Host Immune Response to Enterovirus and Parechovirus Systemic Infections in Children

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Abstract:

Background: Enterovirus (EV) and Parechovirus type A3 (PeV-A3) cause infections ranging from asymptomatic to life-threatening. Host immune responses in children, particularly innate responses to PeV-A3, remain largely unknown. The aim of this study was to determine aspects of the cytokine/chemokine responses to EV and PeV-A3 in cerebrospinal fluid (CSF) and plasma obtained from children with systemic/central nervous system infection.

Methods: A total of 74 salvaged CSF samples (27 with EV, 23 PeV-A3, and 24 neither EV nor PeV-A3) and 35 paired blood samples (10 EV, 14 PeV-A3, and 11 neither) were studied. Concentrations of cytokines and chemokines were measured using a customized 21-plex MILLIPLEX MAP® Human Cytokine/Chemokine Magnetic Bead Panel. Additionally, clinical characteristics data for all the patients were collected from Electronic Medical Records to evaluate the potential association between the immune response and presentations.

Results: We demonstrate that EV and PeV-A3 infections induce different cytokine/chemokine immune responses in children. EV induces more robust responses in CSF with significantly elevated levels of fractalkine, IFN-α2, IFN-γ, IL-1α, IL-4, IL-8, and TNF-α; PeV-A3 induces less robust or absent responses in CSF but robust responses in plasma with significantly higher concentrations of IFN-α2, IL-15, IL-1α, IP-10, and MCP-1.

Conclusions: High cytokine/chemokine concentrations in plasma of PeV-A3 patients compared to EV patients could explain higher/more prolonged fever in PeV-A3 patients, whereas relatively low cytokine/chemokine concentrations in PeV-A3 CSF might explain the absence of CSF pleocytosis.
INTRODUCTION

Viral infections of the central nervous system (CNS) remain a public health issue in developing and developed countries [1]. Enterovirus (EV) and parechovirus (PeV-A), particularly PeV-A3, are reported to be the leading causes of viral CNS infections in much of the world, including the UK, Europe, China, Japan, and the United States [2-9].

EV and PeV-A members of the family Picornaviridae, are non-enveloped viruses with single-stranded positive sense RNA genomes, known or suspected to cause a wide spectrum of clinical manifestations in humans (10). Infections caused by these agents can be asymptomatic but can also be severe, e.g. myocarditis, encephalitis, sepsis-like syndrome, and CNS infections [5, 11, 12]. Neonates and infants are at a higher risk for severe clinical illness and sequelae than are older children and adults [10].

The clinical features of both EV and PeV-A3 can be similar when symptoms do occur. Therefore, distinguishing between EV and PeV-A3 based purely on clinical signs or symptoms is difficult at initial presentation [13, 14]. As the infection evolves, young infants with systemic PeV-A3 infections have higher fever, longer duration of fever, higher heart rate, absence of cerebrospinal fluid (CSF) pleocytosis, plus somewhat higher CSF concentrations of glucose and protein compared to EV-infected infants [5, 15].

The innate arm of the immune system produces important initial responses during viral infections, resulting in both local and systemic release of inflammatory and antiviral molecules, e.g., cytokines. A subgroup of cytokines categorized as chemokines also influence leukocyte migration and trafficking of immune cells [16]. In particular, early production of cytokines induces an antiviral state and triggers the activation of immune cells [17]. Increased levels of cytokines and
chemokines have been observed during systemic viral infections, including dengue fever and influenza [16, 18], and are thought to be associated with aspects of clinical presentations, severity of illness, and degree of abnormalities in laboratory test results.

Clinical characteristics and laboratory findings and their differences in EV- and PeV-A3–infected infants have been documented reasonably well. However, the data for host innate immune responses to EV and PeV-A3 infections in relation to severity of clinical aspects and abnormalities in laboratory test results are still limited [13-14, 36]. Therefore, the objective of our study was to evaluate cytokine and chemokine responses in CSF and plasma of EV vs. PeV-A3 infected vs. control patients with neither EV nor PeV-A3, evaluating for possible differences in innate response between PeV-A3, EV, and controls. Differential results for innate responses could be valuable in further defining pathogenesis and possible outcome markers for EV and PeV-A3 CNS infections.

