Differential Activation of Neuroinflammatory Pathways in Children with Seizures: A Cross-Sectional Study

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Abstract

Background Experimental and clinical findings suggest a crucial role of inflammation in epileptogenesis. We aimed to analyze levels of inflammatory cytokines in plasma and saliva from children with acute seizures and healthy controls and measure their associations with HHV6 and EBV infection.

Methods We analyzed plasma from 36 children within 24 hours of acute 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 ddPCR for viral DNA. Statistical analysis included Wilcoxon Rank Sum test, Fisher's exact test, ANOVA and Spearman correlation.

Results 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 ($p=0.02$) in plasma. 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 ($p=0.001$). CCL11 was higher with 3 or more seizures ($p=0.01$), seizures longer than 10 minutes ($p=0.001$) 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 correlation. Frequency of HHV-6 and EBV detection was similar across seizure types 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 HHV-6 infection.

Background:

Approximately 15 million people worldwide are affected by pharmaco-resistant epilepsy and experience seizures despite complex therapeutic regimens, often burdened by significant side-effects. Current antiepileptic drugs target seizures symptomatically but not underlying pathophysiological mechanisms.[1] Experimental and clinical findings suggest a crucial role of inflammation in epileptogenesis.[2] 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[3] 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 neuronal excitability.[4,5] Previous studies highlighted the role of glial cells (astrocytes and microglia) and neurons in production of inflammatory cytokines.[6]

In a preliminary feasibility study,[7] 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. This 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 PCR for viral DNA and 44 had saliva and 36 had plasma analyzed by Meso Scale Discovery (MSD) immunoassay for neuroinflammatory cytokines (Supplemental Figure 1). 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 PCR for viral DNA and 44 had saliva and 43 had plasma analyzed by MSD for neuroinflammatory cytokines (Supplemental Figure 2). 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 hours (mostly within 1-6 hours, 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 acute seizure in the context of chronic epilepsy; and 3. Febrile seizure, either simple or complex.

Medical records were reviewed for laboratory, imaging and electroencephalogram (EEG) results, if available. 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 PCR. Due to the exploratory/hypothesis generating nature of the study, resources to run all samples in duplicates for cytokine analysis were not available and therefore we opted to randomize a

smaller number of samples and analyze them in duplicates instead of running more samples in 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 2,300 g for 10 minutes immediately after collection and saliva, plasma, and whole blood were aliquoted. Samples were then 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.[7]

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 bamHI (EBV) were selected. Different probes were used to distinguish between HHV-6A and HHV-6B. Ribonuclease P Subunit P30 (RPP30) was used as a cellular housekeeping gene.

Several neuroinflammatory cytokines, including interferon (IFN)g, IL10, IL1β, IL2, IL4. IL6, IL8, tumor necrosis factor (TNF)alpha, CCL11, CCL13, C-X-C motif chemokine (CXCL)10, MCP1, MCP4, macrophage-derived cytokine (MDC), Macrophage inflammatory protein (MIP)1α, thymus activation regulated chemokine (TARC) were analyzed in plasma and saliva by means of a Custom Human V-PLEX Neuroinflammation Panel, Meso Scale Diagnostics after thawing the samples for the first time. Plasma samples were diluted 1:2, while saliva samples remained undiluted prior to analysis. All samples were run in duplicates.

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 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 and clinical variables and HHV-6 viral load 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) and Table 2 (saliva). None of the cases was on ketogenic diet or had a vagus nerve stimulator. Compared to controls, children

with breakthrough seizures (n=18) had higher levels of CCL11 (p<0.001), CCL26 (p<0.001), IL8 (p=0.03), CCL4 (p=0.02) in plasma (Figure 1). Children with new onset seizures (n=13) showed higher levels of CCL11 (p=0.05) and IL6 (p=0.01) (Figure 1). Patients with febrile seizures (n=5) had higher levels of IFNg (p<0.001), IL6 (p<0.001), IL10 (p<0.001), CXCL10 (p=0.001) (Figure 1). CCL11 was higher than controls in children with 3 or more seizures (p=0.01) (Figure 2), in those with seizures longer than 10 minutes (p=0.001) (Figure 3) and when EEG showed focal slowing (p=0.02) (Figure 4).

