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ORIGINAL ARTICLE

Quantitative proteomic analysis of cerebrospinal fluid from patients with idiopathic facial nerve palsy

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Abstract

Background and purpose: Idiopathic facial palsy (IFP) accounts for over 60% of peripheral facial palsy (FP) cases. The cause of IFP remains to be determined. Possible etiologies are nerve swelling due to inflammation and/or viral infection. In this study, we applied an integrative mass spectrometry approach to identify possibly altered protein patterns in the cerebrospinal fluid (CSF) of IFP patients.

Methods: We obtained CSF samples from 34 patients with FP. In four patients, varicella-zoster virus was the cause (VZV-FP). Among the 30 patients diagnosed with IFP, 17 had normal CSF parameters, five had slightly elevated CSF cell counts and normal or elevated CSF protein, and eight had normal CSF cell counts but elevated CSF protein. Five patients with primary headache served as controls. All samples were tested for viral pathogens by PCR and subjected to liquid chromatography tandem mass spectrometry and bioinformatics analysis and multiplex cytokine/chemokine arrays.

Results: All CSF samples, except those from VZV-FP patients, were negative for all tested pathogens. The protein composition of CSF samples from IFP patients with normal CSF was comparable to controls. IFP patients with elevated CSF protein showed dysregulated proteins involved in inflammatory pathways, findings which were similar to those in VZV-FP patients. Multiplex analysis revealed similarly elevated cytokine levels in the CSF of IFP patients with elevated CSF protein and VZV-FP.

Conclusions: Our study revealed a subgroup of IFP patients with elevated CSF protein that showed upregulated inflammatory pathways, suggesting an inflammatory/infectious cause. However, no evidence for an inflammatory cause was found in IFP patients with normal CSF.

KEYWORDS
cerebrospinal fluid, chemokine, facial palsy, proteomics

Uwe Koedel and Frank Schmidt contributed equally to this work.

INTRODUCTION

Spontaneous unilateral/bilateral peripheral facial nerve palsy (FP) is one of the most common cranial nerve affections, with an incidence of 30–60/100,000 [1]. Men and women are equally affected. It has been described in patients of all ages, with a small peak in patients in their 40s [2]. FP is a disfiguring disorder with great impact on everyday life because it may permanently damage facial expression and also affect sense of taste as well as the lacrimal and salivary glands [3]. In approximately two thirds of the cases, no evident cause can be found, the disease is then termed idiopathic FP (IFP), also known as Bell's palsy [1, 4]. Among the remaining third of the cases, the most common causes are herpes simplex virus (HSV) and varicella-zoster virus (VZV) infection, Lyme neuroborreliosis, autoimmune causes (i.e., Miller-Fisher syndrome, sarcoidosis), and compression (i.e., by a tumor) [5]. Because the discovery of a secondary cause has major therapeutic implications, diagnostics including blood tests, computed tomography (CT)/magnetic resonance imaging scan and cerebrospinal fluid (CSF) withdrawal are usually performed [6]. While in symptomatic cases the underlining cause is treated, the primary consensus therapy for IFP consists of steroids [3].

The pathophysiological mechanisms of IFP are still unclear. In the last decades, many hypotheses have been postulated based on experimental evidence, however, none of these could be verified [1, 7]. The most widely examined hypothesis is a herpes simplex infection [8–10], however, the existing evidence is not sufficient to establish a causal relationship to date. Other potential causes with less evidence are other (herpes) viruses, autoimmune causes, ischemia and anatomical factors [7, 11]. Since functional/structural alterations of nervous tissue can be reflected in the protein composition of the CSF, we collected CSF samples of patients from our emergency department who presented with peripheral FP. We applied an integrative proteomic approach to identify possibly altered protein expression patterns in the CSF of IFP patients and searched for pathogen DNA in order to better characterize the pathophysiology of IFP.

MATERIALS AND METHODS

CSF probes

The CSF samples of patients admitted with FP (age >18 years) were collected in the Emergency and Neurology Departments of the University Hospital of Munich, Germany, from 2014 to 2017. We only included patients presenting with peripheral FP as the sole neurological symptom. All samples, as well as clinical data, were collected after obtaining written informed consent according to local ethics guidelines in Munich and the Declaration of Helsinki. Routine diagnosis of IFP was established by excluding secondary causes clinically and in CSF and on cranial CT scan. Additionally, CSF samples from patients with primary tension headache were used as negative controls.

Multiplex PCR

To search for the presence of various pathogens, a Biofire filmarray meningitis/encephalitis (ME) panel (Biomerieux, France) was used. Samples were processed by the Department of Virology at the Max-von-Pettenkofer Institute according to the manufacturer's instructions. The following pathogens were determined with this panel: *Escherichia coli K1*, *Haemophilus influenzae*, *Listeria monocytogenes*, *Neisseria meningitidis*, *Streptococcus agalactiae*, *Streptococcus pneumoniae*, cytomegalovirus, enterovirus, HSV 1, HSV 2, human herpes virus 6, human parechovirus, varicella-zoster virus and *Cryptococcus neoformans/gattii*.