MATERIALS AND METHODS

Sample Collection

This retrospective study was conducted at Children’s Mercy, Kansas City (CMKC) and was approved by the CMKC Institutional Review Board. De-identified clinical specimens (CSF and plasma) were salvaged from neonates and infants ≤3 months of age who underwent sepsis/meningitis evaluation as part of standard of care during January through December 2018. All samples were collected on the day of admission, which was within 2 days of symptom onset. CSF and blood samples were collected within a one hour-interval. All CSF samples and residual plasma samples from each group which had enough sample volume were screened for EV and PeV-A by a two-step RT-PCR targeting the 5’translated region as described previously [15].
Positive CSF samples also underwent bi-directional sequencing targeting the VP1 region for determining the EV and PeV-A genotypes (37, 38). Samples from a total of 71 patients were available for study. Analyzed CSF samples were from 23 patients with EV-positive CSF, 27 patients with PeV-A3 positive CSF, and 24 control patients with CSF negative for both EV and PeV-A3. Overall, 35 CSF samples were paired with a plasma sample obtained from the same patient (10 EV, 14 PeV-A3, and 11 samples from the same patient that had neither EV nor PeV-A3). Clinical and laboratory data were obtained from the patients’ electronic medical records (age, gender, maximum temperature (Tmax), length of hospitalization, peripheral blood white blood cell [WBC] count, CSF WBC, CSF glucose and CSF protein concentration).

Cytokine and Chemokine Analysis

The CSF samples were stored at -70°C prior to analysis. Plasma was separated from blood samples by centrifugation (2000 g for 10 minutes) at 4°C and stored at -70°C until the cytokine analysis. The concentrations of cytokines/chemokines were measured in both CSF and plasma of EV, PeV-A3, and control groups by using the MILLIPLEX MAP® Human Cytokine/Chemokine Magnetic Bead Panel from Millipore Sigma, Burlington, MA. The customized 21-plex cytokine/chemokine panel consisted of the following analytes: Granulocyte-macrophage-colony-stimulating factor (GM-CSF), fractalkine, interferon (IFN)-α2, IFNγ, interleukin (IL)-10, IL-12p40, IL-13, IL-15, IL-17A, IL-1Ra, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IP-10, chemokine ligand 2 (CCL2)/MCP-1, CCL3/MIP-1α, CCL5/RANTES, and tumor necrosis factor (TNF)-α. Samples were measured in duplicate, and the concentration was calculated by reference to the standard curve for each cytokine. The average coefficient of variation was measured between the duplicates, and values less than 30 were considered acceptable. All samples were measured undiluted. The lower detection limit for each cytokine was as follows: 7.5 pg/ml for GM-CSF, 22.7 pg/ml for fractalkine,
2.9 pg/ml for IFN-α2, 0.8 pg/ml for IFNγ, 1.1 pg/ml for IL-10, 7.4 pg/ml for IL-12p40, 1.3 pg/ml for IL-13, 1.2 pg/ml for IL-15, 0.7 pg/ml for IL-17A, 8.3 pg/ml for IL-1Rα, 0.8 pg/ml for IL-1β, 1.0 pg/ml for IL-2, 4.5 pg/ml for IL-4, 0.5 pg/ml for IL-5, 0.9 pg/ml for IL-6, 0.4 pg/ml for IL-8, 8.6 pg/ml for IP-10, 1.9 pg/ml for MCP-1, 2.9 pg/ml for MIP-1α, 1.2 pg/ml for RANTES, and 0.7 pg/ml for TNFα. Cytokines that were below the detection limit were categorized as undetectable.

**Statistical Analysis**

The clinical characteristics of EV, PeV-A3, and control groups are presented as median and interquartile range (IQR). Statistical differences between 2 or multiple groups were analyzed by nonparametric Kruskal-Wallis testing and pairwise post hoc testing in Microsoft Excel 365 with Real Statistics add-in (http://www.real-statistics.com/). P values less than 0.05 were considered statistically significant.

**Discriminant Analysis**

Discriminant analysis (DA) was done using SPSS version 24. The discriminant function aims to create a predictive model of group membership based on a linear combination of variables. Variance-covariance homogeneity was tested using the nonparametric Levene’s test, which is both more robust and suited to non-normal distributed data (19). Stepwise DA was used so that our predictive model would be based on the most discriminating biomarkers for each data set.

