Published in final edited form as:
*J Pediatr Hematol Oncol*. 2010 March ; 32(2): 113–118. doi:10.1097/MPH.0b013e3181c9af84.

# Oxidative Stress and Neurobehavioral Problems in Pediatric Acute Lymphoblastic Leukemia Patients Undergoing Chemotherapy

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## Summary

Neurobehavioral problems after chemotherapy treatment for pediatric acute lymphoblastic leukemia (ALL) have been a recent focus of investigation. This study extended previous research that suggested oxidative stress as a potential mechanism for chemo-therapy-induced central nervous system injury by examining early markers of oxidative stress in relation to subsequent neurobehav-ioral problems. Oxidized and unoxidized components of phosphatidylcholine (PC) were measured in the cerebrospinal fluid of 87 children with ALL at diagnosis, induction, and consolidation. Behavioral assessments were conducted postconsolidation and at the end of chemotherapy. Results revealed a significant association between physiologic reactivity (high vs. low PC changes from diagnosis) and behavioral outcomes (high vs. low pathology). Elevated oxidized PC fraction change was predictive of increased problems with aggression at the end of therapy as well as postconsolidation adaptability. Furthermore, symptoms of hyperactivity systematically changed over time in relation to both unoxidized PC and oxidized PC fraction reactivity. These findings suggest that symptoms of behavioral problems occur early in the course of chemotherapy and that increases in the cerebrospinal fluid PC markers of oxidative stress during induction and consolidation may help to predict certain future behavioral problems.

### Keywords

neurobehavioral outcomes; oxidative stress; pediatric leukemia

Prophylactic central nervous system (CNS) treatment has improved event-free, long-term survival from pediatric acute lymphoblastic leukemia (ALL) to nearly 80%.[1,2] However, many survivors experience adverse acute and late neurocognitive and/or neurobehavioral effects, likely as repercussions of cranial irradiation or intravenous (IV) and intrathecal (IT) methotrexate (MTX) therapy.[3] Previously reported MTX-associated neurocognitive



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deficiencies include problems with visual-motor skills,[4] intelligence,[5,6] and academic abilities.[4,5]

Neurobehavioral problems have also been reported after MTX therapy. Behavioral attention problems, as well as anxiety, depression, and social problems, have been demonstrated in a significant subset of survivors treated only with chemotherapy.[7–9] Attention problems are reportedly related to treatment-associated white matter changes, particularly in the frontal lobes.[10] Although most children experience good behavioral outcomes, the variability across groups is wide, and a significant percentage of patients are negatively affected. Early prediction of which patients will experience adverse outcomes is not currently reliable, and further study is needed to elucidate relevant risk factors for the onset of behavioral problems, and the pattern and duration of symptoms.

Debate continues about the relative roles of physiologic and psychologic factors that contribute to poor neurobehavioral outcomes.[3] Aside from the recognized late effect risk factors including sex, age at diagnosis and treatment, and socioeconomic status, we do not have a clear understanding of the source of individual variability in neurobehavioral outcomes.[11–14] Although risk group and treatment intensity have been shown to play significant roles in the development of late effects, these factors do not always correspond to poor cognitive outcomes.[15,16] Lifestyle behaviors, comorbid medical conditions, and genetic predisposition are the potential predictors of individual variability in outcomes that require further investigation.[3,17,18] Determining the source of neurobehavioral problems will aid in designing appropriate strategies for prevention and early intervention.

Oxidative stress has recently been identified as a potential biologic mechanism for CNS injury and a predictor of subsequent neurocognitive development.[19–22] Phosphatidylcholine (PC) is one of the most abundant phospholipids in the cerebrospinal fluid (CSF).[17] Increased concentrations of CSF phospholipids have been demonstrated to be the potential physiologic markers of therapy-induced CNS injury.[17,18] Specifically, the oxidized PC ratio has been proposed as a marker of CNS cell membrane damage.[19] It has been shown in the rat liver and kidney that intraperitoneal MTX therapy leads to decreased levels of nicotinamide adenine dinucleotide phosphate and glutathione, which are important molecules for cell protection against free radical damage.[20,21] Unsaturated cell membrane phospholipids, including PC, may then serve as key targets of oxidant attack because of their high polyunsaturated fatty acid content.[22] The resulting formation of lipid peroxyl radicals causes more membrane damage, a lipid peroxidation cascade, and ultimately, membrane breakdown.[19,23] A similar mechanism of oxidative stress-mediated injury after high-dose IV and IT MTX therapy may also occur in the CNS of pediatric ALL patients. However, further studies are necessary to elucidate the pathway to injury in this body system.

