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Differential activation of neuroinflammatory pathways in children with seizures: A cross-sectional study

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ABSTRACT

Purpose: Inflammation plays a crucial role in epileptogenesis. We analyzed inflammatory cytokines in plasma and saliva from children with seizures and healthy controls and measured their associations with HHV6 and EBV infection.

Methods: We analyzed plasma from 36 children within 24 h of seizures (cases) and 43 healthy controls and saliva from 44 cases and 44 controls with a multiplex immunoassay. Saliva from all controls and 65 cases and blood from 26 controls and 35 cases were also analyzed by PCR for viral DNA. Primary outcome was cytokine levels in cases vs. controls. Secondary outcomes included detection of HHV-6 and EBV viral DNA in cases vs. controls and viral loads in cases vs. controls. Statistical analysis included the Wilcoxon Rank Sum test, Fisher's exact test, ANOVA, and Spearman correlation.

Results: Compared to controls, patients had higher levels of CCL11 ($p = 0.0018$), CCL26 ($p<0.001$), IL10 ($p = 0.044$), IL6 ($p<0.001$), IL8 ($p = 0.018$), and MIP1β ($p = 0.0012$). CCL11 was higher with 3 or more seizures ($p = 0.01$), seizures longer than 10 min ($p = 0.001$), and when EEG showed focal slowing ($p = 0.02$). In saliva, febrile seizures had higher levels of IL-1β ($n = 7$, $p = 0.04$) and new onset seizures had higher IL-6 ($n = 15$, $p = 0.02$). Plasma and saliva cytokine levels did not show a correlation. The frequency of HHV-6 and EBV detection was similar across groups and not different than controls. We found no correlation between viral load and cytokine levels.

Conclusions: We showed differential activation of neuroinflammatory pathways in plasma from different seizure etiologies compared to controls, unrelated to viral infection.

Introduction

Approximately 50 million people worldwide are affected by epilepsy [1]. Approximately one-third of them are considered pharmaco-resistant [2] and experience seizures despite complex therapeutic regimens, often burdened by significant side-effects. Current antiseizure medications (ASM) target seizures symptomatically but not underlying pathophysiological mechanisms [3]. Experimental and clinical findings suggest a crucial role of inflammation in epileptogenesis [4]. New therapeutic strategies are necessary to improve seizure control and quality of life for people with epilepsy. The first step in developing novel therapies is improving understanding of pathophysiological mechanisms of epileptogenesis.

An emerging hypothesis is that various brain insults, including viral infections, particularly from herpesviruses such as human herpesvirus (HHV)−6 [5] can contribute to epileptogenesis by inducing a cascade of chronic central nervous system (CNS) inflammatory processes and increased blood–brain barrier permeability, leading to enhanced

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neuronal excitability [6, 7]. Previous studies highlighted the role of glial cells (astrocytes and microglia) and neurons in the production of inflammatory cytokines [8].

In a preliminary feasibility study [9], we showed higher levels of IL8 and IL1β in saliva from 32 children with seizures compared to 30 age-matched controls with a febrile illness and no seizures.

With the present exploratory study, we sought to expand the initial data and aimed to investigate the inflammatory response in blood and saliva for different types of seizures in children and elucidate a potential role of HHV-6 infection in triggering activation of a different neuroinflammatory pathway. It is meant as a first step towards the identification of potential novel therapeutic targets.

Methods

Enrollment: In this cross-sectional study, we enrolled children aged 1 month – 18 years at Children's National Medical Center, between October 2017 and February 2019. After an initial screening of 439 subjects, a total of 89 children were enrolled, of whom 65 had saliva, and 35 had whole blood analyzed by droplet digital PCR (ddPCR) for viral DNA; 44 had saliva and 36 had plasma analyzed by Meso Scale Discovery (MSD) immunoassay for inflammatory cytokines (Supplemental Figure 1). In twenty-four children saliva quantity was insufficient for analysis. Controls were healthy children who presented to the Children's Health Center outpatient clinic for a well-child visit. After an initial screening of 140 children, a total of 55 were enrolled, of whom 44 had saliva, and 24 had whole blood analyzed by ddPCR for viral DNA; 44 had saliva and 43 had plasma analyzed by MSD for inflammatory cytokines (Supplemental Figure 2). In eleven children saliva quantity was insufficient for analysis. Children with active or prior neurological, infectious, inflammatory/autoimmune, rheumatological, allergic, or neoplastic diseases were excluded. Only 5 healthy controls had a positive family history of epilepsy. Cases were screened among children presenting to the Emergency Department within 24 h (mostly within 1–6 h, based on emergency medical services records and history obtained from caregivers) of one or multiple seizures of any duration and consisted of three categories: 1. New-onset seizure, defined as first unprovoked seizure; 2. Breakthrough seizure, defined as an acute seizure in the context of chronic epilepsy; and 3. Febrile seizure, either simple or complex.

Medical records were reviewed for clinical variables, laboratory, imaging and electroencephalogram (EEG) results, if available. Duration of seizures was determined by history obtained from caregivers, emergency medical services records and medical records. Study data were collected and managed using a password-protected database (REDCap electronic data capture tools).

Written informed consent was obtained from a parent or legal guardian and written assent from the child, when indicated. Children's National Medical Center Institutional Review Board approved the study.