Sample measurement by shotgun mass spectrometry

Peptides were separated by liquid chromatography (LC) on a nanoAcquity UPLC system (Waters Corporation, USA) coupled to a linear trap quadrupole (LTQ)-Orbitrap Velos mass spectrometer (Thermo Electron Corporation, Germany) equipped with a nano-electrospray ionization source and installed with a Picotip Emmitter (New Objective, USA). For LC separation, the digested peptides were first enriched on a nanoAcquity UPLC 2G-V/Mtrap Symmetry C18 pre-column (2 cm length, 180 µm inner diameter and 5 µm particle size; Waters Corporation) and subsequently separated using a NanoAcquity BEH130 C18 column (10 cm length, 100 µm inner diameter, 1.7 µm particle; Waters Corporation). The separation was achieved with a gradient of 91 min containing buffer A (0.5% DMSO in water with 0.1% acetic acid) and buffer B (5.0% DMSO in acetonitrile with 0.1% acetic acid, gradient, 1%–5% buffer B in 2 min, 5%–25% B in 63 min, 25%–60% B in 25 min, 60%–99% B in 1 min). The peptides were eluted at a flow rate of 400 nl/min. The eluted peptides were analyzed first by Fourier-transform mass spectrometry (MS), operated in positive and profile mode. Next, an MS/MS scan was performed in data-dependent mode to fragment peptides, and data were acquired in the positive, centroid mode. The MS switched automatically between the Orbitrap-MS and LTQ MS/MS acquisition to carry out MS and MS/MS. Survey full scan MS spectra (from m/z 325 to 1525) were acquired in the Orbitrap with resolution R = 30,000, with a target value of 1×10^6. The method allowed sequential isolation of a maximum of the 20 most intense ions, depending on signal intensity, and were subjected to collision-induced dissociation fragmentation with an isolation width of 2 Da and a target value of 1×10^4, or with a maximum ion time of 100 ms. Target ions already selected for MS/MS were dynamically excluded for 60 s. General MS conditions were electrospray voltage, 1.6–1.7 kV; no sheath and auxiliary gas flow, capillary temperature of 300°C. The ion selection threshold was 2000 counts for MS/MS, with an activation time of 10 ms and normalized activation energy of 35%. Only doubly and triply charged ions were triggered for MS/MS analysis.

Shotgun MS and data analysis

Data were analyzed with Genedata Expressionist software (v.13.0.1) and Mascot (v2.6.2). The raw MS data were processed using two Genedata modules: Refiner MS for data pre-processing, and Analyst for data post-processing and basic statistical analyses. Briefly, after noise reduction, LC-MS1 peaks were detected and their properties calculated (m/z and retention time [RT] boundaries, m/z and RT center values, intensity). Chromatograms were further aligned based on the RT spectra. Individual peaks were grouped into clusters and MS/MS data associated to these clusters were annotated with a Mascot MS/MS ions search using a peptide tolerance of 10.0 ppm, an MS/MS tolerance of 0.50 Da, a maximum number of missed cleavages of 2, and the Uniprot database for homo sapiens (20,659 entries). Results were validated by applying a threshold of 1%-corrected normalized false discovery rate. Protein interference was done based on peptide and protein annotations. Redundant proteins were ignored according to the Occam's razor principle, and at least one unique peptide was required for a positive protein identification. Protein intensities were computed using the Hi3 method. Samples were further grouped, and the respective data were post-processed with Genedata Analyst. The statistical analyses included Volcano *t*-tests and bar plot analyses. The relative abundance of proteins was considered significant with a p value ≤0.05 and an effect size ≥2 [12]. Functional enrichment analysis was performed to identify biological functions that were over-represented in differentially expressed proteins with a p value <0.05. Altered expressed proteins, both upregulated and downregulated, were used separately as proteins of interest and the proteins detected from all probes were used as the background set. The proteins were further annotated using Kyoto Encyclopedia of Genes and Genomes (KEGG) and the Gene Ontology for Biological Processes (GO-BP) data, prior to performing Fisher's exact test to determine pathways in which the proteins of interest were significantly over-represented. This analysis was performed on R 3.6.2 using clusterProfiler 3.14.3. GOSemSim was used to eliminate redundant GO-BP results. Only significant over-represented pathways with a p value <0.05 (−log10(p) >1.3) are shown [13].

Cytokine/chemokine multiplex array

Major cytokines and chemokines were measured in the CSF of patients with FP by Luminex multiplex immunoassay using the Human Th1/Th2 Cytokine & Chemokine 20-Plex ProcartaPlex Panel 1 (ThermoFisher Scientific, USA). The following cytokines/chemokines were included: granulocyte macrophage-colony stimulating factor, interferon (IFN) gamma, interleukin (IL)-1 beta IL-2, IL-4, IL-5, IL-6, IL-8, IL-12p70, IL-13, IL-18, tumor necrosis factor alpha, Eotaxin (CCL11), GRO alpha (CXCL1), interferon-gamma-induced protein/CXCL10, monocyte chemoattractant protein-1/CCL2, macrophage inflammatory protein (MIP-1) alpha (CCL3), MIP-1 beta (CCL4), RANTES (CCL5) and stromal cell derived factor alpha (CXCL12).

Statistics (except proteomics)

Statistical tests were performed using PRISM Software (GraphPad). p values <0.05 were considered significant. Differences between independent non-parametrical subgroups were compared by Kruskal–Wallis test, followed by Dunn's multiple comparisons test if more than two subgroups were compared.

RESULTS

Clinical data

In total, 34 patients with acute peripheral FP were included in our study. Most patients presented within 5 days after symptom onset; only two patients came at a later time point (7 and 14 days, respectively). Four patients were diagnosed with VZV-caused FP. Of these, two had considerable pleocytosis (63 and 279/μl, respectively), while the other two had a normal CSF leukocyte count (3 and 4/μl, respectively) but had clinical signs of VZV infection (efflorescence in the dermatomes C2-C6 and *zoster oticus*, respectively). The other 30 patients were clinically diagnosed with IFP due to lack of signs of secondary cause, however, only 17 of those had normal leukocyte count and protein levels (termed IFP). The remaining 13 patients showed slightly abnormal CSF parameters: five showed a slightly increased leukocyte count (6–10 leukocytes/μl, termed IFP with CSF pleocytosis) and eight showed elevated protein levels (59–73 mg/dl) and a normal leukocyte count (termed IFP with elevated CSF protein). As negative controls, five patients with primary tension headache were included in the study. Patient characteristics and CSF parameters are shown in Table 1.

