

Novel CSF biomarkers for facilitating diagnosis of CNS-HLH from other neuroinflammatory disorders

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Key Points

- CSF proteomics identified novel CSF biomarkers that can facilitate the diagnosis of CNS-HLH from other neuroinflammatory disorders.
- CSF CXCL9 is a promising biomarker for distinguishing CNS-HLH from other neuroinflammatory disorders.

Central nervous system (CNS)–hemophagocytic lymphohistiocytosis (HLH) can mimic other neuroinflammatory disorders (ONID), often leading to misdiagnosis. Current diagnostic studies do not reliably distinguish CNS-HLH from ONID. We hypothesized that novel cerebrospinal fluid (CSF) biomarkers identified using unbiased proteomic analysis can facilitate diagnosis of CNS-HLH from ONID. Banked CSF samples were categorized into 3 groups, CNS-HLH, ONID, and controls without CNS inflammation. Proteomic analysis was performed on 25 samples per group (total N = 75). Proteins demonstrating significant changes (>1.5-fold higher and $P < .05$) in the CNS-HLH compared with ONID and associated with HLH pathophysiology were selected as candidate biomarkers. Cross-platform validation of candidate biomarkers, along with known biomarkers CXCL9 and osteopontin, was performed using enzyme-linked immunosorbent assay (ELISA). One hundred twenty-two proteins were identified on CSF proteomic analysis at 99% confidence. Of these, 10 proteins demonstrated a >1.5-fold change with a $P < .05$ in CNS-HLH compared with ONID. SerpinG1, lysozyme, and CD14 were selected as candidate biomarkers. ELISA confirmed significant elevation of all 5 biomarkers in CNS-HLH relative to ONID. Median SerpinG1 levels were 1242.9 ng/mL in CNS-HLH vs 292.2 ng/mL in ONID ($P \leq .001$), lysozyme 222.2 ng/mL vs 65.6 ng/mL ($P = .001$), and CD14 180.3 ng/mL vs 64.5 ng/mL ($P \leq .001$). Median CXCL9 levels were 223.7 ng/mL in CNS-HLH vs 15.5 ng/mL in ONID ($P \leq .001$), and osteopontin levels were 356.5 ng/mL vs 92.9 ng/mL ($P = .001$). These findings demonstrate that novel CSF biomarkers SerpinG1, lysozyme, CD14, and CXCL9 can facilitate diagnosis of CNS-HLH from ONID.

Introduction

Hemophagocytic lymphohistiocytosis (HLH) is a syndrome of pathological immune activation characterized by clinical signs and symptoms of extreme inflammation.¹ HLH can be primary or secondary in association with infections, malignancies, or rheumatologic disorders.¹ Multiple genetic defects

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Deidentified data are available from the corresponding author, Sharat Chandra (sharat.chandra@cchmc.org) on request.

The full-text version of this article contains a data supplement.

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responsible for primary HLH have been described, including PRF1, UNC13D, STX11, STXBP, LYST, and RAB27A, all playing important roles in the granule exocytosis cytotoxicity pathway.^{1,2} Patients harboring these genetic defects usually present early in life with fever, cytopenia, splenomegaly along with elevated ferritin, and soluble interleukin-2 receptor (IL-2R) levels.

Patients with primary HLH can also have central nervous system (CNS) involvement with HLH, that is, CNS-HLH in addition to systemic signs and symptoms.¹ This clinical presentation can mimic several other non-HLH neuroinflammatory disorders (ONID).³ Although diagnosing CNS-HLH in the context of systemic HLH is often feasible, in some patients, CNS involvement may precede or even occur in the absence of systemic disease, posing significant diagnostic challenges.⁴ Current cerebrospinal fluid (CSF) diagnostic studies for CNS-HLH do not reliably distinguish CNS-HLH from ONID. Elevated CSF protein, pleocytosis seen in CNS-HLH can also be seen in ONID. Although the presence of hemophagocytosis in the CSF is a helpful finding, it is seen infrequently.

Blincoe et al described the clinical presentation, treatment, and outcome of 38 patients with isolated CNS-HLH, that is CNS involvement without systemic HLH as the initial manifestation of primary HLH in the largest cohort reported to date.⁵ Patients presented with a wide range of neurological manifestations that included ataxia and gait disturbances, seizures, headache, limb weakness, visual abnormalities, and altered mental status. Magnetic resonance imaging of the brain showed diverse abnormal findings, and CSF studies mostly showed pleocytosis and elevated protein but no evidence of hemophagocytosis. Most patients were initially diagnosed with other diagnoses, that included acute disseminated encephalomyelitis (ADEM), CNS vasculitis, leukodystrophy, multiple sclerosis (MS) and other demyelinating disorders, neuroinflammatory disorders, and encephalitis. The median interval from onset of neurological symptoms to confirmed molecular diagnosis of primary HLH was 19.5 months (range, 1-108 months). Until then, patients received non-HLH directed therapy that allowed disease recurrence or continued progression due to inadequate disease control. The diagnosis of primary HLH was only considered either when disease pattern, imaging, and response to therapy were felt to be atypical, or following genetic investigations for unexplained neurological disease, or following brain biopsy, or after eventual onset of systemic HLH. Furthermore, patients with primary HLH invariably need an allogeneic hematopoietic cell transplant for cure. Delay in diagnosis and inappropriate treatment negatively impacted neurological outcome and led to reduced survival. In patients with isolated CNS-HLH who did not undergo allogeneic hematopoietic cell transplant, only 33% survived. Although brain biopsy carries some procedural risk, it is generally safe, and can be particularly valuable for establishing the diagnosis in patients with suspected isolated CNS-HLH. The only currently used CSF biomarker, neopterin, a catabolic product of GTP, is a nonspecific marker of cellular immune activation and lacks specificity.^{3,6} There is therefore a need for novel biomarkers that can distinguish CNS-HLH from ONID, which in turn could also be valuable in facilitating the diagnosis of isolated CNS-HLH.

CSF is the best matrix to identify novel biomarkers for CNS disorders, due to its direct contact with the brain parenchyma.⁷

Additionally, peripheral blood HLH markers for systemic HLH are not consistently elevated in isolated CNS-HLH. Global protein profiling by mass spectrometry (MS)-based proteomics has identified novel biomarkers in Alzheimer disease and other neurodegenerative disorders.^{8,9} We hypothesized that novel CSF biomarkers identified using an unbiased MS-based proteomics approach can facilitate the diagnosis of CNS-HLH from ONID. The aim of this study was to investigate CSF biomarkers of CNS-HLH by proteomic analysis of banked CSF samples of patients with CNS-HLH, and compare findings with 2 groups: patients with ONID, and controls without CNS inflammation. Cross-platform validation of proteomic findings was performed using enzyme-linked immunosorbent assay (ELISA) analysis. We also investigated 2 known candidate biomarkers of histiocytic disorders, osteopontin and CXCL9,¹⁰⁻¹² by ELISA analysis of banked CSF samples of patients with CNS-HLH, and comparing with ONID and controls without CNS inflammation. We hypothesized that levels of CSF osteopontin and CXCL9 will be higher in CNS-HLH compared with ONID and controls without neuroinflammation.