Sample collection, processing and analysis: For each participant, an attempt was made to obtain simultaneous saliva and blood samples. When saliva quantity was insufficient for all analyses, priority was given to ddPCR. Due to the exploratory/hypothesis-generating nature of the study, resources to run all samples in duplicates for cytokine analysis were not available. Therefore, we opted to randomize a smaller number of samples and analyze them in duplicates instead of running more samples in a single well design. All samples were collected, handled, and processed following standard biosafety procedures. Saliva was collected utilizing a validated pediatric swab (SalivaBio Oral Swab, Salimetrics). Whole blood was collected in EDTA tubes via a venous puncture. Samples were then centrifuged at 2300 g for 10 min immediately after collection and saliva, plasma, and whole blood were aliquoted. Samples were then frozen at - 80° C immediately after processing and shipped on ice to the Viral Immunology Section of the National Institutes of Health for analysis. After thawing the samples, DNA was extracted utilizing a commercially available kit (DNeasy Blood & Tissue Kit, Qiagen) following the manufacturer's protocol for plasma and a previously validated protocol for saliva [9].

HHV-6 and Epstein-Barr Virus (EBV) viral DNA in saliva and whole blood was quantified using digital droplet PCR (ddPCR). Primers from the highly conserved region u57 (HHV-6) and *B*amHI (EBV) were selected. Different probes were used to distinguish between HHV-6A and HHV-6B HHV-6A (u57): Forward: CCGTGGGATCGTCTAAAATTATAGATGT; Reverse: CCACACTAGTCCGGACGGATAA; Probe: CTGGAACTGTATAATAGG. HHV-6B (u57): Forward: CCGTGGGATCGTCTAAAATTATAGATGT; Reverse: CCACACTAGTCCGGACGGATAA; Probe: CTGGAGCTGTACAACAG. Ribonuclease P Subunit P30 (RPP30) was used as a cellular housekeeping gene. ddPCR procedures and analysis of results followed those previously described in our laboratory [10].

Several inflammatory cytokines, which had previously been studied in seizures and epilepsy [11], including interferon (IFN)γ, IL10, IL1β, IL2, IL4. IL6, IL8, tumor necrosis factor TNF, C—C motif chemokine (CCL)11, CCL13, C-X-C motif chemokine (CXCL)10, monocyte chemoattractant protein (MCP)1, MCP4, macrophage-derived cytokine (MDC), Macrophage inflammatory protein (MIP)1α/β, thymus activation regulated chemokine (TARC) were analyzed in plasma and saliva using a Custom Human V-PLEX Neuroinflammation Panel (Meso Scale Diagnostics), after thawing the samples for the first time. This platform was successfully utilized in several previous studies [12-15] for quantification of inflammatory cytokines. Plasma samples were diluted 1:2, while saliva samples remained undiluted prior to analysis. All samples were run in duplicates.

All experiments were performed in accordance with relevant guidelines and regulations governing research at Children's National Medical Center and the National Institutes of Health and were approved by the Institutional Review Board at Children's National Medical Center (Study # Pr000007122).

Statistical analysis and study outcomes

The primary outcome was cytokine levels in cases vs. controls. Secondary outcomes included frequency of detection of HHV-6 and EBV viral DNA in cases vs. controls and viral loads in cases vs. controls.

Statistical analysis was conducted utilizing R version 3.5.3 and included the Pearson Chi-squared test, Fisher's exact test for relative frequencies for HHV-6 detection, Wilcoxon rank-sum test for cytokine analysis, one-way analysis of variance (ANOVA) on ranks and Spearman's correlation for correlations between cytokine levels in blood and saliva and HHV-6 viral load and cytokine levels and clinical variables.

A p-value < 0.05 was considered significant.

Results

Cytokine analysis

Clinical characteristics and cytokine levels are summarized in Table 1 (plasma, subgroups Vs. controls), Table 2 (plasma, all cases Vs. controls) and Supplemental Table 1 (saliva). None of the cases was on ketogenic diet or had a vagus nerve stimulator. Baseline seizure frequency for children presenting with breakthrough seizures in the context of chronic epilepsy was once every 1–2 weeks for 1/18 (5%) patient, once per month for 1/18 (5%), once every two months for 3/18 (17%), once every 4–6 months for 8/18 (45%), and once every 8–14 months for 5/18 (28%). Prior to collection of blood and saliva, 6/13 (46%) of children with new onset of seizures were given at least one dose of benzodiazepine, 3 of them also received IV levetiracetam and 2 of them IV fosphenytoin. Twelve out of 18 (67%) children with breakthrough seizures were given at least one dose of benzodiazepine, 6 of them also received IV fosphenytoin, 1 of them IV valproic acid and 2 of them IV levetiracetam. Compared to controls, patients had higher levels of CCL11 (median, interquartile range (IQR), p-value for Wilcoxon rank-sum test) (145.0 (117.0–196.0) vs. 291.0 (138.0–393.0), $p = 0.0018$),

Table 1
Clinical characteristics of subgroups and cytokine analysis in plasma.

