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Isobaric labeling strategy utilizing 4-plex *N,N*-dimethyl leucine (DiLeu) tags reveals proteomic changes induced by chemotherapy in cerebrospinal fluid of children with B-cell acute lymphoblastic leukemia

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Abstract

The use of mass spectrometry for protein identification and quantification in cerebrospinal fluid (CSF) is at the forefront of research efforts to identify and explore biomarkers for the early diagnosis and prognosis of neurologic disorders. Here, we implemented 4-plex *N,N*-dimethyl leucine (DiLeu) isobaric labeling strategy in a longitudinal study aiming to investigate protein dynamics in children with B-cell acute lymphoblastic leukemia (B-cell ALL) undergoing chemotherapy. The temporal profile of CSF proteome during chemotherapy treatment at weeks 5, 10–14 and 24–28 highlighted many differentially expressed proteins, such as neural cell adhesion molecule, neuronal growth regulator 1, and secretogranin-3, all of which play important roles in neurodegenerative diseases. A total of 63 proteins were significantly altered across all the time

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Author Contributions

QY, CI and LL designed the research. QY performed the experiment and analyzed the data. CD, JSV, MK, KD, CC, NP, MH, MB, KJ, DP and CI conducted patient enrollment and treatment, performed lumbar punctures and provided CSF samples. CI provided IRB approval. QY, CI, and LL prepared the manuscript and all authors provided feedback.

Supporting Information

The Supporting Information is available free of charge on the ACS Publication website.

Figure S1: Dendrograms of protein expression in study participants; Figure S2: Reproducible protein identification and quantification (PDF)

Table S1: Protein concentration for all CSF samples; Table S2: CSF proteins identified across nine patients; Table S3: Proteins significantly altered from Student's t-test; Table S4: Proteins significantly altered from one-way ANOVA test (XLSX)

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points investigated. The most over-represented biological processes from gene ontology analysis included platelet degranulation, complement activation, cell adhesion, fibrinolysis, neuron projection, regeneration and regulation of neuron death. We expect that results from this and future studies will provide means to monitor neurotoxicity and develop strategies to prevent central nervous system (CNS) injury in response to chemotherapy in children.

Graphical Abstract

INTRODUCTION

Survival rates of children and adolescents diagnosed with cancer are steadily increasing.¹ However, childhood cancer survivors often suffer from residual physical, cognitive and psychological impairments, which may significantly affect their daily lives.²⁻³ Neurobehavioral morbidity is drawing increased attention as the cause of limitations in daily functioning. It can affect various aspects of cognitive function, attention, memory, and emotional health.⁴⁻⁵ One of the major contributors to neurobehavioral morbidity is thought to be chemotherapy induced central nervous system (CNS) injury.⁶

Chemotherapy may lead to leukoencephalopathies and low white matter/grey matter volumes in pediatric acute lymphoblastic leukemia (ALL) survivors.⁷⁻⁸ Some insight into the pathomechanisms of neurotoxicity of chemotherapeutics has been gained with the help of preclinical studies, primarily in rodents. Such studies have investigated antimetabolites (methotrexate), alkylating agents (ifosfamide, cyclophosphamide, cisplatin) and vinca alkaloids (vincristine).⁹⁻¹⁰ Similar to what is seen in humans, cytostatic drugs can produce cognitive impairment in small animal models.¹⁰⁻¹² Neuropathological and neurophysiological correlates of CNS toxicity in infant, young and adult rodents included increased markers of apoptosis, decline in neurogenesis, impairment of long-term potentiation, synaptic remodeling, increased blood brain barrier permeability, impaired cell division and migration and oxidative stress.⁹⁻¹⁰

It appears that toxicity induced by cytostatic drugs within the central nervous system utilizes pathways which are also involved in other brain injury syndromes such as hypoxia/ischemia, traumatic injury and neuroinflammation. Oxidative stress is attributed to disruption of mitochondrial DNA leading to formation of reactive oxygen species (ROS). Production of ROS has been demonstrated for various agents, including cyclophosphamide, cytarabin, doxorubicin, methotrexate and carboplatin.¹³⁻²² Disruption of neurogenesis has been shown to occur within the hippocampus following treatment with carmustine, cyclophosphamide,