The purpose of this study was to extend previous work by examining the potential relation between PC markers of oxidative stress and acute neurobehavioral outcomes. Changes in PC markers from CSF samples were compared with the parent ratings of behavior during and shortly after active chemotherapy. It was hypothesized that the increases in CSF PC markers of oxidative stress during induction and from diagnosis through consolidation would help to predict the future behavior problems. Establishment of such an association may add to the development of physiologic risk profiles for neurobehavioral problems, which could then be used to guide preventative interventions. Evidence of agreement between markers of physiologic reactivity and behavioral pathology would also provide rationale for future studies to look at contributing factors and at associations between oxidative stress and behavioral late effects.

## Materials and Methods

### Subjects

The sample included 87 children newly diagnosed with pediatric ALL, who were consecutively recruited at 1 of 2 cancer centers in the southwestern United States. Parents or legal guardians completed the informed consent process, and assent was obtained from patients when appropriate. The study was approved by the necessary institutional review boards. Patients were treated on one of the following Pediatric Oncology Group protocols: 9201, 9406, 9605, 9904, 9905, or 9906. Subjects were excluded if diagnosed with CNS involvement at any time during the course of therapy, if relapsed during therapy, or if treated with cranial irradiation. All children were between 3 and 16 years of age (mean age at diagnosis=7.0y, SD=3.07; 59% female; mean parent education =13.5y). The majority of patients were treated with IV MTX at a $1g/m^2$ dose over a 24-hour period (n=57) or a $2g/m^2$ dose over 4 hours (n=24); 6 patients were treated with a $100\ mg/m^2$ dose over an interval of 10 to 15 minutes. Cumulative IT MTX doses did not differ between patients.

### CSF Sample Phospholipids

CSF samples were harvested from patients at the diagnosis lumbar puncture and during subsequent induction and consolidation lumbar punctures required for IT MTX therapy. Induction and consolidation PC measurements represent mean levels over those treatment phases. Protocols used in the process leading to CSF PC component quantification have been described previously.[19] Briefly, phospholipid classes in the CSF (PC vs. other) were separated using a prepacked silica column and a normal-phase gradient method. After purity information was obtained, a GOLD high-performance liquid chromatography system (Beckman-Coulter) was used to separately quantify unoxidized PC and oxidized PC components via absorption of ultraviolet light at different wavelengths.[24] Oxidized species of PC were not converted to mg/mL due to multiple components (hydroxyperoxides and hydroxyphospholipids) within the peak, dependent upon degree of oxidation. Therefore, an oxidized fraction was obtained for each PC sample [peak area of oxidized PC (measured at 234 nm): peak area of unoxidized PC (measured at 206 nm)]. Mean oxidized PC fractions were generated for each subject by phase of ALL therapy. Therefore, induction and consolidation PC measurements represent mean levels over those treatment phases. Protocol-restricted mean imputation was used to replace outliers (ie, data points greater than 6 standard deviations from the mean).

### Behavioral Measures

The Behavior Assessment System for Children (BASC) parent rating was used to measure behavioral functioning during and shortly after chemotherapy.[25] This questionnaire includes items that assess the following scales: Adaptability, Anxiety, Aggression, Attention Problems, Atypicality, Conduct Problems, Depression, Hyperactivity, Leadership, Social Skills, Somatization, and Withdrawal. For each scale, raw scores are converted to T scores adjusted for age and sex and normalized to a mean of 50 (SD=10) using a large, nationally representative standardization sample. T scores are considered clinically significant when there is more than 1 standard deviation from the mean. BASC evaluations were collected at 2 time points: (1) postconsolidation, 67.5 weeks (SD= 17.95) from diagnosis; and (2) end of therapy, 126.6 weeks (SD=15.51) from diagnosis.

