Clinical significance of occult Central Nervous System localization in adult patients with acutelymphoblastic leukemia. Prospective, multicenter study.
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1.0 Introduction.

In recent decades, several clinical trials have led to increased responses in adult patients with acute lymphoblastic leukemia (ALL).1-3 In this context of improved control of systemic disease, leukemic involvement of the central nervous system (CNS) remains a major factor in limiting the achievement of cure and a major cause of mortality. Leukemic CNS involvement can occur at onset or at relapse. At onset, about 5% of adult patients with ALS have CNS localization, and the overall survival of these patients is significantly shorter than those without such involvement.4,5

Currently, the gold standard for diagnosing CNS involvement in ALS is morphologic examination of the cerebrospinal fluid (CSF). Conventional cytology (CC) is estimated to have >95% specificity for the diagnosis of leukemic localization in the CNS. Unfortunately, however, this method is burdened with low sensitivity (<50%) and consequently is often falsely negative. The low sensitivity of CC is due to the paucity of cells in the CSF and morphological similarities that make it difficult to distinguish benign from neoplastic cells.6,7

Phenotypic study with flow cytofluorimetry (FCM) is a valuable tool for the diagnosis and staging of hematologic disorders involving lymph nodes, peripheral blood, and bone marrow. Over the years, FCM has been implemented in such a way as to allow the detection of abnormal cells representing 0.01% of events (1 cell in 104), the so-called "rare events," and is therefore a useful tool for monitoring minimal residual disease in acute leukemia.

Several recently published studies focused on determining involvement in the CNS during LLA and aggressive early-onset non-Hodgkin lymphomas have demonstrated the superior sensitivity of FCM compared with CC.9,10 In contrast, it is not yet clear whether FCM positivity alone, which can be defined as occult CNS localization, has a real clinical impact or not.

In our previous retrospective study, we evaluated the incidence of occult CNS localization and the impact of such localization on the prognosis of adult patients with ALS at onset. We collected data, between 2007 and 2017, from 240 patients, 103 women and 137 men, median age 44 years (range 17-80). One hundred eighty-four patients (77%) had a B phenotype. Patients were treated according to GIMEMA/NILG protocols or according to the Hyper-CVAD program. One hundred and eight

categories of patients were distinguished: patients with overt CNS localization(CC+FCM+), patients with occult CNS localization (CC-FCM+), and patients with absence of disease in the CNS (SNCneg, CC-FCM-). Overall 179 (75%) patients were SNCneg, 43 (18%) had occult CNS localization and finally 18 (7%) had manifest disease. No cases of morphological positivity and cytofluorimetric negativity were observed. Patients with occult or manifest CNS localization presented higher recurrence rates (systemic and meningeal) than SNCneg patients. In addition, disease-free survival and three-year overall survival were significantly longer in patients with no CNS disease than in patients with occult or manifest CNS localization. On the other hand, no significant differences in survivals were observed between patients with occult or manifest CNS localization, confirming that even a small proportion of leukemic cells at this level can influence patients' prognosis. In multivariate analysis, status at diagnosis of occult or manifest CNS localization and age over 45 years were found to be independently associated with shorter overall survival. In conclusion, our study suggests that in adult patients with LLA, FCM allows detection of occult CNS localization, especially under conditions of leukemic cell scarcity in the CRL, and that the presence of such localization appears to be associated with poor prognosis.

However, given the heterogeneity of the treatments our patients had received and the long observation period, a prospective validation study is needed to confirm these data.

The need for further studies is confirmed by the fact that current international guidelines11-13 do not recommend the use of FCM in the assessment of CSF at onset in adult ALS patients. In addition to confirming the need to perform FCM of CSF at onset to identify patients at higher risk of recurrence, large-scale prospective studies will help to clarify whether or not patients with occult CNS localization should undergo CNS-directed therapy. Understanding this seems particularly important nowadays considering that with the introduction of new drugs (monoclonal antibodies, next-generation tyrosine kinase inhibitors, CAR-T) the therapeutic approach of patients with ALS is increasingly "chemo-free"

Clinical trial proposal and objectives

In light of this, we propose a multicenter prospective study to evaluate the incidence of occult CNS localization and the impact of such localization on clinical outcome.

