**Kynurenine is a cerebrospinal fluid biomarker for bacterial and viral CNS infections**

Running title: Kynurenine as CSF biomarker

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**Importance**

This study of kynurenine and tryptophan concentrations in human CSF revealed marked induction of the kynurenine-tryptophan pathway in bacterial and viral meningitis/encephalitis. It highlights these metabolites as accurate biomarkers, particularly to differentiate among neuroborreliosis, viral meningitis/encephalitis and autoimmune neuroinflammation.
Abstract

Background. The tryptophan-kynurenine-NAD+ pathway is closely associated with regulation of immune cells toward less inflammatory phenotypes and may exert neuroprotective effects. Investigating its regulation in CNS infections would improve our understanding of pathophysiology and end-organ damage, and, furthermore, open doors to its evaluation as a source of diagnostic and/or prognostic biomarkers.

Methods. We measured concentrations of kynurenine (Kyn) and tryptophan (Trp) in 220 cerebrospinal fluid samples from patients with bacterial and viral (herpes simplex, varicella zoster, enteroviruses) meningitis/encephalitis, neuroborreliosis, autoimmune neuroinflammation (anti-NMDA-R encephalitis, multiple sclerosis), and noninflamed controls (Bell’s palsy, normal pressure hydrocephalus, Tourette syndrome).

Results. Kyn concentrations correlated strongly with CSF markers of neuroinflammation (leukocyte count, lactate, and blood-CSF-barrier dysfunction) and were highly increased in bacterial and viral CNS infections, but were low or undetectable in anti-NMDA-R encephalitis, multiple sclerosis, and controls. Trp was decreased mostly in viral CNS infections and neuroborreliosis. Multiple logistic regression analysis revealed combinations of Kyn, Trp and Kyn/Trp ratio with leukocyte count or lactate as accurate classifiers for the clinically important differentiation between neuroborreliosis, viral CNS infections, and autoimmune neuroinflammation.

Conclusions. The Trp-Kyn-NAD+ pathway is activated in CNS infections and provides highly accurate CSF biomarkers, particularly when combined with standard CSF indices of neuroinflammation.

Key words
Biomarkers, Borrelia, central nervous system, diagnosis, infection, kynurenine, metabolites, tryptophan

Abbreviations
AUC, area under the ROC curve; BacM – bacterial meningitis; Bell’s - idiopathic Bell’s palsy; BCB – blood-CSF-barrier; CI, confidence interval; CSF, cerebrospinal fluid; GTS – Gilles de la Tourette syndrome; EntM - enteroviral meningitis, HSE - HSV encephalitis; IDO - indoleamine-2,3-dioxygenase; Kyn, kynurenine; LOD, limit of detection; MS - multiple sclerosis; NMDA – anti-NMDA-receptor encephalitis; NPH - normal pressure hydrocephalus; ROC, receiver operating characteristic; TK pathway - tryptophan-kynurenine-NAD+ pathway; Trp, tryptophan; VZV fac - facial nerve zoster; VZV ME – VZV meningitis/encephalitis; VZV seg - segmental zoster (shingles).
**Introduction**

The tryptophan-kynurenine-NAD+ pathway (TK pathway) is the major pathway for catabolism of endogenous tryptophan (Trp). Its functions go far beyond maintaining Trp homeostasis, as its intermediates exert extensively documented immunomodulatory, neuromodulatory, and both cytoprotective and cytotoxic effects (reviewed in [1, 2]). The enzyme Trp-2,3-dioxygenase regulates the hepatic TK pathway, whereas indoleamine-2,3-dioxygenase (IDO) is the rate-limiting enzyme in kynurenine (Kyn) synthesis at extrahepatic sites including immune cells and the central nervous system (CNS) [1]. Increased IDO activity and subsequent Kyn synthesis are tightly associated with modulation of immune cell function, particularly suppression of activity of macrophages, dendritic cells, and T cells [3]. Dysregulation of the pathway has been implicated in a surprisingly broad variety of CNS disorders, ranging from Tourette syndrome [4] to major depression [5], schizophrenia [6], Alzheimer’s disease [7, 8], Parkinson’s disease [2, 9], Huntington’s disease [10], amyotrophic lateral sclerosis [11], and multiple sclerosis (MS) [6, 12, 13]. In these CNS disorders, it is most plausible that the pathway is activated by inflammation-related processes and modulates pathogenesis by the balance between deleterious (e.g., anthranilic and quinolinic acid, 3-OH-L-Kyn) and protective (kynurenic acid) intermediates, immunosuppressive effects, and the generation of NAD+ for energy metabolism and vitamin B6 synthesis. Of note, modulators of key enzymes in the pathway as well as analogs of TK pathway intermediates have become available, and evidence from animal models has demonstrated their potential to improve outcome of diseases characterized by increased activity of the pathway (MS – reviewed in [13], Huntington’s disease – [10]).

Preliminary evidence from studies in mice [14] and a small human cohort of patients with septic and aseptic meningitis [15] suggested that Kyn concentrations in CNS increased during bacterial meningitis and that inhibiting two key enzymes of the TK pathway exacerbated experimental pneumococcal meningitis in mice [16], suggesting that this pathway exerts a net neuroprotective effect at least in this model. Therefore, investigations into its regulation and relative activity in CNS infections would improve our understanding of pathophysiology and end-organ damage in CNS infections. Moreover, it is conceivable that altered concentrations of TK pathway metabolites might differ among different diseases greatly enough to be used as diagnostic biomarkers, for instance (as suggested by the preliminary results from Coutinho et al. [15]) for the differentiation between septic and aseptic meningitis.

We have therefore performed a detailed analysis of Kyn, Trp, and the Kyn/Trp ratio (as a measure of IDO activity) in CSF samples from patients with bacterial and viral CNS infections, autoimmune neuroinflammatory diseases, and non-inflamed neuropathologies, in order to assess differences in the
induction of the pathway among these disorders and to assess the potential of these three parameters as diagnostic CSF biomarker.

**Study population, materials and methods**

**Study design and population**

The samples and clinical data were obtained at Hannover Medical School during routine lumbar puncture. All samples were processed and stored according to unified standard operating procedures [17]. Briefly, after removing the volume necessary for clinical diagnostics, CSF was centrifuged to separate cells from fluid, and cell-free supernatant was frozen at -80°C until analysis. The study was approved by the Ethics Committee of Hannover Medical School (file no. 2413-2014) and was conducted according to the Helsinki Declaration. Samples from patients with the following diagnoses were analyzed: bacterial meningitis (abbreviated BacM), neuroborreliosis (Borrelia), herpes simplex virus meningitis/encephalitis (HSE), varicella zoster virus (VZV) meningitis/encephalitis (VZV ME), enteroviral meningitis (EntM), facial nerve zoster (VZV fac), segmental zoster (VZV seg; also known as shingles), anti-N-methyl-D-aspartate receptor encephalitis (NMDA), multiple sclerosis (MS), Bell’s palsy (Bell’s), and normal pressure hydrocephalus (NPH). Diagnostic criteria and basic data on disease activity and treatments are summarized in Table S1. The following standard CSF parameters were recorded: leukocyte count, lactate concentration, protein concentration, IgG index, and Q-albumin (CSF/serum albumin ratio). Blood-CSF-barrier dysfunction was scored from 0 (no dysfunction) to 3 (severe dysfunction) using age-corrected Q-albumin [18]. Peripheral blood samples were obtained at the time of lumbar puncture and analyzed the same day in the in-house diagnostic laboratories for the clinically indicated parameters, including complete blood count with differential and C-reactive protein (CRP) concentrations.

**Mass spectrometry**

CSF concentrations of Trp and Kyn were measured as part of a targeted metabolomic screen using the Absolute IDQ® p180 kit (Biocrates Life Sciences AG, Innsbruck, Austria) and liquid chromatography-tandem-mass spectrometry (LC-MS/MS), as described previously [19]. The limits of detection (LOD) for Kyn and Trp (defined as 3x the signal measured with the blank) were found to be 0.15 and 0.25 μM. All values <LOD were replaced by the pseudo value of LOD/2, as recommended by the manufacturer [20]. A separate analysis based on a subset of 88 of the 188 analytes measurable with the p180 kit, but not including Kyn and focusing on CSF biomarkers for varicella zoster virus (VZV) reactivation, has been published separately [19].
Statistical analysis
The Mann-Whitney U test was used to compare differences in median values between two groups. Significance of concentration differences across all sample groups was determined with Kruskal-Wallis analysis with corrections for multiple hypothesis testing. The chi-square test (χ²-test) was used to analyze the difference between category frequencies. Significance of differences was defined as a P value of <0.05 unless stated otherwise. Spearman correlation was used to assess correlations between metabolite parameters (Kyn, Trp, Kyn/Trp ratio) and the standard CSF and blood parameters measured. Receiver operating characteristics (ROC) curve analysis was used to quantify biomarker potential. Significance of areas under the ROC curve (AUCs) was defined by asymptotic P values of <0.05 and lower bound confidence intervals (CI; obtained by 1000 bootstraps) not crossing below 0.5. The leave-one-out (jackknife) method together with logistic regression (forward selection procedure) was used to select the best combination (subset) of metabolite and standard CSF parameters for each diagnostic pair and to assess its prediction potential [21]. The jackknife method was applied to 1000 bootstrap samples in order to estimate the CI of the AUC for the final classifier (i.e. subset of the original input of five parameters). All statistical analyses were performed with the ‘stats’, ‘Biocomb’ [22] and ‘agricolae’ packages of the R Foundation for Statistical Computing (version 3.4.4; from here on referred to as “R”) [23].