### Data Analysis

Study population characteristics, induction-related and consolidation-related changes in PC measures, and BASC outcome measures were summarized with descriptive statistics. Parent education, patient sex, and age at diagnosis were included as patient-related demographic

variables. IV MTX dose (1 or 2 g/m$^2$ only) was included as a treatment variable. Univariate analyses to examine the association of demographic factors and treatment variables with behavioral outcomes were conducted using $\chi^2$, Fisher exact test, or independent samples *t* tests where appropriate.

Patients were clustered into groups of high and low physiologic reactivity and behavioral pathology to explore the association between PC reactivity and behavioral outcomes. To examine physiologic reactivity during induction and during therapy through consolidation, median splits were conducted based on the size of PC marker differences, with patients exhibiting larger increases composing the group of greater physiologic reactivity. BASC outcomes were also dichotomized by median split to create groups of high and low pathology, where patients with more behavioral problems comprised the high pathology group.

Nonparametric agreement analyses were performed to examine significant relationships between high versus low physiologic reactivity and high versus low behavioral pathology. Cohen $\kappa$ was used to measure chance-adjusted patient agreement on physiologic reactivity and behavioral pathology. Kappa values from 0.21 to 0.40 signify fair agreement, and values from 0.41 to 0.60 imply moderate agreement.[26] Logistic regression models were also used to calculate odds ratios of behavioral pathology as predicted by belonging to the high physiologic reactivity group. Mann-Whitney *U* was used to compare median changes in BASC scales from postconsolidation to end of therapy for each of the PC measures. Adjustment for multiple comparisons was not conducted (see discussion).

## Results

### CSF Reactivity Markers

Changes in mean unoxidized PC and oxidized PC fraction from diagnosis to induction and from diagnosis to consolidation are summarized in Table 1. There were no statistically significant differences observed in sex, age, or parent education between high and low physiologic reactivity groups for the PC measures.

### Behavioral Assessments

At all 3 time points, age-adjusted and sex-adjusted mean T scores for most of the individual BASC scales fell within the average range (Table 2). Somatization was elevated during the postconsolidation and end of therapy time points, likely due to treatment-related discomfort. Variability around the mean score on several scales was larger in this sample than expected norms, suggesting that a subset of children displayed more behavioral problems than typical. To study the potential source of increased variability in parent-perceived child behavior problems, analyses involving BASC measures were conducted using the dichotomized variable created by median split. Also of note, although within-group variability seems to increase with increasing time from diagnosis for Aggression, Conduct Problems, and Atypicality, as reflected through the increase in the size of the standard deviations from postconsolidation to the end of therapy, this change is not statistically significant.

### Behavior and Treatment Factors

Pearson $\chi^2$ analysis demonstrated a significant association between IV MTX dose and behavioral pathology group for postconsolidation Adaptability [$\chi^2$ (1, 51) =8.32, $P$<0.01], end of therapy Attention Problems [$\chi^2$(1, 45) = 4.98, $P$<0.05], and end of therapy Leadership Skills [$\chi^2$ (1, 45)=3.96, $P$<0.05]. Also, $\chi^2$ analysis showed an association between sex and postconsolidation Social Skills [$\chi^2$ (1, 59) =6.474, $P$<0.05]. Furthermore, independent samples *t* tests showed significant associations between age at diagnosis and

Aggression at postconsolidation [$t$ (41.1)=3.1, $P$<0.01], and end of therapy [$t$ (42)=3.1, $P$<0.01], with younger age at diagnosis associated with more aggression problems. The remaining behavioral scales did not differ by sex, age at diagnosis, parent education, or IV MTX dose (1 and 2g/m$^2$ groups) (all $P$ values >0.05).

### Association Between Physiologic Reactivity and Behavior

Postinduction PC was related to degree of behavioral symptoms at postconsolidation and end of therapy (Table 3). Greater increases in oxidized PC fraction were significantly associated with higher problems in postconsolidation Adaptability ($\kappa$=0.40, $P$<0.05). The odds of belonging to the high pathology group in Adaptability were significantly greater for patients with high oxidized PC fraction reactivity (odds ratio = 6.0; 95% confidence interval = 1.2-30.7; $P$<0.05).