CSF proteomic analysis

In a first step, we analyzed the proteome of the CSF in all patient groups to check for potential differences and similarities and to identify possible biomarkers for IFP. In total, 555 proteins were detected across all 29 probes. We then performed a principal component analysis (Figure 1). All groups were homogenous and were further analyzed, except the IFP patients with CSF pleocytosis. This group was very heterogenous in the proteome analysis, mainly due to one outlier. Thus, we considered it likely that this group included various etiologies and it was not further processed for a detailed analysis of proteins.

Figure 2 shows a heatmap of the 119/555 proteins that displayed differential concentrations in at least one patient group compared to patients with primary tension headache. IFP patients with normal parameters or CSF pleocytosis showed a similar heatmap profile of mostly unaltered protein concentrations compared to controls, while IFP patients with elevated CSF protein and VZV-FP patients had comparable heatmap profiles.

TABLE 1 Patient characteristics and cerebrospinal fluid parameters for the different patient groups

	Patient characteristics			CSF parameters		
Diagnosis	*n*	Age, years	Sex: female/male	Cells/µl	Protein, mg/dl	Glucose, mg/dl
IFP with normal CSF parameters	17	40 (21–85)	10/7	1 (1–5)	33 (23–50)	63 (53–89)
IFP with elevated CSF protein	8	70 (44–83)	4/4	2 (1–5)	67 (59–73)	75 (56–108)
IFP with CSF pleocytosis	5	51 (38–72)	3/2	6 (6–10)	57 (25–86)	58 (52–70)
VZV-FP	4	41 (34–57)	1/3	34 (3–279)	52 (38–65)	64 (58–145)
Primary tension headache	5	51 (26–58)	4/1	3 (1–4)	37 (27–39)	59 (55–67)

Abbreviations: CSF, cerebrospinal fluid; IFP, idiopathic facial palsy, n, number of patients of each group; VZV-FP, varicella zoster virus-induced facial palsy.

Note: For age, protein and glucose levels and cell count, median with range was calculated.

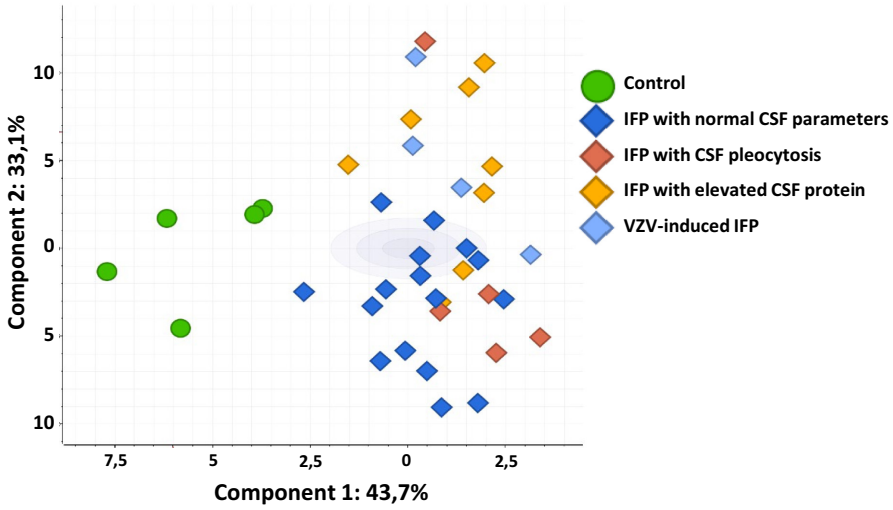


FIGURE 1 Principal component analysis. Principal component analysis was performed for the proteome profile of patients with idiopathic facial palsy (IFP) and normal cerebrospinal fluid (CSF) parameters (dark blue), IFP patients with elevated CSF protein (orange), IFP patients with CSF pleocytosis (red), varicella zoster virus (VZV)-induced facial palsy patients (light blue) and primary tension headache patients (green).

For further analysis, proteins were categorized as downregulated and upregulated between groups based on effect size transformed fold changes. The effect size for each protein was calculated for each group comparison. An effect size greater than ±2 and a p value <0.05 were chosen for significant differences. In the case of VZV-FP patients, while two of them displayed considerable CSF pleocytosis and the other two normal CSF leukocyte count, the group was homogenous, particularly with regard to the upregulated proteins. For IFP patients with normal CSF parameters, none of the proteins, except fibrinogen-α- and β-chain, were significantly upregulated compared to controls (Figure 3a). Both VZV-FP and IFP patients with elevated CSF protein displayed several up- and downregulated proteins compared with controls (Figure 3b,c) and compared with IFP patients with normal CSF parameters (Figure 3d,e). In the comparison between VZV-FP and IFP patients with elevated CSF protein, only four proteins were upregulated in the former group (Figure 3f).

In the next step, we compared the results with two databases, the KEGG and the GO-BP, to look for up- or downregulated biological pathways that were distinct between the FP groups. Compared to IFP patients, VZV-FP patients displayed many inflammation-related upregulated pathways associated with the complement cascade, systemic lupus erythematodes, prion diseases, and *S. aureus* infection pathways according to the KEGG database, while proteins associated with adhesion molecule pathways were downregulated (Figure 4a). Comparison with the GO-BP database produced similar results (Figure 4b). IFP patients with elevated CSF protein showed similar up- and downregulated pathways associated with inflammation to VZV-FP patients, when compared to IFP patients with normal CSF parameters according to the KEGG database (Figure 4c). The GO-BP database reproduced similar results (Figure 4d). Comparing IFP patients with elevated CSF protein and VZV-FP patients, the KEGG database analysis did not return any results, while according to the GO-BP database, the inflammatory pathways elevated in both groups were slightly upregulated in VZV-FP patients (data not shown). Table S1 lists the proteins that were upregulated for each pathway in VZV-FP (A) and IFP patients with elevated CSF protein (B).