Methods

Patient CSF samples and clinical data

CSF samples already banked under an institutional review board-approved protocol (IRB ID: 2021-0378) at Cincinnati Children's Hospital Medical Center and available for analysis were used for this study. CSF samples for analysis were categorized into 3 groups based on underlying diagnosis. The first group included patients with HLH and CNS involvement, that is CNS-HLH. Samples were included if they fulfilled the following criteria: (1) confirmed diagnosis of HLH either by genetic testing and/or if patients met HLH 2004 criteria; (2) evidence of neurological involvement based on abnormal CSF studies, either CSF pleocytosis or elevated CSF protein for age, or abnormal neurological findings on neuroimaging at the time the CSF sample was obtained. The second group included patients with ONID, such as autoimmune encephalitis, ADEM, MS, CNS lupus, or another noninfectious neuroinflammatory disorder. CSF samples from the ONID group were collected during active CNS disease. Samples were included if patients had evidence of neurological involvement based on abnormal CSF studies, either CSF pleocytosis, or elevated CSF protein or abnormal neurological findings on neuroimaging at the time the CSF sample was obtained. Patients with evidence of CNS infection were excluded. The third group included controls without evidence of neuroinflammation, including patients with idiopathic cranial hypertension, patients who underwent lumbar puncture as part of evaluation of a seizure disorder, psychiatric disorder, or cerebral palsy, but had no evidence of neuroinflammation or CNS infection. Patients were included to group 3 only if their CSF studies were normal, that is, no evidence of CSF pleocytosis or elevated CSF protein. CSF protein concentration higher than the upper limit of normal for age was considered as elevated. A CSF white blood cell (WBC) count of $>.004 \times 10^9/L$ was considered as CSF pleocytosis.

Study strategy

The study strategy is shown in [Figure 1](#). Proteomic analysis using liquid chromatography-MS (LC-MS) was performed on 25

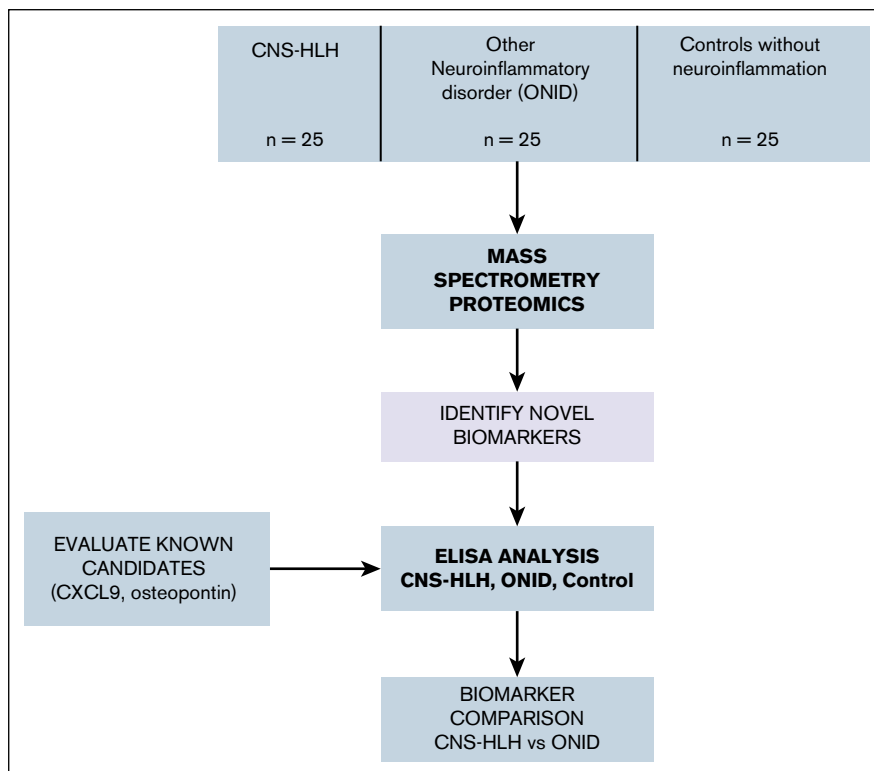


Figure 1. Study strategy. Proteomic analysis using LC-MS was performed on 25 CSF samples in each group (total N = 75). Proteins identified as novel biomarkers were cross-validated by performing ELISA analysis on CSF samples in each group, along with osteopontin and CXCL9 that are known biomarkers for histiocytic disorders. Biomarker comparison was then performed between HLH and ONID groups.

samples in each group (total N = 75) at the University of Cincinnati Proteomics Laboratory.

Protocol for proteomic analysis

A 660-nm assay was performed on all the samples; 20 µg was removed for all samples, then 40 µL Laemmli buffer was added. Samples were then run 1.5 cm into an Invitrogen 4% to 12% B-T gel using MOPS buffer (4-morpholinepropanesulfonic acid) with molecular weight marker lanes in between. Sections were excised, reduced with dithiothreitol, alkylated with iodoacetic acid, and digested overnight with trypsin as described previously.¹³ Peptides were extracted, dried in a speed vac, and resuspended in 50mM triethylammonium bicarbonate. A pooled bridge sample was created from 2 µg (10%) of each sample, and divided into 8 bridge pool samples of 18.75 µg each. Subgroups were also pooled to create a control, ONID, and HLH pool. The 75 samples plus the 3 subgroup pools were tandem mass tag (TMT) labeled following the vendor protocol for 0.2 mg of TMT10plex isobaric labels. The 8 bridge pools were TMT labeled following the vendor protocol for 0.8 mg TMT11-131C isobaric labels using 2 tubes. The specific TMT labels used for each sample are provided in supplemental Table 1. TMT labeled peptides were combined in equal portions (1 µg) for each sample or pool to create the 8 TMT sets. Each of the TMT sets was dried in a speed vac, and reconstituted in 0.1% formic acid; 4.0 µL of each TMT set (1 µg of total peptide for the equal mix) was analyzed by nanoLC-MS/MS (Orbitrap Eclipse) in duplicate. Data acquisition followed a synchronous precursor selection MS3 TMT workflow¹⁴ with specific gradient and MS details reported¹³ and full instrument parameters

provided in the supplemental Information. Results were searched against the homo sapiens database using Proteome discoverer ver 2.4 and the Sequest HT search algorithm (Thermo scientific) using the TMT quantitation workflow reported in the study by Maccorra et al.¹³ Statistics and data visualization are as reported below.