Seizure type	N	Age years mean (SD)	Male N (%)	N Sz	Sz duration (minutes)	Duration epilepsy years mean (SD)	ASMs	EEG	MRI	Etiology
New onset	13	6.1 (5.1)	8 (62%)	1–2: 11 (85%) >2: 2 (15%)	<5: 8 (62%) 5–10: 5 (38%)	N/A	N/A	2 (15%) focal temp slow 2 (15%) gen slow 6 (46%) epilept (3 gen, 3 temp) 1 (8%) sz (temp) 1 (8%) N/A	2 (15%) epileptogenic 10 (77%) normal 1 (8%) N/A	Structural: 2 (15%) Unknown: 11 (85%)
Breakthrough	18	6.0 (5.4)	8 (55%)	1–2: 12 (67%) >2: 6 (33%)	< 5: 12 (66%) 5–10: 3 (17%) >10: 3 (17%)	2.4 (1.4)	LEV: 13 (72%) OXC: 3 (17%) TPM: 2 (11%) LMT: 2 (11%) PB: 3 (17%) VPA: 3 (17%) PHT: 1 (5%) CLB: 4 (22%) LCS: 2 (11%)	9 (41%) focal slow (5 temp, 3 front, 1 occ) 7 (32%) gen slow 11 (50%) epilept (4 gen, 2 front, 4 temp, 1 occ) 3 (13%) sz (2 gen, 1 temp) 1 (4%) N/A	10 (56%) epileptogenic 6 (33%) normal 2 (11%) N/A	Structural: 10 (56%) Dravet: 3 (17%) Other genetic: 3 (17%) Unknown: 2 (11%)
Febrile	5	3.4 (2.1)	5 (100%)	1–2: 3 (60%) >2: 2 (40%)	< 5: 5 (100%)	N/A	N/A	4 (80%) normal 1 (20%) N/A	1 (20%) normal 4 (80%) N/A	N/A
Assay	Controls (n = 43) Mean ± SD	Febrile (n = 5) Mean ± SD	Breakthrough (n = 18) Mean ± SD	New onset (n = 13) Mean ± SD	p-value (ANOVA)					
CCL11	191.3 ± 126.6	174.8 ± 69.8	378.0 ± 236.7	266.8 ± 125.4	<0.001					
CCL26	67.2 ± 76.5	63.1 ± 9.5	248.9 ± 402.0	81.2 ± 31.8	0.01					
IFN-γ	28.1 ± 91.8	1339.5 ± 2298.6	76.5 ± 116.8	12.1 ± 7.4	<0.001					
IL-10	1.6 ± 1.2	17.6 ± 21.7	1.9 ± 1.0	1.4 ± 0.6	<0.001					
IL-13	2.5 ± 1.7	1.2 ± 0.0	2.4 ± 1.3	2.9 ± 1.6	0.19					
IL-1β	0.6 ± 0.9	0.3 ± 0.1	0.5 ± 0.6	0.3 ± 0.1	0.5					
IL-2	1.3 ± 2.2	2.0 ± 2.6	1.5 ± 2.3	0.9 ± 0.3	0.73					
IL-4	0.5 ± 0.4	0.4 ± 0.2	0.4 ± 0.2	0.5 ± 0.3	0.62					
IL-6	1.8 ± 1.7	24.1 ± 14.7	3.3 ± 3.3	3.3 ± 2.7	<0.001					
IL-8	11.1 ± 6.2	16.0 ± 11.5	22.2 ± 20.3	16.4 ± 16.7	0.03					
IP-10	1208.8 ± 4318.7	6084.7 ± 5532.1	637.4 ± 573.8	373.2 ± 171.6	0.02					
MCP-1	223.2 ± 147.4	337.1 ± 281.7	346.9 ± 332.2	223.7 ± 105.6	0.14					
MDC	3019.1 ± 848.3	1974.8 ± 605.6	2822.2 ± 1197.6	2816.4 ± 1553.4	0.23					
MIP-1α	137.8 ± 318.9	26.6 ± 11.1	235.5 ± 730.7	71.0 ± 153.8	0.7					
MIP-1β	139.1 ± 93.6	163.9 ± 53.1	239.0 ± 207.1	149.6 ± 62.9	0.04					
TARC	595.1 ± 910.4	360.4 ± 313.0	528.6 ± 503.3	505.4 ± 416.5	0.91					
TNF-α	5.9 ± 3.5	7.7 ± 2.0	6.0 ± 3.5	5.8 ± 4.0	0.73					

SD, standard deviation; Sz, seizures; ASM, antiseizure medications; N/A, not applicable; focal slow, focal slowing; temp, temporal; front, frontal; occ, occipital; gen, generalized; epilept, epileptiform discharges; LEV, levetiracetam; OXC, oxcarbazepine; TPM, topiramate; LMT, lamotrigine; PB, phenobarbital; VPA, valproic acid; PHT, phenytoin; CLB, clobazam.
Values for cytokines are expressed in pg/mL.

Table 2
Cytokine analysis in plasma.

Assay	Controls ($n = 43$) Median (IQR)	All cases ($n = 36$) Median (IQR)	p-value (Wilcoxon)
CCL11	145.0 (117.0–196.0)	291.0 (138.0–393.0)	0.0018
CCL26	46.4 (36.1–70.0)	74.8 (60.7–106.0)	<0.001
IL-10	1.13 (0.78–2.26)	1.58 (1.24–2.90)	0.044
IL-6	1.00 (0.816–1.86)	2.50 (1.12–6.48)	<0.001
IL-8	9.51 (6.73–14.8)	11.5 (10.3–20.0)	0.018
MIP-1β	109.0 (85.7–151.0)	153.0 (112.0–197.0)	0.012

IQR=Interquartile range
Values for cytokines are expressed in pg/mL.

