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APPLICATIONS OF ANALYTICAL TECHNIQUES TO CLINICAL PRACTICE: ASSESSMENT OF CHEMICAL CHANGES IN PATIENTS WITH ACUTE LYMPHOBLASTIC LEUKEMIA FOLLOWING METHOTREXATE ADMINISTRATION

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The application of the following methods was described for the purpose of exploration of different cerebrospinal fluid biomarkers related to the cognitive impairment in children suffering from acute lymphoblastic leukemia and receiving methotrexate as part of the treatment regimen: gas chromatography/mass spectrometry was employed to measure the levels of homovanillic acid and

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vanilmandelic acid; twodimensional electrophoresis was used to find the changes in protein profiles present in cererebrospinal fluid; and high performance liquid chromatography was utilized to measure the levels of cerebrospinal fluid ceramides. The overall goal of this project was to utilize different analytical techniques in order to monitor chemical changes in cerebrospinal fluid associated with the administered amount of methotrexate.

Introduction

Use of analytical chemistry in clinical setting represents an important contribution to the process of diagnosing, determining severity and following the course and outcome of the pathological condition [1]. An example of the analytical assessment of various chemical changes in the cerebrospinal (CSF) fluid of children suffering with acute lymphoblastic leukemia (ALL) and their correlation with functional outcomes during the course of the treatment is described below.

Acute lymphoblastic leukemia is a malignant disease characterized by an unregulated proliferation of lymphoblasts, which are immature limphoid cells with abnormal morphology. In the pediatric population, ALL is the most common type of malignancy, affecting approximately 2400 children in the US alone [1]. The consequences of the overgrowth of blast cells are suppression of mature white cells, red cells, platelets with an increased susceptibility to infection, anemia, bleeding, and enlargement of the spleen, liver and lymph nodes. Over the past four decades the prognosis of children with ALL has changed dramatically, reaching a five year disease free survival rate of 85 % [2,3]. This improvement is mainly due to recent advances in diagnosis and aggressive treatment of the disease. Central nervous system (CNS) involvement is rare at the time of presentation, but becomes a primary site of relapse in children with ALL [4]. Methotrexate (MTX) is used extensively as a prophylactic agent, either by itself or in combination with other chemotherapeutic agents (cytosine arabinoside and hydrocortisone).

A number of studies have shown that intrathecal chemotherapy and high dose systemic chemotherapy are associated with specific intellectual and academic deficits [5–10]. Recent findings from our laboratory showed that there were statistically significant declines in mathematics and verbal fluency scores in children with ALL undergoing chemotherapeutic treatment with MTX [11]. The results are summarized in Fig. 1. Intellectual and academic abilities were assessed using standardized measures 12 months and 54 months after ALL diagnosis. Declines in intellectual and academic function are thought to be due to treatment-related effects on non-malignant cells in the CNS; however, the specific mechanisms leading to these impairments are not well understood. The purpose of this study was to employ various analytical techniques in order to survey the levels of different biochemically significant molecules in CSF; i.e.

neurotransmitters, proteins and ceramides in patients suffering with ALL and being treated with MTX. The knowledge gained in these studies will broaden the understanding of the injury processes associated with MTX. We have previously published our findings on the levels of major phospholipids in patients undergoing ALL treatment [12]. These results showed that two major phospholipids, phosphatidylcholine and sphingomyelin, were reliable biomarkers for prediction of the extent of therapy-associated cognitive impairments in children undergoing methotrexate treatment. In order to gain more understanding of biochemical processes which might lead to cognitive impairment in ALL patients, we surveyed some key components of CSF to examine changes that occur during ALL therapy. This more comprehensive approach should lead to identification of multiple biomarkers of underlying injury processes leading to cognitive impairments. If such processes can be identified, intervention therapies can be designed to lessen the extent of the side effects of MTX. Three different applications of analytical techniques to analysis of biomolecules in the CSF of the MTX-treated pediatric patients are described and include the use of gas chromatography/mass spectrometry for determination of homovanillic and vanilmandelic acids, the use of two-dimensional electrophoresis for studying proteins and high performance liquid chromatography for measuring ceramide levels.

