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DETERMINATION OF PHOSPHOLIPIDS AS BIOMARKERS OF BRAIN TISSUE DAMAGE IN ACUTE LYMPHOBLASTIC LEUKEMIA PATIENTS

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The application of a high performance liquid chromatography method was described for determination of phospholipid levels in the cerebrospinal fluid of children suffering with acute lymphoblastic leukemia (ALL). The levels of two major membrane phospholipids (phosphatidylcholine and sphingomyelin) were monitored during the course of therapy and compared among three different therapeutic protocols. The monitored levels were also correlated with the results of cognitive testing and assessed as biomarkers of cellular damage associated with the ALL treatment.

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Introduction

Clinical chemistry is one of the most dynamic fields of laboratory science [1]. Over the past decades, the character of this science has changed dramatically from applications of routine manual techniques to a highly sophisticated and equipment intensive discipline. The analytical chemist working in the field of clinical chemistry must be familiar with the preparation and analysis of the specimen, operation and maintenance of the analytical equipment, interpretation of results and finally, be able to make a correlation between the analytical results and the pathological condition(s). Monitoring of different bodily components during disease can help in determining the diagnosis, extent of disease and prognosis of the condition.

Over the past four decades the outlook for children with acute lymphoblastic leukemia (ALL) has changed dramatically, reaching a five-year disease-free survival rate that is now almost 80 % [2]. This improvement is mainly due to recent advances in knowledge about the biology of ALL and to aggressive treatment of the disease. ALL is a malignant disease characterized by unregulated proliferation of lymphoblasts, which are immature lymphocytes. The consequences of the stimulated overgrowth of blast cells are suppression of mature white cells, red cells, and platelets resulting in increased susceptibility to infection, anemia and bleeding [3]. Central nervous system (CNS) involvement is rare at presentation [4]; but it is observed in 75 to 80 % of children who do not receive CNS treatment.

Standard therapy for ALL treatment consists of induction, CNS prophylaxis, consolidation, and maintenance. Induction is the initial phase of ALL treatment. The goal of induction is to rapidly reduce the number of lymphoblasts in the peripheral circulation and bone marrow. Remission is defined as less than 5% lymphoblasts in the bone marrow and is obtained in about 90 % of children. The rate of initial blast-cell kill in blood and bone marrow is a highly significant prognostic indicator for long-term survival [5]. Failure to achieve remission by days 28 – 30 is an indicator of poor survival.

CNS prophylaxis is required to overcome the blood brain barrier and to achieve therapeutic doses of chemotherapy in the brain as this is a sanctuary site for lymphoblasts. CNS treatment usually consists of direct injection (intrathecal administration) of chemotherapeutic agents into the cerebrospinal fluid (CSF). CNS prophylaxis with chemotherapy involves either triple agent chemotherapy with Methotrexate (MTX), Cytosine-Arabinoside (ARA-C) and hydrocortisone (Triple Intrathecal Therapy-TIT) or single agent chemotherapy with MTX (Single Intrathecal Therapy-SIT). Whole brain radiation (RTX) is usually used for CNS disease relapse and is administered in doses ranging from 1800 to 2400 centigray (cGy).

Consolidation therapy is designed to prevent the emergence of new clones of leukemia cells. This phase of therapy typically lasts about 6 months and

involves intermediate to high dose systemic chemotherapy, primarily MTX. At the conclusion of consolidation, cycles of maintenance therapy including vincristine, prednisone, 6-mercaptopurine and MTX are continued 2 to 3 years.

As the number of ALL survivors has increased, the relationship between the damaging effect of cancer therapy (IT chemotherapy, RTX, and intermediate- to high-dose systemic chemotherapy) and the resulting neurocognitive impairments have become more obvious. The major damage is thought to be the disruption of cell membranes of different cell populations. There are several mechanisms for this injury, which may contribute to the tissue damage during and after treatment. Disturbances in cell membranes result in increased permeability and disruption of active transport mechanisms. MTX can impact development of white matter which is an important component of brain development in childhood [6,7]. Intrathecal administration of MTX can interfere with formation of myelin by inhibiting the synthesis of membrane phospholipids [8,9]. ALL treatment can adversely impact general intelligence, visual spatial skills, verbal fluency, academic achievement and memory [10–12].

Whole brain RTX is known to activate the enzymes phospholipase A_2 and C. These enzymes hydrolyze membrane phospholipids forming a pool of free arachidonic acid and other polyunsaturated free fatty acids [13,14]. Once released from membrane phospholipids, arachidonic acid is readily converted to prostaglandins, leukotrienes and oxygen radicals. Another source of the formation of radicals in irradiated tissue is water. After exposure to radiation, the water molecule undergoes heterolytic bond cleavage, leaving an unpaired electron on both the hydrogen and hydroxide [15], as illustrated below.

$$H_2O \xrightarrow{RTX} H\cdot \text{ (hydrogen radical)} + \cdot OH \text{ (hydroxyl radical)}$$

The reactive hydroxyl radical can attack almost all molecules present in human cells, initiating free-radical chain reactions. These reactions include formation of lipid peroxides and radicals. Since antioxidant systems in the brain are not as extensive as in other tissues, damage is significant. Finally, brain cell membranes are very rich in unsaturated fatty acids, which contributes to the extent of the damage [15].

