haematologica
Journal of The Ferrata Storti Foundation

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, Mariagiovanna 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

Haematologica 2019 [Epub ahead of print]

Citation: 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, Mariagiovanna 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. Clinical significance of occult central nervous system disease in adult acute lymphoblastic leukemia. A multicenter report from the campus all network. Haematologica. 2019; 104:xxx doi:10.3324/haematol.2019.231704

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

Maria Ilaria Del Principe[1], Elisa Buzzatti[1], Alfonso Piciocchi[2], Fabio Forghieri[3], Massimiliano Bonifacio[4], Federica Lessi[5], Silvia Imbergamo[5], Enrico Orciuolo[6], Giovanni Rossi[7], Nicola Fracchiolla[8], Silvia Trappolini[9], Benedetta Neri[10], Chiara Sarlo[11], Patrizia Zappasodi[12], Michelina Dargenio[13], Mariagiovanna Cefalo[10], Maria Antonietta Irno-Consalvo[1], Consuelo Conti[14], Giovangiacinto Paterno[1], Gottardo De Angelis[1], Mariarita Sciumè[8], Irene Della Starza[15], Adriano Venditti[1], Robin Foà[15], Anna Rita Guarini[16]

[1]Ematologia, Dipartimento di Biomedicina e Prevenzione, Università degli Studi di Roma "Tor Vergata", Roma; [2]GIMEMA Data Center, Roma; [3] Ematologia Dipartimento di Scienze Mediche e Chirurgiche, Università degli Studi di Modena e Reggio Emilia, Azienda Ospedaliera di Modena, Modena; [4]Dipartimento di Medicina, Sezione di Ematologia, Università di Verona, Verona; [5]Ematologia ed Immunologia Clinica, Azienda Ospedaliera di Padova, Padova; [6]UO Ematologia Univ, Azienda Ospedaliero-Universitaria Pisana, Pisa; [7]U.O. di Ematologia e Trapianto di Cellule Staminali, IRCCS "Casa Sollievo della Sofferenza", San Giovanni Rotondo, Foggia; [8]UOC di Ematologia, Fondazione IRCCS Ca' Granda, Ospedale Maggiore Policlinico, Milano; [9]Clinica di Ematologia, AOU Ospedali Riuniti di Ancona, Ancona; [10]Ematologia, Ospedale Sant'Eugenio, Dipartimento di Biomedicina e Prevenzione, Università degli Studi di Roma "Tor Vergata", Roma; [11]Ematologia, Policlinico Universitario-Campus Biomedico, Roma; [12]Divisione di Ematologica, Fondazione IRCCS Policlinico San Matteo, Università di Pavia, Pavia; [13]Ematologia e Trapianto di Cellule Staminali, Ospedale Vito Fazzi, Lecce; [14]Ematologia, Dipartimento di Onco-Ematologia, Fondazione Policlinico Tor Vergata, Roma; [15]Ematologia, Dipartimento di Medicina di Precisione e Traslazionale, Università "Sapienza", Roma; [16]Dipartimento di Medicina Molecolare, Università "Sapienza", Roma, Italy.

Presented orally in part at the 60th Annual Meeting of the American Society of Hematology, San Diego, CA, December 1-4, 2018

Correspondence to: Maria Ilaria Del Principe, MD, UOSD Mieloproliferative, Fondazione Policlinico Tor Vergata, Viale Oxford 81, 00133 Roma, Italy
Phone: +390620903219; Fax +390620903246; Email: del.principe@med.uniroma2.it

Word count

Abstract: 251 Main text: 2023

Tables: 4 Figures: 3

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 ≥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).[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,[4-6] which translates into a shorter overall survival (OS) duration compared to that of patients without CNS involvement.[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.[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.[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.[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)[14] or NILG (NILG-ALL10/07)[15,16] protocols or the Hyper-CVAD/MTX-ARAC regimen.[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,[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.[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.[19,20] CC positivity was defined as unequivocal, morphologic evidence of leukemic blast in the CSF and/or a CSF WBC count ≥5/µl with less than 10 erythrocytes/µl.[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.[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[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 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^{neg}), while 43 (18%) were OCNSD positive (positive by FCM and negative by CC = $OCNSD^{pos}$) and 18 (7%) were positive by both FCM and CC (manifest CNS disease positive = $MCNSD^{pos}$) (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^{neg}, $OCNSD^{pos}$ and $MCNSD^{pos}$ 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 $OCNSD^{pos}$ and $MCNSD^{pos}$ 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 $OCNSD^{pos}$ and $MCNSD^{pos}$ patients than in CNS^{neg} patients (Table 2) ($p=0.001$). The 3-year DFS was also significantly longer in CNS^{neg}

