Clinical significance of occult central nervous system disease in adult acute lymphoblastic leukemia. A multicenter report from the campus all network

by Maria Ilaria Del Principe, Elisa Buzzatti, Alfonso Piciocchi, Fabio Forghieri, Massimiliano Bonifacio, Federica Lessi, Silvia Imbergamo, Enrico Orciuolo, Giovanni Rossi, Nicola Fracchiolla, Silvia Trappolini, Benedetta Neri, Chiara Sarlo, Patrizia Zappasodi, Michelina Dargenio, Mariangiovanna Cefalo, Maria Antonietta Irno-Consalvo, Consuelo Conti, Giovangiacinto Paterno, Gottardo De Angelis, Mariarita Sciumè, Irene Della Starza, Adriano Venditti, Robin Foà, and Anna Rita Guarini

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Clinical significance of occult central nervous system disease in adult acute lymphoblastic leukemia. A multicenter report from the campus all network

Running head: CNS involvement in adult ALL patients

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ABSTRACT

In acute lymphoblastic leukemia, flow cytometry detects more accurately leukemic cells in patients’ cerebrospinal fluid compared to conventional cytology. However, the clinical significance of flow cytometry positivity with a negative cytology - occult central nervous system disease - is not clear. In the framework of the national Campus ALL program, we retrospectively evaluated the incidence of occult central nervous system disease and its impact on outcome in 240 adult patients with newly diagnosed acute lymphoblastic leukemia. All cerebrospinal fluid samples were investigated by conventional cytology and flow cytometry. The presence of $\geq 10$ phenotypically abnormal events, forming a cluster, was considered as flow cytometry positivity. No central nervous system involvement was documented in 179 patients, while 18 were positive by conventional morphology and 43 were occult central nervous system disease positive. The relapse rate was significantly lower in central nervous system disease negative patients and the disease-free and overall survival were significantly longer in central nervous system disease negative patients than in those with manifest or occult central nervous system disease positive. In multivariate analysis, the status of manifest and occult central nervous system disease positivity was independently associated with a worse overall survival.

In conclusion, we demonstrate that in adult acute lymphoblastic leukemia patients at diagnosis flow cytometry can detect occult central nervous system disease at high sensitivity and that the status of occult central nervous system disease positivity is associated with an adverse outcome. (Clinicaltrials.gov NCT03803670)

Key words: Acute lymphoblastic leukemia, central nervous system, flow cytometry, conventional cytology, prognosis.
INTRODUCTION

Over the last two decades, improved response rates have been reported in adult patients with acute lymphoblastic leukemia (ALL).\textsuperscript{1-3} In this context of a superior systemic disease control, central nervous system (CNS) involvement has become an ever more influential limitation to the achievement of a long-term cure and a main cause of mortality. At diagnosis, about 5-10\% of adult ALL patients have a CNS involvement,\textsuperscript{4-6} which translates into a shorter overall survival (OS) duration compared to that of patients without CNS involvement.\textsuperscript{4}

Conventional cytology (CC) examination of the cerebrospinal fluid (CSF) remains the gold standard for the diagnosis of CNS involvement in ALL; CC is estimated to have a >95\% specificity. However, it has a relatively low sensitivity (<50\%), resulting in frequent false negative determinations. Such a low sensitivity is due to the poor cellularity of CSF and to the difficulties in distinguishing benign from malignant cells on morphologic grounds only.\textsuperscript{7,8}