Complement C8 upregulated in VZV-caused FP and IFP patients with elevated CSF protein: Validation of the proteomic results by ELISA

Since the complement system showed the strongest upregulation in VZV-caused FP and IFP patients with elevated CSF protein, we validated the quantitative proteomic results by determining CSF

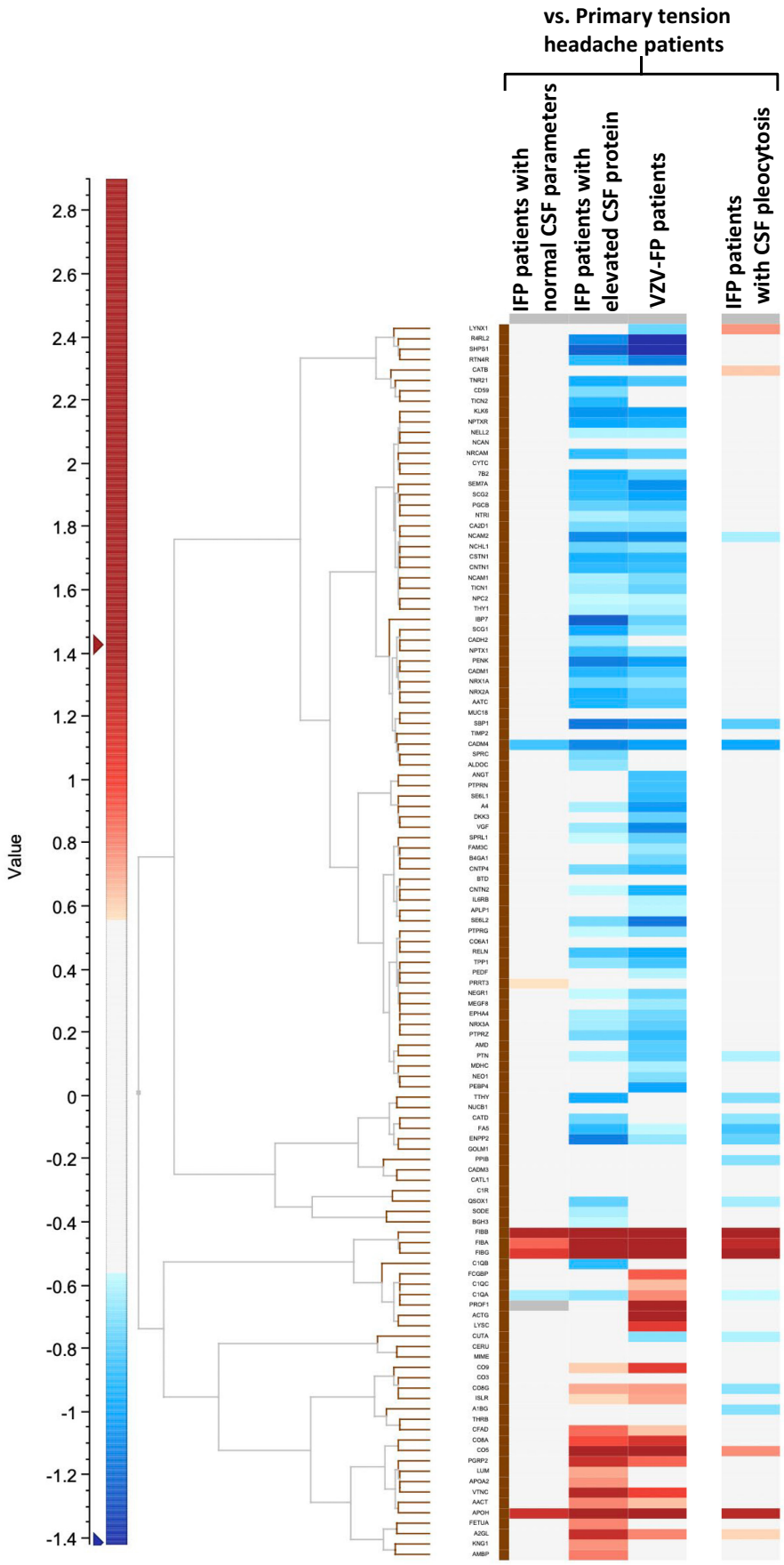


FIGURE 2 Heatmap of altered cerebrospinal fluid (CSF) proteins in facial palsy (FP) patients versus controls. Heatmaps were compiled of 119 proteins found in the CSF proteome of FP patients that had significantly higher/lower concentrations than in primary tension headache patients. The parameters were converted according to x-fold change to a red-white-blue continuum, where red represents higher x-fold change concentrations values and blue low ones. Each column represents one of the following patient groups: patients with idiopathic facial palsy (IFP) and normal CSF parameters; IFP patients with elevated CSF protein; varicella zoster virus-induced (VZV)-FP patients; and IFP patients with CSF pleocytosis.

complement C8-alpha-chain (CO8A) levels by ELISA (Figure 5). In accordance with the proteomics results, VZV-FP and IFP patients with elevated CSF protein had higher CSF CO8A levels than IFP patients with normal CSF parameters and controls.

Cytokine and chemokine profile of FP patients

While the proteome analysis is an overly sensitive method to detect proteins in a probe, it has some limitations. Especially for cytokines and chemokines, whose concentrations in body fluids are relatively low, the sensitivity of proteomics sinks rapidly [14]. In line with this, no major cytokine/chemokine was detected in our proteomic analysis. Since they both play an important role in inflammation, we measured the CSF levels of major cytokines and chemokines in our patients with FP using a multiplex array. Of the 20 cytokines and chemokines tested, eight showed values above the detection limit (Table 2). IFP patients with normal CSF parameters displayed similar values to those of the controls, while in VZV-FP patients, the CSF levels of CXCL1, CXCL10, CCL5 and CXCL8 were significantly higher than in the CSF of IFP patients with normal CSF parameters. IFP patients with elevated CSF protein had higher CSF values for CCL11 and CXCL8 than IFP patients with normal CSF parameters.

