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Chemotherapy-related Changes in Central Nervous System Phospholipids and Neurocognitive Function in Childhood Acute Lymphoblastic Leukemia

Kevin R. Krull¹, Marilyn J. Hockenberry², Petra Miketova³, Marissa Carey⁴, and Ida M. Moore⁵

¹Department of Epidemiology and Cancer Control, St. Jude Children's Research Hospital

²Department of Pediatrics, Baylor College of Medicine

³Cayman Pharma

⁴Department of Behavioral Medicine and Psychiatry, West Virginia University

⁵Biobehavioral Health Science Division, University of Arizona Cancer Center

Abstract

Long-term survivors of childhood leukemia are at risk for neurocognitive impairment, though the neurophysiological basis is not well understood. The purpose of this study was to explore associations between changes in cerebrospinal fluid (CSF) phospholipids and neurocognitive function in children undergoing chemotherapy for acute lymphoblastic leukemia. 76 children were followed prospectively from diagnosis. CSF samples were collected during scheduled lumbar punctures and phospholipids were extracted. Neurocognitive evaluations were conducted annually beginning shortly after diagnosis. Concentrations of sphingomyelin (SM) increased following induction ($p=0.03$) and consolidation ($p=0.04$), while lysophosphatidylcholine (LPC) increased following induction ($p=0.003$). Multivariable analyses demonstrated associations between post-induction SM and motor speed at one ($p<0.001$), two ($p=0.001$), and three ($p=0.02$) years following diagnosis. Post-induction LPC was associated with verbal working memory ($p=0.007$). Results indicate early changes in phospholipids are related to neurocognitive decline and suggest a chemotherapy impact on white matter integrity.

Keywords

Cerebral Spinal Fluid; Childhood Leukemia; Neurocognitive; Phospholipids

Neurocognitive impairment is estimated to occur in 20–40% of survivors of childhood acute lymphoblastic leukemia (ALL), [1,2] and recent reports implicate central nervous system (CNS) treatment with intrathecal (IT) or high-dose intravenous (HD-IV) methotrexate (MTX) chemotherapy. [3,4] Impairment in specific neurocognitive skills have been identified, particularly processing speed, attention, and working memory. [5–7] Furthermore, reduced performance on these specific skills has been reported prior to completion of chemotherapy regimens, and acute neurocognitive changes predict future deficits. [8,9]

Chemotherapy treatment is also associated with neuroimaging abnormalities. [10,11] Prospective brain magnetic resonance imaging (MRI) studies reveal rates of leukoencephalopathy as high as 86% shortly after consolidation therapy involving IT and HD-IV MTX, with a reduction to approximately 30% prevalence by the end of therapy. [10] Notably, the occurrence of acute leukoencephalopathy has been associated with reduced performance on neurocognitive tasks. [12]

Acute changes in integrity of the central nervous system (CNS) can be examined with cerebral spinal fluid (CSF) phospholipids. Sphingomyelin (SM) and lysophosphatidylcholine (LPC) are two phospholipids associated with CNS integrity in a variety of clinical and pre-clinical models. SM is the second most abundant phospholipid found in the CSF, is a major component of myelin sheath and is a sensitive measure of both disrupted myelin development and demyelination. [13] SM is associated with CNS disease, [14] and increased SM is linked to oxidative stress and memory problems in patients with human immunodeficiency virus (HIV). [15,16] LPC is associated with myelin breakdown and increased permeability of the blood brain barrier in adult mice, [17] increased expression of nitric oxide synthase and neuronal concentrations in the adult rat, [18] and delayed myelination in mice pups. [19] However, decreased LPC concentration in CSF has been linked to Alzheimer's disease. [20] To date, the association between LPC or SM and neurocognitive function has not been reported in childhood cancer.