Flow cytometric (FCM) immunophenotyping is a valuable tool for the diagnosis and staging of hematologic disorders involving lymph nodes, blood, bone marrow and other body fluids. Current FCM assays allow to detect phenotypically abnormal cells up to the limit of at least 0.01\% (1 target cell in $10^4$ events), representing therefore a very effective tool for minimal residual disease monitoring in acute leukemia.\textsuperscript{9} Indeed, several recently published experiences have demonstrated the superior sensitivity of FCM over CC for the detection of CNS disease in patients with ALL and non-Hodgkin’s lymphoma.\textsuperscript{10-13} These studies have also contributed to establish a new standard that is the so-called “occult CNS disease” (OCNSD), namely the status of FCM positivity and CC negativity. None of these reports has however clarified whether a condition of OCNSD has an additional prognostic role compared to the well-established negative impact of CC positivity. We therefore conducted a multicenter, retrospective study in the framework of the national Campus ALL program aimed at improving the management of adult ALL patients. The aims of the present study were 1) to evaluate the incidence of OCNSD in a large series of adult patients with ALL, and 2) to assess the impact of OCNSD on the clinical outcome of these patients.
METHODS

STUDY DESIGN AND PATIENTS

Our retrospective analysis included patients seen between January 2007 and December 2017 at 13 Italian Hematology Centers. Cases were documented using a case report form. Variables included the following data: age, gender, ALL onset, genetic/cytogenetic features, B/T phenotype, white blood cell count (WBC) at diagnosis and at the time of lumbar puncture (LP), lactate dehydrogenase (LDH), chemotherapy, date of complete remission (CR), CSF cell count and chemistry, CC and FCM results, date of systemic and/or CNS relapse, allogeneic stem cell transplant (ASCT), date of death or the last follow-up. Personal information was treated in a confidential manner and all sensitive data were anonymously analyzed. Samples were collected at diagnosis. In patients with a high WBC count, which might confound the CSF picture because of the traumatic procedure, the explorative lumbar puncture was performed once the WBC count was reduced below 10x10^9/L by administering steroids.

Patients were treated within or according to GIMEMA (LAL0904, LAL1308, LAL1913, LAL1104)\textsuperscript{14} or NILG (NILG-ALL10/07)\textsuperscript{15,16} protocols or the Hyper-CVAD/MTX-ARAC regimen.\textsuperscript{17,18} In the GIMEMA protocols, CNS prophylaxis consisted in intrathecal injection (IT) of methotrexate (12.5 or 15 mg) alone or combined with steroids once a week for a total of 3-4 administrations during the induction and consolidation cycles, respectively. In LAL0904, cranio-spinal irradiation (CI) was dispensed after the consolidation phase,\textsuperscript{14} while in the other GIMEMA/NILG protocols CI was omitted and all patients received a CNS-crossing agent-based chemotherapy. According to the NILG-ALL10/07 protocol, 12 triple agent (methotrexate 12.5 mg, cytarabine 50 mg, dexamethasone 4 mg) IT injections were given as CNS prophylaxis. Finally, in the Hyper-CVAD/MTX-ARAC program, 16 prophylactic IT were planned.\textsuperscript{17,18} CNS therapy for patients with a CC-positive LP consisted of IT injections of 12 mg methotrexate, 50 mg cytarabine
and 10 mg methylprednisolone twice weekly until CSF blast clearance, and then once weekly for two administrations.

**Cell counts and conventional cytology**

Cytospins for CC examination were prepared as previous detailed.\textsuperscript{19,20} CC positivity was defined as unequivocal, morphologic evidence of leukemic blast in the CSF and/or a CSF WBC count $\geq5/\mu\text{l}$ with less than 10 erythrocytes/µl.\textsuperscript{3,21} Traumatic LP were excluded from the analysis.

**FCM analysis**

All centers involved were selected on the basis of a strict adherence to a standardized approach relying on the same procedures (time elapsed from collection to processing, number of fluorochromes, number of acquired events and analysis). Samples for FCM analysis were locally processed within 60 minutes from harvest, as described elsewhere.\textsuperscript{19} A cocktail of 6-8 monoclonal antibodies was used (Table S1). On average, 1080 events were acquired (range 0-210,000). In agreement with the recommendations for the analysis of rare events, a cluster of at least 10 phenotypically abnormal events was regarded as a proof of CSF infiltration\textsuperscript{10} (Figure 1). Traumatic LP were excluded from the analysis.

**Statistical analysis**

Statistical analysis is described in the Online Supplementary Appendix.