Borreliosis is a major cause of secondary FP. Therefore, we measured CXCL13 levels in our CSF probes, a marker with a good sensitivity and specificity for neuroborreliosis [15, 16]. Antibody index analysis was not available because of lack of blood samples to perform. In IFP patients with normal CSF parameters or elevated CSF protein and controls, CXCL13 was not detectable (Table 2). Three of four VZV-FP patients had substantial CXCL13 CSF levels, while one was negative.

Lack of pathogen DNA in IFP CSF samples

Since a (para)infectious cause is the leading hypothesis for the cause of IFP, CSF samples of all patients with FP were screened for causative pathogens using a multiplex PCR ME panel (see Methods). In patients with VZV-FP, we detected VZV-DNA. In all other samples, neither VZV nor any other pathogens included in the panel could be detected.

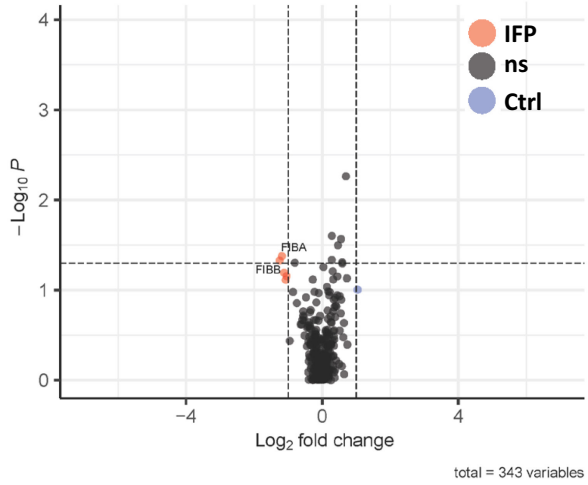
DISCUSSION

In this study, we analyzed CSF samples from patients with IFP in detail by using multiplex pathogen PCR panels, proteomic analysis, and multiplex cytokine assays. Our main findings were: (i) patients with IFP and elevated CSF protein levels showed similar proteomic alterations to those with VZV-associated FP; (ii) in IFP patients with normal CSF parameters, the proteome profile was almost the same as that in control patients, and (iii) pathogens were not detected in any sample from IFP patients.

According to guideline definitions, IFP (Bells' palsy) is a diagnosis by exclusion [17–19]. Secondary causes are excluded by clinical examination, imaging, blood and CSF analysis [19]. The significance of CSF analysis in the IFP diagnosis is still a matter of controversy: guidelines either do not recommend it [17, 18] or recommend it only in selected cases [19]. Consequently, mild CSF abnormalities (pleocytosis, elevated protein) are tolerated in IFP patients with lack of signs of a secondary cause. For example, in a retrospective study, 5% of IFP patients showed a CSF pleocytosis and even 25% an increased CSF-to-serum albumin ratio [20]. Accordingly, in our study, 17% displayed a mild pleocytosis, while 27% exhibited elevated protein levels. We opted to analyze these subgroups separately, unlike authors of previous publications. Indeed, we found substantial differences in the CSF proteomes of IFP patients with regard to CSF protein levels. It is too early to draw conclusions for clinical practice. Further studies are needed to evaluate the relevance of CSF routine abnormalities in IFP patients to clinical course and treatment. Proteomic analysis showed distinct profiles in IFP patients with elevated CSF protein. Proteins associated with immune response pathways were upregulated in an almost identical pattern as that found in VZV-associated FP patients, including complement activation, humoral immune response, lymphocyte activation and acute inflammatory response. Combined with the increase of selected chemokines in the CSF as detected by multiplex analysis, the results are indicative of an inflammatory and possibly infectious process in this IFP patient subgroup. The most widely accepted hypothesis of the cause of IFP is a (para)infectious complication or reactivation of HSV [8, 9]. However, a direct causal link between HSV and FP could not be formed to date. Moreover, antiviral monotherapy failed to show a beneficial therapeutic effect in IFP [21, 22]. In our study, no direct evidence of a viral presence was found in the CSF of IFP. It is still possible that, due to a local reactivation, no viral DNA finds its way into the CSF; however, all four patients with VZV-associated FP tested positive for CSF VZV-DNA regardless of the presence or absence of CSF alterations. Thus, a pathophysiological link between IFP and HSV has yet to be proven. Detecting a possibly infectious cause for a subgroup of IFP patients could have major therapeutic implications, as these patients could benefit from antiviral therapy. Since antiviral agents, when given together with corticosteroids, can prevent the development of long-term sequelae in patients with severe IFP [21, 23], it would be interesting to explore if IFP patients with elevated CSF protein develop more severe long-term deficits than those without and thus would benefit from adjunctive antiviral treatment.