CCL26 (46.4 (36.1–70.0) vs. 74.8 (60.7–106.0), $p<0.001$), IL10 (1.13 (0.78–2.26) vs. 1.58 (1.24–2.90), $p = 0.044$), IL6 (1.00 (0.816–1.86) vs. 2.50 (1.12–6.48), $p<0.001$), IL8 (9.51 (6.73–14.8) vs. 11.5 (10.3–20.0), $p = 0.018$), and MIP1β (109.0 (85.7–151.0) vs. 153.0 (112.0–197.0), $p = 0.0012$) (Fig. 1). Subgroup analysis revealed that children with breakthrough seizures ($n = 18$) had higher levels of CCL11 (mean, standard deviation (SD), p-value for ANOVA test) (378.0 ± 236.7 vs. 191.3 ± 126.6, $p<0.001$), CCL26 (248.9 ± 402.0 vs. 67.2 ± 76.5, $p<0.001$), IL8 (22.2 ± 20.3 vs. 11.1 ± 6.2, $p = 0.03$), CCL4 (239.0 ± 207.1 vs. 139.1 ± 93.6, $p = 0.04$) in plasma. Children with new-onset seizures ($n = 13$) showed higher levels of CCL11 (266.8 ± 125.4 vs. 191.3 ± 126.6, $p = 0.05$), CCL26 (81.2 ± 31.8 vs. 67.2 ± 76.5, $p = 0.012$) and IL6 (3.3 ± 2.7 vs. 1.8 ± 1.7, $p = 0.01$). Patients with febrile seizures ($n = 5$) had higher levels of IFNγ (1339.5 ± 2298.6 vs. 28.1 ± 91.8, $p<0.001$), IL6 (24.1 ± 14.7 vs. 1.8 ± 1.7, $p<0.001$), IL10 (17.6 ± 21.7 vs. 1.6 ± 1.2, $p<0.001$), CXCL10 (6084.7 ± 5532.1 vs. 1208.8 ± 4318.7, $p = 0.001$) (Fig. 2). CCL11 was higher than controls in children with seizures, with stronger difference in case of 3 or more seizures ($p = 0.01$) (Fig. 3). Levels of this cytokine in patients with seizures longer than 10 min were higher than in those with seizures lasting less than 10 min ($p = 0.001$) (Fig. 4). CCL11 was also higher when EEG showed focal slowing ($p = 0.02$) (Supplemental Figure 3).

In saliva, we observed higher levels of IL1β in febrile seizures ($n = 7$, $p = 0.04$) and IL-6 in new-onset seizures ($n = 15$, $p = 0.02$) (Supplemental Figure 4). Cytokine levels in plasma and saliva were not associated with the height of fever. We did not observe a correlation between plasma and saliva cytokine levels (data not shown).

Viral droplet digital PCR

Frequency of HHV-6 and EBV detection was similar across seizure types and not different from controls (Supplemental Table 2). We found no correlation between viral load and cytokine levels (data not shown).

Discussion

In our cross-sectional study, we showed differential activation of inflammatory pathways in plasma from children with different types of acute seizures vs. healthy controls.

A particularly novel aspect of our findings is that we detected higher levels of CCL11 (Eotaxin-1), and its immediate relative CCL26 (Eotaxin-3) in children with seizures. CCL11, a cytokine involved in eosinophil chemotaxis, has been described initially in association with a broad range of allergic conditions such as asthma, rhinitis, and atopic dermatitis [16–18]. Interestingly, levels of CCL11 are also elevated in the sera and CSF of patients with neuroinflammatory disorders such as multiple sclerosis [19], and neurodegenerative diseases such as Alzheimer's disease and Huntington's disease [20]. Inflammatory insults have been observed to stimulate CCL11 secretion in primary cultures of astrocytes, pericytes, and microglia, therefore suggesting a mechanism independent of eosinophil recruitment [21, 22]. CCL11 promotes microglial migration, upregulates nicotinamide adenine dinucleotide phosphate-oxidase 1 (NOX1) in microglia, and increases microglial production of reactive oxygen species (ROS), which potentiates glutamate-induced neurotoxicity [22]. Microglial activation is thought to play a key role in the neuroinflammatory response leading to epileptogenesis [4]. Also, plasma levels of CCL11 correlate with reduced hippocampal neurogenesis after joining an aged mouse to a young partner (heterochronic parabiosis) [23]. Given the pivotal role of alterations in hippocampal signaling and structure in the onset of seizures,

