**0.0001**	0.0526
Tryptophan	0.0024	0.0413	0.8244	0.3199	0.0108	0.0611	0.3465
Kynurenine	**p < 0.0001**	**p < 0.0001**	**0.0003**	**p < 0.0001**	0.0084	**0.0003**	0.039
Kynurenic acid	0.3981	0.5449	**p < 0.0001**	0.0663	0.0008	**p < 0.0001**	0.1404
3-Hydroxy Kynurenine	0.0083	0.0072	0.5737	0.0057	0.0014	0.0017	0.0032
Xanthurenic acid	0.3637	0.0853	0.4058	0.0807	0.9658	0.6370	0.0554
Anthranilic acid	0.0087	0.0176	0.8140	0.0050	0.0410	0.0362	0.0099
3-HAA	0.3206	0.0788	0.0394	0.0373	0.2094	0.0011	0.7646
Quinolinic acid	**p < 0.0001**	**p < 0.0001**	**p < 0.0001**	**p < 0.0001**	0.0031	0.0053	0.0137
Picolinic acid	0.3649	0.2123	0.7307	0.0569	0.6787	0.3509	0.0412
KYN/TRP ratio	**p < 0.0001**	**p < 0.0001**	0.0028	**p < 0.0001**	**p < 0.0001**	**p < 0.0001**	0.0096
KYNA/KYN ratio	**p < 0.0001**	**p < 0.0001**	0.9046	**0.0001**	**p < 0.0001**	**p < 0.0001**	**0.0003**

CP: Cerebral palsy, FND: Functional neurological disorder. Statistically significant findings after Bonferroni correction are presented in bold.

Table 2: **Summary of statistical comparisons using non-parametric (Mann–Whitney U test) pairwise comparisons of neopterin and KP metabolites.**