The purpose of this study was to examine the association between CSF phospholipids associated with white matter integrity (i.e. LPC and SM) and neurocognitive outcomes in children undergoing chemotherapy treatment for ALL. CSF levels of LPC and SM were examined prior to and following various phases of chemotherapy treatment and children were evaluated with a standard battery of neurocognitive measures. We predicted that concentrations of LPC and SM following induction and consolidation therapy referenced to baseline levels would be associated with neurocognitive function over time.

MATERIALS and METHODS

Participants

Study participants were recruited through two pediatric hematology/oncology clinics in the Southwestern United States. Eligibility criteria included newly diagnosed patients who were between 3 and 14 years of age treated on a single therapeutic protocol. Exclusionary criteria were cranial radiation therapy (CRT), a dominant language other than English, and previous diagnoses of neurodevelopmental or neurological disorder. Children diagnosed with CNS disease through analysis of CSF collected at diagnosis were generally treated with CRT and excluded from this study. A total of 88 children were consecutively enrolled at diagnosis between 1999 and 2004 and followed over a four year period. Twelve children were not included in the analysis: five were determined to meet the exclusion criteria after enrollment (four due to primary Spanish language and one due to undiagnosed Turner Syndrome); four children withdrew participation after Baseline due to early relapse, and three children had incomplete data due to later relapse. A final sample of 76 children (86.4%), with a mean (SD) age of 7.0 (3.11) years at diagnosis was obtained.

Chemotherapy was administered according to Children's Oncology Group (COG) protocols for Low (P9201 or P9904), Standard (P9605 or P9905) or High (P9406 or P9906) risk leukemia. [21,22] Risk classification was based on a variety of factors, including age at diagnosis, white blood cell (WBC) count, CNS disease and genetic factors. Children in the current study treated on High risk protocols were diagnosed when > 10 years of age and had WBC/ μ l > 50,000. Induction therapy was standard across children, while consolidation therapy included a short HD-IV MTX infusion (2 g/m² over 4 hours), a long HD-IV MTX

infusion (1 g/m² over 24 hours), or a long IV MTX infusion (100 mg/m² over 24 hours). All patients received CNS prophylaxis with IT MTX at age-titrated doses. The Institutional Review Boards of both institutions approved the research protocol. Informed consent was obtained from all parents and assent was obtained from all age-appropriate children.

CSF Phospholipid Measurement

CSF samples were collected during scheduled lumbar punctures at the time of diagnosis and prior to administration of IT chemotherapy. Children underwent lumbar punctures with the assistance of local anesthesia. CSF samples were related to the previous phase of treatment and averaged for phases that included more than one IT. Concentrations of phospholipids SM and LPC were examined at diagnosis (Diagnosis), following induction (Induction) and consolidation (Consolidation) therapies, and following the first half (Continuation 1) and second half of continuation (Continuation 2) therapy. Samples were placed on dry ice immediately after collection, and centrifuged for 15 min at 3000 rpm to remove any cellular debris. Processed CSF was stored at -80°C until phospholipids were extracted, separated and quantified. Phospholipids were extracted from CSF samples using a modified method developed by Folch and others. [23] Briefly, lipids were extracted from the aqueous CSF with 4 ml of chloroform: methanol (2:1, v/v), vortexed for 1 minute and centrifuged at 10,000 rpm for 20 minutes. The organic phase was collected and stored on ice. A second extraction of the aqueous phase with chloroform-methanol (9:1, v/v) was done to insure complete extraction of the less polar phospholipids. The organic fractions were combined, evaporated to dryness under nitrogen, and resuspended in hexane-isopropanol (1:1, v/v) prior to separation with high-performance liquid chromatography (HPLC). This method recovers approximately 98% of phospholipids. [23,24]