2.0 Experimental design

Adult ALS patients routinely undergo diagnostic lumbar puncture (PL); CSF samples will be studied by investigation of CC and FCM at the time of the first and subsequent diagnostic PLs.

3.1 Objectives

3.1.1 Primary objective.

To assess the incidence of occult CNS localization in adult patients with ALL.

3.1.2 Secondary objectives

a. To examine associations between occult CNS localization and:

b1 overall survival (OS)

b2 recurrence-free survival (DFS)

b3 overall and medullary or extramedullary recurrence rates

b4 cumulative incidence of recurrence (CIR)

b. To evaluate the correlation between occult CNS localization and minimum residual disease (MRD) levels.

c. Determine the status of CNS occult localization also by molecular biology, comparing the results with those of FCM.

4.0 Study population.

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4.1 Sample size and duration of the study.

4.1.1.

All patients with Acute Lymphoblastic Leukemia (ALL) who underwent diagnostic lumbar puncture (PL) in the planned 12 months of enrollment will be included in the study. The sample size was calculated to assess the incidence of occult CNS localization; based on clinical experience14 in order to observe an incidence at diagnosis of 20%, with a 95% confidence interval (two-tailed test) and a width of 0.20, a number of 70 subjects was calculated as the minimum sample size15. Calculations were performed using PASS software.

4.1.2

The duration of the study will be 12 months of enrollment of the first patient and 24 months of follow-up.

4.2 Patient selection.

4.2.1 Inclusion criteria.

- Patients aged ?18 years with diagnosis of ALL at onset undergoing diagnostic-therapeutic PL.
- Signed written informed consent in accordance with ICH/EU/GCP guidelines and national and local laws.

4.2.2 Exclusion criteria.

- Patients <18 years of age
- Diagnosis other than ALL
- Inability to perform PL

5.0 Materials and methods

5.1. Methods

5.1.1 CC

Physical chemical examination and cell count and morphological examination will be performed for each CSF sample as per common clinical practice. Morphological examination of the CSF will be performed by preparation of cytocentrifugate (CC) stained with May-Grunwald-Giemsa. Manifest CNS localization will be defined in the unequivocal presence of leukemic cells in the CSF and/or a mononuclear cell count ≥5/cml.

5.1.2 FCM

For the FCM examination, which is also performed as part of common clinical practice, CSF samples of sufficient volume will have to be taken via PL. The CSF sample to be stored on ice will be processed within 1 hour of collection to avoid cell deterioration or a specific fixative (TransFix/ethylenediaminetetraacetic acid EDTA; Immunostep SL Salamanca, Spain) should be used. The CSF sample will be concentrated by centrifuging it at 300 g for 4 min. The supernatant will be gently aspirated and the pellet resuspended in PBS+BSA 0.2% in varying amounts depending on the number of tubes to be prepared (100?l/tube). One hundred ?l of the suspension will be incubated with monoclonal antibody mix of interest for 20 min in the dark. A wash with 2 ml PBS+BSA 0.2% will then be performed, centrifuge at 300 g for 3 min. The pellet will be resuspended in 700?l of PBS+BSA 0.2%. The phenotypic study will be performed using a panel of 6-8 monoclonal antibodies. The antibody panel will include: CD20-FITC, CD10-PE, CD34-PE, CD19-PerCP, CD45-APC, (Becton Dickinson, Mountain View, CA), for line B; CD3-FITC, CD2-FITC, CD7-PE, CD5-APC, CD8-PE, CD4-FITC, CD45-PerCP (Becton Dickinson, Mountain View, CA) for line T. All samples will be acquired until depletion to obtain the highest number of events. FCM positivity will be defined in the presence of ≥10 cluster-forming events and leukemic phenotype. FCM acquisition will be done locally, but 3 independent, experienced operators will review each case.