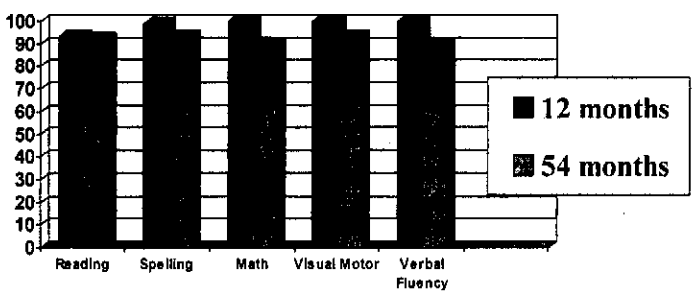
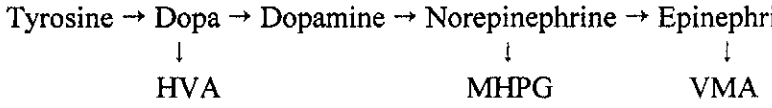


Fig.1 Mean achievement scores of intellectual and academic function in ALL children receiving methotrexate treatment in 12 and 54 moths post-diagnosis. Statistically significant declines in scores were observed in math and verbal fluency

1. Dopamine metabolite measurements by gas chromatography/mass spectrometry (GC/MS) following MTX administration: It has been shown that cerebral biogenic amine synthesis is affected by high doses of MTX [13]; and a significant decrease in homovanillic acid was observed in an ALL patient after receiving high-dose MTX. This observation suggests that MTX alters the monoamine metabolic pathway for dopamine. Homovanillic acid (HVA) is the final product of the monoamine metabolic pathway, and has been found to be the most consistent neurotransmitter-related biomarker in patients with different neurological disorders [14]. Furthermore, due to the acidic properties of homovanillic acid, its ability to equilibrate across the blood brain barrier is extremely limited, making

it an ideal biomarker for injury events originating in the CNS. Vanilmandelic acid (VMA) is the final product of metabolic conversion of norepinephrine and epinephrine. Previously published studies have shown that the pattern of monoamine metabolites is related to clinically important parameters and overall cognitive outcomes [14]. Major steps in catecholamine metabolism are outlined below [14].



Our hypothesis was that following the MTX treatment, the CSF levels of HVA could decrease and the CSF levels of VMA would increase in relation to the amount of methotrexate administered. Such findings would confirm that after MTX administration, the metabolic pathway described above will preferably shift to the right, thus, producing increased levels VMA, while decreasing the production of HVA. These shifts in the neurotransmitter levels likely result in deficiencies in the efficiency and speed of neurotransmission.

2. Profiling of protein molecules in the CSF during ALL treatment using two-dimensional electrophoresis: CSF is a clear aqueous fluid that surrounds the brain and spinal cord providing a unique and highly specialized environment for the complex functions of the CNS. Metabolic products and cofactors originating in the brain are secreted into the interstitial space and transported by bulk flow or net diffusion into the CSF. A protein survey is the most comprehensive approach to explore changes in cell functions in the brain after MTX administration because of physiological functions that are carried out by protein molecules including metabolic reactions (enzymes), transport vehicles and the defense response [15]. The use of protein biomarkers of specific neurological disease(s) has been successfully demonstrated for a variety of different neurological disorders.

In this project, we profiled CSF proteins during the course of intrathecal methotrexate (MTX) CNS treatment in order to define changes in protein patterns and levels of expression in response to the intrathecal MTX CNS chemotherapy. The protein profiling was accomplished by 2D electrophoresis separation. The protein profiles in CSF prior to intrathecal MTX treatment were compared with the protein profiles during the course of therapy. Our hypothesis was that the CSF protein profiles change as a result of MTX treatment, with some specific proteins serving as biomarkers for onset of injury leading to the learning deficiencies.

3. Measuring CSF ceramide levels in CSF using high performance liquid chromatography (HPLC): Ceramides assume a key role in sphingomyelin metabolism and the production of different biologically significant intermediates.

Ceramides are classified as neutral lipids and in the human body are produced during hydrolysis of sphingomyelin by neutral sphingomyelinase [16]. The cellular levels of ceramides were implicated in coordinating cellular responses to various forms of stress, including the exposure to chemotherapeutic agents such as MTX. Increased C16 ceramides have been shown to induce apoptosis, a programmed cell death [17]. In a recent study from our laboratory [18], we showed evidence of apoptosis in postmitotic endothelial cells after treatment with MTX. Following MTX administration to cultured bovine pulmonary artery endothelial cells, there was a significant decline in cell numbers after 1, 3 and 4 days of drug exposure. Fluorescent ApoAlert Enhanced Annexin-V binding demonstrated apoptosis, which was confirmed by a DNA fragmentation assay. These observations confirm that the process of apoptosis occurs after MTX treatment and suggests that brain endothelial cells might also be injured by intrathecal MTX. Based on the findings described above, we hypothesize that following MTX treatment there will be an increase in the CSF ceramide levels (in particular C16 ceramide) that will be responsible for the signaling of the initiation of the apoptosis in the injured CNS cells.