Separation of phospholipids in CSF extracts was done using a Beckman Model 126 Solvent System, 168 Diode Array Detector (206 wavelength), normal phase silica column (Ultrasphere Si-5 microns, 25 cm × 4.6 mm i.d.), and mobile phase gradient. The mobile phase began with hexane:propan-2-ol:water (49.5:49.5:1, v:v) for 5 minutes, changed to hexane:propan-2-ol:water (39:52:9, v:v) over 30 minutes, and returned to hexane:propan-2-ol:water (43:57, v:v) over 15 minutes. The flow rate was 1 ml/minute. This gradient separates phospholipids into component classes based on polarity. Reference standards (Sigma) were used for correct assignment of phospholipid classes based on elution time. Phospholipid standards were used for development of calibration curves for quantifying phospholipids in CSF sample extracts. Typical HPLC chromatogram showing separation of phospholipid classes in volumetric ratios present in CNS cell membranes is shown in Figure 1. The major phospholipid classes include: phosphatidylethanolamine (PE, retention time=20.37 min), phosphatidylinositol (PI, retention time=21.37 min), phosphatidylcholine (PC, retention time=41.18 and 41.57 min), sphingomyelin (SM, retention time=42.20 and 43.32 min), and lysophosphatidylcholine (LPC, retention time=49.55 min).

Neurocognitive Assessments

Each child completed four neurocognitive evaluations during the study. All assessments were conducted during a scheduled visit to the assessment center, and were performed on a day different from when the CSF samples were collected. The initial assessment was scheduled as soon as the child was medically stable, after completion of induction therapy and prior to the beginning of consolidation. Since the treatment regimen employs the same induction protocol for all children, and differed primarily in the consolidation treatment, this initial assessment was considered a “baseline” evaluation. Subsequent evaluations were obtained one year after the initial assessment (Year 1) and following completion of consolidation therapy, two years after the initial assessment (Year 2) and half way through continuation therapy, and three years after the initial assessment (Year 3) and after

completion of chemotherapy. Evaluation times were selected to correspond with typical visits to the clinic when CSF samples were also drawn, and to limit the potential practice effects associated with the administration of intelligence tests at intervals less than one year apart.

General cognitive abilities were assessed with either the *Wechsler Primary and Preschool Scale of Intelligence – Revised (WPPSI-R)* or the *Wechsler Abbreviated Scale of Intelligence (WASI)*. [25,26] Processing speed was evaluated with the *Purdue Pegboard*, and the Processing Speed Index from the Wechsler Intelligence Scale for Children for children 6 years and older. [27,28] Working Memory was assessed with the Memory for Sentences and Bead Memory subtests from the *Stanford-Binet Intelligence Scale – 4th Edition (SBIV)*. [29] Visual-motor integration was evaluated with the *Beery Developmental Test of Visual-Motor Integration – 4th Edition (VMI)*. [30] Academic functions were assessed with the *Woodcock-Johnson Tests of Achievement (WJ-III)*. [31]

Statistical Analyses

A longitudinal study design was used to examine changes over time in CSF markers of membrane injury and neurocognitive function in children with ALL. Descriptive statistics were conducted for relevant demographic and clinical treatment data. Means and standard deviations for concentrations of phospholipids SM and LPC were examined at Diagnosis, Induction, Consolidation, Continuation 1 and Continuation 2. Sample distributions were examined across all time points for normal distribution using Shapiro-Wilk tests. Since SM and LPC were not normally distributed, Wilcoxon Signed Ranks tests were used to examine changes in concentrations over time. SM and LPC values were subjected to log transformations for use in linkage to neurocognitive outcomes. Neurocognitive performance was examined at baseline and at each of the three follow-up times by comparing age-adjusted standard scores to population norms (mean=100, SD=15) using one-sample t-tests. Neurocognitive measures that significantly differed from population norms were used in subsequent analyses. Univariate correlations were conducted between SM and LPC values and neurocognitive outcomes that were collected in conjunction with or at a time following the CSF sampling. Multivariable analyses were conducted for each significant neurocognitive outcome using all SM and LPC values that were significant in univariate associations at $p < 0.10$, adjusting for baseline neurocognitive functioning. Finally, groups were developed using median splits for SM and LPC values at each phase that demonstrated a significant change and t-tests were performed for neurocognitive variables that demonstrated an association in multiple variable modeling.