patients compared to $OCNSD^{pos}$ or $MCNSD^{pos}$ 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) ***(Table 2)***. On the contrary, the 3-year DFS was not different between $OCNSD^{pos}$ and $MCNSD^{pos}$ patients (p=0.3) (Figure 2).

The 3-year OS in CNS^{neg}, $OCNSD^{pos}$ and $MCNSD^{pos}$ 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 $OCNSD^{pos}$ and $MCNSD^{pos}$ patients (p=0.2) (Figure 3).

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 $OCNSD^{pos}$ (HR=1.82, 95% CI 1.15 to 5.92 p=.01) or $MCNSD^{pos}$ 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).

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. [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.[24] FCM allows to detect an hematologic disease in CSF specimens even when the cellularity is very low.[9,25] This peculiarity has been confirmed in pediatric ALL patients where FCM was able to substantially

ameliorate recognition of occult CSF involvement.[26-28] In agreement with pediatric reports[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.[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.[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.[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.[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[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.[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[34-36] can be responsible of disease recurrence once the leukemic cells, surviving systemic chemotherapy within the CNS sanctuary, migrate to the circulation.[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

1) Thomas X, Boiron JM, Huguet F, et al. Outcome of treatment in adults with acute lymphoblastic leukemia: Analysis of LALA-94 trial. J Clin Oncol. 2004;22(20):4075-4086.
2) Kantarjian HM, O'Brien S, Smith TL, et al. Results of treatment with Hyper-CVAD, a dose-intensive regimen in adult acute lymphocytic leukemia. J Clin Oncol. 2000;18(3):547-561.
3) Thomas X, Le QH. Central Nervous system involvement in adult acute lymphoblastic leukemia. Hematology. 2008;13(5):293-302.
4) Lazarus HM, Richards SM, Chopra R, et al. Medical Research Council (MRC)/National Cancer Research Institute (NCRI) Adult Leukaemia Working Party of the United Kingdom and the Eastern Cooperative Oncology Group. Central Nervous system involvement in adult acute lymphoblastic leukemia at diagnosis: results from international ALL trial MRC UKALL XII/ECOG E2993. Blood. 2006;108(2):65-72.
5) Jabbour E, Thomas D, Cortes J, Kantarjian H, O'Brien S. Central Nervous System Prophylaxis in Adults with Acute Lymphoblastic Leukemia. Cancer. 2010;116(10):2290-2300.
6) Larson RA. Managing CNS disease in adults with acute lymphoblastic leukemia. Leuk Lymphoma. 2018;59:3-13.
7) Bromberg JE, Breems DA, Kraan J, et al. CSF flow cytometry greatly improves diagnostic accuracy in CNS hematologic malignancies. Neurology. 2007;68(20):1674-1679.
8) Kaplan JG, , DeSouza TG, Farkash A, et al. Leptomeningeal metastases: comparison of clinical features and laboratory data of solid tumors, lymphomas and leukemias. J Neurooncol. 1990;9(3):225-229.
9) Craig F, Foon KA. Flow cytometric immunophenotyping for hematologic neoplasms. Blood. 2008;111(8):3941-3967.
10) Quijano S, Lopez A, Manuel Sancho J, et al. Spanish Group for the Study of CNS disease in NHL. Identification of leptomeningeal disease in aggressive B-cell non Hogkin's lymphoma: improved sensitivity of flow cytometry. J Clin Oncol. 2009;27(9):1462-1469.
11) de Graaf MT, de Jongste AH, Kraan J, Boonstra JG, Sillevis Smitt PA, Gratama JW. Flow cytometric characterization of cerebrospinal fluid cells. Cytometry B Clin Cytom. 2011;80(5):271-281.
12) Zeiser R, Burger JA, Bley TA, Windfuhr-Blum M, Schulte-Monting J, Behringer DM. Clinical follow-up indicates differential accuracy of magnetic resonance imaging and immunocytology of the cerebral spinal fluid for the diagnosis of neoplastic meningitis-a single centre experience. Br J Haematol. 2004;124(6):762-768.