In summary: in this work, we used 1) gas chromatography/mass spectrometry to measure the levels of homovanillic acid and vanilylmandelic acid; both are metabolic products of major neurotransmitters, epinephrine and norepinephrine. Lower levels of the neurotransmitting molecules result in less efficient and slower transfer of nerve impulses, and therefore result in the cognitive impairments experienced by ALL patients; 2) two dimensional electrophoresis to profile the changes in protein profiles generated in CSF followed the administration of MTX in order to search for cell injury-specific protein biomarkers, which would have predictive value for the type and extent of the CNS injury. Such biomarkers would also provide new information about the mechanism(s) of injury to non-malignant cells in the CNS; and 3) high performance liquid chromatography to measure the levels of ceramides in CSF; ceramides act as signaling molecules for the initiation of apoptosis and represent a possible new mechanism of action of MTX on the CNS cells. With deeper understanding of events preceding cognitive impairments, new pharmaceutical interventions can be designed that would reduce the devastating impact of the MTX on cells in CNS tissue without compromising the efficiency of the chemotherapeutic properties. This would, in the end, improve the quality of life for the survivors of the ALL and allow for less traumatic consequences of the cancer treatment.

Experimental

1. Dopamine metabolites project

Study Sample: Three different patients participating in this study have been diagnosed with acute lymphoblastic leukemia. The type of prophylaxis is selected using patient's prognosis and risk classification. Patients with low risk receive the least amount of MTX, while patients with high risk protocol are administered the highest amount of MTX. Patients from following regiments were used in this study: POG 9904-low risk protocol, POG 9905-standard risk protocol, and POG 9406-high risk protocol. The mean age of subjects was 88.33 months (80, 74 and 111 months); two patients were males and one patient was female.

Analytical Methods

Extraction of neurotransmitters from CSF: Using an extraction procedure described previously [19], 1 ml of CSF sample was transferred to the extraction tube and 0.5 ml of hydrochloric acid was added. Next, 2.5 ml of ethyl acetate was added to each tube and the mixture was vortexed for 10 min at 3000 rpm. The top organic layer containing both HVA and VMA was collected into conical vials and the solvent was evaporated under a steady stream of nitrogen. Dry samples were derivatized with tetramethylsilylate (TMS) reagent and introduced to the CG/MS.

Gas chromatography/mass spectrometry: GC/MS analyses were performed using HP 5970 series mass selective detector in conjunction with HP 5890 gas chromatograph. The chromatographic separation was accomplished using fused silica capillary SPB-5 column (Supelco, Bellefonte, PA, USA): 30 m × 0.25 mm × 0.25 μm film thickness. The heating gradient was initiated at 120 °C and the temperature was increased by 10 °C min^{-1} to 250 °C. The temperature of the injector was 250 °C and detector 290 °C. The concentration of both HVA and VMA were determined using calibration curves constructed by analyzing known amounts of each purchased as standard from Sigma, St. Louis, MO, USA.

2. Protein Project

Study sample: One ALL subject (age: 54 months, Pediatric Oncology Protocol 9605) was followed for one year during the MTX treatment and CSF was collected for the protein analysis.

Analytical Methods

Protein Extraction: Proteins were precipitated from CSF using cooled acetone in large excess. Precipitate was centrifuged into a pallet and used for electrophoresis analysis.

2-D Electrophoresis: Two-dimensional (2-D) electrophoresis was performed according to the method of O'Farrel *et al.* [20]. Isoelectric focusing was carried out in the glass tube (ID = 2 mm) using 2 % pH 3.5 – 10 ampholines (Amershan Pharmacia Biotech, Piscataway, NJ, USA) for 9600 volt-hours. One mg of internal standard, tropomyosin, was added to each sample. This protein migrates as a doublet with a lower polypeptide spot of MW 33,000 and pI 5.2; arrows on the stained gels mark its position. SDS slab electrophoresis was carried out for 4 hrs at 12.5 mA gel^{-1}. The following proteins were added as MW standards: myosin (220,000), phosphorylase A (94,000), catalase (60,000), actin (43,000), carbonic anhydrase (29,000) and lysozyme (14,000). These standards appear as lines across the Coomassie Brilliant Blue R250 stained gels. The total CSF protein concentration was determined using bicinchoninic assay (Pierce, USA). A calibration curve was constructed by using known amounts of albumin (0.25 – 2.000 $\mu g\ ml^{-1}$).

3. Ceramide Project

Study sample: Three different patients participating in this study have been diagnosed with ALL. Patients from the following regiments were used in this study: POG 9904-low risk protocol, POG 9905-standard risk protocol, and POG 9406-high risk protocol. Two patients were females and one patient was male; the mean age of patient was 98.70 months (8, 180 and 108 months).

Analytical Methods

Ceramide Extraction and Derivatization: Ceramides were extracted from CSF with chloroform:methanol (2:1) and chloroform:methanol (9:1) during a two step extraction. The organic layers containing ceramides and other lipids were combined and brought to dryness under nitrogen. In order to improve ultraviolet detection during the HPLC separation, the ceramides were derivatized with benzoyl chloride [21]. The extract was dissolved in 0.5 ml of pyridine and 0.05 ml of benzoyl chloride was added. The mixture was heated at 70 °C for 4 hours and dried under a stream of nitrogen. The final product was redissolved in 4 ml of hexanes and washed with methanol (95 %) and 0.6 M HCl in methanol. The

purified ceramide mixture was dried under nitrogen and re-suspended in the mobile phase for HPLC separation. 3-(*p*-phenylbenzoyl)estrone was used as internal standard for accurate quantification measurements.