RESULTS

Relevant demographic and clinical treatment data are presented in Table 1. 59.2% of the sample was female, with an average parent education level of 13.5 years. Standard risk stratification accounted for 50% of the children's treatment regimen, with 67.1% receiving a long HD-IV MTX infusion (1 g/m² over 24 hours).

Concentrations of SM and LPC in the CSF samples collected across sequential phases of chemotherapy treatment are presented in Table 2. SM was observed to significantly increase following induction ($p=0.03$), consolidation ($p=0.04$), and the second half of continuation ($p=0.03$) therapy. LPC was observed to increase from diagnosis to induction ($p=0.003$).

Neurocognitive performance was examined at baseline and at each of the three follow-up times (Table 3). Compared to population norms, patients demonstrated lower verbal ($p < 0.001$) and visual ($p=0.05$) working memory, as well as lower motor speed ($p < 0.001$) at baseline testing. At baseline, patients also demonstrated lower performance on measures of

spelling ($p=0.003$), and this reduced performance continued into the first follow-up evaluation ($p=0.02$). Subsequent spelling evaluations did not differ from norms and were not further examined. Performance on a visual-motor integration test declined over time and was below expectations at each of the three follow-up evaluations (Year 1 $p=0.006$, Year 2 $p=0.05$, Year 3 $p<0.001$).

CSF samples collected following induction therapy demonstrated associations between SM and reduced motor speed at Year 1 ($r=-0.48$; $p<0.001$), Year 2 ($r=-0.61$; $p=0.001$), and Year 3 ($r=-0.49$; $p=0.02$). Post-induction LPC concentrations were associated with verbal working memory at Baseline ($r=0.29$; $p=0.007$), but subsequent years. No SM or LPC associations were demonstrated with visual working memory or visual-motor integration.

To further examine the motor speed and working memory over time, median splits were conducted on the individual distributions of post-induction SM and LPC and performances on neurocognitive outcomes. As depicted in Figure 2, motor speed was lower at Year 1 in patients who demonstrated lower concentrations of post-induction LPC ($p=0.01$) and higher concentration of post-induction SM ($p=0.02$), though no differences were found for subsequent years. Figure 3 demonstrates that patients who had lower post-induction LPC also displayed lower verbal working memory at Year 1 ($p=0.03$) and Year 3 ($p=0.03$). Visual working memory was associated with SM concentrations later in therapy, with significantly lower working memory scores in patients with higher SM following consolidation therapy ($p=0.05$) and during the second half of continuation therapy ($p=0.03$).

DISCUSSION

Compared to values obtained at diagnosis, sphingomyelin (SM) significantly increased following induction, consolidation and during the second half of continuation therapy, while lysophosphatidylcholine (LPC) increased only following induction therapy. Neurocognitive measures of motor speed and working memory were significantly lower than expected at the baseline assessment and all subsequent assessments. Early changes in SM were related to motor speed and early changes in LPC were linked to verbal working memory. Declines in visual working memory over time were associated with later elevations in SM.

The association between SM and motor speed may be connected to white matter integrity. SM is a major component of myelin, [13] and motor speed is dependent upon myelin integrity. [32] Leukoencephalopathy can occur as early as post-induction therapy and may progress through continuation therapy. [10] Although the highest rates of leukoencephalopathy are reported following consolidation therapy, the biochemical reaction resulting in LPC increases are likely to predate observable changes in brain MRI's.