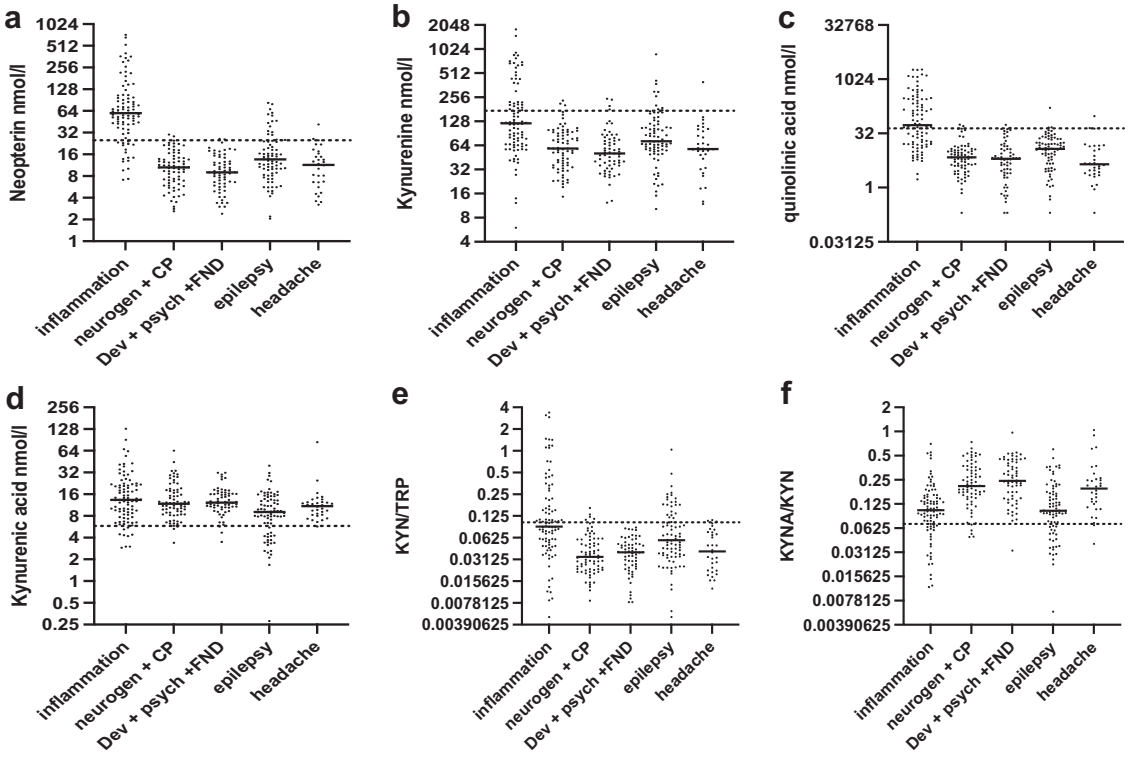


Fig. 2: **Neopterin and kynurenine pathway metabolites in the 5 subgroups**. CSF Neopterin **(a),** Kynurenine **(b),** quinolinic acid **(c),** and kynurenic acid **(d),** plus the ratios of KYN/TRP **(e)** and KYNA/KYN **(f)** are presented as log2 of nmol/l in the two groups of interest (inflammation n = 90 and epilepsy n = 80) and the three control groups (n = 171). The median of the groups is presented as a bar. The 95th centile of the 3 control groups is presented as a dotted line for Neopterin, kynurenine, quinolinic acid and KYN/TRP, and the 5th centile of the 3 control groups is presented as a dotted line for kynurenic acid and KYNA/KYN. As can be seen neopterin is a strongly differentiating biomarker of neuroinflammation, with kynurenine, quinolinic acid and KYN/TRP also differentiating neuroinflammation. In addition, there is evidence of inflammation in some patients with epilepsy. Kynurenic acid is decreased in the epilepsy group **(d),** as is KYNA/KYN **(f)** (all pairwise statistical comparisons are presented in Table 2).

disease states rather than age or gender (supplementary Fig. S3).

Using the 95% threshold, sensitivity and specificity were calculated for all 3 metabolites, with CSF neopterin having the best sensitivity (82%, CI 73–89), then quinolinic acid (57%, CI 47–67), KYN/TRP ratio (47%, CI 36–56) and kynurenine (37%, CI 28–48), and virtually identical 95–96% specificities (Supplementary Table S1). CSF pleocytosis (defined as ≥5 cells/mm^3) was present in 42/79 (53%, CI 42–64) of inflammation group patients with data available.

Based on individual ROC curves generated, CSF neopterin (94.4% CI 91.0–97.7%) and CSF QUIN (87.0% CI 82.2–91.9%) had superior AUCs compared to CSF pleocytosis (84.9% CI 79.5–90.4%) (supplementary Fig. S4). However, based on paired correlation of ROC curves, only the CSF neopterin AUC, was significantly superior to the CSF pleocytosis AUC (p = 0.005). The other biomarker AUC values are presented in supplementary Table S1, and the median and interquartile ranges in each group for the 4 discriminating metabolites are presented in supplementary Table S2.

When the inflammation subgroups were compared, the encephalitis and other inflammation subgroups tended to have higher CSF metabolites than the demyelination subgroup (supplementary Fig. S5), and when exploring the demyelination subgroup, the ADEM and isolated optic neuritis and transverse myelitis patients tended to have elevated metabolites, whereas the multiple sclerosis patients tended to have less elevated CSF metabolic profiles (supplementary Fig. S6).

Epilepsy group

Our secondary group of interest was epilepsy. CSF neopterin and kynurenine were significantly elevated in the epilepsy group compared to the developmental/psychiatry/FND control group (Fig. 2A and B and Table 2). KYNA was significantly decreased in the epilepsy group compared to the developmental/psychiatry/FND control group (Fig. 2D and Table 2). The KYN/TRP ratio was elevated in the epilepsy group compared to 2 of the control groups, and the KYNA/KYN ratio was decreased in the epilepsy group compared to all 3 control groups (Table 2, Fig. 2E and F). When the epilepsy subgroups were compared, the febrile status group tended to have elevated CSF neopterin and KYN/TRP ratio compared to the other groups (Fig. 3A and E) (no exploratory statistical inference procedures performed). KYNA and KYNA/KYN were generally lower in all the epilepsy subgroups compared to the controls (Fig. 3D and F).

Correlations between metabolites

A heatmap was generated to illustrate the distinct metabolic differences between the five subgroups (Fig. 4A).

Between metabolite correlations are presented for the inflammation cohort (Fig. 4B) and the epilepsy cohort (Fig. 4C). All correlations are presented in Fig. 4, but only moderate/strong negative or positive correlations are described here, and no intuitive correlations are mentioned (e.g. KYN with KYN/TRP).

For the inflammation cohort, there were positive correlations: of NEO with KYN, KYNA, QUIN and KYN/TRP; of KYN with KYNA and QUIN; of KYNA with QUIN and KYN/TRP; of AA with QUIN and PIC; and of QUIN with PIC and KYN/TRP.

For the epilepsy cohort, there were generally weaker correlations compared to the inflammation group, although positive correlations: of NEO with QUIN, TRP with KYNA; of KYN with KYNA and QUIN; and of XAN with 3HAA. There was a negative correlation between TRP and 3HAA.

Longitudinal monitoring

Six children had longitudinal samples available for comparison and are presented as monophasic (n = 3), and chronic (n = 3) disorders (Fig. 5). The monophasic disorders included: a 2.4-year-old male with opsoclonus myoclonus ataxia (acute and 12-month follow-up sample), 0.4-year-old male with pneumococcal meningitis (acute and 2-month follow-up), 16-year-old male Neuropsychiatric SLE (acute, 1-month and 2-month follow-up samples). For these monophasic disorders, the values of CSF neopterin, quinolinic acid, kynurenine and KYN/TRP were elevated initially, then normalised (Fig. 5).