**Ethical considerations**

Approval of the local institutional review board and ethics committee was obtained at all participating sites. The trial was registered at [www.clinicaltrials.gov](http://www.clinicaltrials.gov) as NCT03803670.
RESULTS

Patients characteristics

The clinical and laboratory characteristics of the 240 patients are summarized in Table 1. At diagnosis, 179 (75%) CSF samples were negative by both FCM and CC (CNS\textsuperscript{neg}), while 43 (18%) were OCNSD positive (positive by FCM and negative by CC = OCNSD\textsuperscript{pos}) and 18 (7%) were positive by both FCM and CC (manifest CNS disease positive = MCNSD\textsuperscript{pos}) (Table 1). No case proved FCM-negative and CC-positive.

The characteristics of patients belonging to the three groups are listed in Table 1. There was an equal male/female ratio among CNS\textsuperscript{neg}, OCNSD\textsuperscript{pos} and MCNSD\textsuperscript{pos} patients. Also, the median age, median WBC count, B/T lineage, LDH levels did not differ significantly between the three categories. Cytogenetic/genetic data were available in 178/240 cases (74%) and no difference in distribution among the three categories was observed. On the other hand, the status of OCNSD\textsuperscript{pos} and MCNSD\textsuperscript{pos} was significantly associated with a high CSF cellularity (Table 1, p<0.001) and the levels of CSF proteins (Table 1, p=0.023). One hundred and seventy-one patients (71%) were treated within or according to GIMEMA protocols, 37 (15%) with the Hyper-CVAD/MTX-ARAC regimen and 32 (14%) according to the NILG ALL10/07 protocol. Considering the heterogeneity of the chemotherapy regimens utilized, we analyzed our series dividing the patients into three groups on the basis of the intensity of the treatment received. Accordingly, 91 patients (37.9%) underwent a conventional treatment, 120 (50%) an intensified pediatric-inspired regimen and 29 (12.1%), qualified as unfit or frail, were treated with a reduced intensity schedule (Table 1).

Outcome

Of the 232 evaluable patients, 198 (85%) achieved a CR with no significant differences between the three CNS status-based groups (p=0.3). Of these 198 patients, 116 (59%) experienced a relapse; in 18/116 (15%), disease recurrence occurred in the CNS alone or was combined with an hematologic relapse. The relapse rate was significantly higher in OCNSD\textsuperscript{pos} and MCNSD\textsuperscript{pos} patients than in CNS\textsuperscript{neg} patients (Table 2) (p=0.001). The 3-year DFS was also significantly longer in CNS\textsuperscript{neg}
patients compared to OCNSD\textsuperscript{pos} or MCNSD\textsuperscript{pos} patients: 39\% (95\% CI, 31-48) vs. 21\% (95\% CI, 4.5-33.9) vs. 21\% (95\% CI, 7.9-58.4) (p=0.005) \textit{(Table 2)}. On the contrary, the 3-year DFS was not different between OCNSD\textsuperscript{pos} and MCNSD\textsuperscript{pos} patients (p=0.3) (Figure 2).

The 3-year OS in CNS\textsuperscript{neg}, OCNSD\textsuperscript{pos} and MCNSD\textsuperscript{pos} patients was 53\% (95\% CI, 45.5-61.5), 31\% (95\% CI, 19.2-50.5) and 22\% (95\% CI, 9.4-52.7), respectively (p<0.0001) (Table 2). The 3-year OS was not different between OCNSD\textsuperscript{pos} and MCNSD\textsuperscript{pos} patients (p=0.2) (Figure 3).

\textbf{Multivariate analysis}

The clinical impact of the CNS status on OS was also challenged in the multivariate Cox proportional hazard analysis applied to models including age, transplant, sex, WBC count and treatment received. Multivariate analysis confirmed that the OCNSD\textsuperscript{pos} (HR=1.82, 95\% CI 1.15 to 5.92 p=.01) or MCNSD\textsuperscript{pos} status (HR=3.23, 95\% CI 1.76 to 2.89 p<.0001), defined at the time of diagnosis, were factors that independently impacted on OS together with the treatment regimens (intensified vs. conventional vs. reduced intensity for age) (Table 3).