5-fluorouracil or cisplatin.^{11–12, 23–28} Excitotoxic passive neuronal death, caused by excessive stimulation of excitatory amino acid (EAA) receptors and active caspase-mediated cell death or apoptosis occurred in hypoxic and traumatic brain injury as well as in the context of mitochondrial dysfunction.^{29–33} Work by several investigator groups demonstrated that anticancer drugs are also potent neurotoxins *in vitro* and *in vivo*; they activate excitotoxic mechanisms but also trigger active, caspase mediated neuronal death.^{17, 34–36} Systemic inflammation with cytokine release, which may occur in cancer patients as a response to mucositis and systemic infections, may facilitate a process of neuroinflammation, microglial activation and suppression of neurogenesis.^{37–42}

For clinical practice it would be ideal to have biomarkers indicative of the toxicity severity of chemotherapy on the CNS. This would help identify vulnerable patients during early treatment, when there is still time to intervene. Such biomarkers are available and extremely helpful for tracing toxicity on other organs, i.e. kidney, heart, liver, bone marrow. Research on chemotherapy neurotoxicity is limited, especially in children. This is problematic as the brain is most vulnerable during development and the consequences of chemotherapy can be much more severe in children than that in adults. With regard to this specific group, the majority of clinical studies are focused on retrospective or cross-sectional analysis in children with CNS tumors or ALL. Furthermore, most studies were performed after completing the chemotherapy treatment. This limits the opportunity to obtain insights into the timely evolution and potential mechanisms of neurotoxicity which can be crucial for developing preventive strategies at early disease stage.

Advances in mass spectrometry (MS) have stimulated increased interest of its application in neurodegenerative disorders.^{43–45} Given the direct contact with the brain and extracellular space, CSF has been analyzed extensively to help elucidate the physiological and biochemical basis of various neurological diseases.^{46–47} Defining protein biomarkers unique to a disease diagnosis or progression in CSF is currently one of the most exciting areas of research. However, challenges remain when performing proteomic analysis in CSF because of the low abundance and large dynamic range of proteins in body fluids.⁴⁸ Immuno-affinity depletion of abundant proteins prior to CSF protein analysis has been introduced to reduce the sample dynamic range, but this method can also result in decreased precision on the relative quantification results.⁴⁹ Furthermore, many studies have shown the disadvantages of immune-affinity depletion approach. It holds the risk of co-depleting low abundance proteins and hindering their detection.^{50–51} It was also shown that depletion of CSF resulted in the loss of over 20 proteins suggested to be biomarker candidates for neurological diseases.^{52–54}

In the past decades, there have been rapid advancements in the resolution, mass accuracy, sensitivity and scan rate of mass spectrometers for protein analysis.^{55–58} MS-based quantification methods can be divided into two categories, label-free and stable isotope labeling approaches. The label-free strategy injects individual samples directly into mass spectrometer and quantifies the relative abundance of peptides.⁵⁹ Although label-free quantification method benefits from minimal sample handling, various factors could lead to problems with reproducibility and accuracy.⁶⁰ In addition, extensive instrument time required by label-free method is not suitable for large sample set which is typical for biological and clinical studies. On the other hand, the stable isotope labeling approach