The chronic disorders included: a 15.4-year-old male with autoinflammatory disorder (IFIH1) (diagnosis and 3-month follow-up), 10.5-year-old male with SSPE (diagnosis and 2-month follow-up) and 8-year-old female with SSPE (diagnosis, 1 and 2 month follow-up). For the chronic disorders, the CSF metabolites tended to remain elevated or become more elevated over time (Fig. 5).

Discussion

Despite the recent advances in biomarker identification for neuroinflammation, there are still many syndromes that lack specific biomarkers, and CSF pleocytosis is the most commonly used screening biomarker available globally to detect neuroinflammation. Our study investigated the levels of CSF neopterin and KP metabolites as biomarkers of neuroinflammation in a representative spectrum of paediatric neurological disorders, covering the breadth of severe aetiologies.

Using a previous untargeted metabolomics study in encephalitis, we identified that neopterin, KP and nitric oxide pathway metabolites differentiated encephalitis from controls.[35] In order to successfully translate these biomarkers into clinical practise, we developed an LC-MS/MS method to measure the multi-analyte CSF inflammation panel, to be fit for use in an accredited pathology laboratory. The quantitative and reproducible

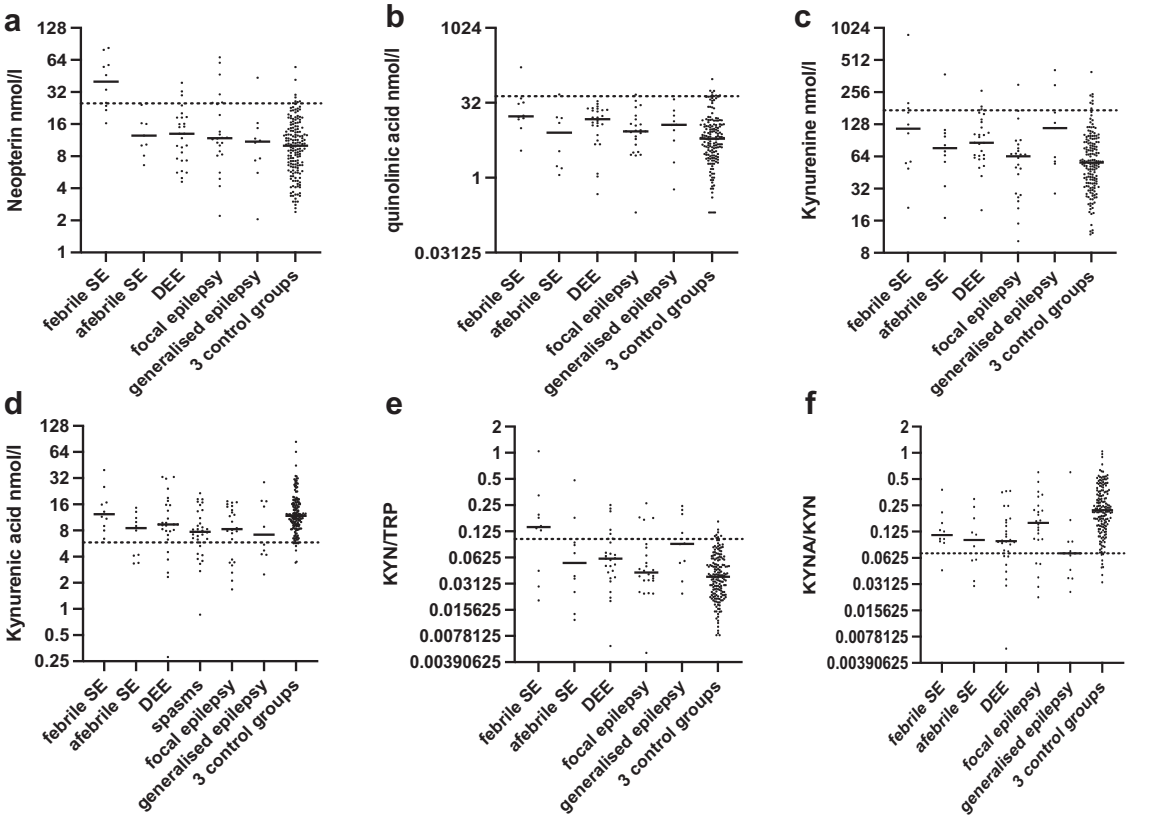
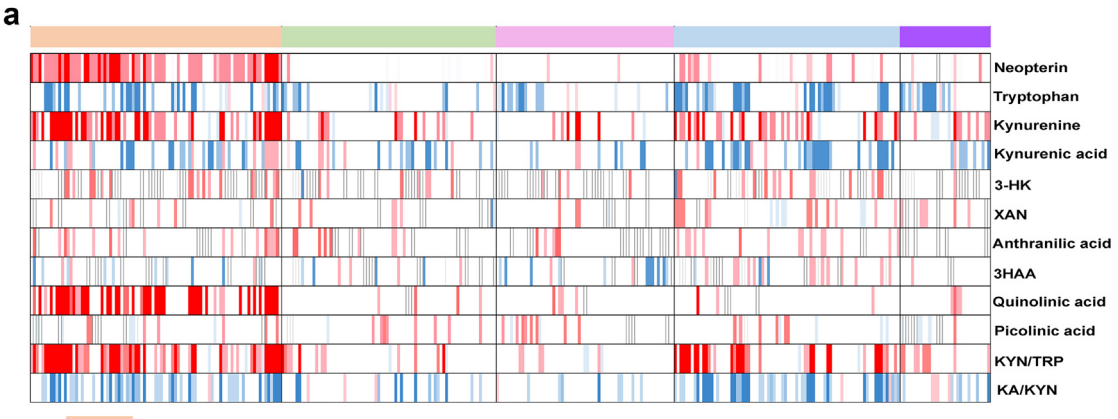