HPLC separation: The HPLC separation was conducted on a high performance liquid chromatography instrument Model 338 Gold (Beckman-Coulter, Fullerton, CA). Normal phase column was used during the separation with the following characteristics: Ultrasphere Si-5 microns, 25 cm × 4.6 mm ID. The mobile phase system was adapted from the method described by Iwamori *et al.* [21] and utilized hexane:ethylacetate (94:6, v:v) solvent system. The flow rate was maintained at 1 ml/min and the separation was monitored by a UV detector at 254 nm.

Results and Discussion

1. Dopamine metabolites project

The HVA and VMA were extracted from all CSF specimens using a simple ethyl acetate extraction. In order to increase the volatility, the crude ethyl acetate extract containing both components of interest was derivatized with TMS reagent. The GC/MS method developed in our laboratory allowed excellent separation of both measured variables and their accurate quantification. In order to explore the reliability of this method, the inter-assay reliability evaluation was conducted. HVA and VMA standards were measured at concentrations of 1 ng/ml in multiplicates. The inter-assay reliability for VMA was found to be 98 % and for HVA 99 %. The table below summarizes the results.

Results / concentration	VMA	HVA
Concentration Prepared	1.00 ng ml^{-1}	1.00 ng ml^{-1}
Concentration Measured (median)	1.02 ng ml^{-1}	1.01 ng ml^{-1}
Number of Measurements	0	0
Standard Deviation	0.00806	0.00316
Standard Error	0.00255	0.00100
Inter-assay Reliability	98 %	99 %

Figure 2 shows the calibration curves for HVA (retention time is 11.62 min) and VMA (retention time is 12.87 min). All of the measured levels of HVA and VMA in the study have fallen into the linear region of each calibration curve. The GC chromatogram of crude acetate extract from the CSF of an ALL patient is shown in Fig. 3. The GC method provided an excellent separation of both components. The identity of each peak was verified by using electron ionization mass spectrometry (EI/MS) detection. Figure 4 shows the EI mass spectra of the

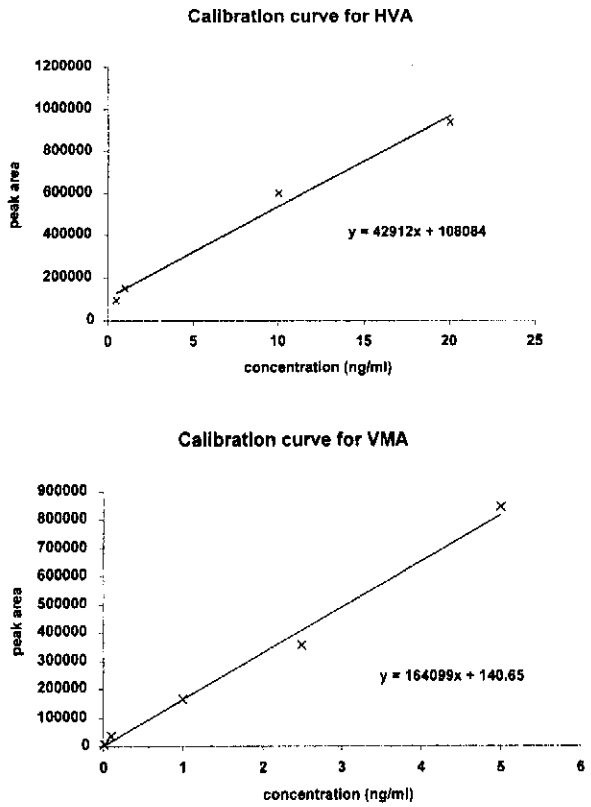


Fig. 2 Calibration curves for HVA and VMA

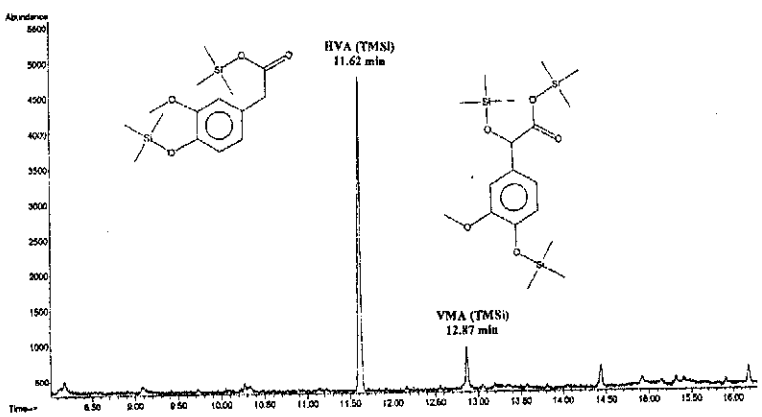


Fig. 3 CG chromatogram of crude acetate extract from ALL patient on the day 8 of MTX treatment. HVA has a retention time of 18.95 min and VMA has a retention time of 20.12 min