Wilks’ Lambda test was used to evaluate the performance of each biomarker in the model. The values of the Wilks’ Lambda statistic range from 0 to 1, with 0 indicating perfect group discrimination and 1 indicating no discrimination at all. The significance of Wilks’ Lambda was tested using the F test (P values < 0.05 were considered statistically significant).
Principal Component Analysis

Principal component analysis (PCA) was performed using either BioNumerics Version 6.6 (Applied Maths, NV, (www.applied-maths.com) or IBM SPSS Statistics for Windows, Version 24.0 (IBM Corp., Armonk, NY). PCA was computed from covariance matrices. To avoid dominance of variables with large values, we normalized the data for each variable by dividing by appropriate variance before principal components computation. Sample adequacy and existence of correlated variables were evaluated by Kaiser-Meyer-Olkin (KMO) measure of sampling adequacy [20, 21] and Bartlett’s test of sphericity [22], respectively; both tests were performed using SPSS. Bartlett’s test of sphericity yields a $P$ value that represents the probability that correlated variables do not exist. For PCA to be meaningful, this null hypothesis must be rejected. Significance of principal components was estimated by Monte Carlo simulation (Parallel Analysis) using the syntax written for SPSS by Brian O’Connor [23].

Biomarker Evaluation Using the Receiver Operating Characteristic Curve Method

The receiver operating characteristic (ROC) curve was generated by plotting specificity minus 1 and sensitivity on the x and y axes, respectively. To simplify the evaluation of any given biomarker, we evaluated the biomarker’s usefulness by the area under the ROC curve (AUC). On such plots, a biomarker with perfect sensitivity and specificity (i.e., both sensitivity and specificity are equal to 100%, and “1-specificity” is equal to “0”) has an AUC equal to 1, while a useless biomarker with sensitivity and specificity of 50% (i.e., using the biomarker is equivalent to flipping a coin) has an AUC equal to 0.5 [24]. Asymptotic significance is measured by calculating a $P$ value that tests the null hypothesis that the true AUC of the relevant biomarker is equal to 0.5. The null hypothesis is rejected at $P$ values lower than 0.05.
To combine biomarkers into the best possible profiles, we used DA canonical functions 1 and 2 as these profiles. These functions were computed to maximize group separation and included only the best performing biomarkers. We used the AUC as a measure of biomarker performance.

RESULTS

Demographics, Clinical and Laboratory Findings of Patients from whom Salvaged Samples Were Obtained

Demographics and laboratory values for overall patient groups (EV, PeV-A3, and controls) are summarized in Supplementary Table 1. There was no statistical difference in length of stay, age, or gender for EV, PeV-A3, and control groups. The median age of EV patients was 4 weeks with 61% male, 3 weeks for PeV-A3 patients with 63% male, and 4 weeks for the control group with 67% male. Maximum temperature was different among groups overall ($P < 0.001$), with Kruskal-Wallis comparison tests showing PeV-A3–infected patients had higher Tmax than EV ($P < 0.001$) and control groups ($P < 0.0001$). There was no significant difference in Tmax between EV-infected patients compared to controls.

Peripheral blood WBC was different among groups overall ($P = 0.001$), with lower counts in PeV-A3–infected groups compared to EV ($P < 0.001$) and control groups ($P < 0.001$). CSF WBC counts were also significantly different among groups overall ($P = 0.0006$), with EV patients having higher counts than control ($P < 0.001$) and PeV-A3 groups ($P < 0.0001$). CSF pleocytosis was present less with PeV-A3 (0/27, 0%) compared to controls (3/24, 12.5%) and EV (8/23, 34.8%) ($P = 0.002$). CSF protein concentrations were not significantly different among groups. CSF glucose concentrations were significantly different among groups ($P = 0.0003$), with
concentrations for the EV group being lower than those for both PeV-A3 and controls ($P < 0.0001$ for both); PeV-A3 and controls were not significantly different.

**Cytokines and Chemokines in Plasma and CSF of EV- and PeV-positive Samples Compared to Control Samples**

Among the 21 cytokines/chemokines analyzed, the cytokine/chemokine concentrations of 5 (GM-CSF, IL-13, IL-17A, IL-2 and IL-5) were below the detection limit in CSF and close to the limit in plasma. The coefficient of variation between the duplicates was acceptable for all duplicate assays on the same sample. No significant differences between groups were detected for RANTES and IL-5 and hence were not included for the additional statistical analysis below (Figure 1a & 1b). Several examples of the analytes that had a statistically significant difference between groups and sample types are shown in Figure 1a & 1b.