\textbf{DISCUSSION}

This retrospective study shows that FCM offers a superior technical support over CC in detecting leukemic cells in the CFS of adult patients with ALL and documents the clinical impact of OCNSD on the outcome of these patients. By introducing FCM analysis, the detection power improved to such an extent that evidence of a CNS involvement increased from 7\% to 25\% of ALL cases at diagnosis. This analysis confirms previous reports that demonstrated the superior sensitivity of FCM over CC.\textsuperscript{10,12,13,22,23} In a large retrospective study of 326 CSF samples collected from patients affected by diffuse large B-cell and Burkitt lymphomas, a CSF involvement was detected by FCM in 33 (13\%) diffuse large B-cell lymphomas and in 9 (11\%) Burkitt lymphomas.\textsuperscript{24} FCM allows to detect an hematologic disease in CSF specimens even when the cellularity is very low.\textsuperscript{9,25} This peculiarity has been confirmed in pediatric ALL patients where FCM was able to substantially
ameliorate recognition of occult CSF involvement.\textsuperscript{26-28} In agreement with pediatric reports\textsuperscript{27}, the 
CNS status of our adults did not correlate with risk factors associated with the risk of relapse, such 
as WBC count at onset, B/T phenotype or cytogenetic/genetic features.

In pediatric ALL, FCM positivity alone in the absence of a positive CC seems to affect clinical 
outcome.\textsuperscript{27-29} Similar observations have been made in patients with high-risk non-Hodgkin 
lymphomas and Burkitt’s lymphomas, in whom FCM positivity of CSF was associated with a 
significantly higher risk of CNS relapse and a worse prognosis.\textsuperscript{24,30}

In adult ALL patients, the role of OCNSD is less clear because of the limited number of studies 
based on small series of patients. By analyzing 168 CSF samples collected from 31 patients with 
ALL, Subira et al.\textsuperscript{31} reported a concordance between FCM and CC with the exception of 10 
samples. All patients with a FCM negative finding remained free from CNS disease. In a small 
population of 38 adults with ALL or lymphoblastic lymphoma, we previously observed that the 
median OS of patients with FCM single positivity was intermediate between double positive or 
negative patients.\textsuperscript{19}

The uncertain clinical significance of the FCM analysis of CSF is confirmed by the discordant 
position of the current guidelines. In fact, while the National Comprehensive Cancer Network 
(NCCN) guidelines\textsuperscript{32} do not indicate that FCM analysis of the CSF should be part of the initial 
work-up, the more recent American pocket guide for the clinician recommends (not strongly) to 
perform this examination at diagnosis.\textsuperscript{33} Based on our large multicenter report, occult CNS status 
indeed has a significant impact on outcome. In fact, patients with OCNSD had a worse DFS and OS 
compared to those who were OCNSD negative. The superimposable duration of OS of OCNSD and 
MCNSD patients indicates that even the presence of few cells in the CNS sanctuary has a clinical 
impact; these few cells can be detected only by using approaches more sensitive than CC, such as 
FCM. The pronounced neurotropism of ALL\textsuperscript{34-36} can be responsible of disease recurrence once the 
leukemic cells, surviving systemic chemotherapy within the CNS sanctuary, migrate to the 
circulation.\textsuperscript{37,38} Thus, the availability of high sensitivity methods capable of accurately defining
whether or not the CSF is colonized by leukemic cells not only offers a refined diagnostic/prognostic work-up, but also helps to personalize CNS prophylaxis through the early identification of patients who may benefit from more aggressive approaches.