allows the mixing of multiple samples at different experimental stages.⁶¹ Among different types of isotope labeling methods, isobaric tag, introducing a single mass addition onto precursors in MS¹ scan but produce discrete reporter ions for quantification in tandem MS (MS²) scan, has recently gained substantial interest.⁶² Previously, a novel 4-plex set of *N,N*-dimethyl leucine (DiLeu) isobaric labeling strategy was developed for high-throughput quantitative proteomics studies.⁶³ Different channels of DiLeu reagents have the identical molecular weight, while various stable isotopes (¹³C, ¹⁵N, ¹⁸O, ²H) are incorporated into reporter group and balance group to produce distinct low-mass reporter ions at *m/z* 115, 116, 117 and 118. During peptide fragmentation in a mass spectrometer, the linker will be fragmented and unique mass reporter ions will be released to indicate relative abundance of the original peptide. Recently, the multiplexing capacity of DiLeu reagents have been increased to 12-plex with high quantitative performance and affordability.⁶⁴ DiLeu reagents have enabled a wide range of applications in various biological systems including proteomic and metabolomic analysis in urine, N-glycoproteomic analysis in breast cancer cell lines and target proteomic analysis in CSF.^{65–68}

Here, we applied DiLeu reagents to globally quantify proteins in CSF samples of children with B-cell ALL undergoing systemic and CNS directed chemotherapy without irradiation. This novel approach enabled a comprehensive investigation of the biochemical changes that accompany chemotherapy and may prospectively allow tracing neurotoxicity of chemotherapy in children and might even help characterize its molecular pathomechanisms.

MATERIALS AND METHODS

Chemicals.

All isotopic reagents required for 4-plex DiLeu reagents synthesis were purchased from ISOTECH Inc (Miamisburg, OH). Urea, Tris base, hydrochloride acid (HCl), trifluoroacetic acid (TFA), reagent-grade formic acid (FA), iodoacetamide (IAA), triethylammonium bicarbonate (TEAB), *N,N*-dimethylformamide (DMF), 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium tetrafluoroborate (DMTMM), *N*-methylmorpholine (NMM), and hydroxylamine solution were purchased from Sigma-Aldrich (St. Louis, MO). MS grade trypsin/Lys-C mix and dithiothreitol (DTT) were purchased from Promega (Madison, WI). All ACS grade and Optima LC/MS grade solvents were acquired from Fisher Scientific (Pittsburgh, PA).

Patient Population.

Samples were collected from 9 pediatric subjects whose demographic information is presented in Table 1. All subjects had B-cell ALL, including four at average risk, one at high risk and one at very high risk. Average risk B-cell ALL was treated according to protocol AALL0932 and high/very high risk patients were treated according to protocol AALL1131 (National Cancer Institute, NCI). All patients received the same treatment for the first 5 weeks, as definitive risk category assignment occurred on day 29. The following chemotherapeutic agents were used: cytarabine, vincristine, dexamethasone, doxorubicine, pegaspargase, methotrexate, mercaptopurine, cyclophosphamide, thioguanine, intrathecal

methotrexate and cytarabine. None of the nine subjects developed significant clinical neurologic complications during chemotherapy.

CSF Collection.

Clear CSF (1–2 ml) research samples were obtained at each lumbar puncture (LP) from pediatric patients with B-cell ALL undergoing routine LPs as part of their treatment protocols. From each subject, 4 CSF samples were collected at weeks 1, 5, 10–14 and 24–28. Week 1 samples were collected before chemotherapy. Week 5 samples were collected at the end of induction chemotherapy which marks the completion of the most intensive phase of treatment. Weeks 10–14 samples were collected during consolidation phase and week-24–28 samples were collected during maintenance phase. Initially, 3–5 ml CSF samples were collected for clinical analysis, the research samples were subsequently placed in a separate tube. Of the 36 samples, 91% had a red blood cell (RBC) count of $< 1/\mu\text{L}$ indicative of no blood contamination. 9% of the samples had a RBC count of 124–4550 red blood cells/ μL in the clinical sample. The University of Wisconsin Institutional Review Board approved all study procedures. All participants provided signed informed consent.

CSF Sample Preparation.