Phospholipids are major components of human cell membranes. Phospholipid levels in CSF increase as a result of disruption of cell membranes. Thus, changes in the levels of the specific phospholipids can provide a tool for measuring the extent of brain damage following treatment with chemotherapy and/or RTX. We have measured the levels of two major phospholipids: phosphatidylcholine (PC), which comprises 61 %, and sphingomyelin (SM), which comprises 24 % of overall phospholipid CSF concentration [16]. In order to

examine the concentration of phospholipid levels in CSF during ALL treatment, the method developed by Folch *et al.* [17] was used. This method involves a chloroform:methanol (2 : 1 v : v) extraction of CSF and achieves recovery rates of 98 % for PC and 89 % for SM. High performance liquid chromatography (HPLC) is the method of choice for separation and quantification of different phospholipid classes. Various HPLC methods for phospholipid analysis were reviewed by Christie [18] and McCluer [19]. In order to achieve a satisfactory separation of phospholipids with diverse polarities, a suitable column and solvent system must be chosen. In normal-phase HPLC the mobile phase is usually non-polar, such as hexane:isopropanol, with the stationary phase being polar (silica gel). The phospholipid classes are then eluted in the order of increasing polarity [20]. Separation of a complex mixture of lipids requires the use of a gradient to achieve optimal separation and resolution. The method developed by Chen *et al.* [21] has been modified for our project and allows separation of all major phospholipid classes. During the HPLC separation, co-elution of some components from the complex biological matrix can occur making the assignment of the individual peaks very difficult. Positive identification of the peaks representing the different classes and species of phospholipids must be confirmed by mass spectrometry.

Experimental

Study Sample. Patients were divided into three groups, depending on the type of therapy received: *Group 1* was comprised of patients who received CNS treatment with TIT. This group consisted of 8 female and 8 male patients with ages at diagnosis ranging from 20 to 206 months. The average age at diagnosis was 88 months. *Group 2* included patients who received SIT CNS prophylaxis. This group included 7 females and 8 males with ages at diagnosis ranging from 27 to 136 months. The average age at diagnosis was 61 months. *Group 3* included patients who were at high risk for disease relapse or had leukemia cells present in the CSF. These patients received either 2400 cGy or 1800 cGy of whole brain radiation administered with a megavoltage linear accelerator. A total of 7 patients were included in this group. There were 3 females and 4 males, their average age at the time of diagnosis being 78 months.

We monitored the levels of PC and SM during the course of treatment and evaluated cognitive and academic performance 9, 24 and 36 months after ALL diagnosis. Measures of cognitive function included: general intelligence, academic achievement (reading, spelling and math), visual motor integration, and verbal fluency.

Analytical Methods

Sample preparation. CSF was obtained during routine lumbar punctures at ALL diagnosis, during evaluation of CNS disease status or for administration of IT chemotherapy. Samples were frozen at - 80 °C prior to extraction. After thawing, 1 ml of CSF was extracted with 4 ml of methanol : chloroform (1 : 2), the extract was centrifuged at 10,000 rpm for 20 minutes, and the organic lower layer collected and stored on ice. The aqueous layer was re-extracted with a second portion of methanol:chloroform (1 : 9). The organic fractions were combined and evaporated to dryness under nitrogen. The residue was resuspended in 100 µl hexane:propan-2-ol : water (39 : 59 : 9) prior to injection into the HPLC system.
HPLC separation. Lipid extracts were separated using a Model 338 Gold HPLC System (Beckman, San Ramon, Ca, USA) using a silica gel column (Ultrasphere Si-5 microns, 25cm × 4.6mm i.d.) with the following mobile phase gradient

Time, min	Flow, ml min^{-1}	Solvent A, %	Solvent B, %
0	1	100	0
5	1	100	0
35	1	0	100
70	1	0	100
75	1	100	0

Solvent A composition : hexane:propan-2-ol (50 : 50)
Solvent B composition : hexane:propan-2-ol:water (39 : 52 :9)

The elution of components was monitored with a UV detector at 206nm, with response at 0.4 absorption units full scale. The baseline declined to negative absorption values because of the increase in the concentration of water in the mobile phase. This is due to the lower UV absorption of water relative to hexane : propan-2-ol. Calibration curves using known amounts of reference standards (Sigma, St. Louis, MO, USA) were used to determine concentrations of PC and SM (Fig. 1) and dissolved in mobile phase A in order to achieve required concentrations. All of the measured concentrations of both PC and SM were in the range defined by calibration curves; thus, we have not observed any measured value, which would provide a negative concentration. The fact that the calibration curve is not passing the zero point is contributed to the systematic error of the employed instrumentation.
Mass spectrometry. Mass spectrometry was used to confirm phospholipid class and to characterize specific molecular species within PC and SM classes. HPLC fractions were collected from the beginning to the end of the chromatographic