Results
Description of the study population
Table 1 summarizes demographic data and results of blood and standard CSF parameters. All results were consistent with known information about the natural history of the diagnoses studied. In particular, CSF leukocyte count, lactate, protein, and blood-CSF-barrier dysfunction were highest in bacterial meningitis, followed by neuroborreliosis, HSE, and VZV meningitis/encephalitis. All standard parameters were essentially normal in NPH, Tourette syndrome, and Bell’s palsy, thus supporting their use as non-inflammatory controls. Causative pathogens in bacterial meningitis corresponded to the clinical spectrum expected in a tertiary care center in central Europe (summarized in Table S1).

Differences in Kyn and Trp concentrations across the diagnostic groups
Overall, Kyn concentrations were <LOD in many of the non-inflamed and less inflamed samples, but this metabolite was detected >LOD with increasing frequency as the degree of inflammation increased, and the percentage of samples with concentrations >LOD was highest in bacterial meningitis, HSE, and neuroborreliosis (Fig. 1A left). Measured Kyn concentrations increased following a similar pattern (Fig. 1A right) and were markedly increased in the infected groups, with mean values varying between 3.7–fold
(enterovirus meningitis) and 24-fold (bacterial meningitis) higher than in the non-inflamed diagnoses. There were two noteworthy observations: (1) bacterial meningitis, being the clearly most highly inflamed disease, had median Kyn concentrations similar to neuroborreliosis, HSE, and VZV meningitis/encephalitis, whereas mean Kyn concentrations were highest in bacterial meningitis due to outliers with extremely high values (shown in Fig. S1A); (2) in spite of the well-documented inflammatory nature of MS [24] and elevated indices of inflammation (cell count, IgG index, each \( P < 0.001 \) with respect to NPH; Table 1), Kyn concentrations were <LOD in nearly all MS samples, even though most samples had been obtained from patients with active disease during relapse (Table S1). There was a tendency of Trp concentrations to decrease in the non-bacterial etiologies with increasing inflammation, but this trend was not observed in bacterial meningitis and neuroborreliosis (Fig. 1B left). The Kyn/Trp ratio was highest in the five CNS infections, although differences compared to the non-infected groups were not as pronounced as in the case of Kyn, and among these five diagnoses its median value was lowest in bacterial meningitis (Fig. 1B right). Incremental increases in Kyn concentration, accompanied by decreases in Trp concentration and increases in Kyn/Trp ratio, were seen in the three clinical forms of VZV reactivation in the order segmental zoster < facial zoster < VZV meningitis/encephalitis, i.e. along increasing neuroinflammation and involvement of the CNS.

**Kyn and Trp concentrations correlate differentially with the extent of neuroinflammation**

We then assessed the association of increased Kyn and Trp concentrations with CSF inflammation (≥5 cells/mm\(^3\)) (Fig. 2). Kyn concentrations were markedly (median = 5.6-fold, mean = 9.4-fold) and highly significantly elevated in the inflamed (infected and non-infected) compared to the non-inflamed samples, and in ROC analysis Kyn concentration distinguished accurately between the inflamed and non-inflamed samples (Fig. 2A left). Trp concentrations were significantly lower in the inflamed samples, but the differences were much smaller compared to Kyn, and Trp discriminated less accurately between inflamed and non-inflamed samples (Fig. 2A right). Thus, increased Kyn concentrations were significantly more strongly associated with CSF inflammation than decreased Trp concentrations. Nonetheless, the association of the Kyn/Trp ratio with CNS inflammation was similar to that of Kyn concentrations alone (Fig. 2B).

To test whether there was a preferential association between the Kyn-Trp parameters and any of the standard diagnostic CSF parameters, we then performed a correlation analysis between the three metabolite parameters and the six standard CSF indices, as well as peripheral blood C-reactive protein (CRP) concentration (Fig. 3 and Fig. S2). Kyn correlated most positively with CSF leukocyte count (\( \rho = 0.76, P = 4.9^{43} \)), but also to a slightly lesser extent with lactate, protein, and blood-CSF-barrier dysfunction,
whereas only weak (albeit significant) correlations were detected with IgG index and blood CRP (Fig. 3A left). Of note, this strong correlation with leukocyte count existed even though the highest Kyn concentrations were measured in a small number of samples with comparatively low leukocyte counts (Fig. 3A right). For Trp, only a weak negative correlation with cell count ($r = -0.30$, $P = 6.2 \times 10^{-06}$) and a weak positive correlation with blood CRP were observed (Fig 3A left). Consistent with this, Kyn/Trp ratio correlations with these parameters were less pronounced than Kyn correlations.

When the same correlations were performed with the individual diagnostic groups, major differences became apparent (Fig. 3B - D left, Fig. S2). Notably, the clearest reciprocal correlations of Kyn and Trp with CSF cell count were seen in the three viral CNS infections, whereas these parameters did not correlate with cell count in bacterial meningitis (Fig. 3B left). Kyn and Trp concentrations were therefore plotted against cell count in this diagnosis only (Fig. 3B right). This analysis revealed several samples with relatively low cell counts but unusually high Kyn or Trp concentrations, which corresponded to the outliers identified in Fig. 3A (right). Thus, induction of Kyn synthesis is relatively uncoupled from CSF inflammation in a small subgroup of bacterial meningitis patients. As shown in Fig. 3C left, the correlations with lactate differed in that there was a positive correlation in bacterial meningitis and that there was a tendency ($r_{\text{Kyn}} = -0.58$ [$P = 0.1$] and $r_{\text{Trp}} = 0.59$ [$P = 0.09$]) toward an inversion of the correlation (now negative for Kyn and positive for Trp) in the case of HSE. The four samples with the highest Kyn values were more evenly distributed across the spectrum of lactate concentrations compared to leukocyte count (compare Fig. 3C right and 3B right). The correlations with the extent of blood-CSF-barrier dysfunction resembled those with lactate, particularly in that the inverted correlation in HSE was also observed (Fig. 3D left). However, only Kyn (but not Trp) correlated significantly with blood-CSF-barrier dysfunction in bacterial meningitis, and there were additional significant correlations with Kyn and Trp in facial zoster. A plot of Kyn concentrations vs. severity of blood-CSF-barrier dysfunction in bacterial meningitis supported the strong association between elevated Kyn concentrations and blood-CSF-barrier dysfunction and also revealed that the samples with high Kyn concentrations but low cell counts identified in Fig. 3B right fell into the group with the highest degree of blood-CSF-barrier dysfunction (Fig. 3D right, arrows). Correlations similar to those with lactate were observed with CSF protein (Fig. S2A left) and, to a lesser extent, with Q-albumin as continuous variable (Fig. S2A right).

There were no significant correlations with IgG index (Fig. S2B left). Of note, no significant correlations with any of the CSF parameters were detected in the autoimmune diagnoses anti-NMDA-R encephalitis and MS. Taken together, these results suggest that Kyn synthesis is strongly associated with various aspects of neuroinflammation in bacterial and viral CNS infections, that it is driven strongly by cellular infiltration in the CNS infection groups except bacterial meningitis, but that there also is a remarkable
absence of induction of the TK pathway in the autoimmune inflammatory disorders in spite of measurable CNS inflammation.

Kyn, Trp and Kyn/Trp ratio are biomarkers for the differentiation between viral CNS infection, neuroborreliosis and autoimmune neuroinflammation

Considering the strong association with neuroinflammation revealed above, ROC analysis was applied to evaluate Kyn, Trp, and Kyn/Trp ratio as biomarkers for the major categories of diagnoses, i.e. bacterial/viral CNS infections, autoimmune inflammation, and non-inflamed controls. Of the three metabolite parameters, Kyn demonstrated highest discriminatory potential for the distinction between bacterial or viral infection vs. the two non-infected groups (Fig. 4A, left, lower triangle in the grid), but was somewhat inferior to CSF cell count (Fig. 4A right, upper triangle). However, evaluating the metabolite markers in mixed models with cell count (Fig. 4B left, lower triangle) showed that the combination of Kyn and Trp greatly improved differentiation between viral infections and autoimmune inflammation, as evidenced by an AUC increase from 0.74 to 0.95, signifying nearly perfect differentiation. The diagnostic information gained in terms of sensitivity, specificity, and positive and negative predictive value for the differentiation between viral CNS infections and the autoimmune inflammatory diseases is summarized in Table 2. Addition of lactate led to only marginal further improvement.

In order to quantify biomarker potential for the differentiation between specific diagnoses, the same analysis was then applied to all possible paired comparisons within the 12 diagnoses (Fig. 5). Again, Kyn was a substantially more accurate classifier than Trp as measured by the higher number of significant AUCs and higher mean AUCs (Fig. 5C), and it performed best for the distinctions between infected and non-infected diagnoses (Fig. 5A left, lower triangle). Kyn/Trp ratio was essentially as accurate as Kyn alone (Fig. 5C), but performed better for several comparisons of VZV meningitis/encephalitis vs. autoimmune and non-inflamed diagnoses (Fig. 5A right, lower triangle). As in the group-wise comparisons shown in Fig. 4, leukocyte count was the best single classifier (Fig. 5C; Fig. 5A right, upper triangle). However, the mixed model consisting of metabolites and cell count led to a remarkable increase in classification performance compared to leukocyte count alone, both in the total number of significant AUCs (62 of the 66 possible comparisons, 94%) and mean AUCs (Fig. 5C). Now there were significant AUCs of >0.83 for all the distinctions between the CNS infections and anti-NMDA-R encephalitis or MS (Fig. 5B left). Remarkably, there now was perfect discrimination (AUC, 1.0) between neuroborreliosis and each of the three viral CNS infections. Other clinically relevant comparisons with
high AUCs were: HSE vs. anti-NMDA-R encephalitis (1.0), facial zoster vs. Bell’s palsy (0.94), and anti-NMDA-R encephalitis vs. MS (1.0). Addition of lactate to the model led to further improvement in the differentiation between bacterial meningitis and the other CNS infections. The lowest AUC (bacterial meningitis vs. VZV meningitis/encephalitis) was now 0.91, and even for the distinction between bacterial meningitis and neuroborreliosis it was 0.93 (Fig. 5B left, upper triangle). Of note, lactate alone was selected as the best classifier in these latter comparisons.