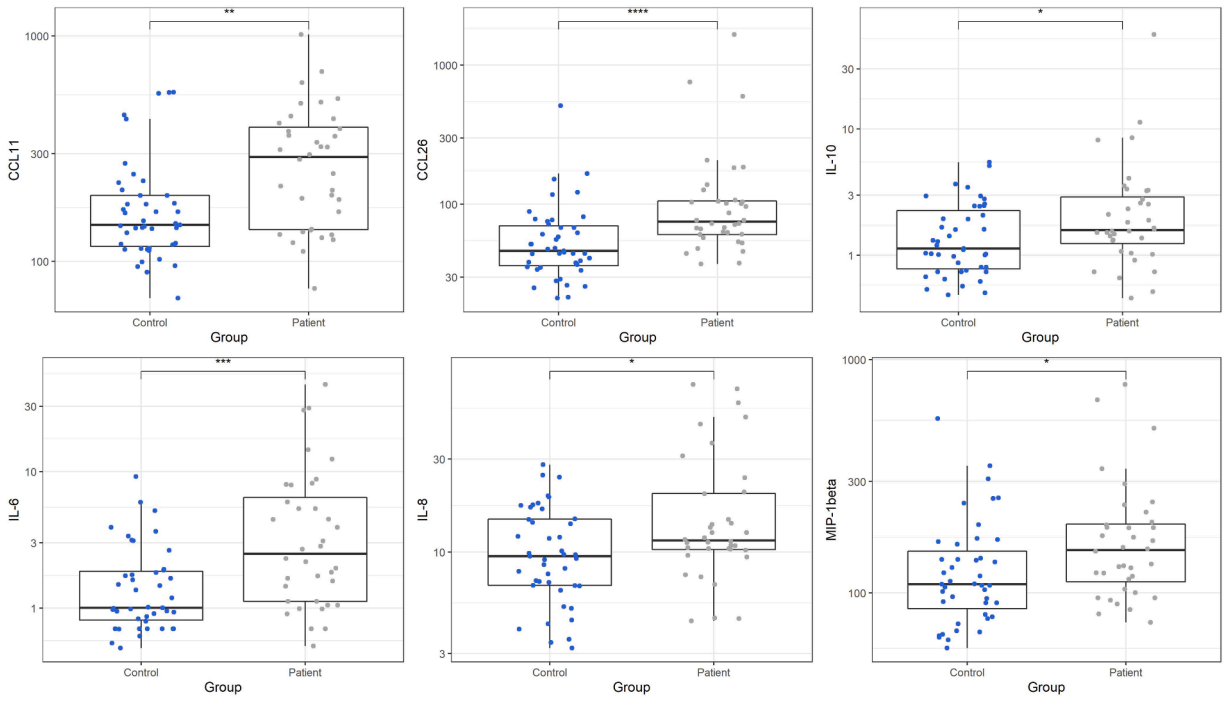

Fig. 1. Cytokine levels in plasma. All cases vs. controls. Levels (pg/mL; median, IQR) for cytokines in plasma from all children with seizures vs. controls. Compared to controls ($n = 43$), cases ($n = 36$) had higher levels of CCL11 ($p = 0.0018$), CCL26 ($p<0.001$), IL10 ($p = 0.044$), IL6 ($p<0.001$), IL8 ($p = 0.018$), and MIP1β (Wilcoxon rank-sum test, $p = 0.0012$). Y-axis is shown as log10 transformed.

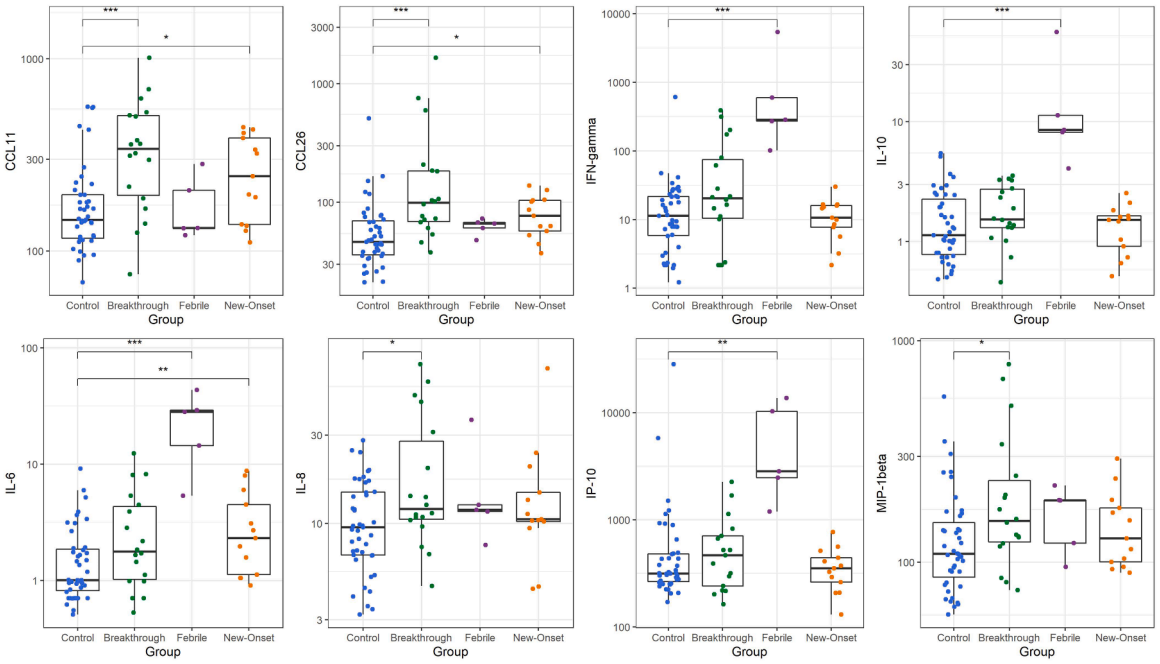

Fig. 2. Cytokine levels in plasma by seizure type. Levels (pg/mL; mean, SD) for selected cytokines in plasma from children with different seizure types (breakthrough seizures in chronic epilepsy ($n = 18$), green; febrile seizures ($n = 5$), purple; and new onset seizures ($n = 13$), orange) vs. controls (blue). Compared to controls, children with breakthrough seizures ($n = 18$) had higher levels of CCL11 ($p<0.001$), CCL26 ($p<0.001$), IL-8 ($p = 0.03$), CCL4 ($p = 0.02$). Children with new onset seizures ($n = 13$) showed higher levels of CCL11 ($p = 0.05$) and IL-6 ($p = 0.01$). Patients with febrile seizures ($n = 5$) had higher levels of IFNγ ($p<0.001$), IL-6 ($p<0.001$), IL-10 ($p<0.001$), CXCL10 (ANOVA test, $p = 0.001$). Y-axis is shown as log10 transformed.

especially of temporal lobe onset [24], it is reasonable to conclude that CCL11 may be involved in ictogenesis. Further longitudinal studies are needed to assess whether this cytokine may also be involved in epileptogenesis. Interestingly, studies have shown that in resected epileptogenic tissue, hippocampal CCL11 levels are higher than those in the entorhinal and temporal cortices [25], potentially suggesting a role in epileptogenesis and warranting further investigation. Interestingly, we also observed that CCL11 levels positively correlated with clinical variables of severity such as seizure duration and number of seizures and with focal slowing on EEG, which is a common finding in the context of focal epilepsy. Other EEG features, such as generalized slowing and epileptiform discharges had no such correlation, possibly indicating that CCL11 may be a biomarker of localized brain dysfunction rather than diffuse or excitatory processes.