The EV group had increased CSF fractalkine, IFN-α2, IFN-γ, IL-1Rα, IL-4, IL-8, and TNF-α when compared to both PeV-A3 and controls, Figure 1a & 1b. Levels of IL-6, IL-10 and TNF-α are high in EV group, but undetectable in PeV-A3 group. Likewise, the PeV-A3 group’s CSF concentrations for fractalkine, IFN-α2, IL-15, IL-4, IP-10, and MCP-1 were significantly higher compared to controls, Figure 1a & 1b. The PeV-A3 group’s CSF IFN-γ, IL-6, MIP-1α, and RANTES concentrations, while lower than controls, were not significantly different than controls.

The innate immune response in plasma of the EV group as determined by plasma concentrations of cytokines/chemokines was less intense than the innate immune responses identified in the PeV-A3 and control groups. Compared to PeV-A3 plasma concentrations, EV plasma concentrations were increased only for IFN-γ and TNF-α, but neither increase was significant. Compared to the
control plasma concentrations, EV plasma concentrations were significantly increased only for IP-10.

In contrast, the PeV-A3 group had a more robust response in plasma, with significantly higher IFN-α2, IL-15, IL-1Rα, IP-10, and MCP-1 concentrations compared to EV or control groups, Figure 1a & 1b.

**Determination of the Most Discriminating Biomarkers**

We tested CSF and plasma biomarkers separately and in combination. The number of biomarkers that contributed significantly to the model varied between data sets. Six, two, and eight biomarkers were significant using CSF, plasma, or both, respectively. Discrimination between EV-infected, PeV-A3–infected, and control subjects was substantially better when using combined CSF-plasma biomarkers.

Better discrimination was evident by visually inspecting canonical function plots (Figure 2), as well as by comparing the rates of correct classification (RCC) (Table 1). Canonical functions 1 and 2 were used to create a coordinate system in which individual participants were plotted and group centroids were marked (squares in Figure 2). The most spread of group centroids and least inter-group overlap were seen using a CSF-plasma combined biomarker profile. Furthermore, the smallest (best) Wilks’ Lambda values were obtained with this combined profile, indicating that the combination provided best performance within the model as well as best model performance (Figure 2). Consistent with these data, we saw the highest RCC fidelity using the combined CSF-plasma biomarker profile, with group RCC ranging between 91% and 100% (Table 1).
Exploring Natural Partitioning Using PCA

We performed PCA using either all biomarkers or only the significant biomarkers identified in our DA studies. Clearer group separation again occurred with combination of CSF with plasma biomarkers and also restriction of analysis to the DA-identified biomarkers (Figure S1).

Evaluation of Biomarkers’ Performance Using ROC Curves

High CSF cytokine AUC values were more frequent for EV-infected patients, while high plasma AUC values were more frequent for PeV-A3–infected patients, Table 2, i.e. blue shaded values in first 4 columns. Generally, multivariate biomarker profiles performed better than individual biomarkers as indicated by the higher AUC values in Table 2, Disc1 values. Multivariate profiles had the highest and fourth highest AUC values for PeV-A3 and EV, respectively. The very highest AUC values for both EV- and PeV-A3–infected subjects were obtained using CSF-plasma combined profiles, Table 2 (columns 5 and 6), highlighting the potential advantage of using combined profiles.

DISCUSSION

The immune system is a complex and coordinated network. Upon invasion of the host by microorganisms, inflammatory mediators are generated in “signaling cascades” through a series of pathways that result in subsequent release of pro-inflammatory and anti-inflammatory cytokines/chemokines intended to inhibit pathogen replication and promote adaptive immunity [14, 25]. While immune responses to EV have been studied in some detail, knowledge gaps persist to a greater extent about PeV-A3, particularly innate responses [1, 14, 26-31].

This is the first US study that describes diverse immune profiles in paired CSF/plasma of EV vs. PeV-A3–infected patients and how differential production of selected molecules might contribute
to the differences in certain clinical or laboratory findings of EV vs. PeV-A3 in young infants. We confirmed that our current patient groups, from which CSF and plasma were obtained, shared demographic and clinical characteristics with previously reported patients infected with PeV-A3 and EV infections [5, 6]. Such characteristics include PeV-A3–infected infants having higher Tmax, longer duration of fever, and less frequent CSF pleocytosis compared to EV-infected infants.