**Discussion**

This study provides the first comprehensive analysis of Kyn and Trp concentrations in CSF from patients with a broad spectrum of infectious, autoimmune inflammatory, and non-inflammatory CNS disorders. The results provide first evidence from analysis of human CSF samples that there is a major induction of the TK pathway in CNS infections, regardless of bacterial or viral etiology, and that there are strong correlations with the standard parameters of neuroinflammation and blood-CSF-barrier dysfunction. Since our study was not designed to assess clinical outcomes, it remains to be studied to what extent induction of the TK pathway contributes to the balance between cytoprotective, cytotoxic and neuromodulatory processes in human CNS infections. Work in a mouse model of pneumococcal meningoencephalitis has suggested that the net effect of this pathway in this model is protective, presumably due to increased generation of NAD+ [16]. Future studies should be geared toward measuring all key products of the pathway in CSF in order to gauge the balance between protective and deleterious effector molecules. Conceivably, the respective key enzymes are regulated differentially depending on etiology or in patient subgroups with different clinical presentations or outcomes. Surprisingly, we did not observe any increase in Kyn or Trp/Kyn ratio in MS, even though most samples were obtained from patients with active disease. This is in clear contrast to extensive work conducted in the experimental autoimmune encephalitis mouse model of MS, which has documented induction of this pathway [13]. It is unclear which difference between this model and the human disease is responsible for this discrepancy. Nonetheless, it is tempting to speculate that lack of IDO induction in MS may constitute a factor contributing to the immune dysregulation underlying this disease, as IDO activity in dendritic cells can inhibit lymphocyte function [25]. In contrast to Rickards et al. [4], who reported activation of the TK pathway in peripheral blood from patients with Tourette syndrome, we did not find any evidence of activation of the pathway in CSF, suggesting that its activation in Tourette syndrome is mostly limited to the peripheral immune system.
The rise in Kyn/Trp ratio was most pronounced in the three viral CNS infections, providing the clearest evidence that increased Kyn concentrations in these disorders are due to induction of IDO, the key enzyme in this pathway at extrahepatic locations. Curiously, the expected increase in Kyn/Trp ratio was not universally seen in bacterial meningitis, mostly because of unexpectedly high Trp concentrations in a subgroup of samples with elevated Kyn concentrations. This curious observation might be explained by differences in intracerebral or systemic synthesis of Trp, differences in its transport across the blood-CSF-barrier, release of Trp from damaged cells, or even release from pathogenic bacteria.

In terms of diagnostic biomarker potential, Kyn, Trp or Kyn/Trp ratio were inadequate markers for the distinction between bacterial and viral CNS infections. However, they did demonstrate good discriminatory value for the clinically important distinction between infectious and noninfectious etiologies of neuroinflammation, in particular between viral infections and non-infectious etiologies (anti-NMDA-R encephalitis and MS). Remarkable discrimination was found between the clinically potentially similar HSE and anti-NMDA-R encephalitis. Clearly, this intriguing observation needs to be verified in analyses of additional samples.

As in our previous study of metabolite biomarkers for VZV reactivation [19], we found that combining metabolite biomarkers with standard indices of neuroinflammation in logistic regression analysis led to further improvement of diagnostic accuracy for several clinically important distinctions. In the present study, this was most pronounced for the differentiation between neuroborreliosis and viral CNS infections, and between the viral CNS infections and the autoimmune inflammatory disorders. This is clinically highly relevant because it may be difficult to differentiate between neuroborreliosis and viral CNS infections or, for instance, HSE and anti-NMDA-R encephalitis, based on clinical examination and standard CSF parameters. Moreover, depending on the laboratory infrastructure, turn-around time to definitive diagnostics (e.g., pathogen PCR, anti-NMDA-R serology) may be several days. The pronounced diagnostic synergy is best explained by the notion that in patients where CSF cell count is less elevated than expected, increased Kyn concentration can be used as an additional marker to correctly identify samples that would be misclassified based on cell count alone, and vice versa. [in particular in those samples where Kyn was <LOD in both groups compared]. Further studies should be directed at validating the diagnostic utility of such biomarker combinations. Considering that simple immunoassays for the detection of Kyn [26] and Trp [27] are commercially available, it would be technically feasible to implement two-step diagnostics based on standard parameters and Kyn and/or Trp concentrations in routine clinical laboratory practice. Future studies should be directed at obtaining additional data in larger cohorts that can be used to implement biomarker combinations and devise diagnostic algorithms incorporating these parameters.
The lack of Kyn elevation in Bell’s palsy, compared to facial nerve zoster, lends further support to the non-inflammatory etiology of Bell’s palsy and suggests that Kyn could potentially serve as an additional biomarker to distinguish between these clinically very similar disorders. The incremental increase of Kyn concentrations from segmental zoster, over facial nerve zoster to VZV meningitis/encephalitis is consistent with our previous findings that the degree of metabolic reprogramming in CSF in VZV reactivation increases with the degree of neuroinflammation and proximity to/involvement of the CNS [19].

This study is limited by the lack of clinical follow-up information about the patients, thus not allowing to assess changes in Kyn and Trp concentrations as prognostic tools. Some groups were small, and even though statistical significance was supported by bootstrapping and internal cross-validations, an external validation with additional samples would greatly strengthen the data. Major strengths of the study are (1) that this is the first study of Kyn/Trp regulation in CNS infections featuring human samples with diagnoses verified by pathogen detection and comparisons with a spectrum of clinically important disease controls and (2) that all samples were collected prospectively according to unified SOPs.
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Conflict of interest: The authors declare that they have no conflict of interest relating to conduct of the study or publication of the manuscript.

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Figure legends

Figure 1. Detection efficiency and differences in Kyn and Trp concentrations across the 12 diagnostic groups. A left, Percentage of samples per diagnosis in which Kyn or Trp concentrations were ≥LOD. A and B - Differences in Kyn (A right) and Trp (B left) concentrations and Kyn/Trp ratio (B right) across the diagnostic groups. The boxes correspond to interquartile distance, bottom and top whiskers to 10th and 90th percentile, respectively. P values (FDR corrected) were obtained by Kruskal-Wallis analysis. A graph including all outliers is shown in Fig. S1. Median values (min.-max.) of Kyn, Trp concentrations and Kyn/Trp ratio in all 12 diagnostic groups are listed in Table S2. Abbreviations: Bell’s - idiopathic Bell’s palsy; EntM - enteroviral meningitis, GTS – Tourette syndrome; HSE - HSV encephalitis; MS - multiple sclerosis; NMDA – anti-NMDA-receptor encephalitis; NPH - normal pressure hydrocephalus; VZV fac - facial nerve zoster; VZV ME – VZV meningitis/encephalitis; VZV seg - segmental zoster.

Figure 2. Kyn and Trp concentrations are differentially associated with neuroinflammation. A-B, Box plots and ROC curves based on Kyn (A left) and Trp (A right) concentrations in CSF and Kyn/Trp ratio (B) in inflamed (CSF leukocyte count, ≥5/mm³, n=121) and non-inflamed (n=99) samples. All differences of medians were highly significant (p<0.001, Mann-Whitney U test). Dark blue horizontal lines, medians; red dots, means; blue shaded areas of ROC curves = upper and lower CI. AUC values (lower and upper CI) are shown under each ROC curve.

Figure 3. Correlations of Kyn, Trp and Kyn/Trp ratio with parameters of neuroinflammation. Red circles, Spearman correlation ρ with p <0.05; unfilled circles, p>0.05. A left, Correlations with six CSF parameters and blood CRP across all samples (n=220). A right, Correlations of Kyn and Trp with leukocyte count across all samples. The red rectangles define the space outside of which only bacterial meningitis values are found. B left, Correlations with leukocyte count within each diagnosis. B right, Correlations with leukocyte count in bacterial meningitis. C left, Correlations with lactate within each diagnosis. C right, Correlations with lactate in bacterial meningitis. D left, Correlations with blood-CSF-barrier dysfunction within each diagnosis. D right, Kyn concentrations in bacterial meningitis depending on the degree of blood-CSF-barrier dysfunction (black horizontal lines, median; red symbols, mean). Abbreviations: Bell’s - idiopathic Bell’s palsy; BCB - blood-CSF-barrier dysfunction, based on age-adjusted Q-albumin as defined in Table 1 (none = 0, mild = 1, moderate = 2, severe = 3); EntM - enteroviral meningitis, GTS – Tourette syndrome; HSE - HSV encephalitis; MS - multiple sclerosis; NMDA – anti-NMDA-receptor encephalitis;
NPH - normal pressure hydrocephalus; VZV fac - facial nerve zoster; VZV ME – VZV meningitis/encephalitis; VZV seg - segmental zoster.