Biomarker selection and statistical analysis

Pair-wise normalization and relative quantification across the samples set was accomplished using the common (bridge) mixture of all samples from the TMT11-131C channel for cross-comparison of groups beyond each 10-plex run. Total ion chromatograph (TIC) profiles provide overall abundance profiles for consistency among the sample sets, whereas box and whisker plots show comparative sample abundance normalized to all peptides. Comparisons were made using sample abundance, and *P* values calculated by the analysis of variance method using Tukey post hoc test. Proteomic data analysis was performed by in-house bioinformatic pipeline (all scripts available at github.com/schuti/SVVATH-D) using R packages.^{15,16} Data normalization was performed by variance stabilization normalization function, whereas missing values were imputed by deterministic minimum imputation^{3,6} algorithm. Heat map with unsupervised clustering was performed by correlation distance and complete linkage. Principal component analysis (PCA) and volcano plot depicting 1.5-fold change in proteins with *P* < .05 between HLH, ONID, and control samples were evaluated. Proteins related to HLH pathological mechanisms with >1.5-fold change in abundance in CNS-HLH samples with *P* < .05 compared with ONID samples and control samples were selected as candidate biomarkers for CNS-HLH.

Unadjusted *P* values were calculated using Wilcoxon rank-sum test for independence. Benjamini-Hochberg procedure was applied to control the false discovery rate (FDR) across the full set of protein comparisons between HLH, ONID, and control groups.

Univariate testing of CSF biomarker values obtained from ELISA analysis and CSF characteristics was performed, and *P* values calculated using Wilcoxon rank-sum test for independence. After adjusting for age-based normal range, dichotomized univariate testing of CSF protein and WBC count was performed, and *P* values calculated using Fisher exact test of independence.

Results

Patient demographics and diagnoses in each group are shown in Table 1. In the CNS-HLH group, patients predominantly had primary HLH with CNS involvement. Clinical and CSF characteristics of the patients in the CNS-HLH group are shown in Table 2. Two patients (HLH2 and HLH9) presented with isolated CNS-HLH. The remainder of the patients presented with systemic HLH meeting HLH 2004 criteria; however, 5 of these patients (HLH6, HLH7, HLH16, HLH19, HLH22) did not have elevated systemic HLH markers (ferritin, soluble IL-2R) at time of CSF analysis. Therefore, a total of 7 patients (28% of the HLH cohort) had CNS involvement in the absence of systemic inflammation. In the control group, most patients were diagnosed with idiopathic intracranial hypertension. Patients in the ONID group had heterogeneous diagnoses, with autoimmune encephalitis and CNS lupus being the most common diagnoses, followed by MS and ADEM.

CSF proteomic analysis

Comparative profiling of the relative abundance of the TMT-tagged proteins across all sample sets demonstrated good reproducibility across each TMT sample set and from the duplicate injections based on an overlay of the TIC profiles from the LC-MS (supplemental Figure 1A). From the collective analysis across the data set, 122 proteins were identified with at least a single peptide

and 99% confidence, each with normalized abundance values across the samples set (supplemental Table 2). Consistent with the TIC profiles, the sample abundance plot also shows minimal variability in abundance of all the samples, scaled to the bridge pool (supplemental Figure 1B). PCA (Figure 2) showed little or no clustering of the samples from the 3 groups, suggesting a limited ability to uniquely parse the groups based on the whole proteome expression levels using PCA. Bioinformatic data analysis was performed to detect significant quantitative changes. A volcano plot of significant differentially expressed proteins (*P* < .05 and >1.5-fold change) illustrates the upregulated and downregulated proteins identified in HLH to control (Figure 3A), and HLH to ONID (Figure 3B). Thirty-four proteins were significantly differentially expressed in the HLH vs control analysis (supplemental Table 3). Ten proteins were significantly differentially expressed in the HLH vs ONID analysis (supplemental Table 4) by at least 1.5-fold change in abundance with a *P* value <.05.

A heat map/cluster analysis of 53 proteins with significant changes between all the groups (*P* < .05) shows that the 3 different sample groups show some clustering based on these protein changes (supplemental Figure 2A), whereas the 10 proteins with significant differences between HLH and ONID showed substantial clustering between the groups, thus meriting further validation (supplemental Figure 2B).

Biomarker selection for ELISA analysis

Differentially expressed proteins SerpinG1, lysozyme, and CD14 with unadjusted *P*-values <.05 were selected as potential biomarkers for cross-validation by ELISA due to potential association with HLH pathological mechanisms. Due to the relatively small sample size, fewer than half of the proteins remained statistically significant after correction for multiple comparisons (supplemental Tables 3 and 4). SerpinG1 remained statistically significant in both HLH vs ONID (FDR-adjusted *P* < .001) and HLH vs controls (FDR-adjusted *P* = .049), lysozyme remained significant between HLH vs controls (FDR-adjusted *P* < .001), but not between HLH vs ONID

Table 1. Patient demographics and diagnoses of CSF specimens in each group

Group 1, HLH	n = 25	Group 2, ONID	n = 25	Group 3, Control	n = 25
Male	18	Male	11	Male	9
Female	7	Female	14	Female	16
Median age 1.5 years (range, 0.4-6)		Median age 14 years (range, 1.0-19)		Median age 14 years (range, 0.1-20)	
Primary HLH	20	Autoimmune encephalitis	4	Idiopathic intracranial hypertension	10
UNC13D	10	CNS lupus	4	Seizure disorder	4
RAB27	2	MS	3	Syndromic disorder	3
PRF1	2	ADEM	3	Psychiatric disorder	3
SH2D1A (XLP1)	2	Anti-NMDAR encephalitis	2	R/O Meningitis	2
XIAP	2	Neuromyelitis optica spectrum disorder	2	Migraine	2
STXBP2	1	GBS/OMS	2	Cerebral palsy	1
Undefined gene	1	MOGAD	2		
EBV-HLH	3	Undefined neuroinflammatory disorder	2		
Secondary HLH	2	Neurosarcoidosis	1		

Patient demographics and diagnoses of CSF specimens in HLH, ONID, and control groups (n = 25 in each group).
EBV, Epstein-Barr virus; GBS, Guillain-Barré syndrome; MOGAD, myelin oligodendrocyte glycoprotein antibody-associated disease; NMDAR, N-methyl-D-aspartate receptor; OMS, opsoclonus myoclonus syndrome; R/O, rule out.