5.1.3 Ancillary study. Molecular biology.

Given the scarce data in the literature regarding the role of molecular diagnostics in the diagnosis of occult localization in the CNS, the study will include an ancillary part to evaluate this role by comparing molecular findings with FCM. The success of the molecular study will be related to the number of leukemic blasts present in the CSF, so a morphological study will be needed on the starting material that will be able to give accurate information about the material being processed. All cases will need to be processed within one hour of collection of the material to avoid degradation. It will then be necessary to centrifuge the sample immediately upon arrival for 10' at 1500g. A portion of the supernatant will be discarded but the portion of the liquid closest to the bottom will be retained. Given that at the time of performing PL in most patients the positivity or non-positivity for a molecular marker is known, one may proceed in the following ways.
If the patient has a known molecular marker, the pellet will be resuspended in trizol for preservation and RNA extraction. If, on the other hand, the patient does not have a known molecular marker, the pellet will be stored at -20°C.
The ancillary study will be performed by the standardized molecular biology laboratories that will be involved in the study (Rome-Policlinico Tor Vergata, Rome-Policlinico Umberto I, Bologna-Istituto Seragnoli, Bergamo- Giovanni XIII Hospital, Palermo-Ospedali Riuniti)

5.2 Data collection sheet.

Cases will be documented using data collection sheets. A sequential identification number will be assigned to each patient registered in the study. This number will identify the patient and will be included in the data collection sheet thus allowing the patient's anonymity to be maintained. Variables collected will be age, gender, date of ALS diagnosis, immunophenotype at onset, genetic and cytogenetic features of ALS, stratification by risk group, extramedullary localizations, type of chemotherapy, date of first PL, white blood cells at onset and at the time of PL, lacticodehydrogenase levels at onset and at the time of PL, CRL cell count, CRL protein count, CRL cytospin outcome, date of complete remission. Subsequent data collection sheets will be provided for patient follow-up, at 12 and 24 months after enrollment, in which the number of PLs performed, MMR evaluation, MMR assessment, outcome, date of any recurrence, any SNC radiotherapy, salvage chemotherapy, date of last follow-up, date of death will be reported.

5.3 Statistical considerations.

Patient characteristics will be described by frequency tables for qualitative variables and position indicators for quantitative variables. Associations of clinical-biological parameters with CNS location will be analyzed by Chi-square or Fisher's exact test for qualitative variables and Wilcoxon or Kruskal-Wallis test for quantitative variables. OS will be calculated from the date of treatment initiation to the date of death from any cause; subjects alive at the last follow-up will be censored. DFS will be calculated from the date of achieving complete remission to the date of relapse or death from any cause; subjects alive without progression at last follow-up will be censored. CIR will be estimated via via nonparametric method considering death as competitive risk. OS and DFS probabilities will be estimated by Kaplan Meier method; log rank test will be used to compare subgroups. Confidence intervals will be calculated at 95%, all tests will be two-tailed, and differences with $p < 0.05$ will be considered statistically significant. R software (A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL https://www.R-project.org/).

6.0 Ethical aspects

The study will be conducted in adherence with Good Clinical Practice guidelines, as described in:

1. ICH Guidelines of Good Clinical Practice, 1996. CPMP/ICH/135/95
2. EU Directives and 2001/20/EC, 2005/28/EC.

3. Declaration of Helsinki (1964, and its amendments and subsequent clarifications).

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Approval of the local ethics committee will be required to conduct the study: Ethics Committee of the Fondazione Policlinico Tor Vergata in Rome.
Patients will be required to sign informed consent to process the data.
Investigators will facilitate monitoring, verification, inspection of regulatory bodies by providing direct access to original data/documents.

7.0 Dissemination of results.

The results obtained will be the subject of one or more publications in leading international scientific journals dealing with topics related to hematology and cytofluorimetry. In addition, the results will be submitted for presentation at major national and international hematology congresses (American Society of Hematology, European Hematology Association, Italian Society of Hematology, Italian Society of Experimental Hematology).

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