Protein concentration was measured with bicinchoninic acid (BCA) protein assay (Thermo Scientific Pierce, Rockford, IL) and summarized in Table S1 for each CSF sample. CSF sample comparison was normalized by equal protein amount and 30 μg CSF proteins from each sample were dissolved in 8 M urea/50 mM Tris HCl (pH 8), reduced with 100 mM DTT for 1h, alkylated with 200 mM IAA for 30 minutes before quenching with 100 mM DTT. Protein samples were then diluted with 50 mM Tris HCl until reaching a urea concentration of < 1 M before adding trypsin/Lys-C in a 50:1 (protein:enzyme) ratio. Proteins were digested at 37°C for 16 h. Digests were quenched by lowering the pH to < 3 with 10% TFA. Peptides were desalted with SepPak C18 solid-phase extraction (SPE) cartridges (Waters, Milford, MA). All samples were dried in vacuo and reconstituted in 0.5M TEAB prior to DiLeu labeling.

Protein Digest Labeling.

Synthesis of DiLeu tags was conducted according to protocol described in detail by Xiang et al.⁶³ DiLeu tag of 0.6 mg was activated in anhydrous DMF combined with 0.6 mg DMTMM, 0.2 mg NMM and vortexed at room temperature for 45 minutes. After centrifugation, the supernatant was used immediately for peptide labeling. DiLeu labeling was performed by addition of labeling solution to CSF peptide digest and vortex at room temperature for 2 h. The labeling reaction was quenched by addition of hydroxylamine to a concentration of 0.25%, and the labeled peptide samples were dried in vacuo. The samples were combined and cleaned with SCX SpinTips (Protea Biosciences, Morgantown, WV), and desalted with Omix C18 pipet tips (Agilent, Santa Clara, CA).

LC-MS² Acquisition.

Samples were dissolved in 0.1% formic acid and analyzed on the Orbitrap Q-Exactive HF Mass Spectrometer (Thermo Fisher Scientific, Bremen, Germany) coupled to a Dionex

UPLC system. The chromatographic separation was carried out via mobile phase A that consists of 0.1% FA in water and mobile phase B consisting of 0.1% FA in ACN. Peptides were loaded onto a 75 $\mu\text{m} \times 15\text{ cm}$ homemade column packed with 1.7 μm , 130 Å, BEH C18 material obtained from a Waters UPLC column (part no. 186004661). Emitter tips were pulled from capillary tubing 75 μm id (Polymicro Technologies, Phoenix, AZ) using a model P-2000 laser puller (Sutter Instrument Co., Novato, CA). The LC gradient was 4%-35% B over 90 min at a flow rate of 300 nL/min. Survey scans of peptide precursors were performed with a scan range from 350 to 1800 m/z at a resolving power of 30 K (at m/z 200) with an automatic gain control (AGC) target of 1×10^6 and a maximum injection time of 100 ms. The top 20 precursors were then selected for higher-energy C-trap dissociation tandem mass spectrometry (HCD MS²) analysis with an isolation width of 1.4 Da, a normalized collision energy (NCE) of 27, a resolving power of 15 K (at m/z 200), an AGC target of 2×10^5 , a maximum injection time of 130 ms, and a lower mass limit of 110 m/z . Precursors were subjected to dynamic exclusion for 40 s with a 10 ppm tolerance. Each sample was acquired in technical triplicates.

Data Analysis.