No alterations in CSF parameters were found in the majority of IFP patients. These patients showed a CSF proteome profile almost identical to that of controls, except that fibrinogen α- and β-chain were upregulated, possibly as markers for tissue remodeling after injury of the facial nerve. In particular, the proteome profile showed no signs of inflammation, contrary to that of IFP patients with elevated CSF protein. This suggests a strictly local pathology of the facial nerve that has no effect on CSF protein composition. Overall, the facial nerve appears to be susceptible to damage due to its long and tortuous course through the temporal bone and within the Fallopian

(a)



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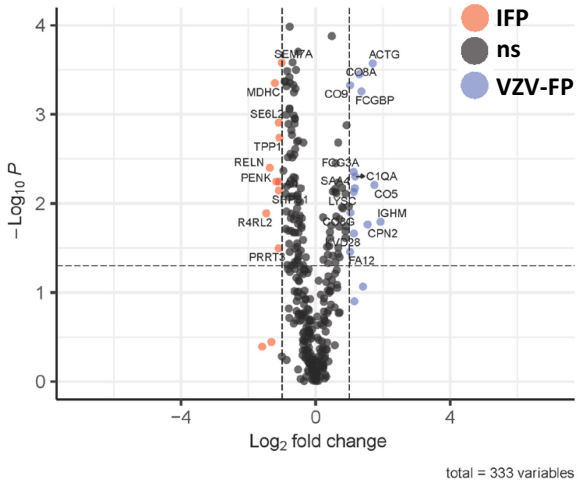

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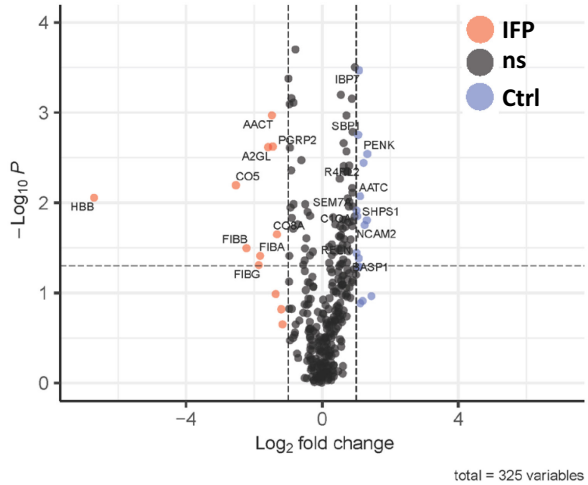

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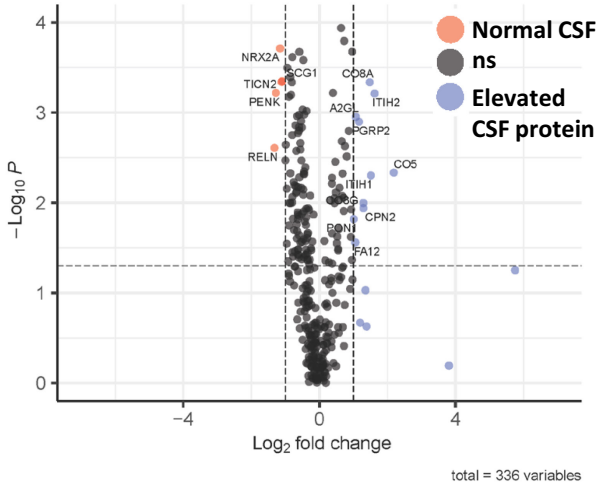

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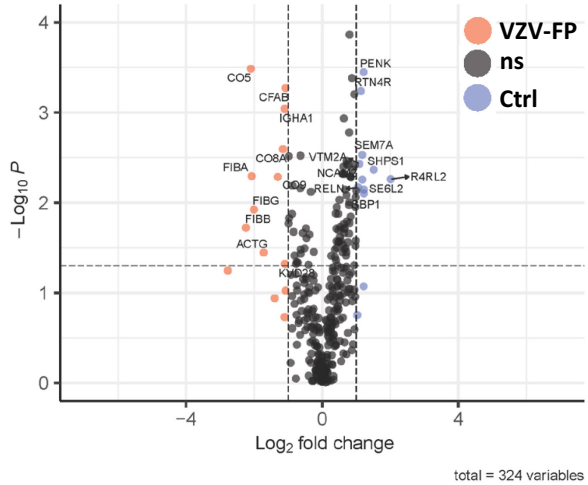

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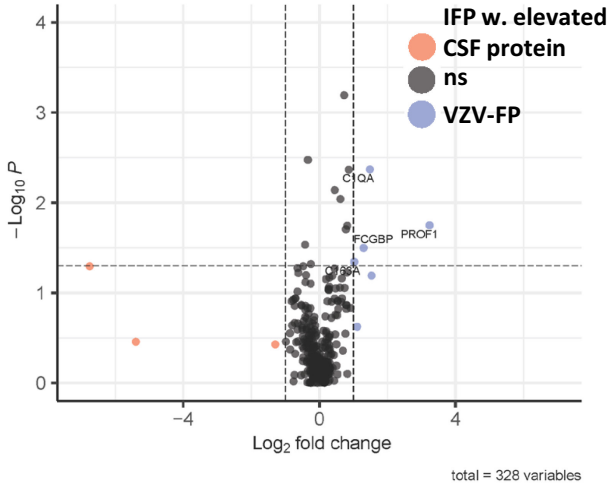

FIGURE 3 Comparison of proteomic profiles of facial palsy (FP) patients. (a–f). Volcano plots depict the effect size of the relative abundance of cerebrospinal fluid (CSF) proteins between the compared groups. The horizontal dotted line represents the significance cut-off of $p = -\log_{(10)}0.05 = 1{,}3$. The vertical dotted lines represent the defined cut-off of effect size > ±2. Each dot represents one protein. Black dots are proteins with an effect size < ±2, colored dots (red or blue) are upregulated proteins with an effect size > ±2 for the designated patient group. Ctrl, control; IFP, idiopathic facial palsy; ns, not significant; VZV-FP, varicella zoster virus-induced facial palsy.