With the limitations of its retrospective nature, the results of our study demonstrate that in adult patients with ALL, FCM allows to more precisely identify and quantify the number of patients with a CNS involvement at diagnosis and that this impacts significantly on the clinical course and outcome of the disease, thus enabling a further refinement of the current diagnostic risk-stratification process. This refined CNS evaluation should become a routine tool for the work-up of ALL patients at presentation. Further and larger prospective studies are needed to further standardize the procedures and permit an optimal clinical application of this technique.

**Acknowledgments**

The authors would like to thank all participants of the Campus ALL program.
References


Table 1. Clinical characteristics of patients according to the CNS status

<table>
<thead>
<tr>
<th></th>
<th>ALL</th>
<th>CNS\textsuperscript{neg}</th>
<th>OCNSD\textsuperscript{pos}</th>
<th>MCNSD\textsuperscript{pos}</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>240</td>
<td>179</td>
<td>43</td>
<td>18</td>
<td></td>
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<tr>
<td>Sex (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F</td>
<td>103 (42.9)</td>
<td>76 (42.5)</td>
<td>20 (46.5)</td>
<td>7 (38.9)</td>
<td>0.835</td>
</tr>
<tr>
<td>M</td>
<td>137 (57.1)</td>
<td>103 (57.5)</td>
<td>23 (53.5)</td>
<td>11 (61.1)</td>
<td></td>
</tr>
<tr>
<td>Age (median [range])</td>
<td></td>
<td>45.00 [17.00, 80.00]</td>
<td>45.00 [17.00, 80.00]</td>
<td>46.00 [17.00, 72.00]</td>
<td>36.50 [18.00, 73.00]</td>
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<tr>
<td>Lineage (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>184 (76.7)</td>
<td>140 (78.2)</td>
<td>34 (79.1)</td>
<td>10 (55.6)</td>
<td>0.088</td>
</tr>
<tr>
<td>T</td>
<td>56 (23.3)</td>
<td>39 (21.8)</td>
<td>9 (20.9)</td>
<td>8 (44.4)</td>
<td></td>
</tr>
<tr>
<td>WBC (median [range])</td>
<td></td>
<td>11000.00 [140.00, 573000.00]</td>
<td>11300.00 [140.00, 573000.00]</td>
<td>10600.00 [1440.00, 291500.00]</td>
<td>9400.00 [400.00, 133840.00]</td>
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<td>Cytogenetic (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Abnormal</td>
<td>118 (64.5)</td>
<td>91 (63.6)</td>
<td>20 (69.0)</td>
<td>7 (63.6)</td>
<td>0.860</td>
</tr>
<tr>
<td>Normal</td>
<td>65 (35.5)</td>
<td>52 (36.4)</td>
<td>9 (31.0)</td>
<td>4 (36.4)</td>
<td></td>
</tr>
<tr>
<td>Treatment (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Conventional</td>
<td>91 (37.9)</td>
<td>70 (39.1)</td>
<td>15 (34.9)</td>
<td>6 (33.3)</td>
<td>0.400</td>
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<tr>
<td>Intensified</td>
<td>120 (50.0)</td>
<td>85 (47.5)</td>
<td>23 (53.5)</td>
<td>12 (66.7)</td>
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<tr>
<td>Reduced</td>
<td>29 (12.1)</td>
<td>24 (13.4)</td>
<td>5 (11.6)</td>
<td>0 (0.0)</td>
<td></td>
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<td>LDH (median [range])</td>
<td></td>
<td>482.00 [21.00, 8332.00]</td>
<td>478.00 [21.00, 8332.00]</td>
<td>555.50 [55.00, 8332.00]</td>
<td>372.50 [180.00, 4086.00]</td>
</tr>
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<td>CSF WBC (median [range])</td>
<td></td>
<td>1.00 [0.00, 3000.00]</td>
<td>1.00 [0.00, 17.00]</td>
<td>1.00 [0.00, 7.00]</td>
<td>39.00 [7.00, 3000.00]</td>
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<tr>
<td>CSF proteins (median [range])</td>
<td></td>
<td>36.50 [5.90, 326.00]</td>
<td>35.00 [5.90, 94.00]</td>
<td>38.50 [16.00, 161.00]</td>
<td>51.00 [23.00, 326.00]</td>
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</tbody>
</table>