13) Di Noto R, Scalia G, Abate G, et al. Critical role of multidimensional flow cytometry in detecting occult leptomeningeal disease in newly diagnosed aggressive B-cell lymphomas. Leuk Res. 2008:32(8):1196-1199.
14) Annino L, Vignetti M, Paoloni FP, et al. Treatment of adolescents and young adults with acute lymphoblastic leukemia (ALL): an update of the GIMEMA experience. Blood. 2009;114(22):3097.
15) Bassan R, Masciulli A, Intermesoli T, et al. Randomizad trial of radiation-free central nervous system prophylaxis comparing intrathecal triple therapy with liposomal cytarabine in acute lymphoblastic leukemia. Haematologica. 2015;100(6):786-793.
16) Bassan R, Masciulli A, Intermesoli T, et al. Final Results of Northern Italy Leukemia Group (NILG) Trial 10/07 Combining Pediatric-Type Therapy with Minimal Residual Disease Study and Risk-Oriented Hematopoietic Cell Transplantation in Adult Acute Lymphoblastic Leukemia (ALL) Blood. 2016;128(22):176.
17) Kantarjian HM, O'Brien S, Smith TL, et al. Results of treatment with hyper-CVAD, a dose-intensive regimen, in adult acute lymphocytic leukemia. J Clin Oncol. 2000;18(3):547-561.
18) Kantarjian H, Thomas D, O'Brien S, et al. Long-term follow-up results of hyperfractionated cyclophosphamide, vincristine, doxorubicin, and dexamethasone (Hyper-CVAD), a dose-intensive regimen, in adult acute lymphocytic leukemia. Cancer. 2004;101(12):2788-2801.
19) Del Principe MI, Buccisano F, Cefalo M, et al. High sensitivity of flow cytometry improves detection of occult leptomeningeal disease in acute lymphoblastic leukemia and lymphoblastic lymphoma. Ann Hematol. 2014;93(9):1509-1513.
20) Del Principe MI, Buccisano F, Soddu S, et al. Involvement of central nervous system in adult patients with acute myeloid leukemia: incidence and impact on outcome. Semin Hematol. 2018;55(4):209-214.
21) Mahmoud HH, Rivera GK, Hancock ML, et al. Low leukocyte counts with blast cells in cerebrospinal fluid of children with newly diagnosed acute lymphoblastic leukemia. N Engl J Med. 1993;329(5):314-319.
22) Roma A, Garcia A, Avagnina A, Rescia C, Elsner B. Lymphoid and myeloid neoplasms involving cerebrospinal fluid: comparison of morphologic examination and imunophenotyping by flow cytometry. Diagn Cytopathol. 2002;27(5):271-275.
23) Mitri Z, Siddiqui MT, EL Rassi F, et al. Sensitivity and specificity of cerebral fluid flow cytometry for the diagnosis of leukemic meningitis in acute lymphoblastic leukemia/lymphoma. Leuk Lymphoma. 2014;55(7):1498-1500.