CCL26 is a chemotactic agent, which is mainly expressed on the surface of eosinophils, mast cells and Th2 lymphocytes. Several pro-inflammatory cytokines, including IL4, TNF, IL-1β and IFN-γ can promote the synthesis of CCL26 in human monocytes, [26] potentially contributing to the pathogenesis of different neurological conditions. Higher levels of CCL26 were reported in the serum of patients with acute ischemic stroke compared with that observed in healthy subjects [27]. Increased CSF levels of CCL26 have been reported in association with the progression of mild cognitive impairment in Alzheimer's disease [28]. Data indicate that overexpression of CCL26 may also play a role in the acute stage of experimental autoimmune encephalomyelitis, and promote the progression of the disease and tissue damage [29]. Overexpression of this cytokine was also shown to contribute to the pathogenesis of secondary progressive multiple sclerosis [30]. Data in epilepsy are lacking.

The only cytokine in our study that was consistently elevated in both plasma and saliva in children with new onset of seizures was IL6. In previous studies, this mediator is increased within 24 h after generalized tonic-clonic seizures and febrile seizures but is not changed after seizures in patients with chronic focal epilepsy [31]. At 6 hours after focal unaware or secondary generalized tonic–clonic seizures in patients with MTLE or extratemporal epilepsies, only the MTLE group showed a significant rise in plasma levels of IL6 [32]. Saliva may represent a less invasive and less expensive method for quantification of this biomarker and further studies are needed to validate this finding. While prior evidence suggests that salivary components may originate from the salivary glands or may be derived from the blood by passive diffusion or active transport [33], studies reveal mixed results when comparing blood and salivary cytokines both in physiologic and pathologic conditions. Some reveal no cross-talk between the two compartments [34], while others find positive correlations only for few cytokines, including IL6, similarly to our study [35], IL1β [36], IL2, IL12, and IFNγ [37]. These differences may be owing to several factors such as a different half-life of cytokines in different biological compartments, factors influencing the biome of the oral cavity (such as oral hygiene, presence of oral pathology, etc.) resulting in faster degradation of cytokines or falsely increased levels, and finally insufficient extravasation of cytokines into saliva or very low local production. Data on proinflammatory cytokine profiles in saliva from children with systemic, and in particular with neurological diseases are lacking.

We also observed elevation in plasma of other cytokines such as IL8, especially in children with chronic epilepsy. Several studies have shown that this cytokine is increased after seizures, including focal, generalized tonic–clonic, myoclonic, atypical absence, and typical absence seizures in serum and CSF of patients with epilepsy [38] and febrile status epilepticus [39]. A study in adults showed that this cytokine is elevated interictally in patients with severe epilepsy [40]. In a previous study with different patients, [9] we found higher levels of this cytokine in saliva from children with epilepsy, but this time we could not confirm this finding in the same biological compartment.

Also, we did not observe an elevation of other cytokines, which were previously reported to be elevated in different types of seizures. Previous pediatric studies that analyzed blood and CSF of children with seizures and animal models have described a potential role of IL1β in the genesis of seizures and later development of epilepsy [41–43]. In our study, IL1β levels were overall low and not different between cases and controls