**Figure 4.** Diagnostic biomarker performance of Kyn, Trp and Kyn/Trp ratio across the 4 major diagnostic groups. Values correspond to significant AUCs (binary ROC analysis) as defined by p<0.05 and lower CI >0.5. A left, Kyn (lower triangle) and Trp (upper). A right, Trp/Kyn ratio (lower) and leukocyte count (upper). B left, Mixed model consisting of Kyn, Trp, Kyn/Trp ratio and leukocyte count (lower) and the same model plus lactate (upper). The following merged diagnostic groups were assessed: bacterial CNS infections (BacM, Borrelia, n=66), viral CNS infections (HSE, VZV ME, EntM, n=100); autoimmune neuroinflammation; NMDA, MS; n=25); non-inflamed controls (Bell’s, Tourette, NPH; n=66). B right, Display of the best combinations of biomarkers identified in B left. Color scheme for selected best type of marker: green - leukocytes or lactate; blue - leukocytes, lactate plus Kyn, Trp, Kyn/Trp ratio; orange, - metabolites. Abbreviations: L – leukocytes; A - lactate; K – Kyn; T – Trp; R – Kyn/Trp ratio. Bell’s - idiopathic Bell’s palsy; EntM - enteroviral meningitis, GTS – Tourette syndrome; HSE - HSV encephalitis; MS - multiple sclerosis; NMDA - anti-NMDA-receptor encephalitis; NPH - normal pressure hydrocephalus; VZV fac - facial nerve zoster; VZV ME – VZV meningitis/encephalitis; VZV seg - segmental zoster.

**Figure 5.** Biomarker performance of Kyn, Trp and Kyn/Trp ratio across the 12 individual diagnostic groups. Values correspond to significant AUCs (binary ROC analysis) as defined by p<0.05 and lower CI >0.5. A left, Kyn (lower triangle) and Trp (upper). A right, Trp/Kyn ratio (lower) and leukocyte count (upper). B left, Mixed model consisting of Kyn, Trp, Kyn/Trp ratio and leukocyte count (lower) and the same mixed model plus lactate (upper). B right, Display of the classifiers identified in C. C left, Number of significant AUCs for each classifier in the 66 possible paired comparisons. C right, Mean values of all AUCs (grey bars) and significant AUCs only (cross-hatched bars). Color scheme for selected best type of marker: green - leukocytes and/or lactate; blue - leukocytes and/or lactate plus Kyn, Trp, Kyn/Trp ratio; orange, - metabolite(s). Abbreviations: L – leukocytes; A - lactate; K – Kyn; T – Trp; R – Kyn/Trp ratio. Bell’s - idiopathic Bell’s palsy; EntM - enteroviral meningitis, GTS – Tourette syndrome; HSE - HSV encephalitis; MS - multiple sclerosis; NMDA – anti-NMDA-receptor encephalitis; NPH - normal pressure hydrocephalus; VZV fac - facial nerve zoster; VZV ME – VZV meningitis/encephalitis; VZV seg - segmental zoster.
Supplemental figure legends

Figure S1. Differences in Kyn and Trp concentrations across the 12 diagnostic groups. Same data as shown in Fig. 1, but y-axes are scaled so that all outlying values can be shown. A left, Kyn; A right, Trp; B, Kyn/Trp ratio.

Figure S2. Correlations of Kyn, Trp and Kyn/Trp ratio with those parameters of (neuro)inflammation that are not included in Fig. 3. Red circles, Spearman correlation $\rho$ with $p <0.05$; unfilled circles, $p >0.05$. A left, CSF protein; A right, Q-albumin; B left, IgG index; B right, peripheral blood CRP.

Figure S3. Diagnostic biomarker performance of Kyn, Trp and Kyn/Trp ratio across the 4 major diagnostic groups (bacterial CNS infections, viral CNS infections, autoimmune neuroinflammation, non-inflamed controls). A, Number of significant AUCs for each classifier in the 6 possible paired comparisons. B, Mean values of all AUCs (grey bars) and significant AUCs only (cross-hatched bars).

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Kynurenine is a cerebrospinal fluid biomarker for bacterial and viral CNS infections

Running title: Kynurenine as CSF biomarker

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Importance
This study of kynurenine and tryptophan concentrations in human CSF revealed marked induction of the kynurenine-tryptophan pathway in bacterial and viral meningitis/encephalitis. It highlights these metabolites as accurate biomarkers, particularly to differentiate among neuroborreliosis, viral meningitis/encephalitis and autoimmune neuroinflammation.
Abstract

Background. The tryptophan-kynurenine-NAD+ pathway is closely associated with regulation of immune cells toward less inflammatory phenotypes and may exert neuroprotective effects. Investigating its regulation in CNS infections would improve our understanding of pathophysiology and end-organ damage, and, furthermore, open doors to its evaluation as a source of diagnostic and/or prognostic biomarkers.

Methods. We measured concentrations of kynurenine (Kyn) and tryptophan (Trp) in 220 cerebrospinal fluid samples from patients with bacterial and viral (herpes simplex, varicella zoster, enteroviruses) meningitis/encephalitis, neuroborreliosis, autoimmune neuroinflammation (anti-NMDA-R encephalitis, multiple sclerosis), and noninflamed controls (Bell’s palsy, normal pressure hydrocephalus, Tourette syndrome).

Results. Kyn concentrations correlated strongly with CSF markers of neuroinflammation (leukocyte count, lactate, and blood-CSF-barrier dysfunction) and were highly increased in bacterial and viral CNS infections, but were low or undetectable in anti-NMDA-R encephalitis, multiple sclerosis, and controls. Trp was decreased mostly in viral CNS infections and neuroborreliosis. Multiple logistic regression analysis revealed combinations of Kyn, Trp and Kyn/Trp ratio with leukocyte count or lactate as accurate classifiers for the clinically important differentiation between neuroborreliosis, viral CNS infections, and autoimmune neuroinflammation.

Conclusions. The Trp-Kyn-NAD+ pathway is activated in CNS infections and provides highly accurate CSF biomarkers, particularly when combined with standard CSF indices of neuroinflammation.

Key words
Biomarkers, Borrelia, central nervous system, diagnosis, infection, kynurenine, metabolites, tryptophan

Abbreviations
AUC, area under the ROC curve; BacM – bacterial meningitis; Bell’s - idiopathic Bell’s palsy; BCB – blood-CSF-barrier; CI, confidence interval; CSF, cerebrospinal fluid; GTS – Gilles de la Tourette syndrome; EntM - enteroviral meningitis, HSE - HSV encephalitis; IDO - indoleamine-2,3-dioxygenase; Kyn, kynurenine; LOD, limit of detection; MS - multiple sclerosis; NMDA – anti-NMDA-receptor encephalitis; NPH - normal pressure hydrocephalus; ROC, receiver operating characteristic; TK pathway - tryptophan-kynurenine-NAD+ pathway; Trp, tryptophan; VZV fac - facial nerve zoster; VZV ME – VZV meningitis/encephalitis; VZV seg - segmental zoster (shingles).
Introduction

The tryptophan-kynurenine-NAD+ pathway (TK pathway) is the major pathway for catabolism of endogenous tryptophan (Trp). Its functions go far beyond maintaining Trp homeostasis, as its intermediates exert extensively documented immunomodulatory, neuromodulatory, and both cytoprotective and cytotoxic effects (reviewed in [1, 2]). The enzyme Trp-2,3-dioxygenase regulates the hepatic TK pathway, whereas indoleamine-2,3-dioxygenase (IDO) is the rate-limiting enzyme in kynurenine (Kyn) synthesis at extrahepatic sites including immune cells and the central nervous system (CNS) [1]. Increased IDO activity and subsequent Kyn synthesis are tightly associated with modulation of immune cell function, particularly suppression of activity of macrophages, dendritic cells, and T cells [3]. Dysregulation of the pathway has been implicated in a surprisingly broad variety of CNS disorders, ranging from Tourette syndrome [4] to major depression [5], schizophrenia [6], Alzheimer’s disease [7, 8], Parkinson’s disease [2, 9], Huntington’s disease [10], amyotrophic lateral sclerosis [11], and multiple sclerosis (MS) [6, 12, 13]. In these CNS disorders, it is most plausible that the pathway is activated by inflammation-related processes and modulates pathogenesis by the balance between deleterious (e.g., anthranilic and quinolinic acid, 3-OH-L-Kyn) and protective (kynurenic acid) intermediates, immunosuppressive effects, and the generation of NAD+ for energy metabolism and vitamin B6 synthesis. Of note, modulators of key enzymes in the pathway as well as analogs of TK pathway intermediates have become available, and evidence from animal models has demonstrated their potential to improve outcome of diseases characterized by increased activity of the pathway (MS – reviewed in [13], Huntington’s disease – [10]).

Preliminary evidence from studies in mice [14] and a small human cohort of patients with septic and aseptic meningitis [15] suggested that Kyn concentrations in CNS increased during bacterial meningitis and that inhibiting two key enzymes of the TK pathway exacerbated experimental pneumococcal meningitis in mice [16], suggesting that this pathway exerts a net neuroprotective effect at least in this model. Therefore, investigations into its regulation and relative activity in CNS infections would improve our understanding of pathophysiology and end-organ damage in CNS infections. Moreover, it is conceivable that altered concentrations of TK pathway metabolites might differ among different diseases greatly enough to be used as diagnostic biomarkers, for instance (as suggested by the preliminary results from Coutinho et al. [15]) for the differentiation between septic and aseptic meningitis.