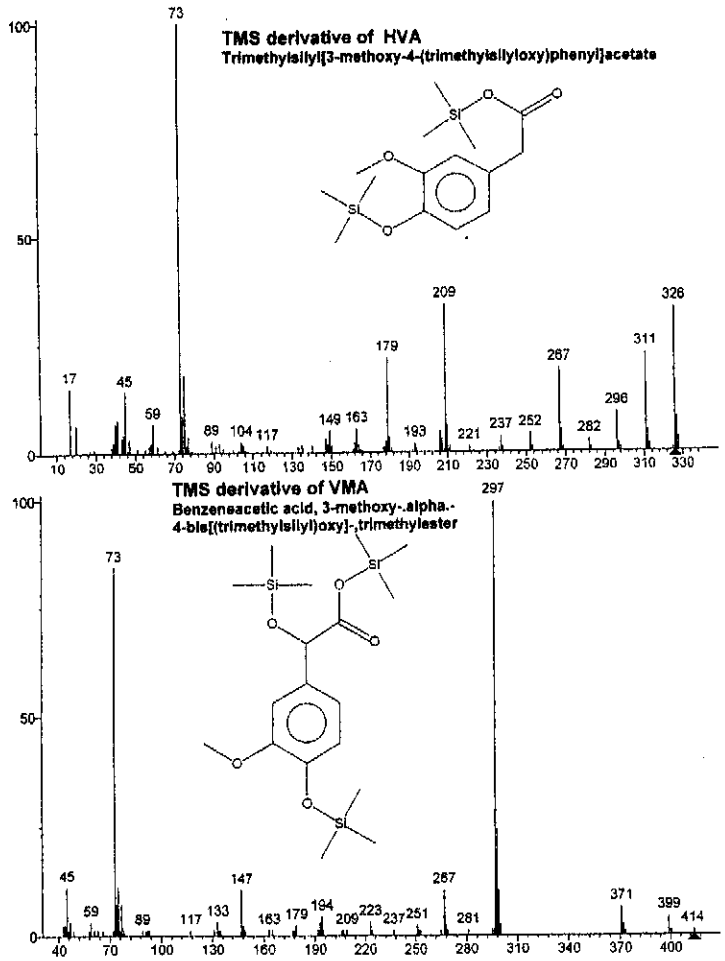


Fig.4 GC/MS spectra of HVA and VMA TMSi-derivates. The black triangles on the x-axes indicate the molecular ion (m/z = 326 for HVA and m/z = 414 for VMA).

derivatized HVA (molecular ion at m/z = 414) and VMA (molecular ion at m/z = 326).

The patients included in this study can be classified based on the relative risk of reoccurrence of the cancer after the initial remission. The higher the ALL risk protocol, the greater amount of MTX administered. The results of ceramide analyses in ALL patients are shown in Fig. 5. Patients on the standard and low risk protocols showed a similar trend in the levels of HVA during the first year of MTX treatment. The levels decreased from baseline during the first 20 weeks due to the high intensity of the treatment. The patient on the standard risk protocol experienced the return of HVA levels to the pre-treatment levels (week 0), while the patient on the low risk ALL protocol showed a systemic decline over time. The