24) Wilson W, Bromberg J, Stetler-Stevenson M, et al. Detection and outcome of occult leptomeningeal disease in diffuse large B-cell lymphoma and Burkitt Lymphoma. Haematologica. 2014;99(7):1228-1235.
25) Nuckel H, Novotny JR, Noppeney R, Savidou I, Duhrsen U. Detection of malignant haematopoietic cells in the cerebrospinal fluid by conventional cytology and flow cytometry. Clin Lab Haem. 2006;28(1):22-29.
26) Sayed D, Badrawy H, Ali AM, Shaker S. Immunophenotyping .and immunoglobulin heavy chain gene rearrangement analysis in cerebrospinal fluid of pediatric patients with acute lymphoblastic leukemia. Leuk Res. 2009;33(5):655-661.
27) Cancela CSP, Murao M, Assumpção JG, et al. Immunophenotyping of the cerebrospinal fluid as a prognostic factor at diagnosis of acute lymphoblastic leukemia in children and adolescents. Pediatr Hematol Oncol. 2017;34(2):53-65.
28) Ranta S, Nilsson F, Harila-Saari A, et al. Detection of central nervous system involvement in childhood acute lymphoblastic leukemia by cytomorphology and flow cytometry of the cerebrospinal fluid. Pediatr Blood Cancer. 2015;62(6):951-956.
29) Martinez-Laperche C, Gomez-Garcia AM, Lassaletta A, et al. Detection of occult cerebrospinal fluid involvement during maintenance therapy identifies a group of children with acute lymphoblastic leukemia at high risk for relapse. Am J Hematol. 2013;88(5):360-365.
30) Benevolo G, Stacchini A, Spina M, et al. Final results of a multicenter trial addressing role of CSF flow cytometric analysis in NHL patients at high risk for CNS dissemination. Blood. 2012;120(16):3222-3228.
31) Subira D, Castanon S, Roman A, et al. Flow cytometry and the study of central nervous disease in patients with acute leukaemia. Br J Haematol. 2001;112(2):381-384.
32) Alvarnas JC , Brown PA, Aoun P, et al. Acute Lymphoblastic Leukemia, Version 2.2015. J Natl Compr Canc Netw. 2015;13(10):1240-1279.
33) Arber DA, Borowitz MJ, Cessna M, et al. Initial Diagnostic Workup of Acute Leukemia: Guideline From the College of American Pathologists and the American Society of Hematology. Arch Pathol Lab Med. 2017;141(10):1342-1393.
34) Akers SM, O'Leary HA, Minnear FL, et al. VE-cadherin and PECAM-1 enhance ALL migration across brain microvascular endothelial cell monolayers. Exp Hematol. 2010;38(9):733-743.
35) Akers SM, Rellick SL, Fortney JE, Gibson LF. Cellular elements of the subarachnoid space promote ALL survival during chemotherapy. Leuk Res. 2011;35(6):705-711.

36) van der Velden VH, de Launaij D, de Vries JF, et al. New cellular markers at diagnosis are associated with isolated central nervous system relapse in paediatric B-cell precursor acute lymphoblastic leukaemia. Br J Haematol. 2016;172(5):769-781.

37) Frishman-Levy L, Izraeli S. Advances in understanding the pathogenesis of CNS acute lymphoblastic leukaemia and potential for therapy. Br J Haematol. 2017;176(2):157-167.

38) Pui CH, Howard SC. Current management and challenges of malignant disease in the CNS in paediatric leukaemia. Lancet Oncol. 2008;9(3):257-268.

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

	level	ALL	CNS^{neg}	$OCNSD^{pos}$	$MCNSD^{pos}$	p
n		240	179	43	18	
Sex (%)	**F**	103 (42.9)	76 (42.5)	20 (46.5)	7 (38.9)	0.835
	M	137 (57.1)	103 (57.5)	23 (53.5)	11 (61.1)	
Age (median [range])		45.00 [17.00, 80.00]	45.00 [17.00, 80.00]	46.00 [17.00, 72.00]	36.50 [18.00, 73.00]	0.302
Lineage (%)	**B**	184 (76.7)	140 (78.2)	34 (79.1)	10 (55.6)	0.088
	T	56 (23.3)	39 (21.8)	9 (20.9)	8 (44.4)	
WBC (median [range])		11000.00 [140.00, 573000.00]	11300.00 [140.00, 573000.00]	10600.00 [1440.00, 291500.00]	9400.00 [400.00, 133840.00]	0.799
Cytogenetic (%)	**Abnormal**	118 (64.5)	91 (63.6)	20 (69.0)	7 (63.6)	0.860
	Normal	65 (35.5)	52 (36.4)	9 (31.0)	4 (36.4)	
Treatment (%)	**Conventional**	91 (37.9)	70 (39.1)	15 (34.9)	6 (33.3)	0.400
	Intensified	120 (50.0)	85 (47.5)	23 (53.5)	12 (66.7)	
	Reduced	29 (12.1)	24 (13.4)	5 (11.6)	0 (0.0)	
LDH (median [range])		482.00 [21.00, 8332.00]	478.00 [21.00, 8332.00]	555.50 [55.00, 5532.00]	372.50 [180.00, 4086.00]	0.806
CSF WBC (median [range])		1.00 [0.00, 3000.00]	1.00 [0.00, 17.00]	1.00 [0.00, 7.00]	39.00 [7.00, 3000.00]	<0.001
CSF proteins (median [range])		36.50 [5.90, 326.00]	35.00 [5.90, 94.00]	38.50 [16.00, 161.00]	51.00 [23.00, 326.00]	0.023