We analyzed concentrations of 21 cytokines in plasma and CSF by using DA, PCA, RCC, and AUC methodology. We demonstrate that innate immune responses in CSF and in plasma differ in CNS EV infections compared to PeV-A3. The differential concentrations in CSF vs. plasma could explain differences in the rates of CSF pleocytosis for PeV-A3 (rare) vs. EV (common) CNS infections, and higher, more prolonged fever in young PeV-A3–infected infants.

Our use of DA or PCA analyses condensed multiple variables into a smaller number of linear combinations or functions. The key difference between the 2 techniques is that DA computes these functions to optimize group prediction; therefore, DA optimizes discrimination between predefined groups, e.g., infection by a certain pathogen. PCA, however, ignores investigator-defined groups and aims to maximize the amount of variance accounted for by the smallest number of principal components. Therefore, plotting participants using the PCs obtained from a PCA enabled examination of the unbiased natural partitioning of groups in a data set.

Using multiple analytic tools allowed exploration of distinctions between pathogens and among compartments using data (multiple cytokines/chemokines from differing compartments) from relatively small numbers of patients.
Many cytokines/chemokines (IL-6, IL-8, IL-10, IP-10, MCP-1, IL-1Rα, fractalkine, and GM-CSF) were elevated in CSF of EV patients but were present in lower concentrations or undetectable in plasma. These cytokines appear to play an important role in early control of both viral replication and also potential tissue injury due to infection [25]. Our data in part support the hypothesis that pro-inflammatory cytokine production in the CSF during EV infection attracts WBCs into the CNS, but anti-inflammatory cytokine production terminates the pleocytosis after elimination of virus [27].

Chemokines such as IL-8 and IP-10 that are involved in regulation of leukocyte migration and inflammation were also more elevated in EV-infected CSF than in plasma. One prior study showed that IL-8 and IP-10 in CSF are principally produced by the CNS microglia or astrocytes, rather than immune cells circulating in the blood in EV-71 infections [32]. IP-10 is known to be a common response in CSF and may be a non-specific response to any CNS viral infection [13]. That said, these chemokines enhance the production of downstream anti-inflammatory cytokines such as IL-10, IL-1Rα, IL-4, and IL-13, which are important in modulating the inflammatory process in viral meningitis [27].

In contrast to EV infections, innate responses in plasma from PeV-A3–infected patients were more robust than in CSF. In PeV-A3 infected infants, plasma concentrations of pro-inflammatory cytokines (IL-15, fractalkine, and IL-12p40) and chemokines (MCP-1 and IP-10), as well as anti-inflammatory cytokines (IFN-α2, IL-1Rα, and IL-10), were elevated. Yet CSF concentrations of the same cytokines/chemokines were relatively low or were undetectable. The relatively high concentrations of pro-inflammatory cytokines in plasma of PeV-A3 patients versus EV or control patients could explain the intensity of some clinical manifestations such as higher and longer duration of fever. On the other hand, relatively low chemoattractant cytokine/chemokine
concentrations in PeV-A3 CSF could help explain the absence of CSF pleocytosis, a classic finding in PeV-A3–infected children.

Our new data suggest that combined compartment (plasma combined with CSF) biomarker profiles more than individual compartment biomarkers are distinctive and characteristic of EV vs. PeV-A3 infections in young infants. This notion is supported by other studies [7, 8] but needs to be confirmed in studies with larger numbers of patients.

Overall, prior studies lend credence to our data. A recent study by Habukha et al., from Japan investigated expression of 22 cytokines and chemokines in CSF and plasma of infants infected with EV and PeV-A3 (36). Sixteen biomarkers overlapped between their study panel (n=22) and ours (n=21). Seven of 16 biomarkers evaluated in both studies (IL-6, IL-10, IL-1Rα, IFN-α2, IL-8, TNF-α and IL-15) were elevated in EV CSF; while our study identified an additional six biomarkers (fractalkine, IFN-γ, GM-CSF, MIP-1α, IL-12p40 and IL-1β) in EV-CSF samples (Figure 1a & 1b). Similarly, in PeV-A3 plasma 5/16 overlapping biomarkers (IFN-α2, fractalkine, IL-15, IL-10, IL-1Rα) were elevated in both studies; and two additional biomarkers (MCP-1 and IP-10) were additionally identified by our study. In studies using only CSF samples, pro-inflammatory CSF cytokines (TNF-α, IL-1, and IL-6) and anti-inflammatory cytokines (IL-4 and IL-10,) were significantly lower in PeV-A than in EV CNS infections [14]. Another study evaluating only CSF cytokines in infants showed that some cytokines (IL-1β, IL-5, IL-6, IL-12, and IL-17) were higher in EV infection; while some cytokines (IL-2, IL-4, IL-7, and IL-13) were detected in higher concentrations in PeV-A3 infection [33].