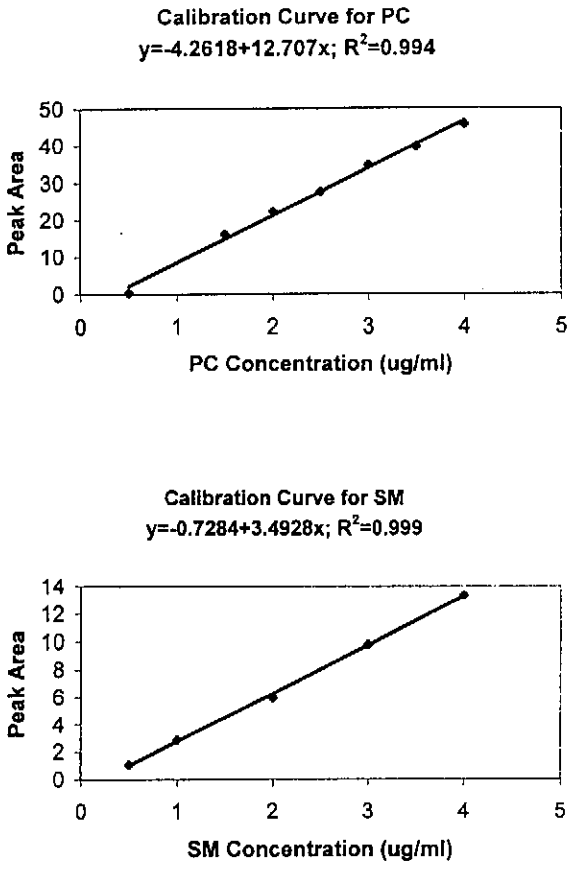


Fig. 1 Calibration curves for PC (top) and SM (bottom)

peak, taken to dryness using a SAVANT evaporating system (Savant Instruments, Inc., Farmingdale, NY, USA) and dissolved in 10 µl methanol. The electrospray (ESI) mass spectral analysis was performed using the PE Sciex (Perkin Elmer, Wellesley, MA, USA) with orifice voltage 50 – 80V. The interface temperature was maintained at 52 °C. The mass range was scanned from m/z 100 – 2000 Daltons.

Results and Discussion

A typical chromatogram of the methanol:chloroform extract of CSF is shown in Fig. 2. All major membrane phospholipid classes are identified: phosphatidylethanolamine (PE, RT = 25.01min), phosphatidylinositol (PI, RT = 27.67min), phosphatidylcholine (PC doublet, RT=39.12min and 39.43min), sphingomyelin (SM,

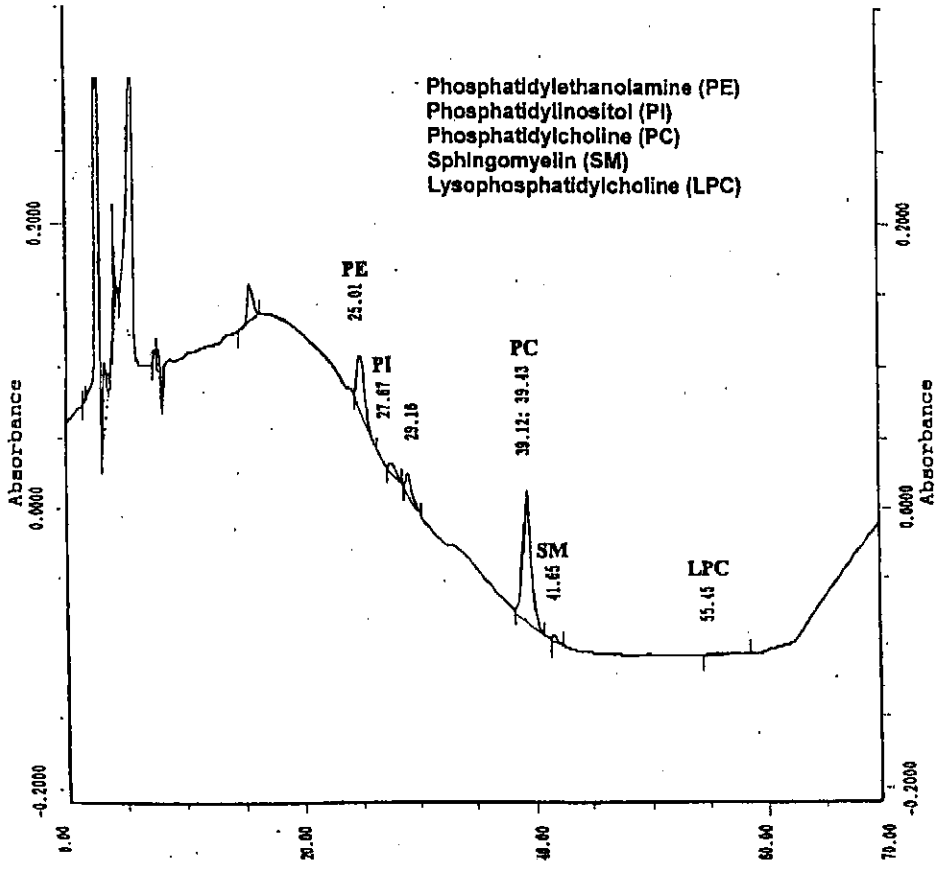


Fig. 2 A normal-phase HPLC chromatogram of a CSF phospholipid extract from a patient suffering with acute lymphoblastic leukemia at the start of the therapy. The major phospholipid classes identified are: phosphatidylethanolamine (PE, RT = 25.01 min), phosphatidylinositol (PI, RT = 27.67 min), phosphatidylcholine (PC doublet, RT = 39.12 min and 39.43 min), sphingomyelin (SM, RT = 41.65 min), and lysophosphatidylcholine (LPC, RT = 55.45 min)