In saliva, we observed higher levels of IL1b in febrile seizures (n=7, p=0.04) and IL-6 in new onset seizures (n=15, p=0.02) (Supplemental Figure 3). 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 1). We found no correlation between viral load and cytokine levels.

Discussion:

In our cross-sectional study we analyzed blood and saliva samples from children with different types of acute seizures to investigate the levels of inflammatory cytokines and the presence of HHV-6 and EBV viral DNA. We showed differential activation of inflammatory pathways in plasma from different seizure etiologies vs. controls. Children with febrile seizures had activation of the IFNg/CXCL10/IL10 pathway, highlighting a potential link with viral infection,[8,9] possibly other than HHV-6 or EBV, as we did not detect these viruses differently in specimens from cases and controls.

Viral infections can trigger a strong activation of innate and adaptive immunity, resulting in significant production of IFNg. This cytokine mediates several immunological effects including activation of macrophages and induction of class II MHC molecule expression.[10] CXCL10 is secreted by monocytes, fibroblasts and endothelial cells in response to IFNg, and therefore their activity is tightly connected.[11] Because we found higher levels of IFNg/CXCL10 in our young children with febrile seizures irrespective of the height of 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 than controls, suggesting that a different virus, or more than one virus, may be implicated in the pathogenesis of febrile seizures. Our results are different than previous studies, which have described an association between HHV-6 detection and febrile seizures and febrile status epilepticus.[12-14] While our cohort of children with febrile seizures is small, a strength of our observations consists in the fact that we analyzed both saliva and blood and compared our results with simultaneous samples from a fairly large population of healthy children. Nevertheless, given the low *n*, results need to be interpreted with caution and replicated in a larger cohort.

Blood from 4/26 (15%) of our controls tested positive for HHV-6 DNA, which is not different than 54/169 (32%) previously reported for a larger multi-center cohort of children with febrile status epilepticus.[12] Importantly, we observed an even higher positivity rate in saliva (28/44, 63%). Other studies are in agreement with our findings of similar detection rates of HHV-6 DNA when comparing children with febrile seizures and simultaneous controls.[15,16] Other investigators[17] showed a potential role of rhinoviruses, adenoviruses and enteroviruses in the onset of febrile seizures and a lesser role of HHV-6, frequently in the context of co-infection.

CCL11 (Eotaxin-1), 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.[18-20] Interestingly, levels of CCL11 are also elevated in the sera and CSF of patients with neuroinflammatory disorders such as multiple sclerosis,[21] and neurodegenerative diseases such as Alzheimer's disease and Huntington's disease.[22] Inflammatory insults have been observed to stimulate CCL11 secretion in primary cultures of astrocytes, pericytes, and microglia therefore suggesting a mechanism independent of eosinophil recruitement.[23] 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.[24] Microglial activation is thought to play a key role in the neuroinflammatory response leading to epileptogenesis.[2] Also, plasma levels of CCL11 correlate with reduced hippocampal neurogenesis after joining an aged mouse to a young partner (heterochronic parabiosis).[25] Given the pivotal role of alterations in hippocampal signaling and structure in the onset of seizures, especially of temporal lobe onset,[26] it is reasonable to conclude that CCL11 may be involved in epileptogenesis. This hypothesis has been corroborated by studies that have shown that in resected epileptogenic tissue, hippocampal CCL11 levels are higher than those in the entorhinal and temporal cortices.[27] In our study, we observed higher levels of CCL11, and of the other Eotaxin family member CCL26, in children with chronic epilepsy and breakthrough seizures and to a lesser extent in new onset of seizures. This finding may also suggest that Eotaxins may contribute to epileptogenesis and warrant further studies. 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.

We also observed elevation in other cytokines such as IL8 in blood from 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.[28-30] In a previous study[7] 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 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 IL-1β in the genesis of seizures and later development of epilepsy.[31-33] In our study, IL-1β levels were overall low and not different between cases and controls (Table 1). Similarly, while expression of MIP1α originating from neurons and microglia increases following soman-induced status epilepticus in rats,[34] we observed similar concentration of this cytokine in our cohorts. These differences could be the result of timing of collection, biological compartment analyzed, or could be related to the immunoassay that was utilized and warrant further investigation.