CNS^{neg}: CSF samples negative by both FCM and CC; $OCNSD^{pos}$: CSF samples positive by FCM and negative by CC; $MCNSD^{pos}$: 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

	level	ALL	CNS^{neg}	$OCNSD^{pos}$	$MCNSD^{pos}$	p
n		240	179	43	18	
Hematological response (%)	**CR**	198 (85.3)	152 (87.4)	32 (80.0)	14 (77.8)	0.317
	No CR	34 (14.7)	22 (12.6)	8 (20.0)	4 (22.2)	
ASCT (%)	**No**	88 (44.9)	65 (44.2)	17 (47.2)	6 (46.2)	0.944
	Yes	108 (55.1)	82 (55.8)	19 (52.8)	7 (53.8)	
Relapse (%)	**No**	78 (40.2)	70 (47.0)	7 (22.6)	1 (7.1)	0.001
	Yes	116 (59.8)	79 (53.0)	24 (77.4)	13 (92.9)	
Relapse site (%)	**CNS**	16 (16.8)	8 (12.7)	7 (31.8)	1 (10.0)	0.099
	BM	79 (83.2)	55 (87.3)	15 (68.2)	9 (90.0)	
OS 3 years	**estimate (95% CI)**	46.4(40.1-53.8)	52.9(45.5-61.5)	31.1(19.2-50.5)	22.2(9.4-52.7)	<0.001
DFS 3 years	**estimate (95% CI)**	34.3(27.9-42.2)	38.6(31-48)	20.6(10.2-41.9)	21.4(7.9-58.4)	0.005

CNS^{neg}: CSF samples negative by both FCM and CC; $OCNSD^{pos}$: CSF samples positive by FCM and negative by CC; $MCNSD^{pos}$: 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

Table 3. Univariate and multivariate analysis of all variables associated with survival