CNS\textsuperscript{neg}: CSF samples negative by both FCM and CC; OCNSD\textsuperscript{pos}: CSF samples positive by FCM and negative by CC; MCNSD\textsuperscript{pos}: CSF positive by both FCM and CC; WBC: white blood cells; LDH: lactate dehydrogenase; CSF: cerebrospinal fluid.
### Table 2 Correlation between CNS status and outcome

<table>
<thead>
<tr>
<th></th>
<th>level</th>
<th>ALL</th>
<th>CNS\textsuperscript{neg}</th>
<th>OCNSD\textsuperscript{pos}</th>
<th>MCNSD\textsuperscript{pos}</th>
<th>p</th>
</tr>
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<td>n</td>
<td>240</td>
<td>179</td>
<td>43</td>
<td>18</td>
<td></td>
</tr>
<tr>
<td>Hematological response (%)</td>
<td>CR</td>
<td>198 (85.3)</td>
<td>152 (87.4)</td>
<td>32 (80.0)</td>
<td>14 (77.8)</td>
<td>0.317</td>
</tr>
<tr>
<td></td>
<td>No CR</td>
<td>34 (14.7)</td>
<td>22 (12.6)</td>
<td>8 (20.0)</td>
<td>4 (22.2)</td>
<td></td>
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<tr>
<td>ASCT (%)</td>
<td>No</td>
<td>88 (44.9)</td>
<td>65 (44.2)</td>
<td>17 (47.2)</td>
<td>6 (46.2)</td>
<td>0.944</td>
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<td></td>
<td>Yes</td>
<td>108 (55.1)</td>
<td>82 (55.8)</td>
<td>19 (52.8)</td>
<td>7 (53.8)</td>
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<tr>
<td>Relapse (%)</td>
<td>No</td>
<td>78 (40.2)</td>
<td>70 (47.0)</td>
<td>7 (22.6)</td>
<td>1 (7.1)</td>
<td>0.001</td>
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<tr>
<td></td>
<td>Yes</td>
<td>116 (59.8)</td>
<td>79 (53.0)</td>
<td>24 (77.4)</td>
<td>13 (92.9)</td>
<td></td>
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<tr>
<td>Relapse site (%)</td>
<td>CNS</td>
<td>16 (16.8)</td>
<td>8 (12.7)</td>
<td>7 (31.8)</td>
<td>1 (10.0)</td>
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<td></td>
<td>BM</td>
<td>79 (83.2)</td>
<td>55 (87.3)</td>
<td>15 (68.2)</td>
<td>9 (90.0)</td>
<td></td>
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<tr>
<td>OS 3 years estimate (95% CI)</td>
<td>46.4 (40.1-53.8)</td>
<td>52.9 (45.5-61.5)</td>
<td>31.1 (19.2-50.5)</td>
<td>22.2 (9.4-52.7)</td>
<td>&lt;0.001</td>
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<tr>
<td>DFS 3 years estimate (95% CI)</td>
<td>34.3 (27.9-42.2)</td>
<td>38.6 (31.48)</td>
<td>20.6 (10.2-41.9)</td>
<td>21.4 (7.9-58.4)</td>
<td>0.005</td>
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CNS\textsuperscript{neg}: CSF samples negative by both FCM and CC; OCNSD\textsuperscript{pos}: CSF samples positive by FCM and negative by CC; MCNSD\textsuperscript{pos}: CSF positive by both FCM and CC; WBC: white blood cells; ASCT: Allogeneic Stem Cell Transplant; CR: complete remission; OS: overall survival; DFS: disease free survival; CI: confidential interval
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<th></th>
<th>Univariate analysis</th>
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<th>Multivariate analysis</th>
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<td>HR</td>
<td>Lower 95% CI</td>
<td>Higher 95% CI</td>
<td>P</td>
<td>HR</td>
<td>Lower 95% CI</td>
<td>Higher 95% CI</td>
<td>P</td>
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<td>Lineage T vs B</td>
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<td>Normal vs Abnormal</td>
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<td>Treatment:</td>
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<td>OCNSD\textsuperscript{pos} vs. CNS\textsuperscript{neg}</td>
<td>1.915</td>
<td>1.259</td>
<td>2.913</td>
<td>0.0024</td>
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<td>1.333</td>
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<td>MCNSD\textsuperscript{pos} vs. CNS\textsuperscript{neg}</td>
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<td>1.73</td>
<td>4.817</td>
<td>&lt;.0001</td>
<td>3.392</td>
<td>2.015</td>
<td>5.71</td>
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</table>