We have therefore performed a detailed analysis of Kyn, Trp, and the Kyn/Trp ratio (as a measure of IDO activity) in CSF samples from patients with bacterial and viral CNS infections, autoimmune neuroinflammatory diseases, and non-inflamed neuropathologies, in order to assess differences in the
induction of the pathway among these disorders and to assess the potential of these three parameters as diagnostic CSF biomarker.

**Study population, materials and methods**

**Study design and population**

The samples and clinical data were obtained at Hannover Medical School during routine lumbar puncture. All samples were processed and stored according to unified standard operating procedures [17]. Briefly, after removing the volume necessary for clinical diagnostics, CSF was centrifuged and cell-free supernatant was frozen at -80°C until analysis. The study was approved by the Ethics Committee of Hannover Medical School (file no. 2413-2014) and was conducted according to the Helsinki Declaration. Samples from patients with the following diagnoses were analyzed: bacterial meningitis (abbreviated BacM), neuroborreliosis (Borrelia), herpes simplex virus meningitis/encephalitis (HSE), varicella zoster virus (VZV) meningitis/encephalitis (VZV ME), enteroviral meningitis (EntM), facial nerve zoster (VZV fac), segmental zoster (VZV seg; also known as shingles), anti-N-methyl-D-aspartate receptor encephalitis (NMDA), multiple sclerosis (MS), Bell's palsy (Bell's), and normal pressure hydrocephalus (NPH).

Diagnostic criteria and basic data on disease activity and treatments are summarized in Table S1. The following standard CSF parameters were recorded: leukocyte count, lactate concentration, protein concentration, IgG index, and Q-albumin (CSF/serum albumin ratio). Blood-CSF-barrier dysfunction was scored from 0 (no dysfunction) to 3 (severe dysfunction) using age-corrected Q-albumin [18]. Peripheral blood samples were obtained at the time of lumbar puncture and analyzed the same day in the in-house diagnostic laboratories for the clinically indicated parameters, including complete blood count with differential and C-reactive protein (CRP) concentrations.

**Mass spectrometry**

CSF concentrations of Trp and Kyn were measured as part of a targeted metabolomic screen using the Absolute IDQ® p180 kit (Biocrates Life Sciences AG, Innsbruck, Austria) and liquid chromatography-tandem-mass spectrometry (LC-MS/MS), as described previously [19]. The limits of detection (LOD) for Kyn and Trp (defined as 3x the signal measured with the blank) were found to be 0.15 and 0.25 μM. All values <LOD were replaced by the pseudo value of LOD/2, as recommended by the manufacturer [20]. A separate analysis based on a subset of 88 of the 188 analytes measurable with the p180 kit, but not including Kyn and focusing on CSF biomarkers for varicella zoster virus (VZV) reactivation, has been published separately [19].

**Statistical analysis**
The Mann-Whitney U test was used to compare differences in median values between two groups. Significance of concentration differences across all sample groups was determined with Kruskal-Wallis analysis with corrections for multiple hypothesis testing. The chi-square test ($\chi^2$-test) was used to analyze the difference between category frequencies. Significance of differences was defined as a $P$ value of <0.05 unless stated otherwise. Spearman correlation was used to assess correlations between metabolite parameters (Kyn, Trp, Kyn/Trp ratio) and the standard CSF and blood parameters measured. Receiver operating characteristics (ROC) curve analysis was used to quantify biomarker potential. Significance of areas under the ROC curve (AUCs) was defined by asymptotic $P$ values of <0.05 and lower bound confidence intervals (CI; obtained by 1000 bootstraps) not crossing below 0.5. The leave-one-out (jackknife) method together with logistic regression (forward selection procedure) was used to select the best combination (subset) of metabolite and standard CSF parameters for each diagnostic pair and to assess its prediction potential [21]. The jackknife method was applied to 1000 bootstrap samples in order to estimate the CI of the AUC for the final classifier (i.e. subset of the original input of five parameters).

All statistical analyses were performed with the ‘stats’, ‘Biocomb’ [22] and ‘agricolae’ packages of the R Foundation for Statistical Computing (version 3.4.4; from here on referred to as “R”) [23].

Results

Description of the study population

Table 1 summarizes demographic data and results of blood and standard CSF parameters. All results were consistent with known information about the natural history of the diagnoses studied. In particular, CSF leukocyte count, lactate, protein, and blood-CSF-barrier dysfunction were highest in bacterial meningitis, followed by neuroborreliosis, HSE, and VZV meningitis/encephalitis. All standard parameters were essentially normal in NPH, Tourette syndrome, and Bell’s palsy, thus supporting their use as non-inflammatory controls. Causative pathogens in bacterial meningitis corresponded to the clinical spectrum expected in a tertiary care center in central Europe (summarized in Table S1).

Differences in Kyn and Trp concentrations across the diagnostic groups

Overall, Kyn concentrations were <LOD in many of the non-inflamed and less inflamed samples, but this metabolite was detected >LOD with increasing frequency as the degree of inflammation increased, and the percentage of samples with concentrations >LOD was highest in bacterial meningitis, HSE, and neuroborreliosis (Fig. 1A left). Measured Kyn concentrations increased following a similar pattern (Fig. 1A right) and were markedly increased in the infected groups, with mean values varying between 3.7–fold (enterovirus meningitis) and 24-fold (bacterial meningitis) higher than in the non-inflamed diagnoses.
There were two noteworthy observations: (1) bacterial meningitis, being the clearly most highly inflamed disease, had median Kyn concentrations similar to neuroborreliosis, HSE, and VZV meningitis/encephalitis, whereas mean Kyn concentrations were highest in bacterial meningitis due to outliers with extremely high values (shown in Fig. S1A); (2) in spite of the well-documented inflammatory nature of MS [24] and elevated indices of inflammation (cell count, IgG index, each \( P < 0.001 \) with respect to NPH; Table 1), Kyn concentrations were <LOD in nearly all MS samples, even though most samples had been obtained from patients with active disease during relapse (Table S1). There was a tendency of Trp concentrations to decrease in the non-bacterial etiologies with increasing inflammation, but this trend was not observed in bacterial meningitis and neuroborreliosis (Fig. 1B left). The Kyn/Trp ratio was highest in the five CNS infections, although differences compared to the non-infected groups were not as pronounced as in the case of Kyn, and among these five diagnoses its median value was lowest in bacterial meningitis (Fig. 1B right). Incremental increases in Kyn concentration, accompanied by decreases in Trp concentration and increases in Kyn/Trp ratio, were seen in the three clinical forms of VZV reactivation in the order segmental zoster < facial zoster < VZV meningitis/encephalitis, i.e. along increasing neuroinflammation and involvement of the CNS.

**Kyn and Trp concentrations correlate differentially with the extent of neuroinflammation**

We then assessed the association of increased Kyn and Trp concentrations with CSF inflammation (≥5 cells/mm\(^3\)) (Fig. 2). Kyn concentrations were markedly (median = 5.6-fold, mean = 9.4-fold) and highly significantly elevated in the inflamed (infected and non-infected) compared to the non-infamed samples, and in ROC analysis Kyn concentration distinguished accurately between the inflamed and non-inflamed samples (Fig. 2A left). Trp concentrations were significantly lower in the inflamed samples, but the differences were much smaller compared to Kyn, and Trp discriminated less accurately between inflamed and non-inflamed samples (Fig. 2A right). Thus, increased Kyn concentrations were significantly more strongly associated with CSF inflammation than decreased Trp concentrations. Nonetheless, the association of the Kyn/Trp ratio with CNS inflammation was similar to that of Kyn concentrations alone (Fig. 2B).

To test whether there was a preferential association between the Kyn-Trp parameters and any of the standard diagnostic CSF parameters, we then performed a correlation analysis between the three metabolite parameters and the six standard CSF indices, as well as peripheral blood C-reactive protein (CRP) concentration (Fig. 3 and Fig. S2). Kyn correlated most positively with CSF leukocyte count (\( \rho = 0.76, P = 4.9^{-43} \)), but also to a slightly lesser extent with lactate, protein, and blood-CSF-barrier dysfunction, whereas only weak (albeit significant) correlations were detected with IgG index and blood CRP (Fig. 3A...
left). Of note, this strong correlation with leukocyte count existed even though the highest Kyn concentrations were measured in a small number of samples with comparatively low leukocyte counts (Fig. 3A right). For Trp, only a weak negative correlation with cell count ($p=-0.30$, $P=6.2 \times 10^{-6}$) and a weak positive correlation with blood CRP were observed (Fig 3A left). Consistent with this, Kyn/Trp ratio correlations with these parameters were less pronounced than Kyn correlations.