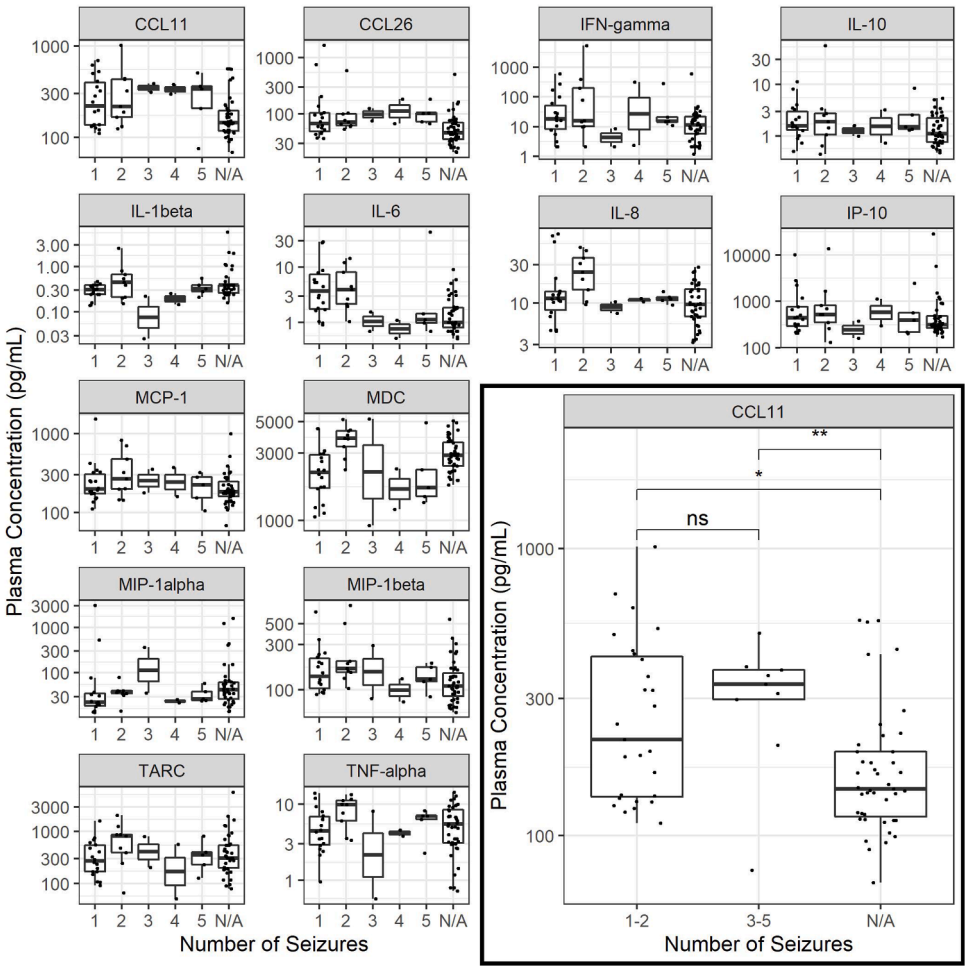

Fig. 3. Cytokine levels in plasma by seizure number. Cytokine levels in plasma (pg/mL; mean, SD) grouped by number of seizures, showing higher CCL11 levels than controls in 10 children with 3 or more seizures (top left plot and blow-up box, bottom right; ANOVA test, $p = 0.01$). Y-axis is shown as log10 transformed.

(Table 1). Similarly, while expression of MIP1α originating from neurons and microglia increases following soman-induced status epilepticus in rats [44], we observed similar concentration of this cytokine in our cohorts. These differences could be the result of the timing of collection, biological compartment analyzed or could be related to the immunoassay that was utilized and warrant further investigation. Interestingly, we observed higher levels of MIP1β, a cytokine that was previously described in MTLE samples [45]. While these two chemokines belong to the same superfamily, MIP1α has a broader range of concentration-dependent chemoattractant characteristics, while MIP1β attracts more selectively CD4+ T lymphocytes, with some preference for CD45RA T cells [46].

Our small cohort of children with febrile seizures had activation of the IFNγ/CXCL10/IL10 pathway, differently than other seizure types. This finding highlights a potential link with a viral infection [47, 48], possibly other than HHV-6 or EBV, as we did not detect these viruses differently in specimens from cases and controls and we found no correlation between viral load and IFNγ levels. Viral infections can trigger a strong activation of innate and adaptive immunity, resulting in significant production of IFNγ. This cytokine mediates several immunological effects including activation of macrophages and induction of class II MHC molecule expression [49]. CXCL10 is secreted by monocytes, fibroblasts and endothelial cells in response to IFNγ, and therefore their activity is tightly connected [50]. Because we found higher levels of IFNγ/CXCL10 in our young children with febrile seizures irrespective of the height of the fever, and not in other patients with new onset of seizures or chronic epilepsy, we speculate that this inflammatory pathway is triggered by a viral infection and not by seizures themselves. The frequency of detection of HHV-6 and EBV in these children was similar to controls, suggesting that a different virus, or more than one virus, may be implicated in the pathogenesis of febrile seizures.

A previous study in adults showed no differences in interictal cytokine levels among patients with different types of epilepsy, but revealed that interictal concentrations of serum IL-6, IFNγ, IL-17a, IFNλ3, and CSF IL-6, IL-17a, IFNλ3 were significant biomarkers for patients with severe epilepsy [40]. Our data from serum specimens collected acutely after a seizure show that of the cytokines analyzed in the interictal study, IL6 is also elevated ictally, while CCL11 and CCL26 were not analyzed interictally and therefore no comparison can be made. The differences in these results need to be interpreted with caution, also taking into account the significant difference in age of the study populations, but highlight the complex interplay that proinflammatory cytokines may exert on ictogenesis and epileptogenesis.

The main strength of our study is that we examined a population of young children with seizures and analyzed samples from different biological compartments for the presence of viral DNA from common viruses and at the same time we studied the levels of a pool of cytokines that are associated with neuroinflammation. We also included several clinical variables in our analysis and compared the results with simultaneous age-balanced healthy controls. The main limitation of our study is the sample size, especially for febrile seizures, which will need to be expanded in further studies to validate our preliminary findings. In addition, we were not able to match all blood and saliva samples for PCR and cytokine analysis, we did not perform PCR for other viruses than