Fig. 3: **CSF metabolites in epilepsy subgroups**. The same metabolites and ratios are presented as for Fig. 2. The three control groups are presented as one large group (n = 171), and the 95th centile (for neopterin, quinolinic acid, kynurenine, and KYN/TRP) and the 5th centile (for kynurenic acid and KYNA/KYN) are presented as dotted lines. The subgroups of the epilepsy group (n = 80) are presented, as per Table 2. As can be seen, most of the inflammatory signal (as per neopterin) is generated by the febrile status group **(a).** Kynurenic acid **(d)** and KYNA/KYN **(f)** is generally low in all epilepsy subgroups, compared to controls. The median of the groups is presented as a bar.

nature of the assay is the cornerstone for clinical applications and translational purposes. The use of simple sample preparation methods and quantification using the mass spectrometry approach presents many strengths in an acute setting including; a rapid patient sample turnaround time of 4 h and individualised testing which is particularly important for patients with clinical urgency, such as acute encephalopathy of unknown cause.

CSF pleocytosis is an important biomarker for the assessment of neuroinflammation and serves as an initial point of evaluation to guide subsequent clinical decision-making. An elevated CSF leukocyte counts is an indicator for the presence of inflammation or infection, however it cannot provide a definitive diagnosis.[44,45] In addition, the absence of CSF pleocytosis does not exclude neuroinflammation, and in our study only 53% of children with a final diagnosis of neuroinflammation had CSF pleocytosis. In our study, at 95% specificity CSF neopterin and quinolinic acid showed better sensitivity for detecting neuroinflammation, compared to CSF pleocytosis. And the area under the curve (ROC) was statistically significantly greater for CSF neopterin compared to CSF pleocytosis. Therefore, CSF metabolites may be useful in some acute encephalopathy syndromes and chronic diseases such as Aicardi-Goutières syndrome and hemophagocytic lymphohistiocytosis, when pleocytosis is often absent (as shown in results). Further comparisons are required to determine the utility of the 4-marker metabolite test compared to CSF neopterin alone.

Neopterin is produced by activated macrophages and dendritic cells in response to cellular immune activation following stimulation by interferon species (Fig. 1), and neopterin has been previously shown to be a valuable clinical marker in a broad range of acute and chronic inflammatory disorders.[27,46] Neopterin showed the highest sensitivity in encephalitis and to a lesser extent in demyelination, particularly for the MS subgroup, suggesting there is less interferon activity in MS compared to other inflammatory groups. In the epilepsy group, neopterin was elevated in the febrile status