Yet another study evaluated only serum cytokines from 12 patients with PeV-A3–induced sepsis-like syndrome vs. 28 healthy children. Serum IL-6, neopterin, sTNFR-I, sTNFR-II, and IL-18 concentrations increased rapidly in early infection but decreased to those of healthy children during
recovery (34). There is also a case report of very high serum IL-6 concentrations in an adult with severe epidemic myalgia due to a PeV-A strain that was not typed [35].

Our study has limitations. We collected clinical data retrospectively, so some clinical data may not be completely accurate. We did not have a paired plasma sample for every CSF sample, so our numbers were relatively small for paired analysis. We did not have sufficient paired plasma from all the negative CSF patients to perform EV and PeV-A RT-PCR. Despite small patient numbers, multiple analyses and modeling, including DA, PCA, and RCC, provided separation of profiles and discrimination between groups. There were multiple detected EV serotypes, but all PeV-A were PeV-A3. EV types can produce different degrees of illness severity, so systemically less virulent and/or more neurotropic EV strains may have contributed to differences in innate responses compared to PeV-A3 infections. We evaluated data for a single year, so years in which differing EV circulate may produce fewer differences in cytokine profiles. Also, we do not know the pathogen that caused the infection leading to a sepsis workup in the control group, so this group may have a mix of pathogens with varying virulence.

Despite these limitations, our data confirm that an infant less than 3 months old presenting with high fever, leukopenia, and lack of CSF pleocytosis is likely to have a PeV-A3 systemic/CNS infection, and suggest the likelihood of higher plasma but lower CSF concentrations of IP-10, MCP-1, IL-15, IL-1Rα, and IFN-α2 compared to EV-infected infants.

In summary, we found that innate immune responses in different compartments (CSF vs. plasma) vary depending on the viral agent. Variations in cytokine and chemokine profiles in patients with EV and PeV-A3 infection may contribute to the pathogenesis, variations in clinical presentation, and disease outcomes of each virus. Analysis of innate immune profiles could be potentially useful to design interventions involving immune-prophylaxis and immunotherapy.
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TABLE 1. RATE OF CORRECT CLASSIFICATION (RCC) BASED ON DISCRIMINANT ANALYSIS (DA) MODELS.

|                  | Predicted Classification |
|------------------|--------------------------|
|                  | Control | EV    | PeV   |
| **Original**     |          |       |       |
| Classification   |          |       |       |
| Control          | 87.5 (21) | 4.2 (1) | 8.3 (2) |
| EV               | 8.7 (2)   | **60.9 (14)** | 30.4 (7) |
| PeV              | 14.8 (4)  | 3.7 (1) | **81.5 (22)** |

Plasma - **Overall RCC: 71.4%**

|                  | Predicted Classification |
|------------------|--------------------------|
|                  | Control | EV    | PeV   |
| **Original**     |          |       |       |
| Classification   |          |       |       |
| Control          | 72.7 (8)  | 27.3 (3) | 0.0 (0) |
| EV               | 30.0 (3)  | **50.0 (5)** | 20.0 (2) |
| PeV              | 0.0 (0)   | 14.3 (2) | **85.7 (12)** |

CSF & Plasma - **Overall RCC: 97.1%**

|                  | Predicted Classification |
|------------------|--------------------------|
|                  | Control | EV    | PeV   |
| **Original**     |          |       |       |
| Classification   |          |       |       |
| Control          | **90.9 (10)** | 0.0 (0) | 9.1 (1) |
| EV               | 0.0 (0)   | **100.0 (10)** | 0.0 (0) |
| PeV              | 0.0 (0)   | 0.0 (0) | **100.0 (14)** |

Rates of correct and incorrect classification are expressed as percentage (%) of group totals. RCCs are in bold, and incorrect classification rates are in regular font. The number of patients per category is in parentheses.
### TABLE 2. EVALUATION OF BIOMARKERS AND BIOMARKER PROFILES USING THE AREA UNDER A ROC CURVE (AUC) METHOD.