Protein and peptide identification and quantification were conducted through Proteome Discoverer 2.1 (Thermo Scientific). Raw files were searched against the Uniprot *H. sapiens* reviewed database (August, 2016; 20152 entries) using the Sequest HT algorithm. Trypsin was selected as the enzyme with at most two missed cleavage. Searches were performed with precursor and fragment mass tolerance of 20 ppm and 0.02 Da, respectively. Static modifications included carbamidomethylation of cysteine residues (+57.02146 Da) and DiLeu labeling on N-terminus and lysine residues (+145.12801 Da). Dynamic modifications included oxidation of methionine residues (+15.99492 Da) and deamidation of asparagine and glutamine residues (+0.98402 Da). Peptide spectral matches (PSMs) were validated based on q-values set to a false discovery rate (FDR) of 1% using Percolator. Quantitation was performed in Proteome Discoverer with a reporter ion integration tolerance of 20 ppm for the most confident centroid. PSMs with all four channels present were considered valid for quantification. Reporter ion intensities summed from three technical replicates were normalized by the total peptide amount. Proteins with at least one unique peptide were considered valid matches if identified in at least five biological replicates in all groups to allow for quantitative comparison across different groups. To ensure accurate quantification, batch effects resulted from multiple batches of isobaric labeling were removed using proBatch package in R through quantile normalization and ComBat.^{69–70} Quantified proteins were subjected to paired two-sample unequal variance Student's *t*-test with a two-tailed distribution for binary comparison of chemotherapy-treated samples (weeks 5, 10–14, 24–28) versus untreated samples (week 1). One-way ANOVA analysis was conducted for comparison across all four groups (weeks 1, 5, 10–14 and 24–28 of chemotherapy) to explore dynamic changes resulting from chemotherapy. All the protein *p*-values were further corrected by Benjamini-Hochberg correction for multiple testing. Significantly altered proteins with adjusted *p*-value (FDR) < 0.1 were subjected to GO analysis and annotations utilizing DAVID Bioinformatics Resources.⁷¹

Data Deposition.

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD017415.⁷²

RESULTS AND DISCUSSION

Reproducible Protein Identification and Quantification.

To investigate protein dynamics induced by chemotherapy, we compared the CSF proteome of nine individuals before and during chemotherapy treatment using 4-plex DiLeu labeling strategy without immuno-affinity depletion, which greatly reduces sample preparation time and improves analytical throughput for quantitative proteomics. The workflow of the experiment is shown in Figure 1. For each individual, CSF was collected before chemotherapy at week 1 and at weeks 5, 10–14, 24–28 during chemotherapy. Equal amounts of proteins were subjected to in-solution digestion, labeled with 4-plex DiLeu, respectively, and combined for sample clean-up with strong cation exchange (SCX) SpinTips. Nine batches of 4-plex DiLeu labeled peptides were analyzed by LC-MS/MS and each batch was acquired in technical triplicates. This resulted in 27 LC-MS/MS runs and all raw data was searched in Proteome Discoverer for protein identification and quantification. We first compared protein abundance of the 9 subjects at each timepoint to study whether age, gender, or risk group of B-cell ALL is a disease-associated factor. Dendrograms from hierarchical clustering analysis of protein abundance presented no significant clusters among the nine subjects within the same age, gender or risk group (Figure S1). In the dendrograms several of the patients moved in the position of the clusters as a function of time, but these movements presented no significant clusters among the nine subjects within the same age, gender or risk group. This indicates the variation of these factors are not able to induce obvious alteration, and thus the 9 subjects were considered as biological replicates for following quantification analysis between different treatment stages. A total number of 3285 peptides derived from 341 proteins (FDR < 1%) were identified across 9 patients (Figure S2A, Table S2). Among them, proteins identified in at least five biological replicates in all groups were considered quantifiable. This led to 3043 peptides from 258 proteins (FDR < 1%) being quantified. We also examined the reproducibility of protein abundance across technical replicates and biological replicates using Perseus software (Figure S2B). Overall, pairwise comparisons of all technical replicates showed excellent quantitative reproducibility with a median Pearson correlation of 0.97. Lower median correlation ($r=0.91$) between biological replicates was observed indicating a larger variation in biological replicates than that in technical replicates.

Protein Alterations Induced by Chemotherapy.

To determine proteins with significant alterations associated with the chemotherapy treatment, all quantified proteins were subjected to Student's *t*-test for binary comparison between chemotherapy-treated samples versus untreated samples. Results indicated that 51, 21, 17 proteins were significantly changed during chemotherapy treatment at weeks 5, 10–14 and 24–28 respectively, compared with week 1 samples. (Table S3). Volcano plots were constructed to graphically display the *t*-test data (Figure 2). Points above the non-axial horizontal lines represent proteins with significant abundance change (adjusted *p*-value <

0.1). Green points to the left of the non-axial vertical line denote proteins with decreased levels, while red points to the right of the line denote proteins with increased levels. Notably, the majority of proteins with significant alterations showed 1.5- to 2-fold change in abundance. Significantly altered proteins (adjusted p -value < 0.1) and specific proteins with high expression in brain were labeled with their gene names.