RT = 41.65 min), and lysophosphatidylcholine (LPC, RT = 55.45 min). The changes in concentrations of CSF PC and SM during ALL treatment are summarized in Fig. 3 and are discussed below by CNS treatment group. The presence of PC and SM in the collected fractions during the CSF lipid separation was confirmed by electrospray mass spectral analysis. Figure 4 shows the electrospray mass spectrum of a collected PC and SM fraction during the HPLC analysis from an ALL patient during the course of treatment. PC with fatty acid side chains C16 : 0 (16 identifies the number of carbons and 0 indicates absence of double bonds) and C18 : 1; and SM with fatty acid side chains C16 : 0 and

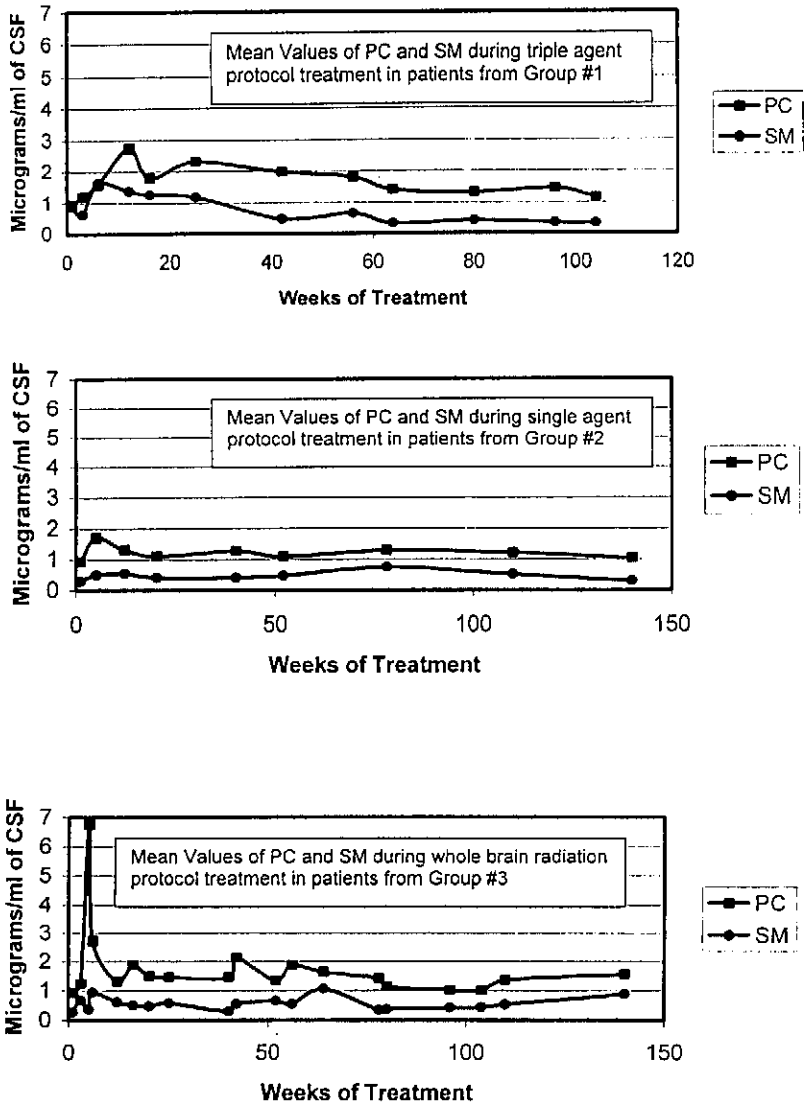


Fig. 3 Mean values of PC and SM in the CSF during the course of treatment of ALL patients who received triple agent intrathecal therapy (Group 1); patients who received single agent intrathecal therapy (Group 2); and patients who received whole brain radiation (Group 3)

C16 : 1 were identified as major phospholipids present in this fraction.

Group 1. CSF PC levels were monitored in all the patients and analyzed by weeks of ALL treatment. Data points that were available on the majority of subjects were included in the statistical analysis. These weeks included: 1, 3, 6, 12, 16, 25, 42, 56,

Table I Mean values and standard deviation of PC and SM during the course of treatment of Group 1 (top), Group 2 (middle) and Group 3 (bottom).

Week	PC Mean	Std. Dev. PC	SM Mean	Std. Dev. SM
1	0.954	0.197	0.860	1.130
3	1.198	2.270	0.630	0.700
6	1.580	0.820	1.630	2.030
12	2.760	2.370	1.380	1.620
16	1.800	1.740	1.260	1.310
25	2.320	2.160	1.190	1.430
42	1.980	1.700	0.480	0.360
56	1.820	1.090	0.660	0.540
64	1.410	0.750	0.330	0.090
80	1.330	0.860	0.410	0.280
96	1.460	1.060	0.340	0.180
104	1.160	0.440	0.330	0.090

Week	PC Mean	Std. Dev. PC	SM Mean	Std. Dev. SM
1	0.920	0.078	0.280	0.110
5	1.700	1.060	0.500	0.290
12	1.310	0.260	0.540	0.230
20	1.100	0.320	0.410	0.160
40	1.280	0.300	0.410	0.190
52	1.090	0.350	0.470	0.250
78	1.300	0.350	0.750	0.860
110	1.220	0.430	0.520	0.340
140	1.050	0.350	0.290	0.030