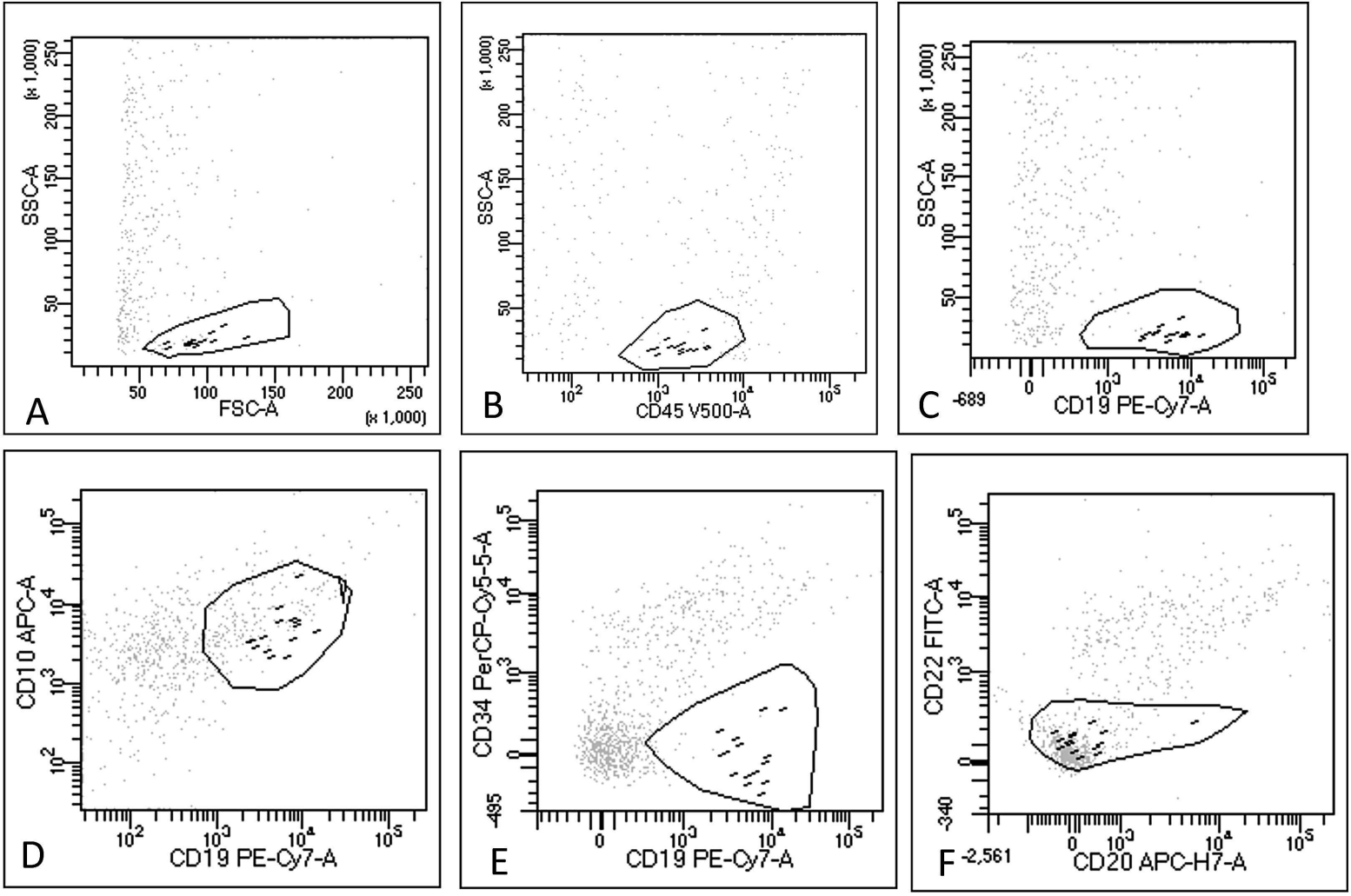
	Univariate analysis				Multivariate analysis			
	HR	**Lower 95%CI**	**Higher 95%CI**	**p**	**HR**	**Lower 95%CI**	**Higher 95%CI**	**P**
Age	1.01	1	1.03	0.0062				
Sex: M vs F	1.044	0.739	1.474	0.8076				
Lineage T vs B	0.957	0.6367	14.375	0.8313				
WBC	1	1	1	0.3764				
Cytogenetic: Normal vs Abnormal	1.15	0.76	1.74	0.5101				
Treatment: Conventional vs intensified	0.61	0.42	0.88	0.0089	0.584	0.403	0.848	0.0047
Treatment: Conventional vs reduced	1.47	0.89	2.44	0.1295	1.708	1.027	2.842	0.0393
LDH	1	1	1	0.908				
CSF_WBC	1	1	1.001	0.5719				
CSF_proteins	1.008	1.002	1.013	0.0071				
ASCT yes vs no	0.564	0.387	0.823	0.0029				
OCNSD^{pos} vs. CNS^{neg}	1.915	1.259	2.913	0.0024	2.03	1.333	3.093	0.001
MCNSD^{pos} vs. CNS^{neg}	2.887	1.73	4.817	<.0001	3.392	2.015	5.71	<.0001

HR: hazard ratio; CI: confidential interval**;** CNS^{neg}: CSF samples negative by both FCM and CC; OCNSD^{pos}: CSF samples positive by FCM and negative by CC; MCNSD^{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.