Table 2. Clinical and laboratory characteristics of patients in CNS-HLH group

Patient	Age, y	Sex	Systemic HLH (at diagnosis)	Elevated ferritin (at time of CSF)	Elevated sIL-2R (at time of CSF)	CSF protein, mg/dL	CSF glucose, mg/dL	CSF WBC count, mm ³	CSF Lymph, %	CSF Monos, %	CSF neopterin, nmol/L	Abnormal MRI findings	Neurological signs/symptoms
HLH1	1.5	M	Y	Y	Y	337	N/A	2	5	92	572.0	Yes	Seizures, AMS
HLH2	1	M	N	N	N	28	52	2	96	4	250.0	Yes	Seizures, AMS
HLH3	2	F	Y	N	Y	70	57	88	94	6	239.9	Yes	Seizures, fever
HLH4	4	M	Y	Y	Y	37	76	1	77	23	29.8	Yes	Fever
HLH5	3	F	Y	Y	Y	26	45	2	88	6	161.7	Yes	Lethargy
HLH6	0.8	F	Y	N	N	48	44	4	69	11	154.7	Yes	Facial droop
HLH7	6	M	Y	N	N	31	42	3	48	52	151.8	Yes	Seizures, fever
HLH8	1	M	Y	Y	N	39	72	9	8	0	112.7	Yes	AMS, regression of milestones
HLH9	3	M	N	N	N	126	35	18	85	15	103.4	Yes	L eye esotropia
HLH10	9	M	Y	Y	Y	57	43	1	82	9	135.3	Yes	Seizures
HLH11	1.5	F	Y	Y	Y	35	43	15	78	22	54.5	Yes	Seizures
HLH12	0.6	F	Y	Y	N	35	48	5	88	10	31.2	No	AMS
HLH13	11	M	Y	Y	N	49	74	3	92	8	39.3	Yes	Seizures
HLH14	0.6	F	Y	Y	N	74	65	40	44	55	31.2	Yes	AMS
HLH15	0.4	M	Y	Y	Y	90	75	17	86	8	36.4	Yes	Seizures
HLH16	1.5	M	Y	N	N	24	78	6	91	9	26.4	No	None
HLH17	4	M	Y	Y	Y	54	86	7	30	10	26.1	Yes	AMS
HLH18	3	M	Y	Y	Y	26	73	8	93	6	17.4	Yes	Headache, drowsiness
HLH19	1.5	M	Y	N	N	274	66	9	20	80	8.4	No	Surveillance LP
HLH20	1	M	Y	Y	Y	27	76	1	0	100	203.7	No	Fever, HLH flare
HLH21	1.5	M	Y	Y	Y	40	74	0	25	75	421.8	Yes	AMS
HLH22	1	M	Y	N	N	166	59	4	94	6	135.4	Yes	Fever, HLH flare
HLH23	6	M	Y	Y	Y	80	50	36	96	4	670	Yes	Seizures
HLH24	2	F	Y	Y	Y	38	44	6	88	11	439.8	Yes	Seizures
HLH25	1	M	Y	Y	Y	68	30	10	93	7	613.2	Yes	Seizures

Normal range of CSF neopterin, <16.5 nmol/L.

AMS, altered mental status; F, female; LP, lumbar puncture; Lymph, lymphocyte; M, male; Monos, monocytes, MRI, magnetic resonance imaging; N/A, not applicable; sIL-2R, soluble IL-2R; Y, yes.

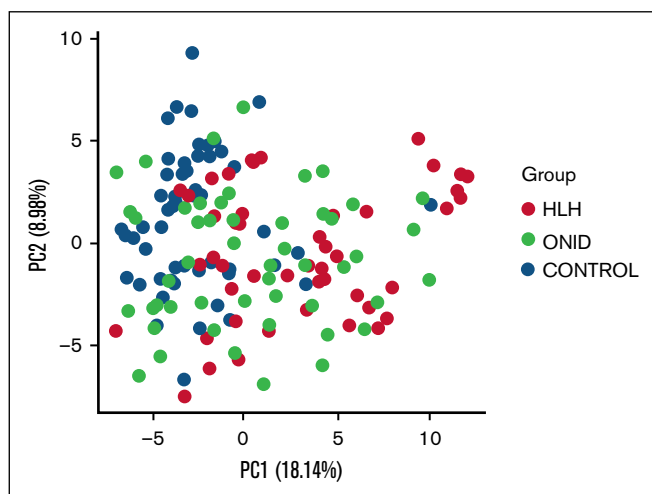


Figure 2. CSF proteomics PCA. PCA showed little or no clustering of the samples from the HLH, ONID, and control groups. PC, principal component.

(FDR-adjusted $P = .067$). CD14 was differentially expressed but did not show statistical difference among groups following correction for multiple comparisons. CD14 was selected because it is a marker of activation during macrophage differentiation, and macrophage activation is a feature of HLH.¹⁷ SerpinG1 is mainly synthesized and secreted by hepatocytes, but is also produced by monocytes and macrophages, and biosynthesis can be stimulated by cytokines, especially interferon gamma.¹⁸ Lysozyme is a regulator of IL-2-activated T-cell proliferation. Further, lysozyme in serum is produced by the monocyte/macrophage system, making it a potential marker for macrophage activation.¹⁹ Osteopontin and CXCL9 were not identified by proteomic analysis, but were selected for ELISA analysis as known candidate biomarkers and because they are both biologically plausible based on HLH pathological mechanisms. CSF osteopontin is a promising biomarker for CNS-Langerhans cell histiocytosis (LCH), another histiocytic disorder, and is also elevated in CNS-HLH.¹⁰ HLH is associated with T-cell activation and interferon gamma upregulation, and CXCL9 is a chemokine that is associated with interferon gamma upregulation. Further CXCL9 levels are significantly elevated in the blood in HLH.^{11,12}

Cross-platform validation by ELISA analysis

SerpinG1, lysozyme, CD14, CXCL9, and osteopontin were measured by ELISA assays performed on banked CSF specimens of control group ($n = 24$), HLH group ($n = 23$), and ONID group ($n = 23$). CXCL9 ELISA assay could not be performed in 8 CSF specimens (5 HLH samples and 3 ONID samples) due to insufficient CSF volume. Median CSF levels of SerpinG1, lysozyme, CD14, CXCL9, and osteopontin, along with median CSF characteristics across control, ONID, and HLH groups are shown in Figure 4. Median and range values for all biomarkers and CSF characteristics across groups are shown in supplemental Table 5. Notably, CXCL9 was elevated in 83.3% (15/18) of HLH samples, and elevated only in 5% (1/20) of ONID samples. CXCL9 was not elevated in any of the control samples.

Correlation heat map for all CSF characteristics and biomarkers is shown in Figure 5, with the size of marker demonstrating significance of correlation. All biomarkers appeared to be strongly

correlated, whereas there was some correlation seen between WBC and osteopontin and CSF protein and SerpinG1. Other CSF variables did not correlate with CSF biomarkers.