Table I – continued

Week	PC Mean	Std. Dev. PC	SM Mean	Std. Dev. SM
1	0.925	0.092	0.263	0.037
3	1.243	0.180	0.674	0.047
5	6.770	undefined	0.360	undefined
6	2.733	3.092	0.937	0.851
12	1.309	0.382	0.606	0.456
16	1.878	1.001	0.498	0.004
20	1.505	0.389	0.466	undefined
25	1.463	0.286	0.572	0.307
40	1.460	0.333	0.297	0.054
42	2.130	0.884	0.548	0.363
52	1.348	0.230	0.651	0.188
56	1.885	0.233	0.541	0.452
64	1.646	0.537	1.071	1.734
78	1.425	0.189	0.340	0.079
80	1.150	0.134	0.372	0.933
96	1.014	0.136	0.424	0.162
104	1.000	0.256	0.432	0.260
110	1.363	0.328	0.529	0.418
140	1.570	0.083	0.872	0.845

64, 80, 96 and 104. Mean PC and SM concentrations, including standard deviation values, from patients in this group are summarized in the top portion of Table I. The mean value of PC ranged between 0.954 $\mu g\ ml^{-1}$ to 2.76 $\mu g\ ml^{-1}$; mean SM values ranged from 0.33 $\mu g\ ml^{-1}$ to 1.38 $\mu g\ ml^{-1}$ of CSF.

Correlational analyses were used to determine the relationship between the maximum value of PC and a particular week of treatment. Criteria for significance included correlation coefficients > 0.8 and level of statistical significance < 0.05. The results of these statistical evaluations are shown in Table II. The early interval includes weeks 1 – 16 and the highest level of PC in this time period is marked as PCMXER. The medium interval of treatment follows from weeks 17 – 79 and the highest level of PC in this interval is marked as PCMXMD. The late period of therapy consists of weeks 80 – 104 and the highest level of PC in this period is

Table II Results from correlation statistical analysis of PC and SM levels and the weeks of treatment in the CSF of patients from Group 1.

Subject	Correlation coefficient	Significance	Number of cases
PCMXER × week 12	0.9956	0.000	12
PCMXMD × week 25	0.9255	0.000	14
PCMXLT × week 96	0.9401	0.000	15
PCMAX × week 12	0.9644	0.000	12
SMMXER × week 6	0.9541	0.000	9
SMMXMD × week 25	0.9498	0.000	8
SMMXLT × week 80	0.9651	0.000	10
SMMAX × week 6	0.8295	0.003	9

marked as PCMXLT. The overall maximum value of PC is marked as PCMAX.

Concentrations of CSF SM were also evaluated. As anticipated, the concentrations of SM were lower than PC. SM was not detected in all samples; therefore, only CSF samples from 10 patients were available for analysis of SM concentrations. The time intervals for SM were assigned in the same manner as in the PC measurements and are designed SMMXER, SMMXMD, SMMXLT and SMMAX.

PC reached an overall maximum level in week 12 in all 16 patients. The maximum values for PC were correlated as follows: for the early interval of therapy the overall maximum PC level occurred during week 12, which is identical with the overall maximum value of PC. In the middle interval of treatment, the specific maximum value of PC was observed during the 25th week. During the last period of therapy the individual PC maximum was observed in week 96. Therefore, it is not surprising that the correlation coefficients were very high.

SM levels were evaluated using the same approach. The overall maximum level of SM was observed in the 6th week in 9 of 16 patients (the correlation coefficient was 0.9541). The individual maximum concentrations of SM in the early period occurred in the 6th week, in the middle time interval the highest SM concentration was observed during week 25, and the maximum SM value during the late component of therapy was found in week 80. Correlation coefficients ranged from 0.83 to 0.99, indicating a similar response to therapy across the entire group of patients. The early stage of treatment (week 1 – 16) is characterized by the highest CSF levels of both PC and SM, thus, indicating significant cellular damage in the CNS. Elevated SM levels in CSF are associated with disruption of the myelin sheath, which could adversely effect impulse propagation. The initial