The only cytokine in our current 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 hours after generalized tonic-clonic seizures and febrile seizures but is not changed after seizures in patients with chronic focal epilepsy.[35] 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.[36] 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,[37] 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,[38] while others find positive correlations only for few cytokines, including IL6 similarly to our study,[39] IL1b,[40] IL2, IL12 and IFNg.[41] These differences may possibly be owing to several factors such as 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.

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 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 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 for example high-mobility group box 1 (HMGB1) and IL-18. 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. 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 and therefore our observations may represent indirect measures of inflammatory activation in the periphery.

Conclusions

In our study we showed differential activation of neuroinflammatory pathways in plasma from different seizure etiologies compared to controls, unrelated to HHV-6 infection. Children with febrile seizures had activation of the IFNg/CXCL10/IL10 pathway, highlighting a potential link with viral infection, possibly other than HHV-6 or EBV. Children with new onset seizures and chronic epilepsy had higher levels of CCL11, a cytokine that can enhance microglial neuroinflammation and reduce hippocampal neurogenesis.

Further longitudinal studies are needed to examine 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. By correlating these findings with imaging and EEG, 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.

Abbreviations

CNS, central nervous system

IL, interleukin

CSF, cerebrospinal fluid

PBMCs, peripheral blood mononuclear cells

CCL, C-C Motif Chemokine Ligand

HHV, human herpesvirus

MTLE, mesial temporal lobe epilepsy

MCP, monocyte chemoattractant protein

GFAP, glial fibrillary acidic protein

MSD, Meso Scale Discovery

EEG, electroencephalogram

EBV, Epstein-Barr Virus

ddPCR, digital droplet PCR

RPP30, Ribonuclease P Subunit P30

IFN, interferon

TNF, tumor necrosis factor

MDC, macrophage-derived cytokine

MIP, Macrophage inflammatory protein

TARC, thymus activation regulated chemokine

ANOVA, one-way analysis of variance

NOX, nicotinamide adenine dinucleotide phosphate-oxidase

ROS, reactive oxygen species

HMGB1 = high-mobility group box 1

FOCAL SLOW = focal slowing

GEN SLOW = generalized slowing

EPILEPT = epileptiform discharges

SZ = seizure

LEV = levetiracetam

OXC = oxcarbazepine

TPM = topiramate

LMT = lamotrigine

PB = phenobarbital

VPA = valproic acid

PHT = phenytoin

CLB = clobazam

Declarations

Ethics approval and consent to participate

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.

Consent for publication

Not applicable.

Availability of data and materials:

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

Competing interests:

The authors declare that they have no competing interests.

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Authors contributions:

LB designed the study, contributed to data analysis and interpretation, and drafting and revising of the manuscript for intellectual content.

MM contributed to data analysis and interpretation and drafting and revising of the manuscript for intellectual content.

GN conducted the statistical analysis and contributed to data interpretation and drafting and revising of the manuscript for intellectual content.

BT contributed to data collection and drafting and revising of the manuscript for intellectual content.

AD contributed to data collection and drafting and revising of the manuscript for intellectual content.

EW contributed to data interpretation and drafting and revising of the manuscript for intellectual content.

WS contributed to data collection and drafting and revising of the manuscript for intellectual content.

AB contributed to data collection and drafting and revising of the manuscript for intellectual content.

JC contributed to study design, data analysis and interpretation, and drafting and revising of the manuscript for intellectual content.

WT contributed to study design, data analysis and interpretation, and drafting and revising of the manuscript for intellectual content.

WG contributed to study design, data analysis and interpretation, and drafting and revising of the manuscript for intellectual content.

SJ contributed to study design, data analysis and interpretation, and drafting and revising of the manuscript for intellectual content.