| CSF & Plasma | Plasma | CSF & Plasma | Plasma | CSF & Plasma | Plasma | CSF & Plasma | Plasma | CSF & Plasma | Plasma |
|--------------|--------|--------------|--------|--------------|--------|--------------|--------|--------------|--------|
| TNFa         | 0.88   | Disc2        | 0.866  | Fractalkine  | 0.854  | Disc1        | 0.952  | Disc2        | 0.924  | Disc1 |
| MIP1a        | 0.861  | IL1b         | 0.726  | GMCSF        | 0.774  | IFNa2        | 0.901  | Disc1        | 0.912  | Disc2 |
| IL1b         | 0.844  | IFNg         | 0.686  | IL17A        | 0.718  | IP10         | 0.891  | IL1b         | 0.839  | GMCSF |
| IL1b         | 0.835  | IP10         | 0.632  | MIP1a        | 0.666  | IL12p40      | 0.753  |
| IL1b         | 0.826  | MCP1         | 0.623  | IL6          | 0.66   | IL10         | 0.728  |
| IL1b         | 0.812  | IL10         | 0.61   | IL2          | 0.676  | IL8          | 0.755  |
| IL1b         | 0.774  | RANTES       | 0.593  | IL8          | 0.612  | IL13         | 0.595  |
| IL1b         | 0.765  | IL17A        | 0.548  | TNFa         | 0.604  | IL6          | 0.595  |
| IL1b         | 0.749  | IL13         | 0.543  | IL1Ra        | 0.6    | IL5          | 0.571  |
| IL1b         | 0.702  | IL12p40      | 0.541  | IP10         | 0.564  | IL4          | 0.568  |
| IL1b         | 0.69   | IL1Ra        | 0.54   | IL4          | 0.548  | MIP1a        | 0.556  |
Disc1/Disc2: first/second discriminant function. Analogous to principal components in PCA, discriminant analysis computes discriminants. Both principal components and discriminant functions are eigenvectors that can be viewed as artificial variables comprised of contributions from observed variables. In a multivariate problem, datapoints are plotted in multidimensional space with as many axes as variables. To transform this into a simpler two- or three-dimensional presentation, variables are combined into eigenvectors ranked by the amount of variance they explain. In PCA, the direction of the eigenvector that explains the most variance (i.e. first component) is selected so that it explains the maximum amount of variance that can be explained by one vector (i.e. maximizing amount of variance explained). In discriminant analysis, the eigenvector that explains the most variance (first discriminant function) is selected to maximize group separation. Since these eigenvectors are linear combinations of observed variables, they may be used as a way to combine variables and test them using the AUC method. Here, we selected discriminant functions (Disc1 and Disc2) since they are likely to be superior to principal components given the way they were computed. Heat map correspond to the range of AUC values from the lowest (dark red) to highest (dark blue).
Figure Legends:

**Figure 1a & 1b.** Bar graphs representing cytokine/chemokine concentrations in cerebrospinal fluid (CSF) and plasma of EV, PeV-A3, and control groups. Six analytes demonstrated significantly elevated values in CSF of EV patients when compared to both PeV and control groups; fractalkine, IFN-α2, IFN-γ, IL-1Rα, IL-4, IL-8, and TNF-α. In contrast, values for 5 analytes were significantly higher in plasma of PeV-A3–infected patients when compared to both EV and control groups; IFN-α2, IL-15, IL-1Rα, IP-10, and MCP-1. (*p<0.05, **p<0.005, ***p<0.0005)

**Figure 2.** Discriminant analysis (DA) predictive model. Cerebrospinal fluid (CSF) (control: n = 24, EV: n = 23, PeV: n = 27) and plasma (control: n = 11, EV: n = 10, PeV: n = 14) biomarkers were analyzed separately and in combination. Canonical functions (DSC) 1 and 2 are plotted on the x and y axes, respectively. Respective P values shown in the plots indicate model significance. Wilks’ Lambda values for individual biomarkers and the model as a whole are shown in the tables below the plots. b: plasma biomarker, c: cerebrospinal fluid biomarker.
Figure 1a

The figure shows the levels of various cytokines and chemokines in plasma and cerebrospinal fluid (CSF) samples. The data is presented as mean ± standard deviation.