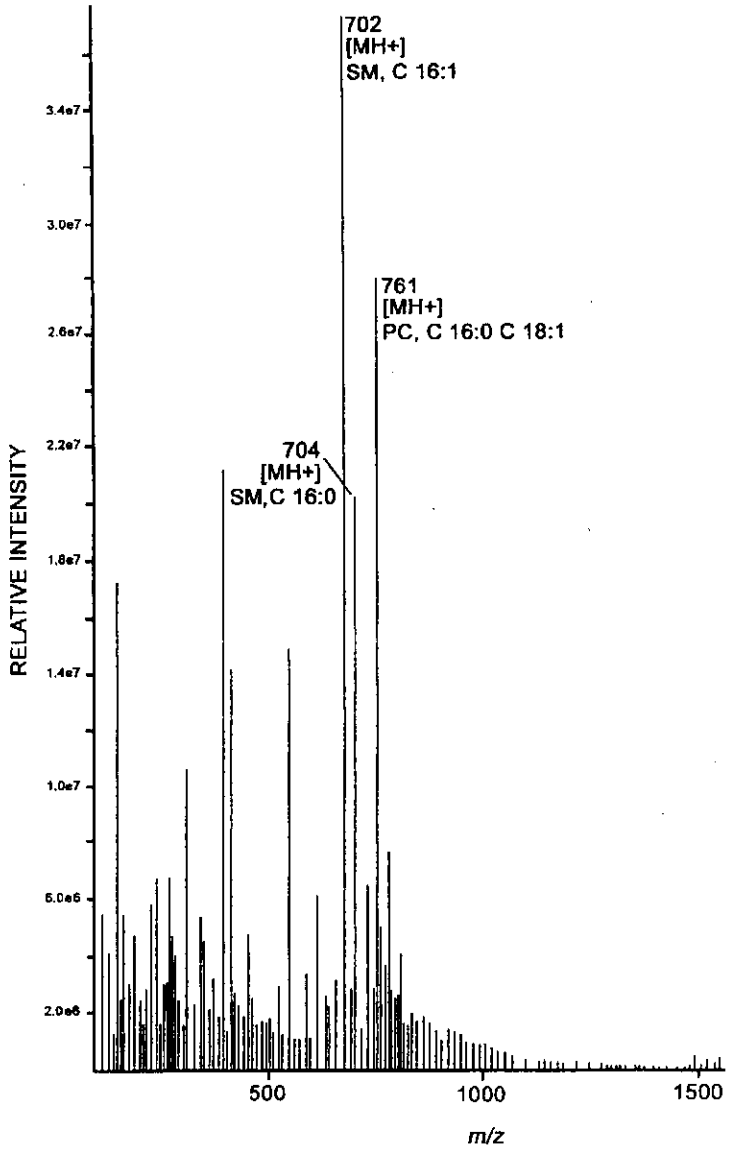


Fig. 4 Electrospray mass spectra of PC and SM fraction collected from an ALL patient during the course of treatment

phase of treatment appears to contribute to chemotherapy-induced injury to the CNS.

Group 2. The profiles of the PC and SM levels during SIT treatment are presented in Fig. 3. PC levels were monitored in all 15 patients, while SM levels were

measured in only 13 patients, as explained previously. Statistical analyses were completed on data points that were available from the majority of subjects. The common weeks of CSF sample collection are determined by the ALL treatment protocol. Therefore the weeks of data collection from subjects in Group 2 are not the same as those from Group 1 subjects. However, due to their close proximity, a comparison can be made between Groups 1 and 2. The weeks of interest were as follows: 1, 5 – 8, 12 – 15, 20 – 24, 40 – 44, 52 – 56, 78 – 82, 110 and 140. The early interval of treatment consists of weeks 1 – 15, with the highest value of PC being PCMXER. The medium interval of treatment includes weeks 16 – 77 and the maximum value of PC is designated as PCMXMD. The last interval of therapy includes weeks 78 – 140 and the maximum value of PC is designated as PCLTMX. The overall maximum value of PC is designated PCMAX. Concentrations of SM were measured during the same time intervals and assigned in the same manner as PC: SMMXER, SMMXMD, SMMXLT and SMMAX. Mean CSF PC and SM concentrations, including standard deviations, from Group 2 subjects are summarized in the middle section of Table I. The mean values of PC ranged between 0.92 $\mu g\,ml^{-1}$ to 1.70 $\mu g\,ml^{-1}$, while the mean values of SM ranged between 0.28 $\mu g\,ml^{-1}$ to 0.75 $\mu g\,ml^{-1}$.

Correlational analyses were also used to determine the relationship between the maximum values of PC and SM with a particular week of treatment. The criteria for significance used in Group 1 were also used in Group 2. Results of the correlations are summarized in Table III. The overall maximum value of PC was observed in the 5th week in 12 patients (the correlation coefficient was 0.9922). The maximum PC value in the early period was reached in week 5, which is identical with the overall maximum value. The highest PC value in the middle interval of treatment was found in week 40, and the maximum value of PC in the late portion of therapy occurred in week 110. In 8 of 15 patients, the overall maximum value of SM was found in week 78. The maximum values for SM in the early, middle and late intervals of therapy occurred in weeks 12, 20 and 78, respectively. Correlation coefficients ranged from 0.9006 to 0.9922.

There was a major difference in the maximum values of PC and SM between Groups 1 (TIT) and 2 (SIT). The maximum values of PC during the initial treatment phase appear approximately 7 weeks earlier in Group 2. This could be due to differences in the induction phase of ALL treatment. The increase in CSF PC found in week 12 in Group 1 may be due to the co-administration of intermediate-dose systemic MTX.

Group 3. CSF was obtained from Group 3 subjects at weeks that were similar to Groups 1 and 2. All points of interest were included for Group 3. Radiation schedules for these subjects were determined by specific treatment protocols, therefore, correlation coefficients were not computed because results could be misleading. The profiles of the PC and SM levels in ALL patients during RTX treatment are presented in Fig. 3. Dramatic increases in the concentrations of both

Table III Results from correlation statistical analysis of PC and SM levels and the weeks of treatment in the CSF of patients from Group 2.

Subject	Correlation coefficient	Significance	Number of cases
PCMXER × week 5	0.9922	0.000	12
PCMXMD × week 40	0.9766	0.000	13
PCMXLT × week 110	0.9006	0.003	7
PCMAX × week 5	0.9922	0.000	12
SMMXER × week 12	0.924	0.000	10
SMMXMD × week 20	0.8581	0.000	12
SMMXLT × week 78	0.9396	0.000	8
SMMAX × week 78	0.9382	0.000	8