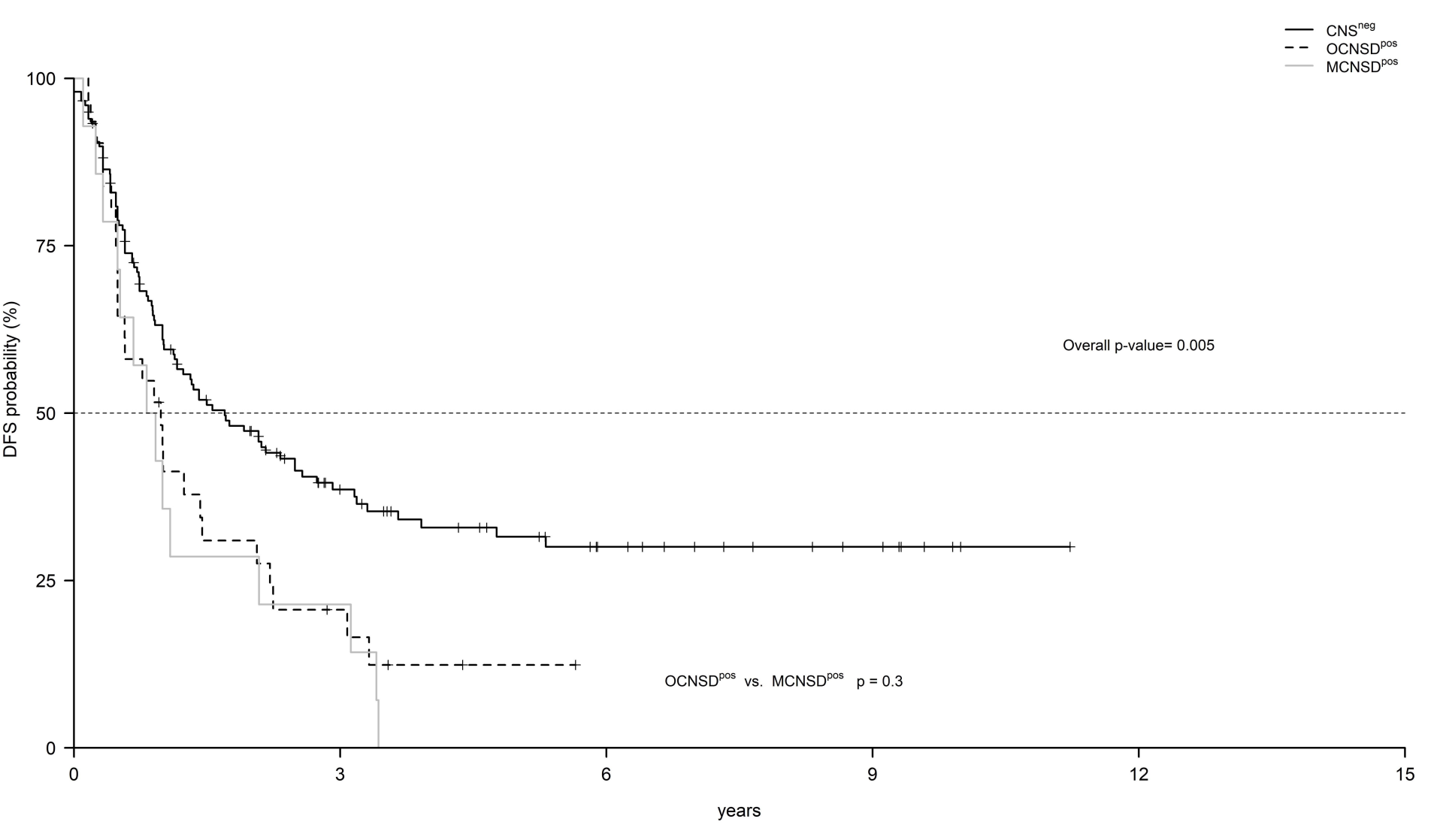
Legend to Figure 1: Flow cytometry detection of occult CNS involvement in a patient with B lineage acute lymphoblastic leukemia. The blast population is depicted in grey which denotes a cluster of few cells CD19 (plot c) and CD10 (plot d) positive, and CD34, CD22 negative (plots e-f) and CD20 weak (plot f)

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

Legend to Figure 3: OS based on the CNS status. Kaplan-Meier plot comparing OS of patients CNS^{neg}, $OCNSD^{pos}$ and $MCNSD^{pos}$.



Population	#Events	%Total
BLAST POPULATION	15	1.5593


CNS^neg
OCNSD^pos
MCNSD^pos
100
75
50
25
0
DFS probability (%)
Overall p-value= 0.005
OCNSD^pos vs. MCNSD^pos p = 0.3
0
3
6
9
12
15
years