At first glance, the positive correlation between LPC and working memory performance seems counterintuitive, as animal studies report associations between LPC and myelin breakdown in adult mice [17] and delayed myelination in pups. [19] However, the working memory functions assessed in the current study are most likely associated with cortical brain functions, and are less dependent than motor speed on white matter integrity. [33] In addition, LPC is found to increase vasodilatation in the CNS, [34] to stimulate activity in immune cells, [35] and to successfully treat serious infection in clinical samples. [36,37] Thus, higher levels of LPC may promote a healthy biological response to infection and/or toxins. In fact, pre-clinical studies identify LPC as neuroprotective in hippocampal ischemic injury models. [38] This neuroprotective effect is demonstrated when peak LPC levels precede ischemic events by up to 72 hours. Since the CSF samples in our study were collected prior to subsequent intrathecal chemotherapy, elevated LPC levels found post-induction actually immediately precede the initiation of consolidation therapy. Perhaps this

LPC elevation serves to protect the CNS from subsequent toxicity during consolidation. This potential protective effect of LPC may account for the positive outcomes in the current study, which are consistent with the reported findings of lower risk for dementia in an elderly cohort. [20]

Evaluated collectively, these results suggest early CNS biochemical and neurocognitive changes in children undergoing treatment for ALL. Our previous research demonstrated that early changes in motor speed are related to more complex neurocognitive functions after therapy completion. [8] We recently demonstrated that cognitive stimulation during active chemotherapy can prevent declines in working memory skills until at least one year off therapy. [39] The current findings support the need for this early intervention.

This study is not without limitations. Chief among these is the lack of prospective brain imaging data on the current cohort. Such imaging is not a standard of care in childhood ALL, and is a costly and time intensive process. Still, the strong association between two indices of white matter integrity, motor speed and SM, should be confirmed by additional linkage to MRI evidence of leukoencephalopathy. Brain imaging would also assist in determining whether any of the current results were associated with cerebral hemorrhage, seizures of PRES during the course of chemotherapy, though neurologic symptoms consistent with these conditions were not reported by the clinical team. An additional limitation is the retrospective association between CSF samples and CNS chemotherapy. To reduce unnecessary spinal taps, CSF analysis samples are collected immediately prior to intrathecal injection of chemotherapy agents. This requires collected samples to be linked to previous intrathecal injections. Ideally, additional samples collected shortly following treatment would better reflect changes in phospholipids associated with chemotherapy. However, to prevent sample contamination with chemotherapy agents, this would require additional spinal taps, that are not therapeutically warranted. A final limitation is the duration of the follow-up interval. Neurocognitive deficits in survivors of ALL continue to emerge over time, and become more significant as survivor enter long-term follow-up (i.e. 5 years post-diagnosis). [2] Although such follow-up was not feasible during the course of the current NIH-funded study, ongoing efforts include further examination of CSF samples in relation to long-term neurocognitive outcomes and brain imaging.

The current results have implications for follow-up. The connection between SM and leukoencephalopathy should be confirmed with brain imaging studies, particularly diffusion tensor imaging of white matter integrity. If the observed changes in SM precede the onset of leukoencephalopathy, direct and initial impact on myelin integrity associated with an impact on processing speed would be confirmed. This would support the need for early preventative intervention strategies. The potential neuroprotective effects of LPC should also be confirmed and further explored, as it may eventually provide alternative prevention strategies. Again, brain imaging studies would assist in this goal. Related to both previous points, the current results demonstrate individual variability in changes in phospholipids as well as neurocognitive function. This variability in outcomes should be explored through examination of associations with genetic polymorphisms, which may mediate the impact of chemotherapy and/or identify predispositions for such responses.

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Figure 1.

Typical high-performance liquid chromatography pattern showing separation of phospholipid classes in volumetric ratios present in CNS cell membranes. The major phospholipid classes include: phosphatidylethanolamine (PE, retention time=20.37 min), phosphatidylinositol (PI, retention time=21.37 min), phosphatidylcholine (PC, retention time=41.18 and 41.57 min), sphingomyelin (SM, retention time=42.20 and 43.32 min), and lysophosphatidylcholine (LPC, retention time=49.55 min).

Figure 2.

Age-adjusted mean motor speed performance from Baseline to Year 3 follow-up for patients demonstrating post-inductions concentrations of sphingomyelin (SM) and lysophosphatidylcholine (LPC) that fall above (Hi) and below (Lo) the median values. The mean expected performance for the typical child is 100.

Figure 3.