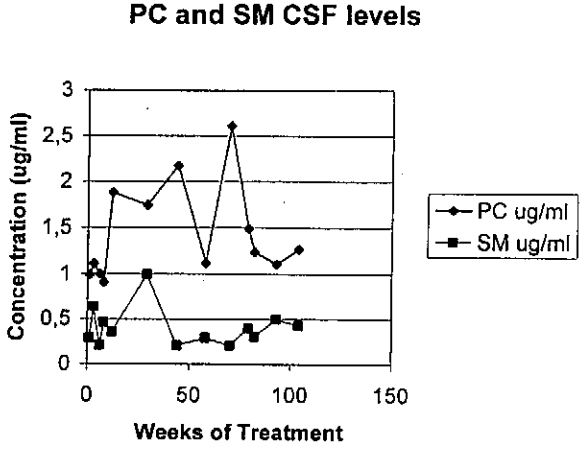


Fig. 5 PC and SM levels during the course of treatment from an ALL patient receiving whole brain radiation therapy. The radiation treatment was administered during weeks 10 and 15

PC and SM were found immediately following radiation therapy in all patients. In most cases, the increase in PC and SM was greater than that observed in patients belonging to Groups 1 and 2. An example of PC and SM levels in the CSF of a radiation patient is presented in Fig. 5. The mean PC values ranged from 0.76 $\mu g\ ml^{-1}$ to 8.24 $\mu g\ ml^{-1}$; the mean SM levels ranged from 0.126 $\mu g\ ml^{-1}$ to 4.171

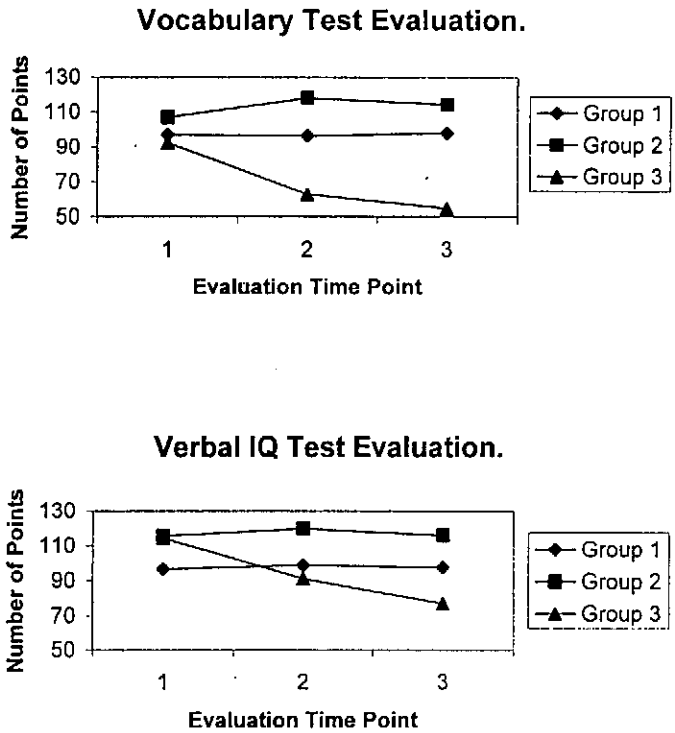


Fig. 6 Results of cognitive test evaluation (Vocabulary Test and Verbal Intelligence Quotient Test) from T1 = 3 months, T2 = 24 months and T3 = 36 months post CNS treatment

$\mu g\,ml^{-1}$. These findings suggest that RTX may be more damaging to cell membranes than is chemotherapy, and that RTX may initiate more than one mechanism of membrane injury. As discussed previously, radiation activates phospholipases A_2 and C, which are responsible for phospholipid hydrolysis. Phospholipid hydrolysis can further disrupt the integrity of cell membranes. Furthermore, the formation of free radicals contributes to brain cell injury, which could be explained by the observed increases in the concentrations of free phospholipids in the CSF. It is important to note that for all Groups of subjects there was considerable variation in the concentrations of CSF PC and SM. This is reflected in the large standard deviations associated with the mean values (Table I).

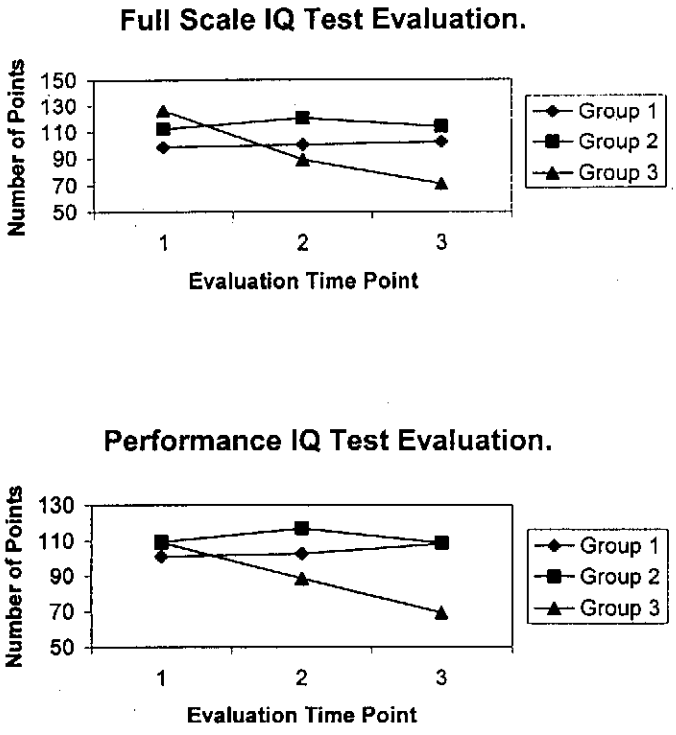
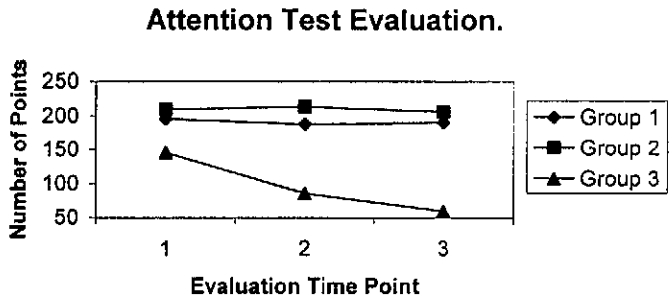