To compare protein abundance across multiple timepoints (at weeks 1, 5, 10–14 and 24–28), one-way ANOVA analysis was conducted and identified 63 proteins with significant alterations (adjusted p -value < 0.1 , Table S4). Then we performed hierarchical clustering using Perseus software based on z-score normalized protein intensities to further explore the profile of these 63 proteins at different stages of chemotherapy treatment (Figure 3A). Heatmap demonstrated column-wise clustering of nine biological replicates in each group and this indicated that intergroup experimental variation was greater than intragroup biological variation. Furthermore, the untreated group was clearly separated from the other three chemotherapy-treated groups indicating the change of patients' CSF protein profile during the treatment. In addition, profiles from the three chemotherapy-treated groups also showed different trends in protein abundance among themselves, which likely reflects the long- and short-term effects of chemotherapy on CSF proteins. Some of them such as neural cell adhesion molecule 2 (NCAM2) may directly reflect neurotoxicity of chemotherapy drugs and showed increased or decreased levels at week 5 (end of induction phase), while some indirect effects such as immune dysregulation may lead to delay in changes of protein abundance until week 24–28. Figure 3B depicts 2 clusters of proteins based on their relative abundance across 4 different timepoints. Generally, cluster 1 included 28 proteins showed decreased levels during chemotherapy treatment compared with untreated group. In cluster 2, 36 proteins showed increased levels associated with the treatment.

Gene Ontology Analysis.

To explore the biological processes modulated by the 63 altered proteins with statistical significance, we performed GO analysis and annotations of each cluster utilizing DAVID Bioinformatics Resources (Figure 4). To pinpoint specific proteins in the most over-represented biological processes, we highlighted top 5 hits and correlated them with proteins significantly altered in one-way ANOVA analysis across multiple timepoints. Uniprot accession numbers were converted into gene names and the chord diagram was generated from GOpilot.⁷³ In each chord, enriched GO biological processes are shown on the right, and differentially expressed proteins contributing to this enrichment are shown on the left. Each protein was represented by rectangles with green-to-red color which correlated to the value of protein $\log_2$ -transformed fold change (FC). The outer layer represents FC of week 5/week 1, the middle one is week 10–14/week 1 and the inner layer is week 24–28/week 1. Results showed that for each cluster there were distinct and significantly enriched biological processes (Fisher Exact p -value < 0.05). For example, fibrinolysis is enriched with high significance from proteins with decreased levels present in cluster 1 (Figure 4A). Fibrinolysis, the removal process of intravascular fibrin clots, is an essential component of normal hemostasis.⁷⁴ Although the origin of physiological and disease-induced fibrinolysis in the CNS remains controversial, it is highly related to neurological inflammatory disorders. This result is also consistent with previous findings that anti-cancer drugs may facilitate a

process of neuroinflammation, microglial activation and suppression of neurogenesis in adults.^{37–42} For cluster 2 (Figure 4B), in which proteins mainly showed increased levels, one prominent biological process is neuron projection and regeneration which means regrowth of neuronal processes (axons or dendrites) in response to their loss or damage.⁷⁵ The increased activity of proteins regulating neuronal regeneration may represent the response of brain or spinal cord injury due to chemotherapy treatment. Previous studies also showed disruption of neurogenesis occurred within the hippocampus following treatment with carmustine, cyclophosphamide, 5-fluorouracil or cisplatin.^{11–12, 23–28} Positive regulation of neuron death is another enriched process involved in cluster 2 which is highly related with findings that anticancer drugs can trigger active, caspase mediated neuronal death in children with leukemia.^{17, 34–36} In some biological processes such as negative regulation of endopeptidase activity and positive regulation of neurofibrillary tangle assembly, proteins were exclusively decreased or increased during chemotherapy treatment. While in the biological process of complement activation, both proteins with increased and decreased levels were identified, revealing intricate physiological changes in CNS associated with chemotherapy treatment. Moreover, apolipoprotein E (APOE) and clusterin (CLU) were recurrent in many of these biological processes. In consistency with previous studies where distinct alterations of these two proteins in neurological conditions including neurodegenerative diseases have been reported,^{76–80} both of them showed remarkable elevated levels during the chemotherapy in this study.