Age-adjusted mean verbal working memory performance from Baseline to Year 3 follow-up for patients demonstrating post-inductions concentrations of sphingomyelin (SM) and lysophosphatidylcholine (LPC) that fall above (Hi) and below (Lo) the median values. The mean expected performance for the typical child is 100.

Table 1

Demographic and Treatment Characteristics

Demographic Characteristics	Mean	SD
Age @ Diagnosis	7.0	3.11
Sex	N	%
Female	45	59.2
Male	31	40.8
Grade @ study recruitment	2.9	2.82
Mother's Education (yrs)	13.5	2.57
Father's Education (yrs)	13.5	2.93
Household Income (\$)	57,143	41,519
Treatment Characteristics		
Risk Stratification	N	%
Low	30	39.5
Standard	38	50.0
High	8	10.5
IV MTX treatment arm	N	%
100mg/m ²	4	5.3
1gm/m ²	51	67.1
2gm/m ²	21	27.6

Table 2

Cerebral Spinal Fluid Phospholipids over time

CSF Phospholipids	Diagnosis		Induction		Consolidation		Continuation 1		Continuation 2	
	Mean (SD)	p	Mean (SD)	p	Mean (SD)	p	Mean (SD)	p	Mean (SD)	p
Sphingomyelin	0.47 (1.57)	0.03	0.69 (1.19)	0.03	0.54 (1.44)	0.04	0.55 (1.04)	0.09	0.48 (0.73)	0.03
Lysophosphatidylcholine	23.23 (22.51)	0.003	39.15 (61.50)	0.003	24.66 (39.38)	0.60	18.94 (30.77)	0.27	20.24 (29.74)	0.37

Note: Due to non-normal distribution of CSF phospholipids, Wilcoxon Signed Ranks test were conducted to examine change in respective phospholipid value from diagnosis to each subsequent phase of chemotherapy treatment.

Table 3

Age-Adjusted Neurocognitive Performance over Time

	Baseline	Y1		Y2		Y3	
	Mean (SD)	p [/]	Mean (SD)	p [/]	Mean (SD)	p [/]	p [/]
Full Scale IQ	100.1 (14.91)	0.94	100.6 (15.12)	0.73	101.9 (12.01)	0.32	100.6 (11.07)
Academics							
Reading	98.6 (15.90)	0.44	102.3 (14.80)	0.19	107.7 (18.50)	0.01	104.4 (12.08)
Calculation	100.7 (17.54)	0.79	99.5 (17.31)	0.83	100.8 (17.33)	0.79	96.6 (16.19)
Applied Mathematics	103.6 (15.60)	0.04	105.9 (14.20)	0.001	107.8 (13.21)	<0.001	108.9 (15.25)
Spelling	94.9 (15.09)	0.003	95.6 (15.20)	0.02	97.4 (15.10)	0.28	94.6 (14.83)
Working Memory							
Verbal	92.2 (15.18)	< 0.001	94.4 (11.02)	< 0.001	93.9 (10.64)	0.001	94.3 (11.32)
Visual	95.9 (18.62)	0.05	95.5 (14.83)	0.01	95.7 (14.34)	0.06	93.3 (14.65)
Processing Speed							
Coding	100.7 (19.25)	0.82	103.9 (16.14)	0.09	103.4 (16.78)	0.25	100.0 (14.55)
Symbol Search	108.7 (17.24)	0.001	110.9 (15.99)	<0.001	111.3 (16.3)	<0.001	107.9 (15.77)
Visual-Motor							
Motor Speed	88.2 (22.54)	< 0.001	91.6 (17.54)	< 0.001	93.4 (17.24)	0.02	89.1 (15.50)
Visual Motor Integration	98.5 (13.23)	0.32	95.6 (15.20)	0.006	95.5 (13.85)	0.05	89.6 (8.06)
							< 0.001

[/] p value for one sample t-tests comparing patient performance to population norms.

Bold values represent scores that are significantly below the average values for the standardization sample.