Biomarker comparison between ONID and HLH groups

Univariate testing of all continuous forms of CSF biomarkers and CSF characteristics was performed, and the results are shown in Table 3. In addition to SerpinG1, lysozyme, CD14, CXCL9, and osteopontin, CSF protein, glucose, CSF WBC count, neutrophils (segs), CSF lymphocyte and monocyte counts were compared between ONID and HLH groups. Levels of all CSF biomarkers (SerpinG1, lysozyme, CD14, CXCL9, and osteopontin) were significantly elevated in HLH compared with ONID. Although CSF protein was also significantly elevated, this did not account for the fact that CSF protein levels are age dependent, with normal range being higher in infants. After adjusting for age-based normal range, dichotomized univariate testing of CSF protein showed no significant difference between the 2 groups ($P = .21$), as shown in Table 4. Dichotomized univariate testing of CSF WBC count also showed no significant difference between the 2 groups ($P = .24$), as shown in Table 4. None of the other CSF parameters was significantly different in HLH vs ONID. Boxplot comparison of biomarkers between HLH and ONID is shown in Figure 6.

Further, we measured CD14, SerpinG1, and lysozyme levels by ELISA in banked peripheral blood samples from 10 patients with CNS-HLH (8 of whom are included in this study), and compared with 10 pre-bone marrow transplant (BMT) malignancy controls (banked samples drawn just prior to start of BMT). Pre-BMT malignancy controls were chosen because they are not expected to have elevated systemic HLH markers. We did not have access to any blood samples from patients with ONID. Levels of CD14, SerpinG1, and lysozyme were not significantly different between HLH and pre-BMT malignancy control groups (supplemental Figure 3A). Within the HLH cohort, 6 patients had normal systemic HLH markers (ferritin and soluble IL-2R), including 2 patients with isolated CNS-HLH, and 4 patients had elevated HLH markers at the time of sample collection. CD14 levels were significantly higher in patients with elevated systemic markers compared with those with normal systemic markers (median, 3793 ng/mL vs 1657 ng/mL; $P = .04$), whereas SerpinG1 and lysozyme levels were similar in both groups (supplemental Figure 3B).

Discussion

CSF proteomic approaches have improved in sensitivity, speed, and practicability over the years,^{9,20} leading to the discovery of an enormous number of biomarker candidates in neurodegenerative disorders.^{7,21,22} Our study shows that an unbiased CSF proteomic approach using MS can also identify CSF biomarker candidates for diagnosis of CNS-HLH from ONID. We identified 3 novel CSF biomarkers (SerpinG1, lysozyme, and CD14) using MS-based proteomics for facilitating diagnosis of CNS-HLH from ONID. The pathological mechanisms of HLH in the CNS remain poorly characterized, but likely relate to the hyperinflammation and dys-regulated immune response observed systemically.^{23,24} Although SerpinG1 is produced mainly by the liver, it is also produced by monocytes and macrophages, and its biosynthesis is stimulated by cytokines, especially interferon gamma, which is also the

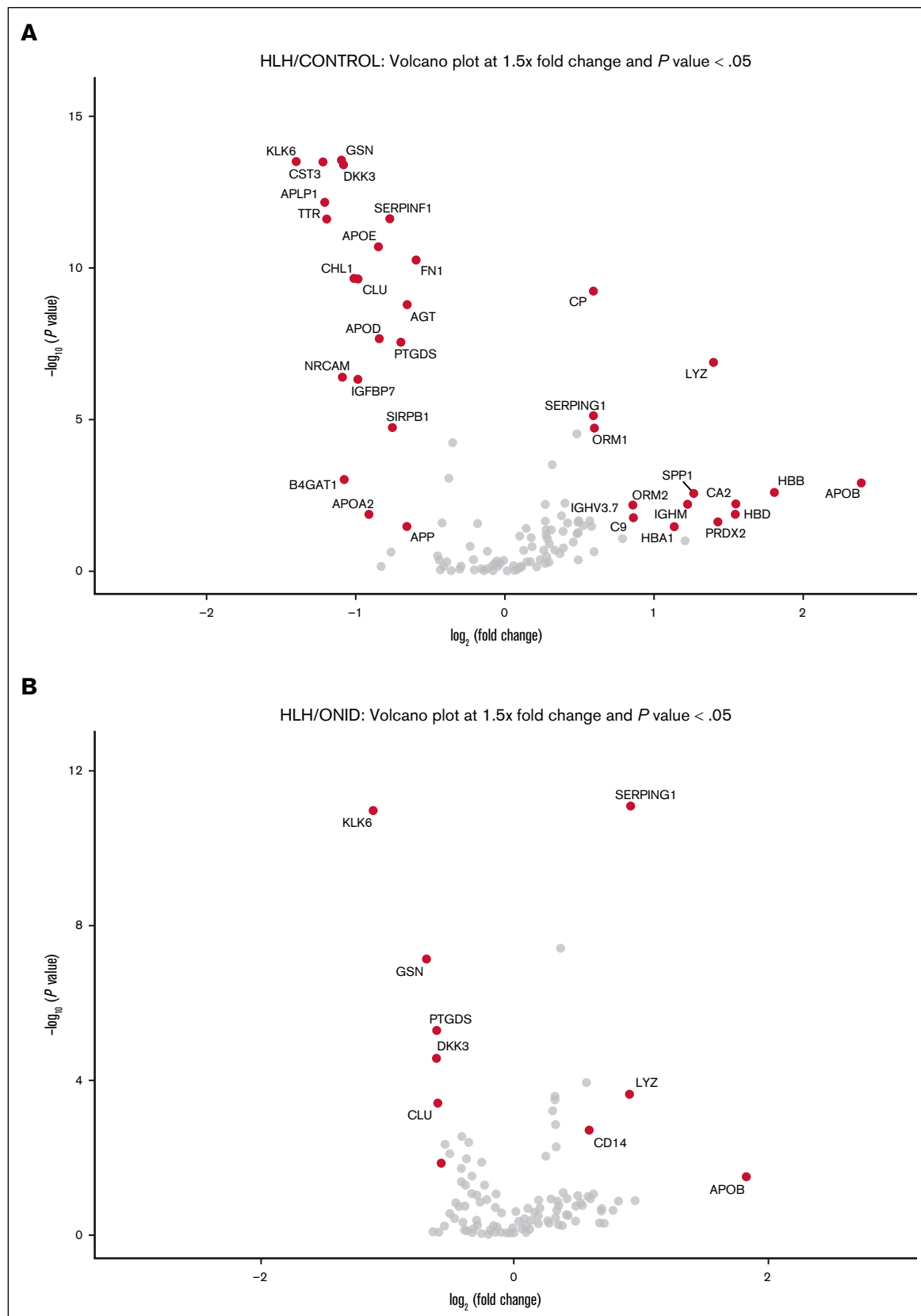


Figure 3. CSF proteomics volcano plots. (A) HLH vs control. Volcano plot of significant differentially expressed proteins ($P < .05$ and >1.5 -fold change) illustrates the upregulated and downregulated proteins identified in HLH compared with control. (B) HLH vs ONID. Volcano plot of significant differentially expressed proteins ($P < .05$ and >1.5 -fold change) illustrates the upregulated and downregulated proteins identified in HLH compared with ONID.