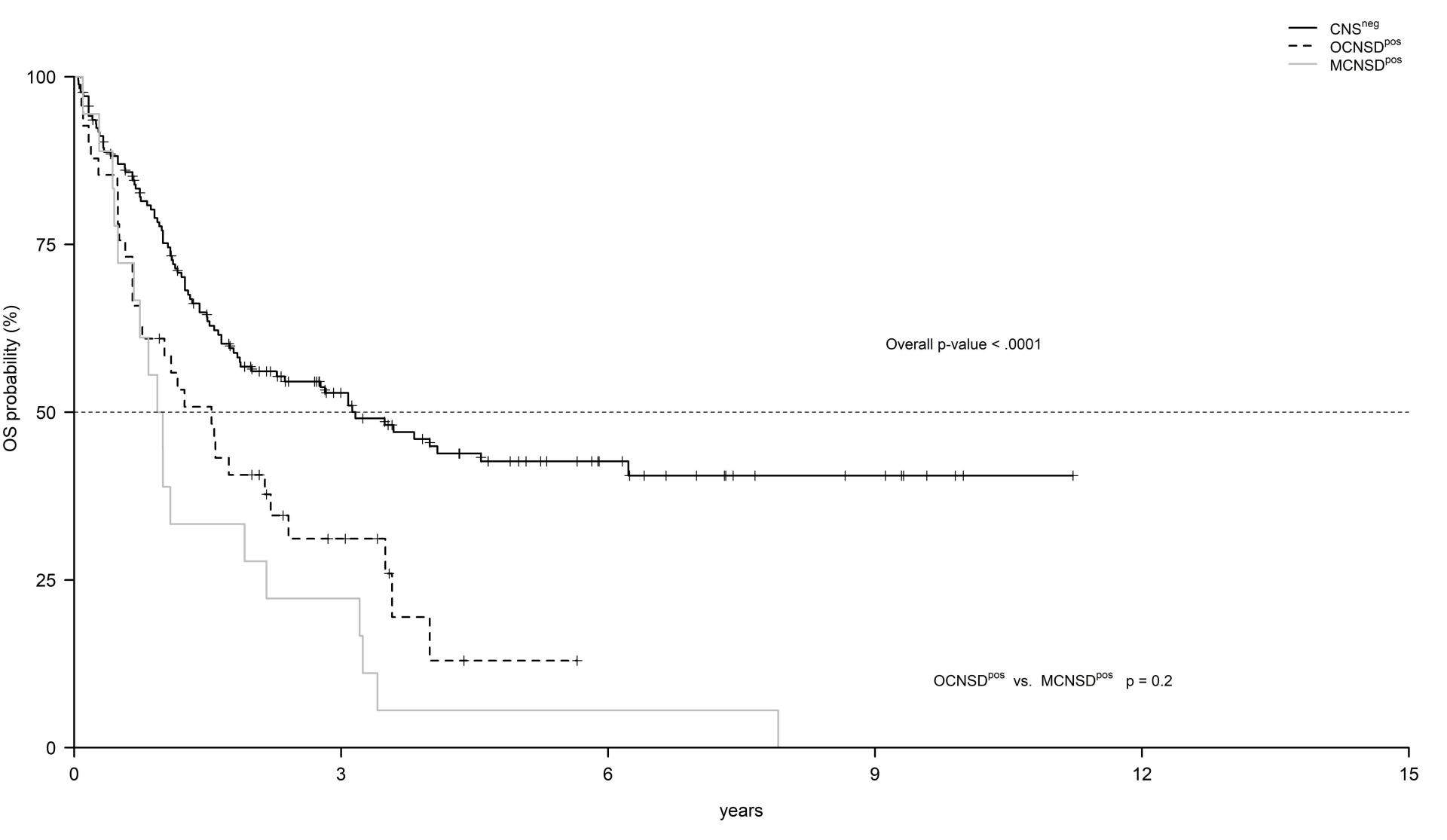

CNS^neg
OCNSD^pos
MCNSD^pos
100
75
50
25
0
OS probability (%)
Overall p-value < .0001
OCNSD^pos vs. MCNSD^pos p = 0.2
0
3
6
9
12
15
years

Table S1. Immunophenotype Panel

FLUOROCHROMES	**FITC**	**PE**	**PERCPCy5.5**	**PE CY7**	**APC**	**APC CY7**	**V450**	**V500**
B LINEAGE	CD10	CD22 or CD58	CD38	CD19	CD34	CD20	-	CD45
T LINEAGE	CD7 or CD2	CD99	CD3	CD4	CD1	CD8	CD5	CD45

FITC: Fluorescein Isothiocyanate; PE: Phycoerythrin; PERCP Cy5.5: Petidinin chlorophyll protein cyanine 5.5; PECy7: Phycoerythrin cyanine-7;; APC: allophycocyanin; APCCy7: 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.