HR: hazard ratio; CI: confidential interval; CNS\textsuperscript{neg}: CSF samples negative by both FCM and CC; OCNSD\textsuperscript{pos}: CSF samples positive by FCM and negative by CC; MCNSD\textsuperscript{pos}: CSF positive by both FCM and CC; WBC: white blood cells count; ASCT: Allogeneic Stem Cell Transplant; WBC: white blood cells; LDH: lactate dehydrogenase; CSF: cerebrospinal fluid.
**Legend to Figure 1:** Flow cytometry detection of occult CNS involvement in a patient with B lineage acute lymphoblastic leukemia. The blast population is depicted in grey which denotes a cluster of few cells CD19 (plot c) and CD10 (plot d) positive, and CD34, CD22 negative (plots e-f) and CD20 weak (plot f).

**Legend to Figure 2:** DFS based on the CNS status. Kaplan-Meier plot comparing DFS of patients CNS\(^{\text{neg}}\), occult OCNSD\(^{\text{pos}}\) and MCNSD\(^{\text{pos}}\).

**Legend to Figure 3:** OS based on the CNS status. Kaplan-Meier plot comparing OS of patients CNS\(^{\text{neg}}\), OCNSD\(^{\text{pos}}\) and MCNSD\(^{\text{pos}}\).
Overall p-value = 0.005

OCNSD$^{pos}$ vs. MCNSD$^{pos}$ p = 0.3
Overall p-value < .0001

OCNSD<sup>pos</sup> vs. MCNSD<sup>pos</sup> p = 0.2
### Table S1. Immunophenotype Panel

<table>
<thead>
<tr>
<th>FLUOROCHROMES</th>
<th>FITC</th>
<th>PE</th>
<th>PERCPCy5.5</th>
<th>PE CY7</th>
<th>APC</th>
<th>APC CY7</th>
<th>V450</th>
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<tr>
<td><strong>B LINEAGE</strong></td>
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<td>CD38</td>
<td>CD19</td>
<td>CD34</td>
<td>CD20</td>
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<tr>
<td><strong>T LINEAGE</strong></td>
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<td>CD99</td>
<td>CD3</td>
<td>CD4</td>
<td>CD1</td>
<td>CD8</td>
<td>CD5</td>
<td>CD45</td>
</tr>
</tbody>
</table>

FITC: Fluorescein Isothiocyanate; PE: Phycoerythrin; PERCP Cy5.5: Petidinin chlorophyll protein cyanine 5.5; PE Cy7: Phycoerythrin cyanine-7; APC: allophycocyanin; APC Cy7: allophycocyanin cyanina7
**Statistical analysis**

Comparisons between groups were performed to assess differences in biologic and clinical data using the Chi-squared test or Fisher’s exact test for categorical data and the Mann-Whitney and Kruskal-Wallis tests in case of continuous variables. OS (time elapsed from therapy start to death) and disease-free survival (DFS - time elapsed from complete remission to relapse or death in remission) were calculated using the Kaplan-Meier method. Multivariate analysis was performed according to the Cox model. All tests were 2-sided, accepting p <0.05 as indicating a statistically significant difference and confidence intervals were calculated at 95% level. All analyses were performed using SAS, release 9.4.