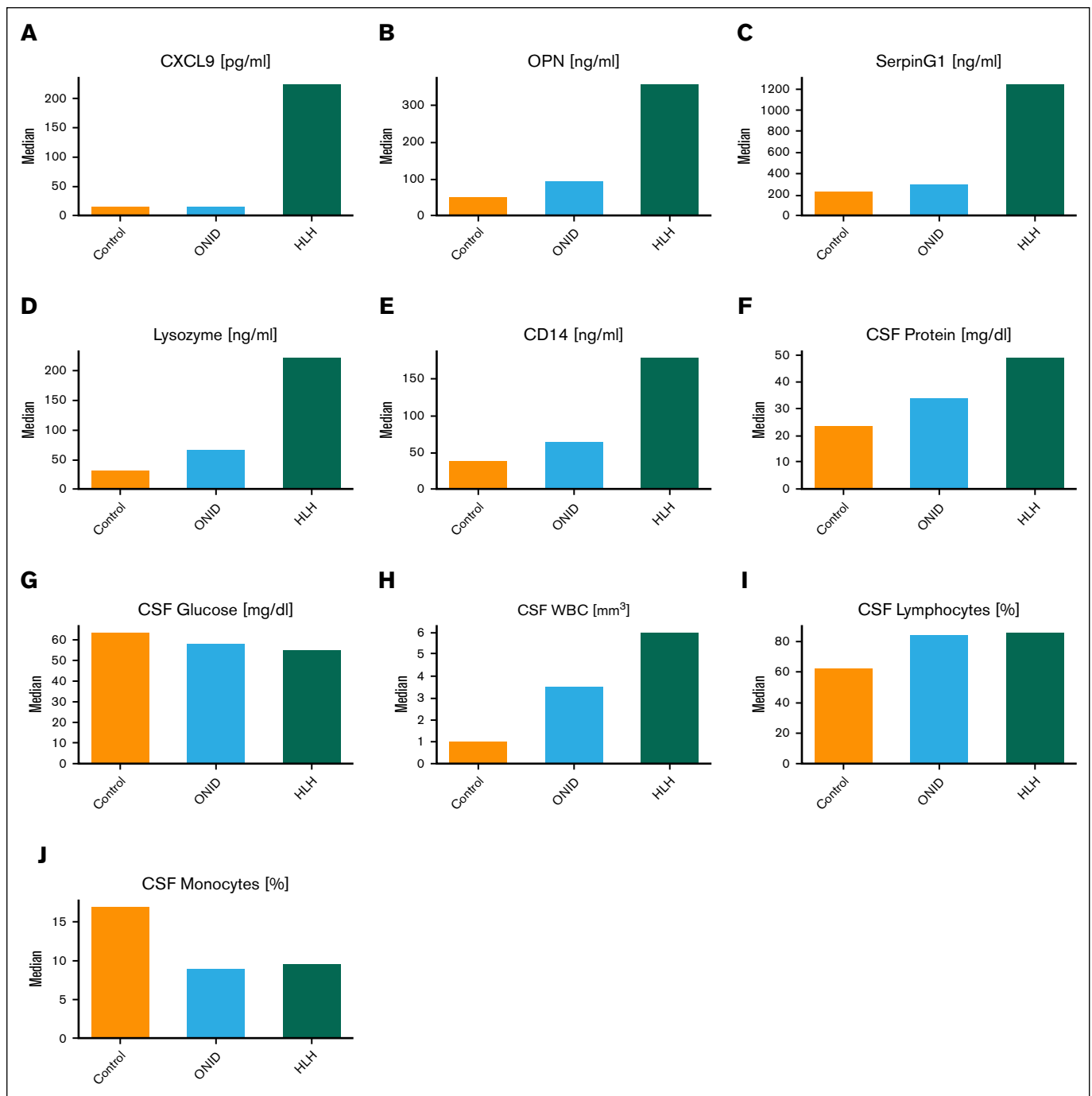


Figure 4. CSF characteristics and biomarker profiles in CNS-HLH, ONID, and control groups. Median levels of biomarkers measured by ELISA analysis of CSF and CSF characteristics are shown for patients with CNS-HLH (green), ONID (blue), and controls (orange). Panels include: (A) CXCL9, (B) OPN, (C) SerpinG1, (D) lysozyme, (E) CD14, (F) CSF protein, (G) CSF glucose, (H) CSF WBC count, (I) CSF lymphocytes, and (J) CSF monocytes. Bars represent group medians. CXCL9, OPN, SerpinG1, lysozyme, and CD14 were notably elevated in CNS-HLH compared with ONID and controls. OPN, osteopontin.

predominant cytokine upregulated in HLH.^{18,25,26} Lysozyme is an inducible marker of macrophage activation and a regulator of IL-2-activated T-cell proliferation.^{19,27,28} Serum lysozyme is a known marker of monocyte/macrophage activation in rheumatoid arthritis.¹⁹ CD14 is present on most monocytes and tissue macrophages, and is regarded as a key marker of, and facilitator for,

proinflammatory macrophage function.¹⁷ CD14 expression on the cell surface significantly increases when a macrophage is stimulated and becomes activated.²⁹ Notably, all 3 CSF biomarkers are associated with macrophage activation. HLH is a disorder of uncontrolled T-cell activation that leads to excessive macrophage activation.² Although HLH is associated with excess T-cell

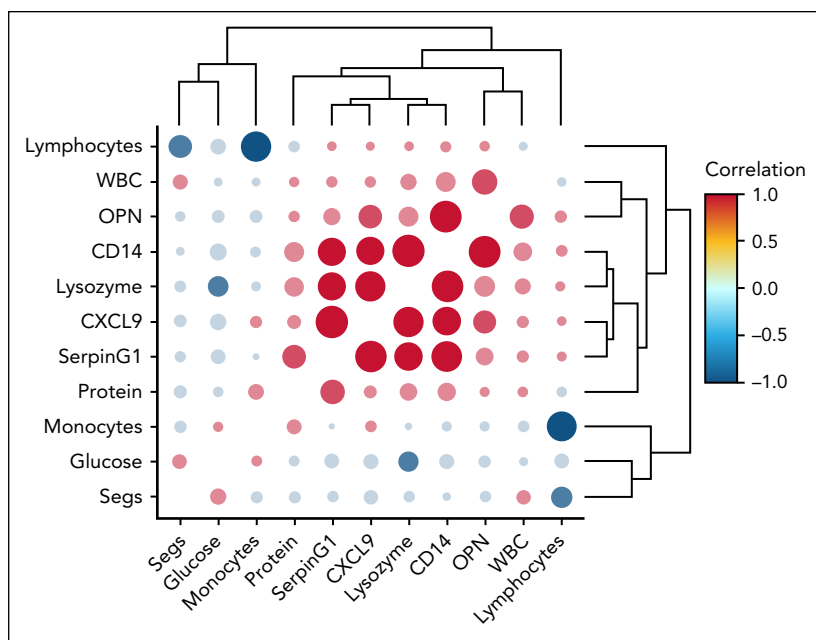


Figure 5. Correlation heat map for all CSF characteristics and biomarkers. Size of marker demonstrates significance of correlation. Larger markers have lower *P*-values, and smaller markers have higher *P* values. The color of the marker denotes correlation metrics where -1 is negatively correlated and 1 is positively correlated. All biomarkers appear to be highly correlated, and there is also some correlation seen between WBC count and OPN, and CSF protein and SerpinG1. OPN, osteopontin.