Postconsolidation PC was also related to more behavioral problems. Highly oxidized PC fraction reactivity was related to greater problems with Aggression at the end of therapy ($\kappa$=0.49, $P$<0.01). The odds of belonging to the high pathology group in Aggression were significantly greater for patients with high oxidized PC fraction reactivity (odds ratio=10.8; 95% confidence interval=1.8-65.6; $P$<0.01).

The relation between high or low physiologic reactivity and median change in behavioral symptoms over time was examined using the Mann-Whitney $U$ test. A significant difference in the change of Hyperactivity symptoms from postconsolidation to the end of therapy was seen between groups with high and low unoxidized PC reactivity from diagnosis to induction [$U$ (27) =45.5, $P$<0.05, effect size $r$=0.41]. The low physiologic reactivity group experienced a decrease in hyperactivity symptoms of 0.6 standard deviations from postconsolidation to the end of therapy, whereas the high reactivity group displayed a nonsignificant change in symptoms. In addition, the degree of change in Hyperactivity symptoms was related to oxidized PC fraction reactivity from diagnosis to consolidation [$U$ (25) = 39.5, $P$<0.05, effect size $r$= 0.40]. The low physiologic reactivity group displayed a decrease in symptoms of 0.3 standard deviations from postconsolidation to the end of therapy, whereas the high reactivity group displayed an increase in symptoms of 0.2 standard deviations over this same time interval.

## Discussion

The oxidized and unoxidized components of PC, an important structural molecule in CNS cell membranes, were used as proxies for oxidative stress and CNS membrane integrity in this study. Increased CSF phospholipid concentration has been proposed as a biologic marker of MTX-induced CNS injury, and a potential molecular mechanism for this injury via oxidative damage has been proposed.[18,27] Furthermore, the oxidized version of PC has previously been shown to play a role in the pathophysiology of atherosclerosis,[28] lung disease,[29] and multiple sclerosis.[30] Although neurobehavioral problems have been described in ALL survivor populations, this is the first study to our knowledge that has examined the association between oxidative damage and such outcomes. Findings from this study suggest that oxidized PC fraction changes during induction and consolidation are predictive of the degree of symptoms observed on future behavioral ratings.

Because normative data does not exist for the change in PC markers of oxidative stress used here, patients were separated by median split into groups of high and low physiologic reactivity to generate a variable for this biologic predictor. In addition, we acknowledge that most of the patients fell within the average range of BASC scores. However, increased variability compared with standardization data suggests that a subset of children deviated

from the group mean. Physiologic reactivity changes from diagnosis to induction and to consolidation were significantly associated with future behavior problems.

A significant proportion of patients in the high physiologic reactivity group during induction were also members of the high pathology group for Adaptability postconsolidation. This finding that decreased Adaptability is among the first neurobehavioral symptoms to manifest in the high reactors may be associated with changes in lifestyle expectations during therapy. Children often experience the transition from full medical care to community-based care and the move back to school postconsolidation. Therefore, one could expect the child's ability to adapt to environmental change to be among the first biologically mediated behaviors observable by parents.

Higher oxidized PC fraction change during induction was also related to Attention and Conduct Problems at the end of therapy. This delayed onset of externalizing behaviors may have a physiologic explanation, but may take longer than Adaptability to manifest after a child's return to a normal environment. A relation was also demonstrated between oxidized PC fraction change from diagnosis to consolidation and Aggression at the end of therapy. The Conduct Problems that manifest postconsolidation, characterized by nonaggressive disobedience as well as decision-based actions, may become more severe over time and transition into Aggression and physical actions in a subset of patients. It is possible that the toxicity threshold for functional change (Conduct Problems) is lower than for that for physical change (Aggression). It is also important to note that this relation and the one between age at diagnosis and end-of-therapy Aggression may reflect 2 individual associations as age is not associated with physiologic reactivity (and therefore unlikely to be a confounder). Younger patients were more likely to experience pathology, consistent with other literature.[31] Overall, this analysis provides evidence that the oxidized PC change—Aggression relation has some physiologic basis.