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Tables

Table 1. Clinical characteristics and cytokine analysis in plasma

Seizure type	N	Age years mean (SD)	Male N (%)	N Sz	Sz duration (minutes)	Duration epilepsy years mean (SD)	AEDs	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 slow 2 (15%) gen slow 6 (46%) epilept 1 (8%) sz 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 7 (32%) gen slow 11 (50%) epilept 3 (13%) sz 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

Table 2. Clinical characteristics and cytokine analysis in saliva

Seizure type	N	Age mean (SD)	Male N (%)	N Sz	Sz duration mean (SD)	EEG	MRI
New onset	15	6.7 (5.2)	7 (47%)	1-2: 13 (86%) >2: 2 (14%)	<10 min: 15 (100%)	2 (13%) focal slow 1 (6%) gen slow 8 (53%) epilept 1 (6%) sz 1 (6%) N/A	1 (6%) epileptogenic 13 (86%) normal 1 (6%) N/A
Breakthrough	22	6.8 (5.5)	10 (46%)	1-2: 17 (77%) >2: 5 (23%)	< 10 min: 18 (82%) > 10 min: 4 (18%)	9 (41%) focal slow 7 (32%) gen slow 11 (50%) epilept 3 (13%) sz 1 (4%) N/A	9 (45%) epileptogenic 10 (45%) normal 3 (14%) N/A
Febrile	7	2.7 (2.3)	7 (100%)	1-2: 5 (72%) >2: 2 (28%)	< 10 min: 6 (86%) > 10 min: 1 (14%)	1 (14%) focal slow 4 (57%) normal 2 (28%) N/A	2 (28%) normal 5 (72%) N/A

Assay	Controls (n=44) Mean ± SD	Febrile (n=7) Mean ± SD	Breakthrough (n=22) Mean ± SD	New onset (n=15) Mean ± SD	p-value (ANOVA)
CCL11	2.1 ± 2.8	1.0 ± 1.7	0.3 ± 0.2	0.7 ± 1.7	**0.03**
CCL26	0.3 ± 0.4	0.2 ± 0.2	0.2 ± 0.3	0.2 ± 0.3	0.66
IFN-γ	0.0 ± 0.1	0.2 ± 0.3	0.0 ± 0.1	0.4 ± 1.1	**0.03**
IL-10	1.8 ± 2.1	1.3 ± 0.5	1.6 ± 0.8	2.3 ± 1.7	0.61
IL-1β	66.0 ± 97.3	110.5 ± 83.3	84.8 ± 80.7	190.0 ± 197.2	**0.02**
IL-2	1.9 ± 1.6	1.5 ± 1.5	2.4 ± 1.5	3.1 ± 1.9	0.09
IL-4	1.4 ± 1.1	1.3 ± 1.3	1.6 ± 1.1	2.1 ± 1.5	0.37
IL-6	10.3 ± 21.3	37.3 ± 52.9	15.4 ± 21.4	18.8 ± 18.0	0.07
IL-8	1219.8 ± 2357.7	1375.7 ± 1224.2	1124.9 ± 951.9	3471.3 ± 5370.1	0.09
IP-10	1413.4 ± 4150.0	2338.4 ± 4230.3	684.8 ± 1671.6	1063.0 ± 2582.4	0.76
MCP-1	215.2 ± 720.0	75.6 ± 147.6	47.2 ± 50.1	1434.5 ± 3859.0	0.09
MIP-1α	2.1 ± 6.3	1.2 ± 1.1	0.2 ± 0.1	0.6 ± 0.9	0.52
MIP-1β	1.6 ± 6.1	0.4 ± 0.9	0.1 ± 0.1	1.5 ± 5.1	0.76
TNF-α	9.2 ± 12.2	9.6 ± 13.3	9.9 ± 8.0	22.0 ± 23.2	0.06