activation, T-cell activation is also a feature of neuroinflammatory disorders, including MS, CNS-lupus, ADEM, anti-*N*-methyl-D-aspartate receptor encephalitis, and myelin oligodendrocyte glycoprotein antibody-associated disease, all of which were represented in our study cohort.³⁰⁻³³ Hence, it is possible that proteins predominantly associated with T-cell activation are not differentially expressed between CNS-HLH and ONID. Although there is some degree of macrophage activation in ONID considering elevation of these biomarkers relative to controls, our data suggest that the extent of macrophage activation is much higher in CNS-HLH compared with ONID, and may be the key distinguishing feature of HLH relative to ONID. Overall, our study identified CSF SerpinG1, lysozyme, and CD14 as novel CSF biomarkers for distinguishing CNS-HLH from ONID. Although our

cohort had a limited number of patients with isolated CNS-HLH, almost one-third of patients had active CNS disease without elevation of systemic HLH markers. Although this represents a small subset, in the context of a rare disease our data are robust enough to provide meaningful insight. These novel biomarkers could potentially be extrapolated to isolated CNS-HLH, although this will need to be confirmed in a larger cohort.

Interferon gamma is essential to the pathophysiology of HLH and is also the predominantly upregulated cytokine in HLH.³⁴ Further, interferon gamma signature in the plasma proteome distinguishes systemic HLH from sepsis and systemic inflammatory response syndrome.¹¹ CXCL9 is a chemokine that is associated with interferon gamma upregulation, and interferon gamma is the only

Table 3. Univariate testing of CSF biomarkers and CSF characteristics

Dependent group		ONID	HLH	<i>P</i> value
CXCL9, pg/mL	Median (IQR)	15.5 (15.5-15.5)	223.7 (64.8-1210.0)	<.001
OPN, ng/mL	Median (IQR)	92.9 (61.0-245.6)	356.5 (186.8-1189.9)	.001
SerpinG1, ng/mL	Median (IQR)	292.2 (238.9-386.1)	1242.9 (837.6-1810.9)	<.001
Lysozyme, ng/mL	Median (IQR)	65.6 (52.2-78.6)	222.2 (104.6-602.6)	.001
CD14, ng/mL	Median (IQR)	64.5 (41.2-79.5)	180.3 (84.8-291.0)	<.001
CSF protein, mg/dL	Median (IQR)	34.0 (25.0-47.5)	49.0 (35.0-77.0)	.02
CSF glucose, mg/dL	Median (IQR)	58.0 (53.5-68.5)	54.5 (44.0-72.8)	.497
CSF WBC, mm ³	Median (IQR)	1.5 (1.0-7.5)	6.0 (3.0-12.5)	.07
CSF segs, %	Median (IQR)	0.0 (0.0-8.5)	0.0 (0.0-1.0)	.78
CSF lymphocytes, %	Median (IQR)	84.0 (72.0-94.0)	86.0 (46.0-92.5)	.45
CSF monocytes, %	Median (IQR)	9.0 (5.2-19.5)	9.5 (6.2-20.2)	.42

Median and range values of CSF biomarkers obtained from ELISA analysis, along with CSF characteristics in HLH and ONID groups. All 5 biomarkers were significantly higher in HLH compared with ONID. *P* values were calculated using Wilcoxon rank-sum test for independence.

IQR, interquartile range; OPN, osteopontin.

Table 4. Dichotomized univariate testing of CSF protein and WBC count

Dependent group	ONID	HLH	P value
Elevated CSF protein			
No	18 (78.3%)	13 (56.5%)	.21
Yes	5 (21.7%)	10 (43.5%)	
Elevated CSF WBC count			
No	14 (60.9%)	9 (39.1%)	.24
Yes	9 (39.1%)	14 (60.9%)	

After adjusting for age-based normal range, there was no significant difference in CSF protein and WBC between HLH and ONID groups. *P* values were calculated using Fisher exact test of independence.

known stimulant for CXCL9 production.³⁵ Notably, CXCL9 was elevated in 85% of CNS-HLH samples, and elevated only in 5% of ONID samples. In 95% of ONID samples, CXCL9 levels were similar to control samples. Although CXCL9 is induced by interferon gamma, the cellular source of CXCL9 itself is typically macrophages/monocytes. In HLH, overactivated cytotoxic T cells secrete large amounts of interferon gamma, which drives macrophage activation and, in turn, leads to CXCL9 production. It is plausible that the extent of interferon gamma production in HLH far exceeds that seen in ONID, which can explain why CXCL9 was

significantly higher in CNS-HLH compared with ONID, despite being a marker of T-cell activation. Our data suggest that CSF CXCL9 is a promising biomarker for distinguishing CNS-HLH from ONID.

In our study, CSF osteopontin levels were also significantly higher in CNS-HLH compared with ONID. Osteopontin is secreted by activated macrophages and T cells, and has been shown to be an important component of early cellular responses and inflammation. CSF osteopontin is a biomarker for another histiocytic disorder, CNS-LCH.¹⁰ In the study by McClain et al, CNS osteopontin levels were elevated in both CNS-LCH and CNS-HLH; however, osteopontin levels were significantly higher in CNS-LCH compared with CNS-HLH. Therefore, barring CNS-LCH, osteopontin can potentially be a biomarker to distinguish CNS-HLH from ONID. Neurofilament light chain is a key biomarker for monitoring MS, Alzheimer disease, CNS-LCH, and other neurodegenerative conditions, as it indicates axonal damage and neuronal loss.^{36,37} Considering neuronal injury is common to the pathophysiology of both CNS-HLH and ONID, neurofilament light chain was not selected as a candidate biomarker for validation by ELISA in our study. Our study also confirmed that commonly used CSF parameters, such as CSF protein, glucose, WBC, lymphocyte, and neutrophil counts cannot distinguish between CNS-HLH and ONID, which further supports using novel biomarkers to distinguish CNS-HLH from ONID.