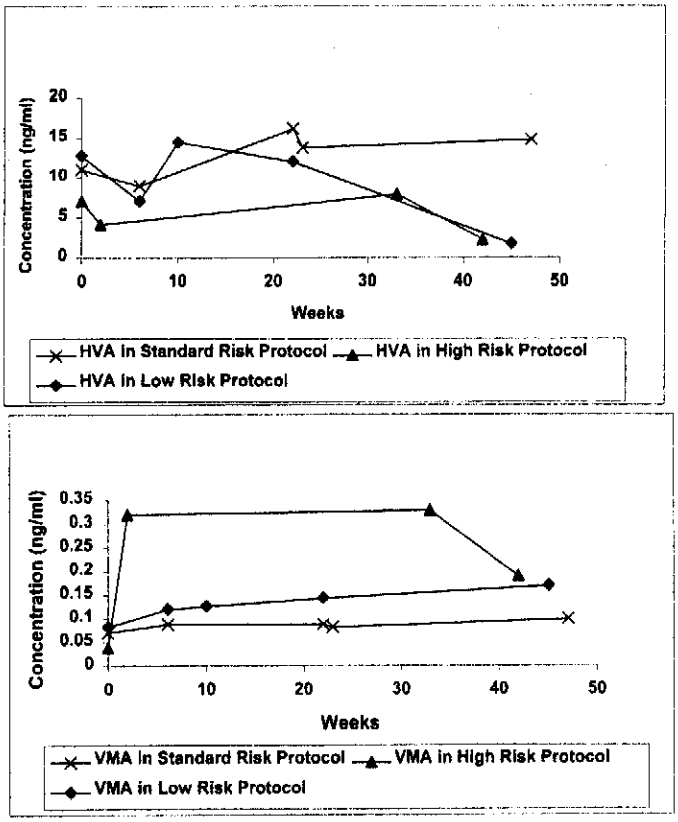


Fig. 5 HVA and VMA CSF levels in low risk protocol patient, standard risk protocol patient and high risk protocol patient

patient on the high risk ALL protocol showed significantly lower HVA levels due to the high dosage of MTX. The HVA levels never returned back to baseline. These results confirmed that there is a MTX dose dependent HVA depletion. With the higher dose of MTX, less HVA is produced, which results in a decrease in the efficiency and the speed of neuronal transmission.

In order to gain more understanding about the consequences of the HVA depletion, we examined the levels of VMA. VMA can be found further down the metabolic scheme for neurotransmitter conversion as illustrated in the Introduction section of this paper. With the decreasing levels of HVA, the metabolism is directed towards increased production of VMA. Our results confirmed the tested hypothesis: with the decreasing levels of HVA, there were increasing concentrations observed for VMA. This was the most significant for the high risk patient, where the VMA levels were three times higher compared to the standard and low risk patients.

In conclusion, the results of this project demonstrated that with increased

doses of MTX, there was greater depletion of HVA. The HVA was converted into VMA, as shown by the increased levels associated with the HVA depletion. Results form our previous study [12] shown that high MTX doses were correlated with more severe cognitive impairments; one possible mechanism of the greater impairments is the reduction of HVA levels and the metabolic conversion into VMA.

2. Protein Project

The CSF protein profiles from an ALL patient were examined during the course of treatment. Proteins were precipitated from CSF using a cooled excess of acetone. The protein pellet was collected and the twodimensional electrophoresis was performed in order to achieve a protein separation according to the method of O'Farrell [20]. The literature indicates that up to 40 different protein bands can be observed in the 2-D electrophoresis separation of CSF proteins [22] isolated from a healthy individual. The number of bands is determined by the sensitivity of the gel dye used to visualize the proteins. Commassie Brilliant Blue dye was used to visualize the protein bands (sensitivity in mg range) because it does not fade over time.

In order to ensure proportional loading onto the electrophoretic gel, the total protein concentration was determined using the bicinchoninic acid (BCA) method (Pierce, USA). Figure 6 shows the total protein concentration in the ALL patient during the first year of treatment. The highest concentration of the total protein reached 289 $\mu g\ ml^{-1}$ of CSF during week 12 of treatment and was likely due to massive activation of immunoproteins and due to over-saturation of plasma proteins as result of inflammation and increased vascular permeability. During the first 12 weeks, patients undergo the most intensive phase of treatment in order to diminish the leukemia load to a minimum. High doses of MTX are used as a preventive treatment to avoid the invasion of the cancerous cells into the CNS system.

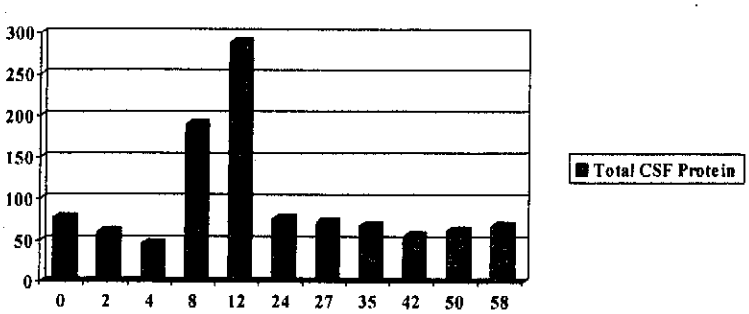


Fig. 6 Total protein concentration in ALL patient during the first year of treatment. The total protein levels were determined using the bicinchoninic acid (BCA) method (Pierce, USA).