Figures

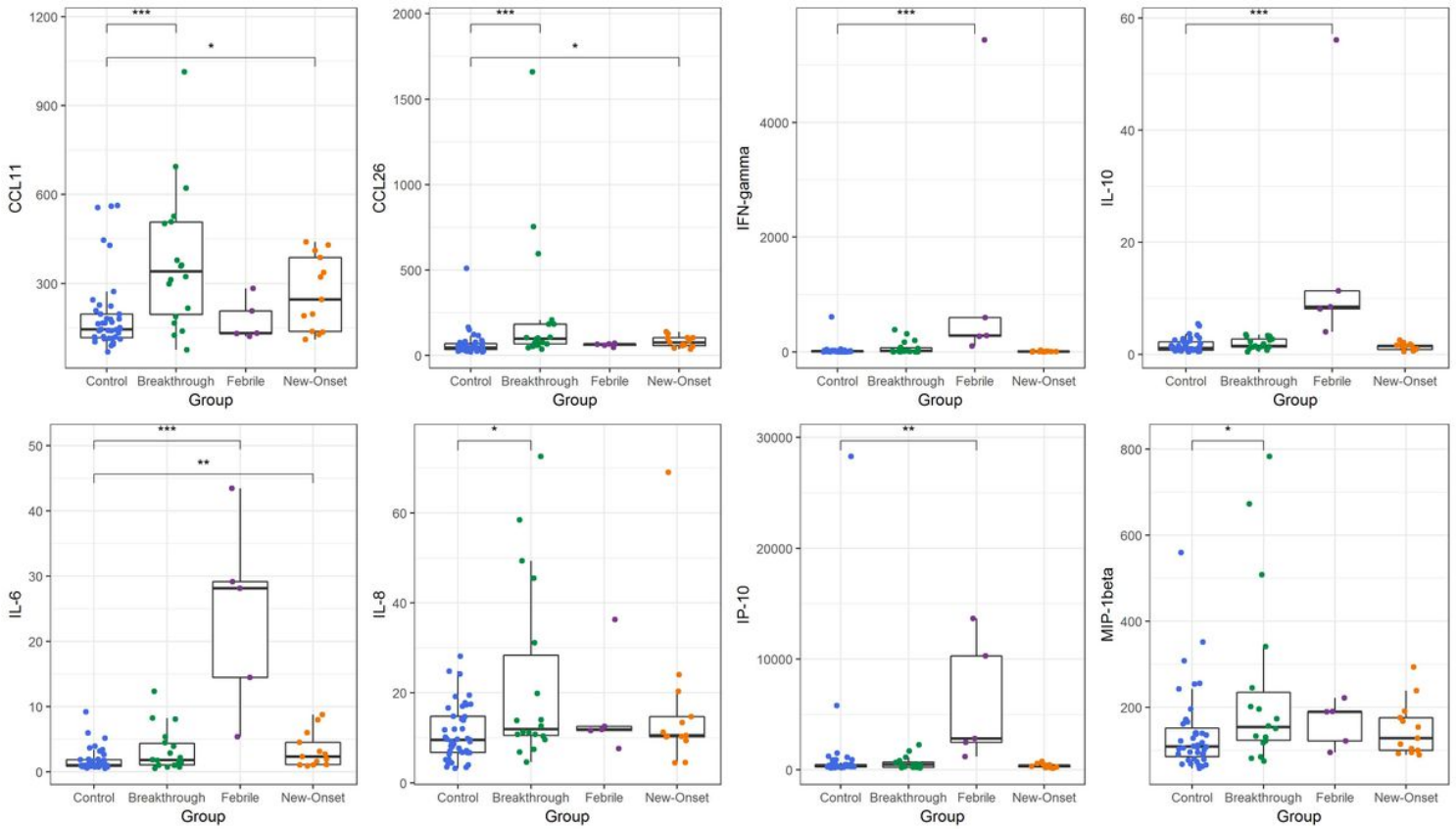

Figure 1

Cytokine levels in plasma by seizure type. Levels (pg/mL) for selected cytokines in plasma from children with different seizure types (breakthrough seizures in chronic epilepsy, green; febrile seizures, purple; and new onset seizures, 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 IFN (p<0.001), IL-6 (p<0.001), IL-10 (p<0.001), CXCL10 (p=0.001).