When the same correlations were performed with the individual diagnostic groups, major differences became apparent (Fig. 3B - D left, Fig. S2). Notably, the clearest reciprocal correlations of Kyn and Trp with CSF cell count were seen in the three viral CNS infections, whereas these parameters did not correlate with cell count in bacterial meningitis (Fig. 3B left). Kyn and Trp concentrations were therefore plotted against cell count in this diagnosis only (Fig. 3B right). This analysis revealed several samples with relatively low cell counts but unusually high Kyn or Trp concentrations, which corresponded to the outliers identified in Fig. 3A (right). Thus, induction of Kyn synthesis is relatively uncoupled from CSF inflammation in a small subgroup of bacterial meningitis patients. As shown in Fig. 3C left, the correlations with lactate differed in that there was a positive correlation in bacterial meningitis and that there was a tendency ($\rho_{\text{Kyn}}=-0.58$ [P=0.1] and $\rho_{\text{Trp}}=0.59$ [P=0.09]) toward an inversion of the correlation (now negative for Kyn and positive for Trp) in the case of HSE. The four samples with the highest Kyn values were more evenly distributed across the spectrum of lactate concentrations compared to leukocyte count (compare Fig. 3C right and 3B right). The correlations with the extent of blood-CSF-barrier dysfunction resembled those with lactate, particularly in that the inverted correlation in HSE was also observed (Fig. 3D left). However, only Kyn (but not Trp) correlated significantly with blood-CSF-barrier dysfunction in bacterial meningitis, and there were additional significant correlations with Kyn and Trp in facial zoster. A plot of Kyn concentrations vs. severity of blood-CSF-barrier dysfunction in bacterial meningitis supported the strong association between elevated Kyn concentrations and blood-CSF-barrier dysfunction and also revealed that the samples with high Kyn concentrations but low cell counts identified in Fig. 3B right fell into the group with the highest degree of blood-CSF-barrier dysfunction (Fig. 3D right, arrows). Correlations similar to those with lactate were observed with CSF protein (Fig. S2A left) and, to a lesser extent, with Q-albumin as continuous variable (Fig. S2A right).

There were no significant correlations with IgG index (Fig. S2B left). Of note, no significant correlations with any of the CSF parameters were detected in the autoimmune diagnoses anti-NMDA-R encephalitis and MS. Taken together, these results suggest that Kyn synthesis is strongly associated with various aspects of neuroinflammation in bacterial and viral CNS infections, that it is driven strongly by cellular infiltration in the CNS infection groups except bacterial meningitis, but that there also is a remarkable
absence of induction of the TK pathway in the autoimmune inflammatory disorders in spite of measurable CNS inflammation.

Kyn, Trp and Kyn/Trp ratio are biomarkers for the differentiation between viral CNS infection, neuroborreliosis and autoimmune neuroinflammation

Considering the strong association with neuroinflammation revealed above, ROC analysis was applied to evaluate Kyn, Trp, and Kyn/Trp ratio as biomarkers for the major categories of diagnoses, i.e. bacterial/viral CNS infections, autoimmune inflammation, and non-inflamed controls. Of the three metabolite parameters, Kyn demonstrated highest discriminatory potential for the distinction between bacterial or viral infection vs. the two non-infected groups (Fig. 4A, left, lower triangle in the grid), but was somewhat inferior to CSF cell count (Fig. 4A right, upper triangle). However, evaluating the metabolite markers in mixed models with cell count (Fig. 4B left, lower triangle) showed that the combination of Kyn and Trp greatly improved differentiation between viral infections and autoimmune inflammation, as evidenced by an AUC increase from 0.74 to 0.95, signifying nearly perfect differentiation. The diagnostic information gained in terms of sensitivity, specificity, and positive and negative predictive value for the differentiation between viral CNS infections and the autoimmune inflammatory diseases is summarized in Table 2. Addition of lactate led to only marginal further improvement.

In order to quantify biomarker potential for the differentiation between specific diagnoses, the same analysis was then applied to all possible paired comparisons within the 12 diagnoses (Fig. 5). Again, Kyn was a substantially more accurate classifier than Trp as measured by the higher number of significant AUCs and higher mean AUCs (Fig. 5C), and it performed best for the distinctions between infected and non-infected diagnoses (Fig. 5A left, lower triangle). Kyn/Trp ratio was essentially as accurate as Kyn alone (Fig. 5C), but performed better for several comparisons of VZV meningitis/encephalitis vs. autoimmune and non-inflamed diagnoses (Fig. 5A right, lower triangle). As in the group-wise comparisons shown in Fig. 4, leukocyte count was the best single classifier (Fig. 5C; Fig. 5A right, upper triangle). However, the mixed model consisting of metabolites and cell count led to a remarkable increase in classification performance compared to leukocyte count alone, both in the total number of significant AUCs (62 of the 66 possible comparisons, 94%) and mean AUCs (Fig. 5C). Now there were significant AUCs of >0.83 for all the distinctions between the CNS infections and anti-NMDA-R encephalitis or MS (Fig. 5B left). Remarkably, there now was perfect discrimination (AUC, 1.0) between neuroborreliosis and each of the three viral CNS infections. Other clinically relevant comparisons with
high AUCs were: HSE vs. anti-NMDA-R encephalitis (1.0), facial zoster vs. Bell’s palsy (0.94), and anti-NMDA-R encephalitis vs. MS (1.0). Addition of lactate to the model led to further improvement in the differentiation between bacterial meningitis and the other CNS infections. The lowest AUC (bacterial meningitis vs. VZV meningitis/encephalitis) was now 0.91, and even for the distinction between bacterial meningitis and neuroborreliosis it was 0.93 (Fig. 5B left, upper triangle). Of note, lactate alone was selected as the best classifier in these latter comparisons.

**Discussion**

This study provides the first comprehensive analysis of Kyn and Trp concentrations in CSF from patients with a broad spectrum of infectious, autoimmune inflammatory, and non-inflammatory CNS disorders. The results provide first evidence from analysis of human CSF samples that there is a major induction of the TK pathway in CNS infections, regardless of bacterial or viral etiology, and that there are strong correlations with the standard parameters of neuroinflammation and blood-CSF-barrier dysfunction. Since our study was not designed to assess clinical outcomes, it remains to be studied to what extent induction of the TK pathway contributes to the balance between cytoprotective, cytotoxic and neuromodulatory processes in human CNS infections. Work in a mouse model of pneumococcal meningoencephalitis has suggested that the net effect of this pathway in this model is protective, presumably due to increased generation of NAD+ [16]. Future studies should be geared toward measuring all key products of the pathway in CSF in order to gauge the balance between protective and deleterious effector molecules. Conceivably, the respective key enzymes are regulated differentially depending on etiology or in patient subgroups with different clinical presentations or outcomes.

Surprisingly, we did not observe any increase in Kyn or Trp/Kyn ratio in MS, even though most samples were obtained from patients with active disease. This is in contrast to extensive work conducted in the experimental autoimmune encephalitis mouse model of MS, which has documented induction of this pathway [13]. It is unclear which difference between this model and the human disease is responsible for this discrepancy. Nonetheless, it is tempting to speculate that lack of IDO induction in MS may constitute a factor contributing to the immune dysregulation underlying this disease, as IDO activity in dendritic cells can inhibit lymphocyte function [25]. In contrast to Rickards et al. [4], who reported activation of the TK pathway in peripheral blood from patients with Tourette syndrome, we did not find any evidence of activation of the pathway in CSF, suggesting that its activation in Tourette syndrome is mostly limited to the peripheral immune system.
The rise in Kyn/Trp ratio was most pronounced in the three viral CNS infections, providing the clearest evidence that increased Kyn concentrations in these disorders are due to induction of IDO, the key enzyme in this pathway at extrahepatic locations. Curiously, the expected increase in Kyn/Trp ratio was not universally seen in bacterial meningitis, mostly because of unexpectedly high Trp concentrations in a subgroup of samples with elevated Kyn concentrations. This might be explained by differences in intracerebral or systemic synthesis of Trp, differences in its transport across the blood-CSF-barrier, release of Trp from damaged cells, or even release from pathogenic bacteria.

As in our previous study of metabolite biomarkers for VZV reactivation [19], we found that combining metabolite biomarkers with standard indices of neuroinflammation in logistic regression analysis led to further improvement of diagnostic accuracy. In the present study, this was most pronounced for the differentiation between neuroborreliosis, viral CNS infections, and the autoimmune inflammatory disorders. This is clinically highly relevant because it may be difficult to differentiate between neuroborreliosis and viral CNS infections or, for instance, HSE and anti-NMDA-R encephalitis, based on clinical examination and standard CSF parameters. Moreover, depending on the laboratory infrastructure, turn-around time to definitive diagnostics (e.g., pathogen PCR, anti-NMDA-R serology) may be several days. The pronounced diagnostic synergy is best explained by the notion that in patients where CSF cell count is less elevated than expected, increased Kyn concentration can be used as an additional marker to correctly identify samples that would be misclassified based on cell count alone, and vice versa. Considering that simple immunoassays for the detection of Kyn [26] and Trp [27] are commercially available, it would be technically feasible to implement two-step diagnostics based on standard parameters and Kyn and/or Trp concentrations in routine clinical laboratory practice. Future studies should be directed at obtaining additional data in larger cohorts that can be used to implement biomarker combinations and devise diagnostic algorithms incorporating these parameters.

The lack of Kyn elevation in Bell’s palsy, compared to facial nerve zoster, lends further support to the non-inflammatory etiology of Bell’s palsy and suggests that Kyn could potentially serve as an additional biomarker to distinguish between these clinically very similar disorders. The incremental increase of Kyn concentrations from segmental zoster, over facial nerve zoster to VZV meningitis/encephalitis is consistent with our previous findings that the degree of metabolic reprogramming in CSF in VZV reactivation increases with the degree of neuroinflammation and proximity to/involvement of the CNS [19].