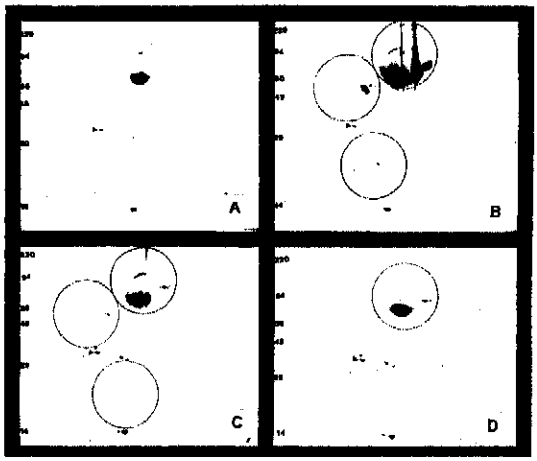


Fig. 7 2-D electrophoresis profile from the ALL patient at the time of diagnosis (A), week 8 (B), week 12 (C) and week 63 (D) of treatment

Following the total protein determination, the 2-D electrophoresis was conducted. Figure 7 shows a 2-D electrophoresis profile from the ALL patient at the time of diagnosis (A), week 8 (B), week 12 (C) and week 63 (D) of treatment. The changes in the profiles are marked with circles and are striking especially during week 8, which marks the end of the induction period of treatment (the initial phase of ALL therapy designed to rapidly decrease the number of circulating leukemia cells). The most significant changes in the pattern are observed in the 70 kDa region, suggesting the generation of the heat shock proteins. Also, additional spots are observed in the following regions: 100 – 200 kDa, 40 – 60 kDa and 25 kDa. Such an increase is likely due to activation of immunoproteins and heat shock proteins as a response to intensive MTX exposure. In conclusion, there were significant changes in the protein profiles during the course of methotrexate treatment. Also, the greatest increase in protein spots and the maximum protein levels in CSF occur simultaneously, at 8 to 12 weeks after the initiation of MTX treatment, when the intensity of the treatment is at its highest. In addition, significant changes in protein patterns were observed in the 70 kDa region. We are in the process of identifying specific protein spots by using matrix assisted laser desorption (MALDI) mass spectrometry.

3. Ceramide Project

The ceramides were isolated the lipid fraction of CSF sample using methanol:chloroform solvent system. After derivatization with benzoyl chloride, the ceramides were introduced onto the HPLC system for isocratic separation. The

typical HPLC chromatogram of such separation is shown in Figure 8. The retention time for ceramides was 7.1 min, while the retention time for internal standard, 3-(p-phenylbenzoyl)estrone, was 14.5 min. Peaks in the 5-5 min regions are byproducts of the derivatization reaction. The C16 ceramide was a major component of the ceramide peak and its identity was confirmed by electrospray mass spectrometry. The quantification of C16 ceramide was not attempted due to the fact that minor multiple species of ceramide with different numbers of carbons in the fatty acid chain were also present in the HPLC peak. In order to express the change in the CSF ceramide levels, we used the area under the peak units as an expression of the total ceramide fraction. Figure 9 summarizes the results for the three patients on different risk protocols during the first 6 months of MTX treatment. There was a large increase in the total ceramide levels within the first three months of MTX treatment regardless of the risk group protocol. The most significant increase was observed in the high risk ALL protocol patient.

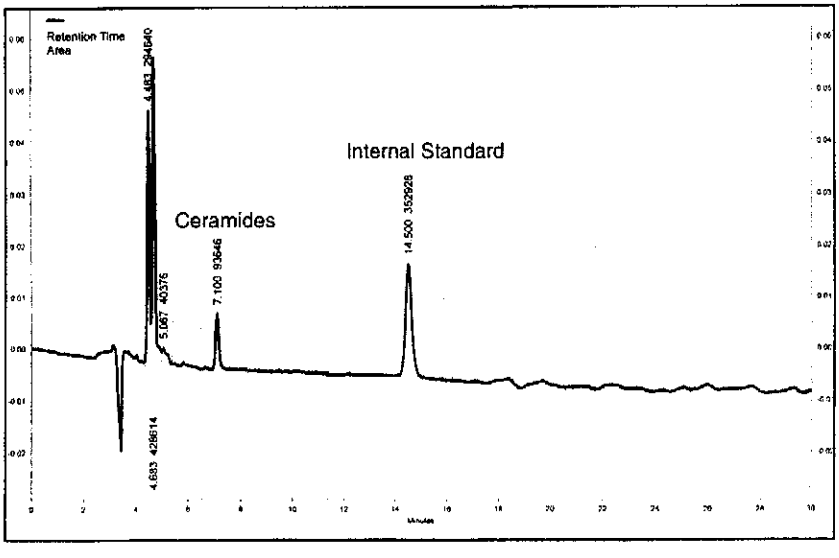


Fig. 8 HPLC chromatogram of ceramides (retention time is 7.1 min) and internal standard (retention time is 14.5 min)

In order to compare cumulative values of the total ceramide fraction in all patients, the area under the curve that was produced by the changing ceramide levels over the six months of MTX treatment was calculated. Area under the curve (AUC) is a mathematical summary of changes in a variable during a selected time period. The values of the same time points shared by all three patients (months 1 – 6) were graphed in 2-dimensional space and the area under the resulting curve was summarized using the NCSS 2000 statistical software. The result of such analysis provides a single number expressing the summarized value of the total ceramide fraction for each patient. The high risk patient AUC was calculated to be