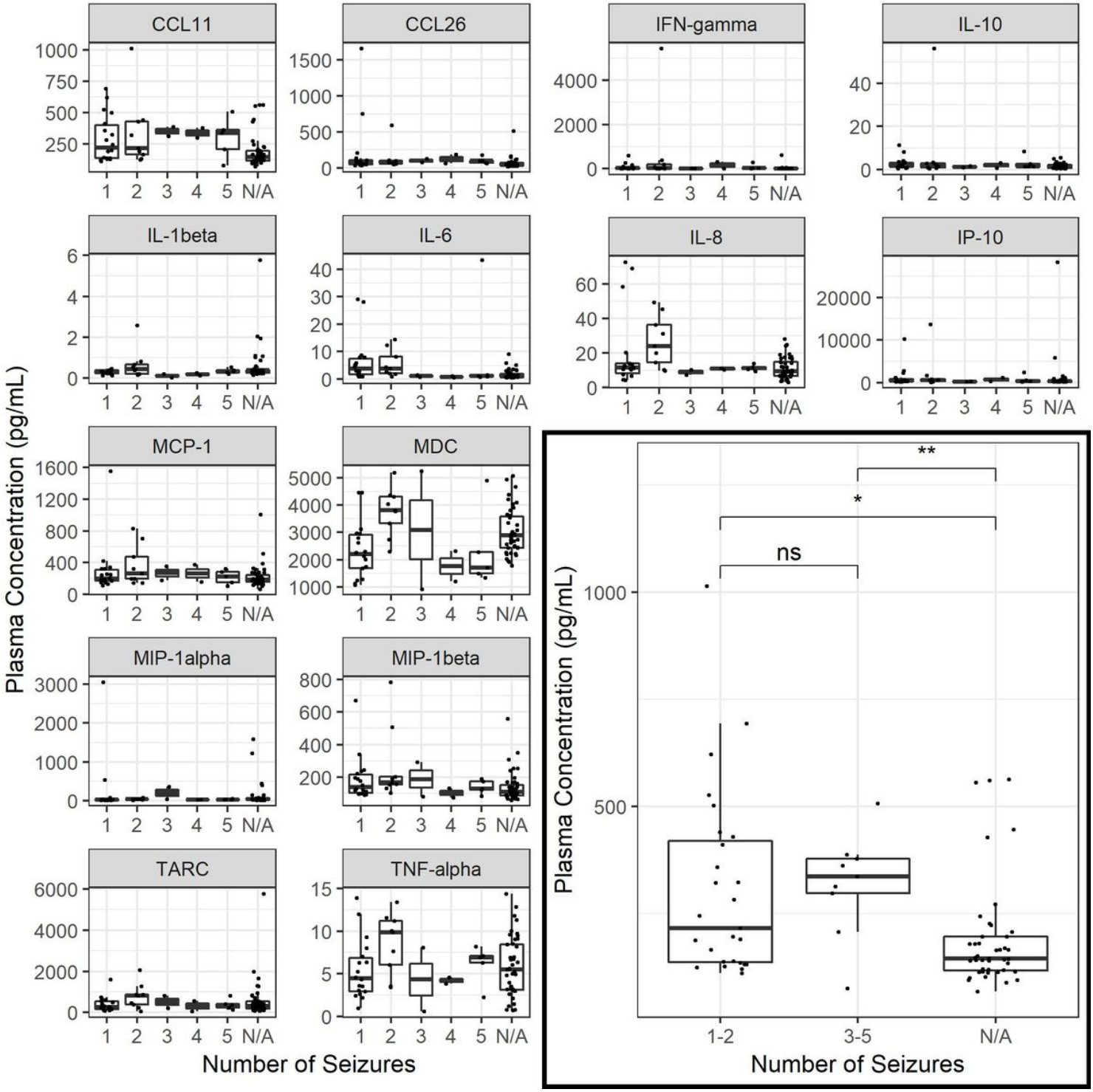

Figure 2

Cytokine levels in plasma by seizure number. Cytokine levels in plasma (pg/mL) grouped by number of seizures, showing higher CCL11 levels than controls in children with 3 or more seizures (top left plot and blow-up box, bottom right; p=0.01). N/A = not applicable.