b

	Gender	Age at CSF sampling, months	Broad diagnostic group	NEO, nm	TRP, nm	KYN, nm	KYNA, nm	3HK, nm	XAN, nm	AA, nm	3HAA, nm	QUIN, nm	PIC, nm	KYN/TRP ratio	KYNA/KYN ratio
Gender	1.000	-0.093	0.054	-0.089	-0.257	-0.109	-0.109	-0.072	0.104	0.015	-0.060	-0.131	-0.155	0.037	0.048
Age at CSF sampling, months	-0.093	1.000	-0.259	-0.296	-0.106	-0.287	-0.438	-0.025	0.074	-0.204	0.103	-0.185	-0.168	-0.172	0.006
Broad diagnostic group	0.054	-0.259	1.000	0.106	0.236	0.085	0.154	-0.037	-0.362	0.409	-0.103	0.090	0.264	-0.045	-0.064
NEO, nm	-0.089	-0.296	0.106	1.000	-0.173	0.655	0.624	0.208	-0.178	0.374	-0.049	0.676	0.375	0.607	-0.262
TRP, nm	-0.257	0.106	0.236	-0.173	1.000	-0.101	0.138	-0.014	-0.023	0.044	-0.185	-0.034	0.087	-0.586	0.321
KYN, nm	-0.109	-0.287	0.085	0.655	-0.101	1.000	0.662	0.261	-0.071	0.350	-0.004	0.582	0.315	0.826	-0.588
KYNA, nm	-0.109	-0.438	0.154	0.624	0.138	0.662	1.000	0.212	-0.134	0.299	-0.106	0.523	0.397	0.449	0.119
3HK, nm	-0.072	-0.025	-0.037	0.208	-0.014	0.261	0.212	1.000	-0.342	0.306	-0.024	0.176	0.320	0.151	-0.122
XAN, nm	0.104	0.074	-0.362	-0.178	-0.023	-0.071	-0.134	-0.342	1.000	-0.301	0.144	-0.003	-0.285	-0.001	0.001
AA, nm	0.015	-0.204	0.409	0.374	0.044	0.350	0.299	0.306	-0.301	1.000	-0.107	0.411	0.538	0.176	-0.137
3HAA, nm	-0.060	0.103	-0.103	-0.049	-0.185	-0.004	-0.106	-0.024	0.144	-0.107	1.000	-0.030	-0.159	0.087	-0.151
QUIN, nm	-0.131	-0.185	0.090	0.676	-0.034	0.582	0.523	0.176	-0.003	0.411	-0.030	1.000	0.417	0.448	-0.244
PIC, nm	-0.155	-0.168	0.264	0.375	0.087	0.315	0.397	0.320	-0.285	0.538	-0.159	0.417	1.000	0.134	-0.085
KYN/TRP ratio	0.037	-0.172	-0.045	0.607	-0.586	0.826	0.449	0.151	-0.001	0.176	0.087	0.448	0.134	1.000	-0.631
KYNA/KYN ratio	0.045	0.006	0.054	-0.262	0.321	-0.588	0.119	-0.122	0.001	-0.137	-0.151	-0.244	-0.085	-0.631	1.000

c

	Gender	Age at CSF sampling, months	Broad diagnostic group	NEO, nm	TRP, nm	KYN, nm	KYNA, nm	3HK, nm	XAN, nm	AA, nm	3HAA, nm	QUIN, nm	PIC, nm	KYN/TRP ratio	KYNA/KYN ratio
Gender	1.000	-0.008	-0.099	0.191	-0.028	0.137	0.090	0.182	0.159	-0.084	-0.039	0.101	-0.078	0.118	-0.037
Age at CSF sampling, months	-0.008	1.000	0.123	-0.226	-0.062	-0.205	-0.377	-0.215	-0.057	-0.417	0.181	-0.233	0.025	-0.046	-0.132
Broad diagnostic group	-0.099	0.123	1.000	0.090	0.305	-0.008	0.091	-0.032	-0.319	0.050	-0.316	0.273	0.311	-0.207	0.099
NEO, nm	0.191	-0.226	0.090	1.000	-0.204	0.372	0.114	0.066	0.243	0.193	0.102	0.476	-0.069	0.378	-0.192
TRP, nm	-0.028	-0.062	0.305	-0.204	1.000	0.178	0.497	-0.076	-0.164	-0.163	-0.516	0.162	0.150	-0.597	0.312
KYN, nm	0.137	-0.205	-0.008	0.372	0.178	1.000	0.419	-0.031	0.289	0.051	-0.047	0.542	0.112	0.636	-0.572
KYNA, nm	0.090	-0.377	0.091	0.114	0.497	0.419	1.000	-0.016	-0.050	-0.172	-0.343	0.269	0.085	-0.059	0.412
3HK, nm	0.182	-0.215	-0.032	0.066	-0.076	-0.031	-0.016	1.000	-0.007	-0.094	0.152	0.324	0.117	0.026	0.001
XAN, nm	0.159	-0.057	-0.319	0.243	-0.164	0.289	-0.050	-0.007	1.000	0.109	0.565	0.116	-0.233	0.318	-0.265
AA, nm	-0.084	-0.417	0.050	-0.193	-0.163	0.051	-0.172	-0.094	0.109	1.000	0.078	0.213	-0.138	0.112	-0.138
3HAA, nm	-0.039	0.181	-0.316	0.102	-0.516	-0.047	-0.343	0.152	0.565	0.078	1.000	-0.087	-0.227	0.297	-0.279
QUIN, nm	0.101	-0.233	0.273	0.476	0.162	0.542	0.269	0.324	0.116	0.213	-0.087	1.000	0.117	0.247	-0.223
PIC, nm	-0.078	0.025	0.311	-0.069	0.150	0.112	0.085	0.117	-0.233	-0.138	-0.227	0.117	1.000	0.008	-0.008
KYN/TRP ratio	0.118	-0.046	-0.207	0.378	-0.597	0.636	-0.059	0.026	0.318	0.112	0.297	0.247	0.008	1.000	-0.731
KYNA/KYN ratio	-0.037	-0.132	0.099	-0.192	0.312	-0.572	0.412	0.001	-0.265	-0.138	-0.279	-0.223	-0.008	-0.731	1.000