**Fractalkine**
- Plasma: EV: 800 pg/ml, PeV-A: 600 pg/ml, Control: 200 pg/ml
- CSF: EV: 600 pg/ml, PeV-A: 500 pg/ml, Control: 200 pg/ml

**IFN-γ**
- Plasma: EV: 40 pg/ml, PeV-A: 30 pg/ml, Control: 20 pg/ml
- CSF: EV: 30 pg/ml, PeV-A: 20 pg/ml, Control: 10 pg/ml

**TNF-α**
- Plasma: EV: 150 pg/ml, PeV-A: 120 pg/ml, Control: 100 pg/ml
- CSF: EV: 120 pg/ml, PeV-A: 100 pg/ml, Control: 80 pg/ml

**IL-6**
- Plasma: EV: 800 pg/ml, PeV-A: 600 pg/ml, Control: 200 pg/ml
- CSF: EV: 600 pg/ml, PeV-A: 500 pg/ml, Control: 200 pg/ml

**GM-CSF**
- Plasma: EV: 15 pg/ml, PeV-A: 10 pg/ml, Control: 5 pg/ml
- CSF: EV: 10 pg/ml, PeV-A: 5 pg/ml, Control: 5 pg/ml

**IL-15**
- Plasma: EV: 150 pg/ml, PeV-A: 120 pg/ml, Control: 100 pg/ml
- CSF: EV: 120 pg/ml, PeV-A: 100 pg/ml, Control: 80 pg/ml

**IFN-α2**
- Plasma: EV: 200 pg/ml, PeV-A: 180 pg/ml, Control: 150 pg/ml
- CSF: EV: 180 pg/ml, PeV-A: 160 pg/ml, Control: 150 pg/ml

**IL-1Ra**
- Plasma: EV: 5000 pg/ml, PeV-A: 4000 pg/ml, Control: 2000 pg/ml
- CSF: EV: 4000 pg/ml, PeV-A: 3000 pg/ml, Control: 2000 pg/ml

**IL-10**
- Plasma: EV: 200 pg/ml, PeV-A: 150 pg/ml, Control: 100 pg/ml
- CSF: EV: 150 pg/ml, PeV-A: 100 pg/ml, Control: 50 pg/ml

**IL-8**
- Plasma: EV: 2000 pg/ml, PeV-A: 1500 pg/ml, Control: 1000 pg/ml
- CSF: EV: 1500 pg/ml, PeV-A: 1000 pg/ml, Control: 500 pg/ml

**IP-10**
- Plasma: EV: 15000 pg/ml, PeV-A: 10000 pg/ml, Control: 5000 pg/ml
- CSF: EV: 10000 pg/ml, PeV-A: 5000 pg/ml, Control: 2000 pg/ml

**MCP1**
- Plasma: EV: 10000 pg/ml, PeV-A: 8000 pg/ml, Control: 4000 pg/ml
- CSF: EV: 8000 pg/ml, PeV-A: 6000 pg/ml, Control: 4000 pg/ml
Figure 1b

**IL-4**

**MIP-1α**

**IL-12p40**

**IL-1β**

Plasma vs. CSF levels of cytokines in different conditions.

- **IL-4**
  - Plasma: Black bar
  - CSF: Gray bar

- **MIP-1α**
  - Plasma: Black bar
  - PeV-A: Light gray bar
  - Control: Dark gray bar

- **IL-12p40**
  - Plasma: Black bar
  - CSF: Gray bar

- **IL-1β**
  - Plasma: Black bar
  - CSF: Gray bar
Figure 2

| Biomarker   | Wilks’ Lambda | Biomarker   | Wilks’ Lambda | Biomarker   | Wilks’ Lambda |
|-------------|---------------|-------------|---------------|-------------|---------------|
| Model       | 0.258         | Model       | 0.382         | Model       | 0.046         |
| Fractalkine | 0.294         | IFN-α2      | 0.500         | Fractalkine | 0.061         |
| MCP-1       | 0.294         | IP-10       | 0.569         | IFN-γ       | 0.069         |
| TNF-α       | 0.309         |             |               | IL-15       | 0.071         |
| GM-CSF      | 0.310         |             |               | IFN-α2      | 0.071         |
| IL-6        | 0.352         |             |               | MCP-1       | 0.077         |
| IP-10       | 0.379         |             |               | IL-6        | 0.079         |
|             |               |             |               | IP-10       | 0.091         |
|             |               |             |               | TNF-α       | 0.148         |

| Function | % of variance | Function | % of variance | Function | % of variance |
|----------|---------------|----------|---------------|----------|---------------|
| 1        | 69.1          | 1        | 97.4          | 1        | 63.0          |
| 2        | 30.9          | 2        | 2.6           | 2        | 37.0          |