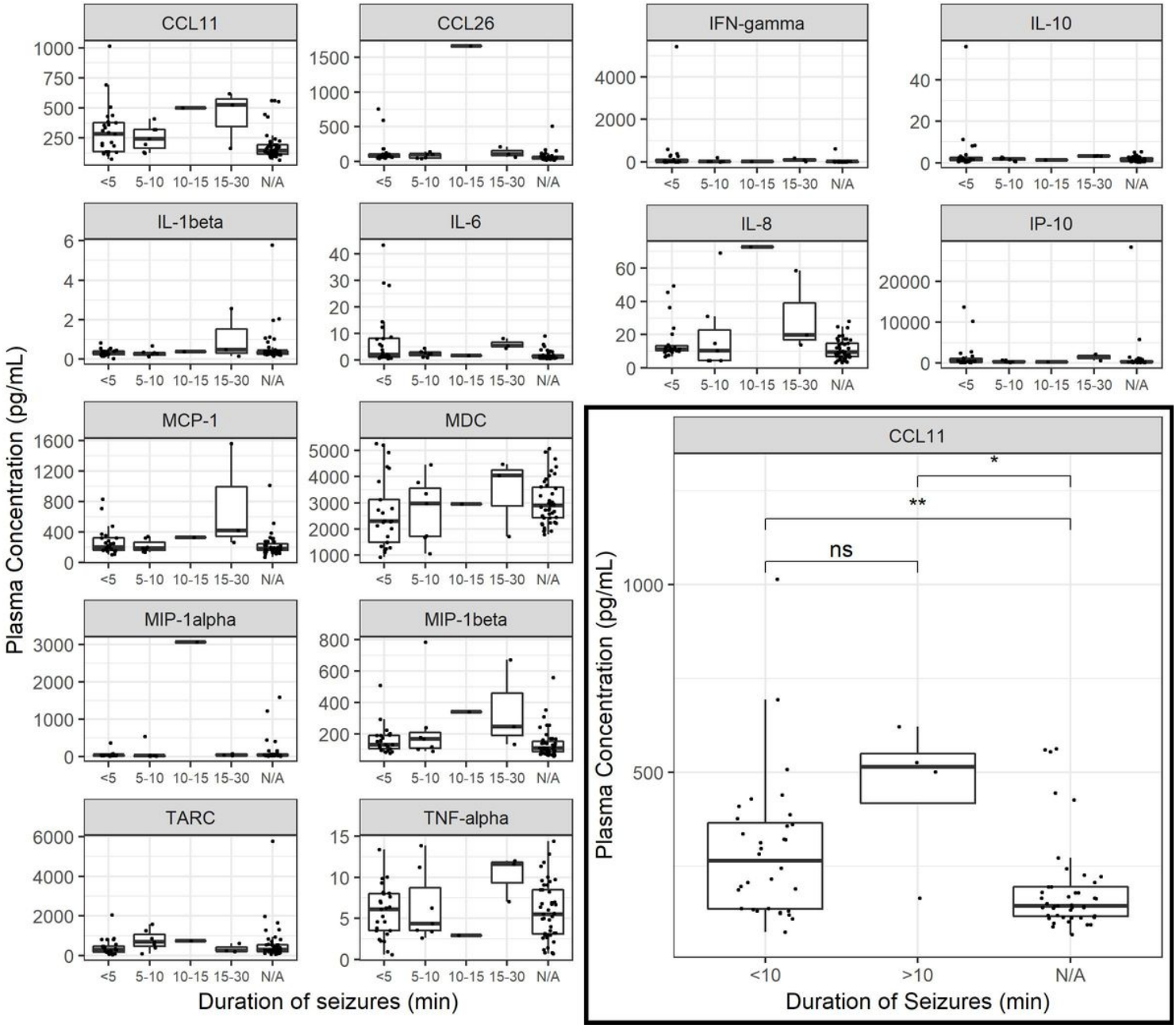

Figure 3

Cytokine levels in plasma by seizure duration. Cytokine levels in plasma (pg/mL) grouped by seizure duration, showing higher CCL11 levels than controls in children with seizures longer than 10 minutes (top left plot and blow-up box, bottom right; p=0.001). N/A = not applicable