Fig. 7 Results of a cognitive test evaluation (Full Scale Intelligence Quotient Test and Performance Intelligence Quotient Test) from T1 = 3 months, T2 = 24 months and T3 = 36 months post CNS treatment

Cognitive Evaluation

Cognitive and academic performance of subjects in Groups 1, 2 and 3 was also evaluated. Subjects were tested three times during therapy: 3, 24 and 36 months after ALL diagnosis. Figures 6 – 9 summarize the performance on cognitive and academic measures by evaluation time point and CNS treatment group.

Regardless of the specific cognitive measure, Group 3 subjects scored significantly lower than those in Groups 1 and 2 at 36 months after ALL diagnosis. Group 2 had the least decline in cognitive and academic performance, while Group 1 patients had a moderate decline in some areas. It is interesting to note that subjects in Group 3 who had the greatest decline in cognitive abilities had the greatest increase in the concentration of CSF PC and SM following radiation. Furthermore, patients in Group 1 who received TIT chemotherapy and intermediate dose systemic MTX had higher PC and SM concentrations that those



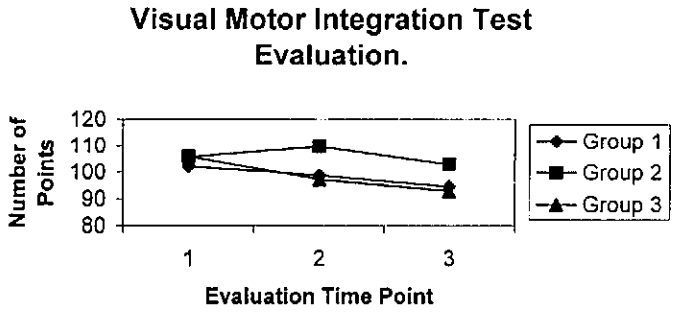


Fig. 8 Results of a cognitive test evaluation (Attention Test and Visual Motor Integration Test) from T1 = 3 months, T2 = 24 months and T3 = 36 months post CNS treatment

who received SIT. Group 1 patients also had a greater decline in cognitive abilities than did those in Group 2. Therefore, CSF concentrations of PC and SM may be good biological markers of overall brain tissue injury associated with ALL therapy.

Conclusion

In summary, we have used HPLC for measuring PC and SM concentrations in CSF obtained from children undergoing three different CNS treatment modalities for ALL. Increased levels of PC and SM were observed in all three Groups following initiation of ALL therapy. Children who received the most intense CNS treatment (whole brain RTX) had the greatest increase in CSF phospholipids and the greatest decline in cognitive and academic performance. Similarly, less intense CNS treatment (SIT) was associated with lower levels of CSF phospholipids and a less dramatic decline in cognitive and academic performance. Current studies involve

measuring changes in the fatty acid composition of specific phospholipids and the contribution of oxidative processes to CNS injury.

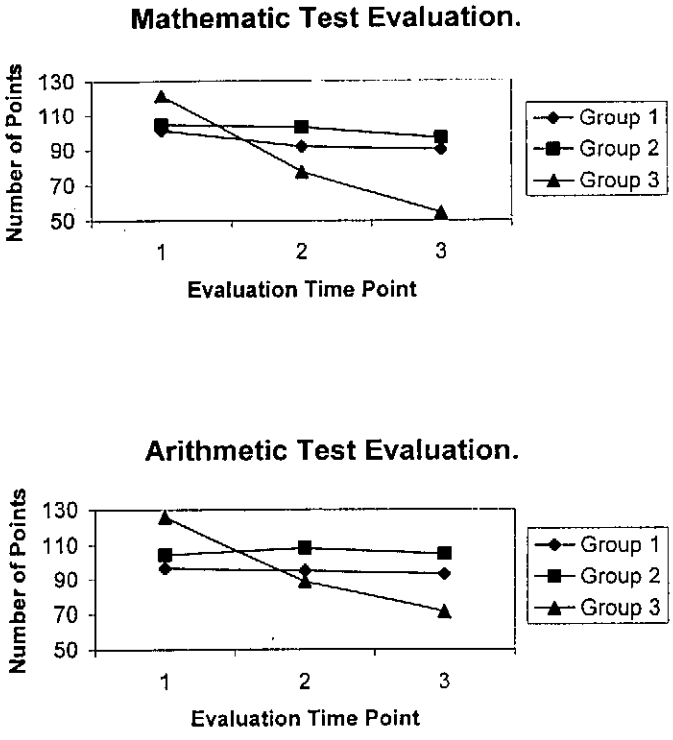


Fig. 9 Results of a cognitive test evaluation (Mathematic Test and Arithmetic Test) from T1 = 3 months, T2 = 24 months and T3 = 36 months post CNS treatment

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