Strong negative	Moderate negative	Weak negative	Very weak neg/pos	Weak positive	Moderate positive	Strong positive
-1.0 -0.9 -0.8	-0.7 -0.6 -0.5	-0.4 -0.3 -0.2	-0.1 0.0 0.1	0.2 0.3 0.4	0.5 0.6 0.7	0.8 0.9 1.0

Fig. 4: **Heat map in all groups, and correlations in inflammation and epilepsy groups. (a)** Heat map shows that most of the elevated metabolite signal (red) and decreased metabolite signal (blue) is from the inflammation group (salmon colour in top bar), and to a lesser extent from the epilepsy group (pale blue colour in top bar). **(b)** Correlations for inflammation group demonstrates the positive correlations between metabolites (red) and negative correlations (blue). **(c)** Correlations for the epilepsy group shows some positive and negative correlations, although generally less than for the inflammation group. For (b) and (c): red cells represent positive correlations, blue cells represent negative correlations, grey font represents non-statistically significant correlation coefficients, the bolded black font represents Spearman's correlation coefficient (R) with $p < 0.05$, and bolded purple font represents R with $p < 0.01$.

subgroup, supporting the presence of neuroinflammation in this syndrome.

The kynurenine pathway is a major route for the metabolism of tryptophan, producing a series of biologically active molecules with a broad range of properties including as oxidants, antioxidants, immunomodulators, neurotoxins and neuroprotectants.[47,48] Disruption of the tryptophan-kynurenine metabolism is strongly linked with neuroinflammation and immune activation.[39] Increased production of pro-inflammatory cytokines results in the activation of the KP regulatory enzyme indoleamine-2,3-dioxygenase (IDO1) and related enzymes, which causes dysregulation of the KP, specifically depletion of tryptophan and imbalanced formation of neuroprotective (kynurenic acid) and neurotoxic metabolites (quinolinic acid, 3-hydroxykynurenine) (Fig. 1).

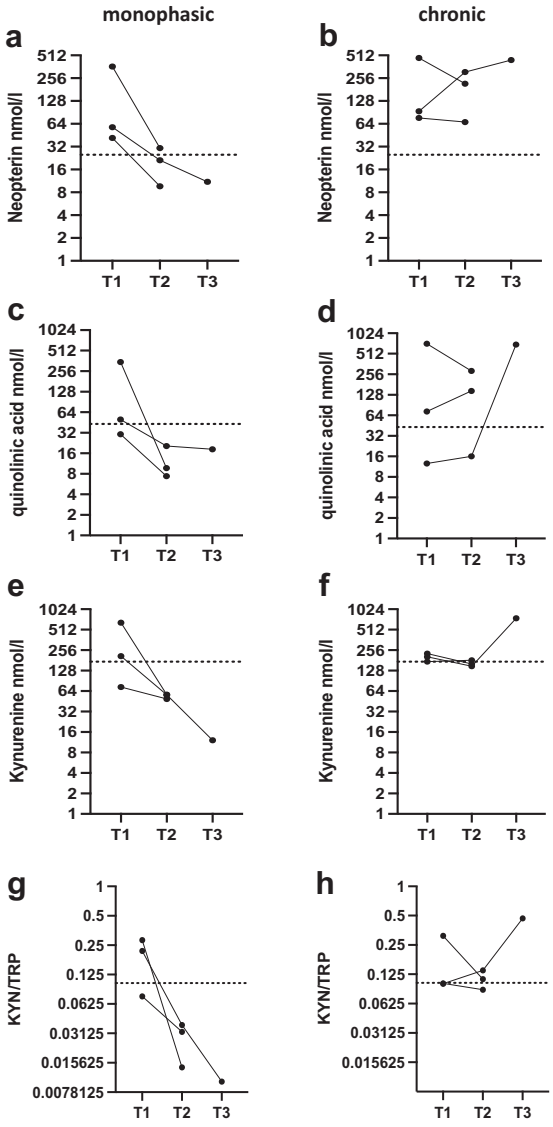


Fig. 5: **Longitudinal monitoring in monophasic and chronic neuroinflammatory conditions**. Longitudinal sampling shows the utility of the main inflammatory metabolites for comparing monophasic inflammation (left column) compared to chronic inflammation (right column). The cases with monophasic inflammation (n = 3) or chronic inflammation (n = 3) measured over 2 or 3 timelines (T1, T2, T3), show that in monophasic inflammation the inflammatory metabolites are initially elevated but then decline and normalize over time **(a, c, e, g).** Whereas the chronic patients had persistently elevated values over the 2 or 3 time points **(b, d, f, h).** All data presented in log2 scale, and the 95th centile of the 3 control groups is presented as a dotted line.