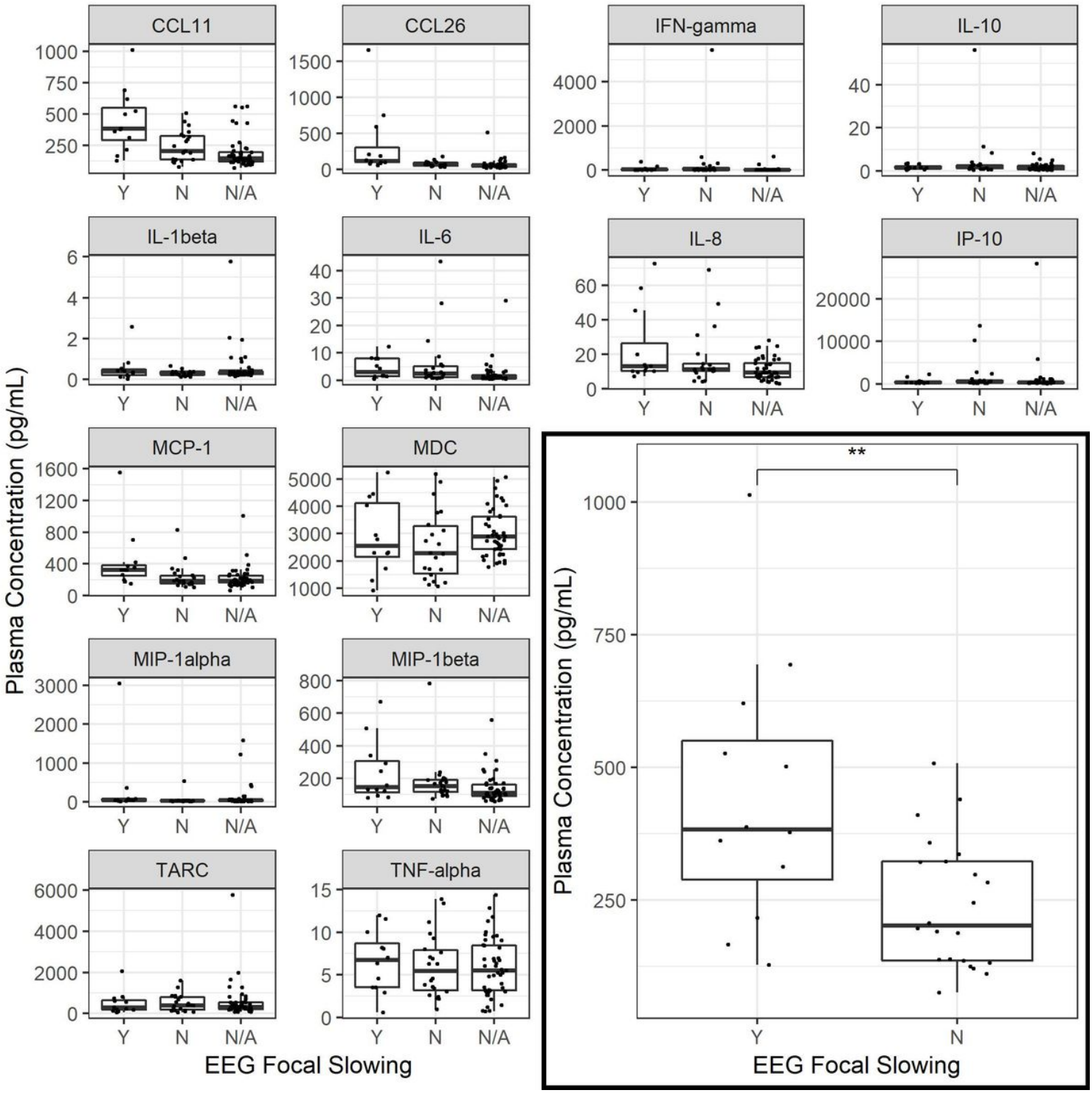

Figure 4

CCL11 level in plasma and EEG focal slowing. Higher CCL11 levels in plasma (pg/mL) than controls were detected in children with focal slowing on EEG (top left plot and blow-up box, bottom right; p=0.02). N/A = not applicable

Supplementary Files

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