Symptoms of Hyperactivity seemed to change over time. Although Hyperactivity scores for high and low physiologic reactivity groups were not significantly different at postconsolidation, symptoms changed differentially from postconsolidation to end of therapy between the 2 groups. The elevated symptoms in the high physiologic reactivity group leveled off, whereas higher symptoms in the low group returned to normal by the end of therapy.

Acute and late treatment effects can significantly impair the quality of life for pediatric ALL survivors. The first step toward preventing negative outcomes is learning to predict which patients are likely to be adversely affected. Overall, these findings support the hypothesized association between phospholipid oxidation, a measurable physiologic marker, and certain acute neurobehavioral problems in this population. The description of such a relation contributes to the characterization of the risk profile for behavioral outcomes. As such, it may eventually allow for earlier intervention in children with high physiologic reactivity during treatment.

This study had several limitations that should be addressed in future investigations. The overall sample size (N= 87) was reduced for specific PC marker analyses due to inadequate quality of some CSF samples, thereby restricting the power to detect effects and the ability to use parametric statistical tests. This study should be replicated in a larger sample with more complete data to see if the same and/or different relationships hold. Another limitation surrounds the use of only a single parent report scale to evaluate child behavioral outcomes. Ideally, multiple independent respondents would be available (ie, teachers) to control for parental expectations and stress. For example, parenting stress has been associated with parent ratings of behavioral adjustment and social skills in children being treated for

cancer.[32] In future studies, it may be possible to obtain self-report ratings for older patients, teacher ratings, a measure of parent stress, and direct observations of patient behavioral functioning. In addition, pretreatment neuropsychologic assessment data were not available for comparison to baseline. Generally, it is not possible to collect pretreatment behavioral data on these subjects due to the short time between diagnosis and start of chemotherapy. Delay of treatment for the purpose of baseline cognitive evaluation was not a priority. Finally, adjustment for multiple comparisons was not conducted, so it is possible that the associations between physiologic and behavioral reactivity were observed by chance. However, adjustment was not done for several reasons. This is the first study of which we are aware that examines the association between physiologic markers of oxidative stress in the CNS and behavioral changes. All potential associations observed were highlighted for confirmation in a larger replication study. Furthermore, we have previously reported these physiologic markers to be associated with treatment intensity,[19] and treatment intensity to be associated with neurobehavioral outcomes.[33] Thus, associations were predicted prospectively. In addition, the primary comparisons are repeated measures analyses of change, which assists in controlling error variance.

Despite these limitations, oxidative damage may prove, after replication, to be a significant component of an individual's risk profile, as this study suggests. If so, it is possible that administration of antioxidants or dietary supplementation with omega-3 fatty acids may help to mitigate the oxidative stress in pediatric ALL patients undergoing chemotherapy and prevent poor neurobehavioral outcomes.[34]

## Acknowledgments

Supported in part by NIH grants HD 37816 (K. Kaemingk) and NR 04905 (I. M. Moore).

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**Table 1**

**Mean and Median Changes in Unoxidized (μg/mL) and Oxidized Phosphatidylcholine Fractions Over 2 Time Intervals: Diagnosis to Induction and Diagnosis to Consolidation**

| | Change From Diagnosis to Induction | | | | Change From Diagnosis to Consolidation | | | |
|---|---|---|---|---|---|---|---|---|
| | N | Mean | SD | Median | N | Mean | SD | Median |
| Unoxidized CSF PC | 59 | 4.0401 | 4.45 | 3.2100 | 69 | 1.4007 | 2.10 | 1.2704 |
| Oxidized CSF PC fraction | 46 | 0.0105 | 0.02 | 0.0082 | 52 | 0.0099 | 0.01 | 0.0098 |

CSF indicates cerebrospinal fluid; PC, phosphatidylcholine; SD, standard deviation.