The biologic effect of KP metabolites depends on the cellular environment, local concentrations, enzymatic activities and metabolic functions, which are under complex positive and negative feedback loops.[49,50] Induction of IDO1 due to neuroinflammation consequently results in the elevation of kynurenine and depletion of tryptophan. The significantly elevated KYN/TRP ratio in inflammatory disorders and febrile status groups infers IDO1 activation in these syndromes and demonstrates KYN/TRP is a useful biomarker of neuroinflammation. Kynurenic acid is produced by astrocytes due to the conversion of kynurenine by the kynurenine aminotransferase[51] (Fig. 1). Interestingly KYNA was lower in the epilepsy group compared to all controls. These findings are in-line with the findings from our previous epileptic spasms study, where we showed that KYNA is lower in epileptic spasms than all controls (including age matched genetic epilepsy group) and we proposed that low KYNA is a potential biomarker of corticosteroid responsiveness.[52] Our data here suggests that KYNA is generally lower in all epilepsy subgroups, and therefore warrants further investigation of therapies that modify the KP (such as the ketogenic diet) in epilepsy.

The levels of quinolinic acid were generally higher in all inflammatory groups. Quinolinic acid is an excitotoxin and *N*-methyl-D-aspartate agonist in the CNS with immune-regulatory and pro-inflammatory properties.[53] There is a growing literature reporting the production of quinolinic acid following inflammation.[54–57] Quinolinic acid is produced by activated macrophages and microglia.[58,59] Quinolinic acid can trigger cell death of neurons, astrocytes and oligodendrocytes, as well as potentiate glutamate excitotoxicity and increase production of proinflammatory cytokines.[59,60]

The longitudinal samples showed how the selected diagnostic metabolites are potentially useful in the longitudinal monitoring of neuroinflammation. The metabolites decline and normalise in monophasic disease over time, whereas remain elevated in chronic neuroinflammatory diseases, suggesting that these biomarkers may be useful for future clinical trials treating neuroinflammation to assess response to treatments. Although we have focused on the positive disease groups of inflammation and epilepsy, this assay can be useful to demonstrate the absence of neuroinflammation in important presentations such as acute psychosis or functional neurological disease.

We used a stringent statistical cut-off for multiple testing (the Bonferroni correction), and consequently, although other metabolites in the KP appeared to be potentially involved in neuroinflammation, they failed to make our statistical thresholds to be diagnostic biomarkers. However, some of the findings such as decreased tryptophan and elevated 3-hydroxykynurenine in inflammation and epilepsy are worthy of further investigation (supplementary Fig. S1).

Our correlation analyses explored how different metabolites associate or interact with each other and demonstrated expected correlations (such as positive correlations of KYN with QUIN), but also unexpected correlations (such as AA with PIC in the inflammation group, and XAN with 3HAA in the epilepsy group).

These metabolites were not the focus of our study yet warrant further investigation in neurological diseases.

Limitations

A first limitation is the absence of cytokine and chemokine analysis in the groups of interest (inflammatory and epilepsy) and three control groups. There is mounting evidence reporting the elevation of pro-inflammatory cytokines (such as interferon-γ (IFN-γ), interleukin-6 (IL-6), IL-8, tumour necrosis factor α, IFN-inducible protein-10) in neuroinflammation.[26,61,62] The measurement of cytokines in conjunction with the inflammation panel would be ideal. However, this is currently impractical given the batch testing requirement of commercial kits, which have been designed for research purposes only rather than clinical practise. More recent studies have shown the quantification of cytokines and chemokines is moving towards proteomic analysis using tandem mass spectrometry,[63–66] which provides a promising future opportunity.

Another limitation of the study was the modest size of our longitudinal samples (n = 6). Substantially larger longitudinal studies are required to monitor changes. A further limitation was the absence of blood samples. Although CSF provides the best tissue for evaluating the brain environment, given the fact that blood is less invasive to collect it would be useful to determine whether blood can adequately reflect the metabolic changes we observed in CSF. Evidence of blood brain barrier (BBB) disruption has been reported to be associated with CNS neuroinflammation,[67,68] and concomitant analysis of both CSF and blood samples will be useful in understanding the origins of the inflammatory process, and whether the metabolite changes are from CSF, or secondary to blood brain barrier disruptions.

Future directions

CSF provides unique insights into brain function without the need for brain tissue biopsy. CSF metabolomics is a powerful tool to fingerprint metabolic profiles, building our knowledge of cellular functions, homeostasis and biomolecular changes implicated in disease onset and progression. With great potential to provide insights into human diseases, metabolomics can explore the effects of environmental exposures on the origins of common brain disorders such as depression and neurodegeneration. The combination of metabolomics with other omics techniques such as transcriptomics, proteomics and microbiomics can lead to a better understanding of biological interactions and yield therapeutic targets for improved treatment options.