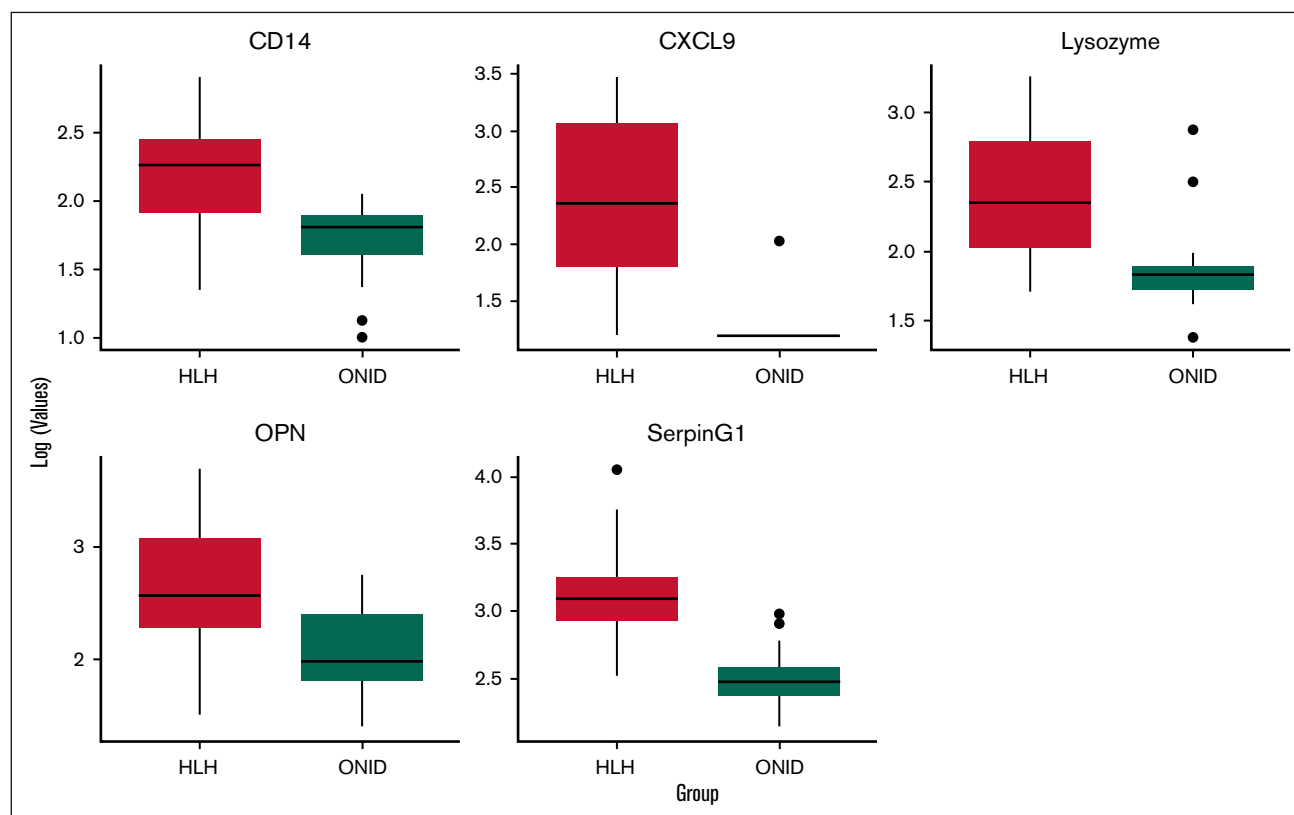


Figure 6. Comparison of CSF biomarkers in HLH vs ONID (ELISA analysis). Data represented as Tukey box-and-whisker plots. Each box is defined by the 25th and 75th percentiles of the data, and represents the interquartile range (IQR). The median is represented by a solid horizontal line. Whiskers are extended from the box to the last value at <1.5 IQR, toward the maximum and the outliers represented as round dots.

Peripheral blood analysis showed that levels of SerpinG1, CD14, and lysozyme did not differ from controls who lack HLH-driven systemic inflammation. In particular, SerpinG1 and lysozyme levels were not elevated in patients with HLH with systemic activity compared with those without. Their lack of association with systemic activity suggests that their CSF elevation is not a spillover effect from the circulation and reflects a CNS-specific process, that is CNS-HLH. In contrast, CD14 was elevated in blood in the context of systemic activation, suggesting potential contributions from both CNS and systemic sources. Importantly, because CD14 was not elevated in blood in patients with HLH without systemic activity, all 3 biomarkers can be relevant in the setting of isolated CNS-HLH. However, because overall blood levels of SerpinG1, CD14, and lysozyme in patients with CNS-HLH did not differ from controls, they cannot be relied upon for diagnosing CNS-HLH from blood alone. CSF analysis therefore remains essential for facilitating diagnosis of CNS-HLH.

Our study has several limitations. We were surprised that proteomic analysis did not identify several chemokines that are typically involved with T-cell and macrophage activation. Chemokines are small molecules, and it is possible they were not present or present at very low concentrations in the CSF, below the level of detection of MS. Alternatively, their identification may have been obscured by high-abundance proteins. In particular, albumin dominated the overall protein profile, which made it difficult to identify subtle differences for other proteins between samples.³⁸ Although high-abundance proteins can be removed by immunodepletion prior to MS analysis, cytokines and chemokines can also be removed, suggesting that this method does not necessarily improve identification of chemokines by MS.³⁹ Heterogeneity of diagnoses in the ONID group was another limitation. Additionally, outliers in the ONID group (eg, in myelin oligodendrocyte glycoprotein antibody-associated disease, Guillain-Barré syndrome, and acute necrotizing encephalopathy) demonstrated biomarker levels (CXCL9, SerpinG1) overlapping with HLH, indicating that certain neuroinflammatory disorders may share biomarker profiles with HLH and thereby limit diagnostic specificity. Because osteopontin expression is age-dependent, and the HLH cohort was substantially younger than the ONID and control groups, the observed increase in osteopontin levels may in part reflect age bias. Although osteopontin remained higher in HLH even among age-matched patients, the small number of very young controls limits definitive conclusions. The elevation of CD14 in blood during systemic activation suggests some degree of spillover into the

CSF, which limits its specificity as a biomarker for CNS-HLH in the presence of systemic HLH.

In conclusion, an unbiased CSF proteomics approach identified SerpinG1, lysozyme, and CD14 as novel CSF biomarkers that can facilitate diagnosis of CNS-HLH from ONID. CSF CXCL9 is also a promising biomarker for distinguishing CNS-HLH from ONID. Our findings could potentially be extrapolated to isolated CNS-HLH, although this will need to be confirmed in a larger cohort.

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Authorship

Contribution: S. Chandra and S.M.D. initiated the project and its initial design; S. Chandra, K.E.P., K.S.F., and K.S. assisted with the collection of cerebrospinal fluid (CSF) samples; K.D.G. and S. Chutipongtanate assisted with the CSF proteomics analysis and analyzed the proteomics data; N.L. assisted with the enzyme-linked immunosorbent assay (ELISA) analysis; A.W. assisted with the statistical analysis for the ELISA and CSF parameter data; S. Chandra wrote the manuscript, with essential input and expertise from S.M.D., K.D.G., S. Chutipongtanate, R.A.M., and M.B.J.; and all authors edited the manuscript and provided essential inputs.

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