This study is limited by the lack of clinical follow-up information, thus not allowing to assess changes in Kyn and Trp concentrations as prognostic tools. Some groups were small, and even though statistical significance was supported by bootstrapping and internal cross-validations, an external validation with
additional samples would greatly strengthen the data. Major strengths of the study are (1) that this is the first study of Kyn/Trp regulation in CNS infections featuring human samples with diagnoses verified by pathogen detection and comparisons with a spectrum of clinically important disease controls and (2) that all samples were collected prospectively according to unified SOPs.
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Figure legends

**Figure 1.** Detection efficiency and differences in Kyn and Trp concentrations across the 12 diagnostic groups. A left, Percentage of samples per diagnosis in which Kyn or Trp concentrations were ≥LOD. A and B - Differences in Kyn (A right) and Trp (B left) concentrations and Kyn/Trp ratio (B right) across the diagnostic groups. The boxes correspond to interquartile distance, bottom and top whiskers to 10th and 90th percentile, respectively. P values (FDR corrected) were obtained by Kruskal-Wallis analysis. A graph including all outliers is shown in Fig. S1. Median values (min.-max.) of Kyn, Trp concentrations and Kyn/Trp ratio in all 12 diagnostic groups are listed in Table S2. Abbreviations: Bell’s - idiopathic Bell’s palsy; EntM - enteroviral meningitis, GTS – Tourette syndrome; HSE - HSV encephalitis; MS - multiple sclerosis; NMDA – anti-NMDA-receptor encephalitis; NPH - normal pressure hydrocephalus; VZV fac - facial nerve zoster; VZV ME – VZV meningitis/encephalitis; VZV seg - segmental zoster.

**Figure 2.** Kyn and Trp concentrations are differentially associated with neuroinflammation. A-B, Box plots and ROC curves based on Kyn (A left) and Trp (A right) concentrations in CSF and Kyn/Trp ratio (B) in inflamed (CSF leukocyte count, ≥5/mm³, n=121) and non-inflamed (n=99) samples. All differences of medians were highly significant (p<0.001, Mann-Whitney U test). Dark blue horizontal lines, medians; red dots, means; blue shaded areas of ROC curves = upper and lower CI. AUC values (lower and upper CI) are shown under each ROC curve.

**Figure 3.** Correlations of Kyn, Trp and Kyn/Trp ratio with parameters of neuroinflammation. Red circles, Spearman correlation ρ with p <0.05; unfilled circles, p>0.05. A left, Correlations with six CSF parameters and blood CRP across all samples (n=220). A right, Correlations of Kyn and Trp with leukocyte count across all samples. The red rectangles define the space outside of which only bacterial meningitis values are found. B left, Correlations with leukocyte count within each diagnosis. B right, Correlations with leukocyte count in bacterial meningitis. C left, Correlations with lactate within each diagnosis. C right, Correlations with lactate in bacterial meningitis. D left, Correlations with blood-CSF-barrier dysfunction within each diagnosis. D right, Kyn concentrations in bacterial meningitis depending on the degree of blood-CSF-barrier dysfunction (black horizontal lines, median; red symbols, mean). Abbreviations: Bell’s - idiopathic Bell’s palsy; BCB - blood-CSF-barrier dysfunction, based on age-adjusted Q-albumin as defined in Table 1 (none = 0, mild = 1, moderate = 2, severe = 3); EntM - enteroviral meningitis, GTS – Tourette syndrome; HSE - HSV encephalitis; MS - multiple sclerosis; NMDA – anti-NMDA-receptor encephalitis;
NPH - normal pressure hydrocephalus; VZV fac - facial nerve zoster; VZV ME – VZV meningitis/encephalitis; VZV seg - segmental zoster.

Figure 4. Diagnostic biomarker performance of Kyn, Trp and Kyn/Trp ratio across the 4 major diagnostic groups. Values correspond to significant AUCs (binary ROC analysis) as defined by p<0.05 and lower CI >0.5. A left, Kyn (lower triangle) and Trp (upper). A right, Trp/Kyn ratio (lower) and leukocyte count (upper). B left, Mixed model consisting of Kyn, Trp, Kyn/Trp ratio and leukocyte count (lower) and the same model plus lactate (upper). The following merged diagnostic groups were assessed: bacterial CNS infections (BacM, Borrelia, n=66), viral CNS infections (HSE, VZV ME, EntM, n=100); autoimmune neuroinflammation; NMDA, MS; n=25); non-inflamed controls (Bell’s, Tourette, NPH; n=66). B right, Display of the best combinations of biomarkers identified in B left. Color scheme for selected best type of marker: green - leukocytes or lactate; blue - leukocytes, lactate plus Kyn, Trp, Kyn/Trp ratio; orange, - metabolites. Abbreviations: L – leukocytes; A - lactate; K – Kyn; T – Trp; R – Kyn/Trp ratio. Bell’s - idiopathic Bell’s palsy; EntM - enteroviral meningitis, GTS – Tourette syndrome; HSE - HSV encephalitis; MS - multiple sclerosis; NMDA - anti-NMDA-receptor encephalitis; NPH - normal pressure hydrocephalus; VZV fac - facial nerve zoster; VZV ME – VZV meningitis/encephalitis; VZV seg - segmental zoster.

Figure 5. Biomarker performance of Kyn, Trp and Kyn/Trp ratio across the 12 individual diagnostic groups. Values correspond to significant AUCs (binary ROC analysis) as defined by p<0.05 and lower CI >0.5. A left, Kyn (lower triangle) and Trp (upper). A right, Trp/Kyn ratio (lower) and leukocyte count (upper). B left, Mixed model consisting of Kyn, Trp, Kyn/Trp ratio and leukocyte count (lower) and the same mixed model plus lactate (upper). B right, Display of the classifiers identified in C. C left, Number of significant AUCs for each classifier in the 66 possible paired comparisons. C right, Mean values of all AUCs (grey bars) and significant AUCs only (cross-hatched bars). Color scheme for selected best type of marker: green - leukocytes and/or lactate; blue - leukocytes and/or lactate plus Kyn, Trp, Kyn/Trp ratio; orange, - metabolite(s). Abbreviations: L – leukocytes; A - lactate; K – Kyn; T – Trp; R – Kyn/Trp ratio. Bell’s - idiopathic Bell’s palsy; EntM - enteroviral meningitis, GTS – Tourette syndrome; HSE - HSV encephalitis; MS - multiple sclerosis; NMDA – anti-NMDA-receptor encephalitis; NPH - normal pressure hydrocephalus; VZV fac - facial nerve zoster; VZV ME – VZV meningitis/encephalitis; VZV seg - segmental zoster.
Supplemental figure legends

**Figure S1.** Differences in Kyn and Trp concentrations across the 12 diagnostic groups. Same data as shown in Fig. 1, but y-axes are scaled so that all outlying values can be shown. A left, Kyn; A right, Trp; B, Kyn/Trp ratio.

**Figure S2.** Correlations of Kyn, Trp and Kyn/Trp ratio with those parameters of (neuro)inflammation that are not included in Fig. 3. Red circles, Spearman correlation $\rho$ with $p < 0.05$; unfilled circles, $p > 0.05$. A left, CSF protein; A right, Q-albumin; B left, IgG index; B right, peripheral blood CRP.

**Figure S3.** Diagnostic biomarker performance of Kyn, Trp and Kyn/Trp ratio across the 4 major diagnostic groups (bacterial CNS infections, viral CNS infections, autoimmune neuroinflammation, non-inflamed controls). A, Number of significant AUCs for each classifier in the 6 possible paired comparisons. B, Mean values of all AUCs (grey bars) and significant AUCs only (cross-hatched bars).

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References


Table 1. Demographic and clinical laboratory characteristics

<table>
<thead>
<tr>
<th>DEMOGRAPHIC</th>
<th>BLOOD</th>
<th>CSF</th>
</tr>
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<tbody>
<tr>
<td>Sex [female</td>
<td>Age [years]</td>
<td>Leukocyte [1000/µL]</td>
</tr>
<tr>
<td>Male</td>
<td>Median (range)</td>
<td>Median (range)</td>
</tr>
<tr>
<td>BacM (n = 32)</td>
<td>47</td>
<td>53</td>
</tr>
<tr>
<td>Borrelia (n = 34)</td>
<td>47</td>
<td>53</td>
</tr>
<tr>
<td>HSE (n = 9)</td>
<td>22</td>
<td>78</td>
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<tr>
<td>VZV ME (n = 15)</td>
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<td>60</td>
</tr>
<tr>
<td>EntM (n = 10)</td>
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<td>60</td>
</tr>
<tr>
<td>VZV fac (n = 16)</td>
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<td>44</td>
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<tr>
<td>VZV seg (n = 14)</td>
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<td>43</td>
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<tr>
<td>NMDA (n = 8)</td>
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<td>30</td>
</tr>
<tr>
<td>MS (n = 17)</td>
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<td>53</td>
</tr>
<tr>
<td>Bell’s (n = 11)</td>
<td>55</td>
<td>45</td>
</tr>
<tr>
<td>GTS (n = 20)</td>
<td>10</td>
<td>90</td>
</tr>
<tr>
<td>NPH (n = 35)</td>
<td>43</td>
<td>57</td>
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</table>

P value (all groups) b

| 1.7e-01 | 9.2e-05 | 6.1e-09 | 5.4e-13 | 0.0 | 1.1e-16 | 7.3e-15 | 0.0 | 2.2e-16 d |

P value (inflamed vs non-inflamed) b

| 0.484 | 0.135 | 0.006 | 0.008 | 0.0 | 1.7e-13 | 1.0e-11 | 6.3e-14 | 1.2e-13 f |

b Calculated by Kruskal-Wallis test unless stated otherwise. c Calculated by chi-square test. Bold numbers: P ≤0.05.
d Inflamed: CSF leukocyte count ≥5/µL; non-inflamed: CSF leukocyte count 0-4/µL.