The changes of neopterin, KP metabolites and adjacent ratios holds great promise in an acute setting. However, at present there is limited consensus regarding the inflammatory regulation of enzymes in the pathway and functions of KP metabolites.[69] The interplay of KP with other metabolic pathways is not well understood, and understanding such interactions may open a window of opportunity for new therapies. Moreover, the effects of diet on tryptophan metabolism,[70,71] and the effects of supplementing kynurenic acid[72] on CSF metabolomic findings should be further explored.

The investigation of the gut microbiota is an option for future research to address the interaction between inflammation, the gut microbiota and human disease. Recent studies have shown manipulation of the microbiome as a potential therapeutic intervention for improved clinical outcomes.[73] Various therapeutic approaches have been developed to modulate and restore the microbiome including prebiotics, probiotics, antibiotics, postbiotics and fecal microbiota transplantation.[74] Although the molecular mechanisms underlying these strategies remain unclear, targeted modulation of the microbiome is becoming a powerful approach in anti-infectious and anti-inflammatory therapies.

Conclusion

CSF metabolomics can provide unique insight into cellular dysfunction associated with host–environment interactions. Using a broad range of brain disorders that affect children, we have demonstrated the diagnostic value of CSF metabolomic analyses that can improve clinical care, using a targeted assay that exhibits speed, sensitivity, specificity and reproducibility. We provide evidence that neopterin and kynurenine metabolites represent valid biomarkers for active neuroinflammation in both acute and chronic neurological conditions. Future studies in adult cohorts using a similar panel, and further discovery of novel biomarkers through untargeted metabolomic studies are warranted.

Contributors

J. Yan: Conceptualization, Investigation, Methodology, Formal Analysis, Validation, Writing - original draft, Writing – reviewing & editing. **K. Kothur:** Validation (phenotyping patients from electronic medical record), Writing – reviewing & editing. **S. Mohammad:** Validation, Writing – reviewing & editing. **J. Chung:** Writing – Validation, reviewing & editing. **S. Patel:** Validation (creation of redcap database for phenotyping), Writing – reviewing & editing. **H.F. Jones:** Validation (phenotyping patients from electronic medical record), Writing – reviewing & editing. **B.A. Keating:** Writing – Validation, reviewing & editing. **V.X. Han:** Validation (phenotyping patients from electronic medical record), Writing – reviewing & editing. **R. Webster:** Validation, Writing – reviewing & editing. **S. Ardern-Holmes:** Validation, Writing – reviewing & editing. **J. Antony:** Validation, Writing – reviewing & editing. **M.P. Menezes:** Validation, Writing – reviewing & editing. **E. Tantsis:** Validation, Writing – reviewing & editing. **D. Gill:** Validation, Writing – reviewing & editing. **S. Gupta:** Validation, Writing – reviewing & editing. **T. Kandula:** Validation, Writing – reviewing & editing. **H. Sampaio:** Validation, Writing – reviewing & editing. **M. Farrar:** Validation, Writing – reviewing & editing. **C. Troedson:** Validation, Writing – reviewing & editing. **P.I. Andrews:** Validation, Writing – reviewing & editing. **S.C. Pillai:** Validation, Writing – reviewing & editing. **B. Heng:** Validation (kynurenine scientific discussions and hypotheses), Writing – reviewing & editing. **G.J. Guillemin:** Validation (kynurenine scientific discussions and hypotheses), Writing – reviewing & editing. **A. Guller:** Validation (statistical correlation

analysis), Writing – reviewing & editing. **S. Bandodkar:** Validation, Verification of underlying data, Supervision, Funding acquisition, Writing – reviewing & editing. **R.C. Dale:** Conceptualization, Investigation, Validation, Verification of underlying data, Supervision, Funding acquisition, Writing - original draft, Writing – reviewing & editing. JY, SB and RCD have directly accessed and verified the underlying data reported in the manuscript. All authors have read and approved the final version of the manuscript.

Data sharing statement

The data supporting the findings of this study and de-identified individual participant data are available in the article and/or supplementary material. Readers are welcome to contact the corresponding author for the raw data used in this work.

Declaration of interests

M Farrar reports grants from NHMRC and Cerebral Palsy Alliance Research Foundation, honoraria for educational presentations from Roche, Biogen and Novartis, participation on advisory board for Novartis Gene therapies and Roche, being medical director for Muscular dystrophy NSW and member of scientific and medical committee of Childhood dementia and Friedreich's ataxia. R Dale reports honorarium from Beijing pediatric neurology conference. The other authors have declared that no conflict of interest exists.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.ebiom.2023.104589.

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