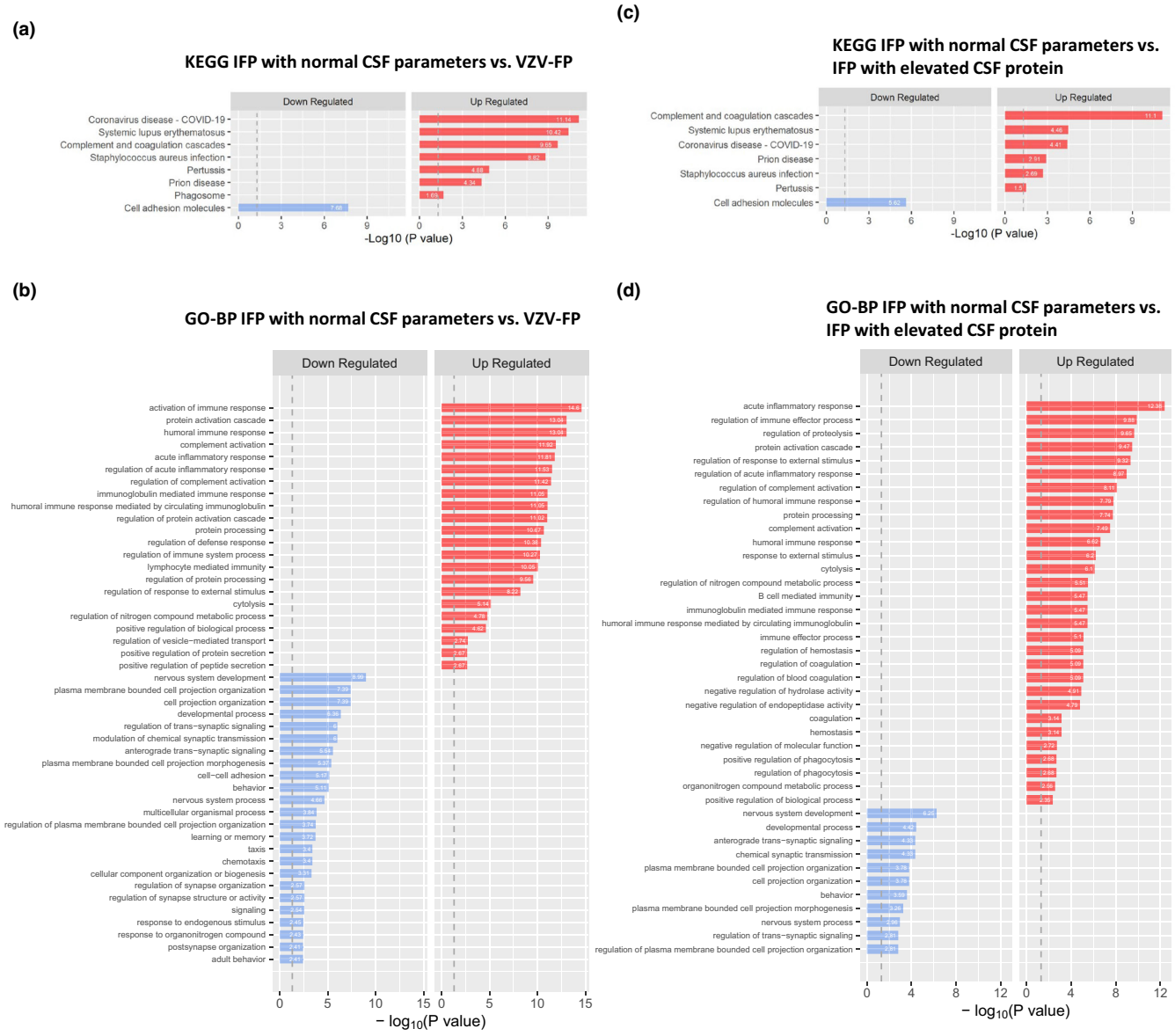

FIGURE 4 Biometric analysis of the cerebrospinal fluid (CSF) proteome of facial palsy (FP) patients. (a, b) The CSF proteome of 17 idiopathic facial palsy (IFP) patients with normal CSF parameters was compared with the CSF proteome of VZV-FP patients using the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (a) and the Gene Ontology for Biological Processes (GO-BP) database (b). Up- (red) or downregulated (blue) pathways in patients with VZV-FP that had a p value <0.05 ($\log_{10}p > 1.3$) are shown. (c, d) The CSF proteome of the 17 IFP patients with CSF pleocytosis was compared with the CSF proteome of IFP patients with elevated CSF protein using the KEGG database (c) and the GO-BP database (d). Up- (red) or downregulated (blue) pathways in IFP patients with elevated CSF protein that had a p value <0.05 ($\log_{10}p > 1.3$) are shown.

canal [24]. Even comparatively small increases in nerve volume, for example, due to endoneurial edema caused by locally reduced perfusion, could lead to paralytic symptoms [25–27]. Accordingly, neuroimaging studies showed that: (i) cross-sectional areas of the facial nerve were larger and those of the internal auditory canal were smaller on the affected sides than the equivalents on the unaffected sides in IFP patients [7, 26, 28]; (ii) the ratio of the diameters of the facial nerve and the facial canal was increased in IFP patients; and