Specific Proteins of Interest.

One goal of this project is to identify candidate biomarkers indicative of CNS toxicity of chemotherapy at an early treatment stage. When interpreting our results, one needs to consider that many proteins are also present in the blood and their blood concentration may change during treatment. Changes in CSF concentrations of the respective proteins likely reflect blood levels and changes in the blood brain barrier permeability. To bypass this confounder, we paid special attention to those proteins which are highly expressed in the brain.

Figure 5 illustrated the distribution of specific proteins in CSF at different stages of chemotherapy across the 9 patients. These proteins were significantly altered during the process and the differential alteration patterns were possibly related to pathological conditions in CNS induced by chemotherapy treatment. Figure 5A and 5B showed changes in neural cell adhesion molecule 2 (NCAM2) and neuronal cell adhesion molecule (NRCAM), two proteins that are critical for neural cell adhesion.^{81–82} Neural cell adhesion molecule is involved in cell-cell interactions during synaptogenesis, morphogenesis, and plasticity of the nervous system. Disturbances in brain cellular function may alter concentrations of neural cell adhesion molecule in the CSF and its elevated level has been reported in CSF from patients with mood disorder.⁸³ In this study, we also found significantly increased NRCAM and NCAM2 levels in patients undergoing chemotherapy. Thus, it is possible that chemotherapy drugs disturbed synaptic restructuring and neural plasticity which lead to the CNS injury. Another two interesting proteins are neuronal growth regulator 1 (NEGR1, Figure 5C) which is involved in neuronal cell growth and differentiation,⁸⁴ and secretogranin-3 (SCG3, Figure 5D) which plays a key role in

neurotransmitter storage and is involved in apoptosis of dopaminergic neurons.⁸⁵ All the proteins above showed significant elevation in the CSF, most prominent on week 5 (end of intensive induction chemotherapy). This increase may reflect CNS injury caused by chemotherapy on neuronal membranes and other structures. It will be interesting but also challenging to study correlation between the early elevation of these proteins in CSF and long-term neurocognitive and neuroimaging outcomes, which could be assessed at a later stage.

When interpreting changes of protein levels, an interesting question to consider is how brain development may affect them. The CSF samples were collected within a short period of 24–28 weeks which is approximately 6 months, so we do not expect dramatic developmental changes to occur in children within such short period of time. Therefore, we attributed the observed protein changes primarily to the effects of chemotherapeutic agents on the CNS. Our essential long-term goal is to correlate changes in protein levels during chemotherapy with structural changes in the brain, neurocognitive data and validate the potential biomarkers. As these patients are still undergoing treatment and sufficient data from long-term outcomes are not available, we are not able to provide such correlations at this point in time. In addition, the number of patients studied so far is very small. In the future, it is our hope that CSF proteome results combined with analysis of other CSF metabolites will enable us to gain deeper insights into pathomechanisms of CNS injury during chemotherapy in children. Although our CSF proteomics investigations are still in an early phase, we are able to establish a solid methodology that enables us to explore longitudinal protein changes in small volumes of CSF in this first pilot study.