Abbreviations: BacM - bacterial meningitis, Bell’s - idiopathic Bell’s palsy, Borrelia - Borrelia burgdorferi neuroborreliosis, EntM - enteroviral meningitis, GTS – Tourette syndrome, HSE - HSV encephalitis, MS - multiple sclerosis, NMDA – anti-NMDA-R encephalitis, NPH - normal pressure hydrocephalus, VZV fac - facial nerve zoster, VZV ME – VZV meningitis/encephalitis, VZV seg - segmental zoster (shingles).
Table 2. Diagnostic performance of the combined classifier Kyn + Trp vs. CSF leukocyte count for the discrimination between viral CNS infections and autoimmune neuroinflammatory disorders

<table>
<thead>
<tr>
<th>Comparison</th>
<th>Classifier</th>
<th>Accuracy *</th>
<th>Sensitivity</th>
<th>Specificity</th>
<th>PPV</th>
<th>NPV</th>
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</thead>
<tbody>
<tr>
<td>Viral vs autoimmune</td>
<td>Leuk. count</td>
<td>0.741</td>
<td>0.735</td>
<td>0.750</td>
<td>0.806</td>
<td>0.667</td>
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<tr>
<td>Viral vs autoimmune</td>
<td>Trp + Kyn</td>
<td>0.915</td>
<td>0.912</td>
<td>0.920</td>
<td>0.939</td>
<td>0.885</td>
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</tbody>
</table>

The data are derived from the ROC analysis shown in Fig. 4B.

Group definitions: viral – HSE, VZV ME, EntM; autoimmune – NMDA, MS

* (sensitivity + specificity) / 2 at the trade-off point in the ROC curve

Abbreviations: Leuk. – leukocyte, PPV – positive predictive value, NPV – negative predictive value
### Table S1. Diagnostic criteria and clinical information

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>Criteria</th>
<th>Disease grade/activity</th>
<th>Systemic treatment at time of lumbar puncture a</th>
</tr>
</thead>
</table>
| Bacterial meningitis (n=32)      | Clinical meningitis, pleocytosis and detection of bacterial pathogen in CSF or blood b | Detected pathogens: *Streptococcus pneumoniae* (44%)  
*Staphylococcus aureus* (16%)  
*Neisseria meningitidis* (9%)  
*Listeria monocytogenes* (9%)  
Others (12%) | Antibiotics (n=4)  
- ceftriaxone, ampicillin  
- ciprofloxacin, metronidazole  
- ceftriaxone, cephazolin  
- gentamycin, ampicillin, ceftazidime |
| Neuroborreliosis (n=34)          | Neurological deficit and inflammatory CSF syndrome; intrathecal synthesis of *Borrelia* IgG or elevated *Borrelia* specific antibody index c | Second-stage neuroborreliosis (100%) (duration of symptoms ≤6 months) | Antibiotics (n=6)  
- ceftriaxone  
- in combination with acyclovir (n=2)  
- corticosteroids (n=1)  
- acyclovir and corticosteroids (n=1) |
| HSV encephalitis (n=9)           | Mental status changes and positive HSV PCR or elevated (>1.5) ASI         | Meningitis, 73%  
Encephalitis, 27% | Acyclovir (n=4)  
- with ampicillin (n=1) |
| VZV meningitis/encephalitis (n=15) | Detection of VZV in CSF by PCR and/or intrathecal synthesis of VZV IgG, clinical meningitis/encephalitis with or without typical zoster rash | Meningitis, 73%  
Encephalitis, 27% | Immunosuppression (n=1)  
- rituximab, bendamustin (3weeks prior to lumbar puncture due to mantle cell lymphoma) |
| Enterovirus meningitis (n=10)    | Clinical meningitis and detection of enterovirus in CSF by PCR             | Acute onset (100%) (symptoms ≤3 months)       | - |
| Facial nerve zoster (n=16)       | Facial palsy with or without typical zoster rash, detection of VZV DNA in CSF by PCR and/or intrathecal synthesis of VZV IgG | - | - |
| Segmental zoster (n=14)          | Typical segmental zoster skin rash, and/or detection of VZV DNA in CSF by PCR and/or intrathecal synthesis of VZV IgG | - | - |
| Anti-NMDA-R encephalitis (n=8)   | Clinical encephalitis and detection of IgG anti-NMDA-R antibodies in CSF | Acute onset (100%) (symptoms ≤3 months)       | - |
| Multiple sclerosis (n=17)        | McDonald 2017 criteria d | Oligoclonal bands CSF (100%)  
Acute flare/relapse (82%)  
MRI gadolinium enhancing lesions (70%)  
Stable (12%) | Corticosteroids (n=2) |
| Tourette syndrome (n=20)         | Criteria according to DSM-5 e | Classified by YGTSS-TTS  
mild (10%)  
moderate (70%)  
severe (20%) | Symptomatic treatment (n=3)  
- Abilify (n=1)  
- Dronabinol (n=1)  
- Sativex (n=1) |
| Bell’s palsy (n=11)              | Facial nerve palsy without evidence of infectious etiology or pleocytosis | - | - |
| Normal pressure hydrocephalus (n=35) | Normal CSF pressure, typical findings on CT or MRI, at least one symptom of Hakim triad f | - | - |

a Excluding antipyretics, analgesics and medications for unrelated conditions  
e American Psychiatric Association (2013) Diagnostic and statistical manual of mental disorders (5th ed.)  

Abbreviations: RRMS = relapsing-remitting multiple sclerosis; SPMS= secondary-progressive multiple sclerosis; YGTSS-TTS = Yale Global Tic Severity Scale Total Tic Score.
Table S2. Kyn and Trp concentrations and Kyn/Trp ratio in the 12 diagnostic groups

<table>
<thead>
<tr>
<th></th>
<th>Kynurenine [µmol/L]</th>
<th>Tryptophan [µmol/L]</th>
<th>Kynurenine / Tryptophan ratio</th>
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<tbody>
<tr>
<td></td>
<td>Median (range)</td>
<td>Median (range)</td>
<td>Median (range)</td>
</tr>
<tr>
<td>BacM (n = 32)</td>
<td>1.27 (0.15-31.4)</td>
<td>2.76 (0.25-84.2)</td>
<td>0.36 (0.03-125.6)</td>
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<tr>
<td>Borrelia (n = 34)</td>
<td>0.98 (0.15-6.15)</td>
<td>2.28 (0.25-2.81)</td>
<td>0.93 (0.06-10.68)</td>
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<tr>
<td>HSE (n = 9)</td>
<td>1.56 (0.15-3.73)</td>
<td>0.51 (0.25-4.65)</td>
<td>2.04 (0.04-14.9)</td>
</tr>
<tr>
<td>VZV ME (n = 15)</td>
<td>1.45 (0.15-6.87)</td>
<td>0.82 (0.25-3.37)</td>
<td>0.57 (0.06-27.48)</td>
</tr>
<tr>
<td>EntM (n = 10)</td>
<td>0.44 (0.15-1.23)</td>
<td>1.44 (0.25-3.63)</td>
<td>0.57 (0.04-4.92)</td>
</tr>
<tr>
<td>VZV fac (n = 16)</td>
<td>0.52 (0.15-2.54)</td>
<td>1.62 (0.25-2.86)</td>
<td>0.26 (0.05-10.16)</td>
</tr>
<tr>
<td>VZV seg (n = 14)</td>
<td>0.15 (0.15-0.37)</td>
<td>2.09 (0.25-3.13)</td>
<td>0.07 (0.05-0.6)</td>
</tr>
<tr>
<td>NMDA (n = 8)</td>
<td>0.15 (0.15-0.81)</td>
<td>1.78 (0.7-2.74)</td>
<td>0.08 (0.05-0.6)</td>
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<tr>
<td>MS (n = 17)</td>
<td>0.15 (0.15-0.15)</td>
<td>2.07 (0.67-3.37)</td>
<td>0.07 (0.04-0.22)</td>
</tr>
<tr>
<td>Bell’s (n = 11)</td>
<td>0.15 (0.15-0.15)</td>
<td>2.19 (1.5-3.18)</td>
<td>0.07 (0.05-0.1)</td>
</tr>
<tr>
<td>GTS (n = 20)</td>
<td>0.15 (0.15-0.43)</td>
<td>2.05 (1.53-3.24)</td>
<td>0.07 (0.05-0.13)</td>
</tr>
<tr>
<td>NPH (n = 35)</td>
<td>0.15 (0.15-1.07)</td>
<td>2.32 (1.4-2.23)</td>
<td>0.06 (0.04-0.47)</td>
</tr>
</tbody>
</table>

P value *(all patient groups)*

|                  | 0.0 | 3.6e-06 | 6.0e-14 |

P value *(inflamed vs non-inflamed b)*

|                  | 0.0 | 7.6e-06 | 0.0     |

*a* Calculated by Kruskal-Wallis test.


Abbreviations: BacM - bacterial meningitis, Bell’s - idiopathic Bell’s palsy, Borrelia - *Borrelia burgdorferi* neuroborreliosis, EntM - enteroviral meningitis, GTS – Gilles de la Tourette syndrome, HSE - HSV encephalitis, MS - multiple sclerosis, NMDA – anti-NMDA-R encephalitis, NPH - normal pressure hydrocephalus, VZV fac - facial nerve zoster, VZV ME – VZV meningitis/encephalitis, VZV seg - segmental zoster (shingles).