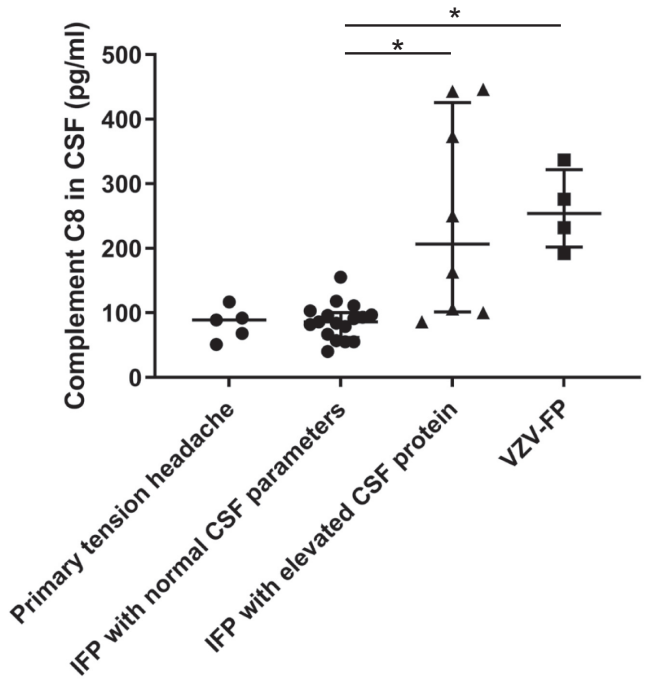

FIGURE 5 Complement C8 cerebrospinal fluid (CSF) levels in patients with facial palsy. Complement CO8 alpha chain levels were measured in the CSF of patients with idiopathic facial palsy (IFP) and normal CSF parameters or elevated CSF protein as well as varicella zoster virus-induced facial palsy (VZV-FP) and primary tension headache patients. Each symbol represents one patient. The mean with standard error was calculated. Statistics was conducted with the Kruskal–Wallis test. * <0.05, ** < 0.01, *** < 0.001

(iii) this correlated with the degree of nerve palsy (as determined by the House-Brackmann score) [7, 26, 28]. It is also worth noting that endoneurial blood supply can vary substantially between the two facial nerves of one individual. As endoneurial capillary density corresponds to the level of sensitivity to ischemic nerve damage [29], this might contribute to side- and individual-dependent differences in the susceptibility of facial nerves.

Our study has some limitations. First, the number of patients included was small; however, our groups were very homogenous except for the IFP group with pleocytosis. This group was consequently excluded because it consisted of only five patients with one outlier with a similar proteome profile to that of VZV-associated FP patients, while the CSF proteome of the others resembled that of IFP patients with normal CSF. Secondly, the groups were not sex- or age-matched. Thirdly, this study only used the KEGG and GO-BP databases, which could lead to biased results. However, we also compared the proteome results with further bioinformatics databases (WIKI, GO), and this produced the same results. Fourth, we did not perform any follow-up observations and consequently could not examine potential prognostic factors. Also, repeatedly obtained CSF samples were not available in our patients, therefore we cannot comment on the CSF alterations over time. Moreover, a clinical score, such as the House-Brackmann Scale, was not routinely used in our clinic, making it almost impossible to correlate CSF observations with paresis severity. When the House-Brackmann Scale was retrospectively applied to the documented clinical findings, all patients were assigned to grades IV and V. As a result, a correlation analysis with the CSF findings cannot be observed in our patient

TABLE 2 Levels of various chemokines and cytokines in patients with idiopathic facial palsy with normal cerebrospinal fluid (CSF) parameters, with CSF pleocytosis and with elevated CSF protein, as well as varicella-zoster-virus-induced facial palsy and primary tension headache

	CSF levels (pg/ml)				statistics
Cytokine/ Chemokine	IFP with normal CSF parameters	IFP with elevated CSF protein	VZV-FP	PTH	*p* value
CXCL12	319 (72–468)	372 (268–449)	507 (313–588)	326 (187–375)	0.09
CXCL10	**133 (77–439)**	240 (124–920)	**3499 (713–6363)**	212 (76–440)	**0.004**
CXCL8	**95 (39–156)**	**177 (111–833)**	**423 (154–1055)**	107 (49–194)	**0.001**
CCL11	**2 (0–3.1)**	**3.8 (0–6.1)**	4.7 (1.7–7.3)	2.5 (1–3.7)	**0.011**
CCL5	**0 (0–1.2)**	0.75 (0–1.8)	**5.4 (1.5–31)**	0 (0–2.1)	**0.0002**
CCL4	0 (0–34)	0 (0–15.3)	0 (0–29)	0 (0–15)	0.994
CCL2	1359 (996–2719)	1590 (1040–3444)	901 (93–3421)	1361 (1016–2349)	0.680
CXCL1	**0 (0–6.6)**	2.1 (0–23.2)	**24 (0–35)**	0 (0–9.8)	**0.019**
CXCL13	**0 (0–0)**	**0 (0–0)**	**98 (0–213)**	**0 (0–0)**	**0.019**

Abbreviations: CSF, cerebrospinal fluid; IFP, idiopathic facial palsy; PTH, primary tension headache; VZV-FP, varicella zoster virus-induced facial palsy.

Note: All cytokines/chemokines were measured by multiplex array but CXCL13, which was measured by single ELISA. The median values with range were calculated for each chemokine/cytokine and patient group. Kruskal–Wallis test was performed between the groups. Highlighted *p* values represent significant values <0.05. Highlighted median values represent group pairs that display significant differences from each other for the respective cytokine/chemokine. In the case of CXCL8, both IFP with elevated CSF protein and VZV-FP patients had higher CSF values than IFP patients with normal CSF parameters. For CXCL13, VZV-FP patients were significantly higher than all other groups.

cohort. Finally, we did not have information on the VZV vaccination status of our patients.

In conclusion, our study reveals distinct subgroups among IFP patients, supporting the hypothesis of different pathomechanisms underlying this disease. If CSF diagnostics reveal increased protein levels, an inflammatory/infectious genesis seems to be likely. This particular group of patients possibly requires different, additional treatment measures versus those with normal CSF.

AUTHOR CONTRIBUTIONS

Ilias Masouris and Uwe Ködel designed the study. Manuela Gesell Salazar performed the protein preparation and MS. Frank Schmidt, Neha Goswami and Fathima Mashood performed the data analysis and visualization of the proteomics. Ilias Masouris, Barbara Angele and Brigitte Groß performed the remaining experiments. Ilias Masouris was involved in recruitment of patients and collected the clinical data. Ilias Masouris wrote the first draft of the paper. Uwe Ködel, Frank Schmidt and Matthias Klein co-wrote the manuscript. All authors discussed the results, reviewed and commented on the manuscript.

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CONFLICT OF INTEREST

All authors report no disclosures relevant to this manuscript.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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