CONCLUSION

Using our custom-developed 4-plex DiLeu labeling reagents, we successfully conducted an exploratory investigation of proteomic changes induced by chemotherapy treatment in CSF samples of children with B-cell-ALL. The multiplexing quantification workflow not only allows for straightforward sample comparison but also reduces analysis time. By comparing CSF protein profiles before and after chemotherapy treatment, we clearly observed the differential alteration of proteins at various stages after the treatment. The GO analysis revealed intricate biological processes linked to protein alterations. Consistent with previous studies, several neurological disorders related proteins, such as neural cell adhesion molecule, neuronal growth regulator 1, and secretogranin-3 were also significantly changed in our study. Although these proteins cannot be directly interpreted as biomarkers for CNS injury without further biological validation, we think that our findings represent a promising first step towards gaining insight into the pathomechanisms of chemotherapy neurotoxicity in children and provide tools for monitoring and possibly minimizing such toxicity. Through this and future studies, protein quantification utilizing DiLeu labeling reagents has the potential to contribute towards better cognitive outcomes and improvement of quality of life for pediatric patients exposed to chemotherapy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Experimental workflow for multiplexed CSF protein quantification. CSF samples were collected from 9 patients before chemotherapy at week 1 and at weeks 5, 10–14, 24–28 during chemotherapy. For each patient, samples were digested with trypsin/Lys-C, labeled with 4-plex DiLeu tags respectively, and combined for sample clean-up with SCX SpinTips. Samples were analyzed using a nanoLC system coupled to a Q-Exactive HF Orbitrap mass spectrometer. Data was searched through Proteome Discoverer and further processed by Perseus and R.

Figure 2.

Volcano plots showed CSF protein level changes between (A) week 5 vs. week 1; (B) week 10–14 vs. week 1; and (C) week 24–28 vs. week 1. Log₂ protein fold changes are plotted against the negative log₁₀ adjusted *p*-values (FDR) from nine biological replicates. Points above the non-axial horizontal line represent significantly altered proteins (lower line, adjusted *p*-value < 0.1; upper line, adjusted *p*-value < 0.05, student's *t*-test). Green dots represent proteins with decreased levels and red dots represent proteins with increased levels.

Figure 3.

(A) Hierarchical clustering of the 63 proteins that exhibited statistically significant expression (ANOVA test, adjusted p -value < 0.1) in the CSF visualized in a heatmap. (B) Expression profile of the two hierarchical clusters, which were statistically different relative to one another (color based on distance from center in Perseus).

Figure 4. Proteins to biological pathway linkages for proteins in (A) cluster 1 and (B) cluster 2 based on hierarchical clustering analysis. The significantly altered proteins (ANOVA, adjusted p -value < 0.1) have been linked with pathways as color-coded ribbons. Green-to-red rectangles next to the altered proteins indicate the magnitude of the log₂ fold change (FC), where the inner layer represents FC of week 1 in comparison with week 5, the middle layer in comparison with week 10–14 and the outer layer in comparison with week 24–28.

Figure 5.

Box plot of the distribution of (A) neural cell adhesion molecule 2; (B) neuronal cell adhesion molecule; (C) neuronal growth regulator 1 and (D) secretogranin-3 in CSF at different stages of chemotherapy. Whiskers extend to data points that are less than 1.5* inter-quartile range away from 1st and 3rd quartile, respectively. The horizontal line shows the median. (*, adjusted p -value < 0.1; **, adjusted p -value < 0.05, Student's t -test)

Table 1.

Demographics and medical data from the current study participants

Subject number	Sex	Age at diagnosis	Diagnosis	Risk category
1	M	3y 6mo	B-cell ALL	Average risk (AR)
2	F	12y 11mo	B-cell ALL	Very high risk (VHR)
3	M	3y 6mo	B-cell ALL	High risk (HR)
4	M	3y 2mo	B-cell ALL	Average risk (AR)
5	F	12y 11mo	B-cell ALL	Very high risk (VHR)
6	M	2y 4mo	B-cell ALL	Average risk (AR)
7	F	2y 11mo	B-cell ALL	Average risk (AR)
8	M	5y 6mo	B-cell ALL	Very high risk (VHR)
9	M	13y 8mo	B-cell ALL	Very high risk (VHR)
6M:3F		6.7 ± 1.6 (SEM)		4AR/1HR/4VHR
		3.2–12.9 y		