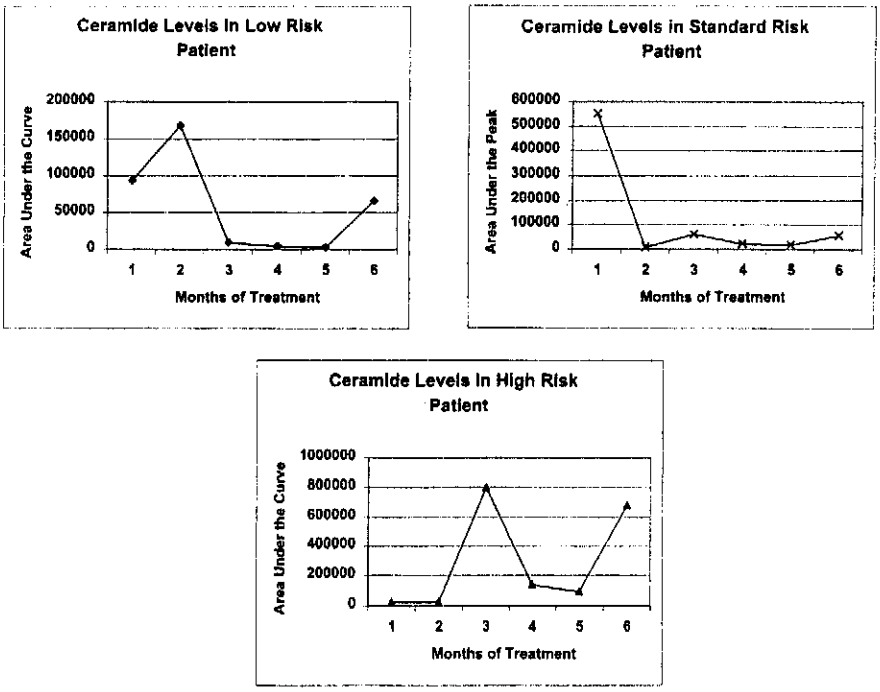


Fig. 9 Ceramide Levels in ALL patients with different risk protocols during the first six months of treatment

5.5 × 106 units, for standard risk patient AUC was determined to be 3.2 × 106, and for the low risk patient AUC was 1.4 × 106. The graphical representation of the AUC results is presented in Figure 10. The results showed that the amount of total ceramide in CSF is directly related to the amount of MTX received. The higher the dose of MTX is administered to the patient, the greater the elevation of total ceramides.

The major component of the ceramide fraction, C16 ceramide, functions as signaling molecule for the initiation of apoptosis. Apoptotic cell death is an active event during which the cell undergoes characteristic cytological changes: cell shrinkage, condensation of nucleoplasm and cytoplasm, and degradation of the nucleus [23]. The mechanism of action of MTX as an antifolate agent has been elucidated in great detail [24]. However, our previous findings [18] and the results from this study support the hypothesis that MTX may activate apoptotic cellular pathways *via* increased levels of C16 ceramide that could contribute to the reported cognitive and structural changes in the CNS.

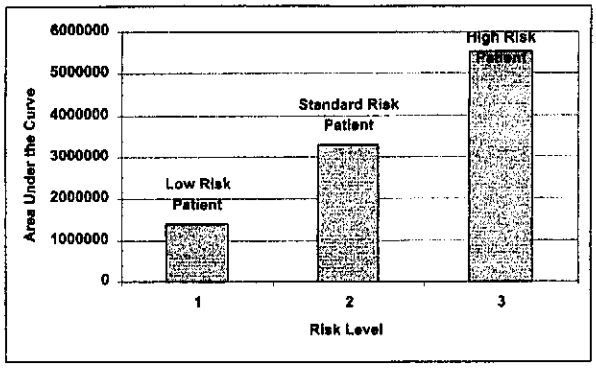


Fig. 10 Area under the curve for ceramide levels in ALL patients with different risk protocols during the first six months of treatment

Conclusion

In this study, we conducted a survey of different CSF biomarkers associated with the cognitive impairment of ALL patients who receive intrathecal MTX as a part of the treatment regiment. The individual projects may appear unrelated due to the large diversity of measure variables, techniques employed and methodologies used. However, the different approaches led to the same main aim of this work, which was to explore changes in different chemical entities of CSF related to one pathological condition, a CNS injury leading to cognitive impairment. The different biomarkers and their relationship to the MTX dosage will help us to understand the processes preceding the cognitive impairments and serve as a base for developing interventions that would reduce the extent of the impairments in ALL children.

Acknowledgements

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