**Table 2**

**Behavioral Rating During Active Chemotherapy**

| | Postconsolidation (N=59) | | | End of Therapy (N=47) | | |
|---|---|---|---|---|---|---|
| **Subscale** | **Mean** | **SD** | ***P*** | **Mean** | **SD** | ***P*** |
| Hyperactivity | 48 | 13.44 | 0.25 | 47.2 | 12.36 | 0.13 |
| Aggression | 47.2 | 10.69 | 0.045 | 47.4 | 12.27 | 0.15 |
| Conduct problems | 46.4 | 8.14 | 0.006 | 48.4 | 11.59 | 0.39 |
| Anxiety | 50.8 | 12.04 | 0.62 | 49.6 | 11.44 | 0.82 |
| Depression | 49.2 | 13.23 | 0.63 | 49.2 | 12.4 | 0.66 |
| Somatization | 61.4 | 15.02 | 0.00 | 61 | 14.75 | 0.00 |
| Atypicality | 48.1 | 11.21 | 0.20 | 49.2 | 13.22 | 0.69 |
| Withdrawal | 47.8 | 9.61 | 0.086 | 50.8 | 12.81 | 0.68 |
| Attention problems | 50.3 | 12.05 | 0.86 | 52.6 | 13.43 | 0.19 |
| Adaptability | 51.8 | 9.3 | 0.16 | 49.2 | 10.26 | 0.65 |
| Social skills | 53.4 | 10.21 | 0.013 | 53.3 | 10.98 | 0.047 |
| Leadership | 53 | 10.36 | 0.068 | 51 | 10.21 | 0.54 |

Note: The scores presented above are T scores with a mean of 50 and standard deviation of 10, normalized to age and sex based on a national standardization sample. *P* values represent results from 1-sample *t* tests, examining if mean scores are statistically significantly different from 50. Of interest here is the degree of variability on certain scales compared with the national norms. This larger variability suggests that some children within the sample are differentially displaying many more symptoms than others within the group.

## Table 3
## Agreement (κ) Between Physiologic Reactivity and Behavioral Outcomes

| | Δ Diagnosis to Induction | | Δ Diagnosis to Consolidation | |
|---|---|---|---|---|
| | **Oxidized PC** | **Unoxidized PC** | **Oxidized PC** | **Unoxidized PC** |
| Postconsolidation | | | | |
| Hyperactivity | 0.029 | − 0.034 | − 0.003 | 0.000 |
| Aggression | 0.207 | −0.245 | 0.273 | − 0.174 |
| Conduct problems | −0.116 | − 0.033 | 0.171 | 0.059 |
| Anxiety | 0.092 | −0.021 | 0.225 | − 0.130 |
| Depression | −0.029 | − 0.160 | − 0.049 | 0.043 |
| Somatization | 0.154 | −0.009 | − 0.049 | − 0.217 |
| Atypicality | 0.092 | − 0.160 | 0.167 | −0.304 * |
| Withdrawal | −0.213 | − 0.021 | 0.056 | 0.217 |
| Attention problems | 0.095 | − 0.051 | 0.119 | 0.087 |
| Adaptability | 0.400 * | 0.176 | − 0.142 | 0.049 |
| Social skills | 0.092 | 0.033 | 0.056 | 0.087 |
| Leadership | −0.161 | 0.029 | 0.038 | 0.118 |
| End of Therapy | | | | |
| Hyperactivity | 0.319 | 0.078 | 0.305 | 0.055 |
| Aggression | 0.254 | 0.201 | 0.488 † | 0.160 |
| Conduct problems | 0.385 * | − 0.115 | 0.148 | 0.124 |
| Anxiety | −0.306 | − 0.007 | −0.055 | − 0.111 |
| Depression | 0.233 | 0.201 | 0.053 | 0.055 |
| Somatization | 0.017 | 0.043 | − 0.120 | −0.105 |
| Atypicality | −0.134 | − 0.045 | 0.053 | − 0.099 |
| Withdrawal | 0.191 | − 0.126 | 0.179 | 0.000 |
| Attention problems | 0.386 * | 0.026 | 0.053 | − 0.105 |
| Adaptability | 0.162 | 0.212 | 0.066 | 0.026 |
| Social skills | 0.274 | − 0.007 | 0.077 | 0.206 |
| Leadership | 0.249 | −0.160 | 0.032 | − 0.181 |

* κ is significant at the α = 0.05 level.

† κ is significant at the α = 0.01 level.

PC indicates phosphatidylcholine.