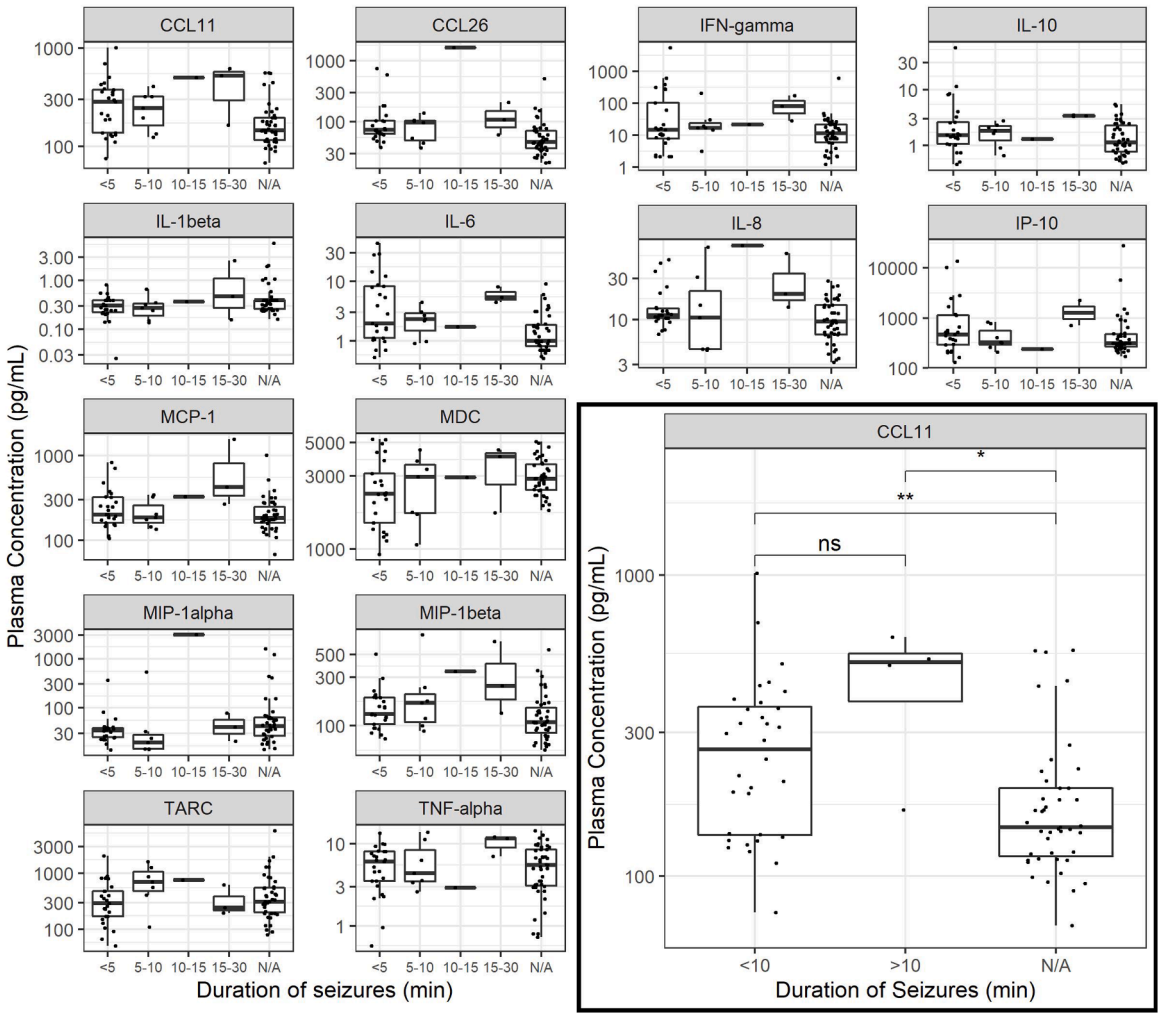

Fig. 4. Cytokine levels in plasma by seizure duration. Cytokine levels in plasma (pg/mL; mean, SD) grouped by seizure duration, showing higher CCL11 levels than controls in 5 children with seizures longer than 10 min (top left plot and blow-up box, bottom right; ANOVA test, $p = 0.001$). Y-axis is shown as log10 transformed.

HHV-6 and EBV, and we did not have serological data. While the pool of cytokines that was tested included many inflammatory mediators that have been previously associated with seizures, some were not tested, such as high-mobility group box 1 (HMGB1) and IL-18 [11]. Due to the cross-sectional nature of this study, we did not examine cytokine profiles at different time points, which may be an interesting aspect to explore in further longitudinal studies. Moreover, while sampling for most patients occurred reasonably within 1–6 h of seizure onset, exact timing cannot be determined. As a result, there might be uneven distribution of the exact time of the sampling, causing a potential bias. In this study, Type-I error and false-positive results were not of primary concern as the focus was on hypothesis generating and exploration. All cytokines that indicate some utility across key outcomes of interest may be further investigated in future trials in a more tightly controlled manner. Also, CSF could not be obtained from study participants; therefore, our observations may represent indirect measures of inflammatory activation in the periphery.

Conclusions

In our study, we analyzed cytokine levels from children with different types of acute seizures and we showed higher serum levels of CCL11/CCL26, IL6, IL8 and MIP1β compared with healthy controls. These findings highlight a potential role of proinflammatory cytokines in ictogenesis. We also observed activation of the IFNγ/CXCL10/IL10 pathway in a small cohort of children with febrile seizures, differently than other seizure types. While this may indicate involvement of a viral pathogen, our HHV-6 and EBV PCR data showed no difference in detection rates between cases and controls and no correlation between cytokine levels and viral loads.

Further longitudinal studies are needed to examine a larger cohort of children with new onset of seizures and those with febrile seizures/status epilepticus and their cytokine profiles over time and possibly include CSF in the analysis. Ideally, such studies should also include multiple cohorts of patients on anti-seizure medication monotherapy to assess whether treatment may influence cytokine levels. By correlating these findings with imaging and EEG, by comparing well-controlled cases versus pharmacoresistant patients, and by expanding the panel of viruses analyzed, we may contribute to shedding light on the pathophysiology of different seizure types and identifying a biomarker of risk of developing epilepsy, with the ultimate aim of selecting a population that may benefit from early immunomodulatory or antiviral therapy.

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writing the manuscript.

Ethics approval

Children's National Medical Center Institutional Review Board approved the study.

Consent to participate

Written informed consent was obtained from a parent or legal guardian and written assent from the child, when indicated.

Availability of data and material

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declaration of Competing Interest

The authors declare that they have no conflicts of interest/competing interests.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.seizure.2021.05.022.

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