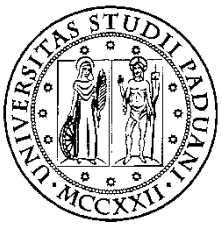

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TESI DI DOTTORATO

Central nervous system involvement in pediatric acute lymphoblastic leukemia detected by 8-color flow cytometry: a prospective study

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Index

Premise……...…5

Central nervous system involvement in pediatric acute lymphoblastic leukemia detected by 8-color flow cytometry: a prospective study

Abstract……...6

Riassunto……9

Introduction……..…… …12

Background……..……15

Aim of the study……28

Methods……29

Results……..……42

Discussion……65

References…….. 74

Hematopoietic Stem Cell Transplantation for Isolated Extramedullary Relapse of Acute Lymphoblastic Leukemia in Children

Abstract……..…..81

Introduction……..83

Patients and method……...84

Results……..87

Discussion……98

References……102

Premise

My PhD was mainly dedicated to children affected by ALL, either as patients during my daily clinical work, either as subjects of my research.

Here I present the two most relevant works I carried out during this period.

The first one, that gives the title to this thesis, it's a project aiming to reveal the clinical significance of central nervous system involvement detected by flow cytometry. My role in this project comprehended ideation of the research project, analysis by flow cytometry of all the samples, recording, collection and analysis of clinical and laboratory data and result discussion.

The second project aimed to define the potential role of autologous hematopoietic stem cell transplantation in pediatric relapsed ALL. Autologous hematopoietic stem cell transplantation (HSCT) was quite frequently performed for the treatment of patient with isolated extramedullary relapse at our institution. We therefore decided to collect data of children with extramedullary relapses who underwent HSCT in Italy. My role is in this project concerned data collection, result discussion and final paper writing (actually submitted).

Central nervous system involvement in pediatric acute lymphoblastic leukemia detected by 8-color flow cytometry: a prospective study

Abstract

Introduction

Acute lymphoblastic leukemia (ALL) may involve central nervous system (CNS) in 3-6% of pediatric patients. Conventional cytology (CC) of cerebrospinal fluid (CSF), together with cell count, is the current standard test to define CNS infiltration, although sensitivity and specificity are low. Flow cytometry (FC) can identify blasts in CSF samples that are negative for cytology with higher sensitivity and specificity. Clinical significance of this occult CNS involvement in children with ALL is still not clearly understood.

The aim of this work is to explore the frequency of CNS involvement by FC analysis of CSF at diagnosis and at each lumbar puncture during therapy in primary and relapsed ALL. Moreover, we want to study prospectively its clinical significance in comparison with cytology and cell count.

Patient and methods

From 12.09.2013 to 12.09.2016 we included all consecutive patients (aged 1-18 years) with Philadelphia negative ALL and with ALL isolated bone marrow (iBM) relapse diagnosed at our Institution. Parent's informed consent was acquired and the study was approved by the local ethical committee. Treatment schedule and definition of CNS involvement were as per AIEOP-BFM ALL 2009 Protocol.

Relapsed patients were mainly treated according to AIEOP ALL REC 2003 protocol.

At each time point of intrathecal therapy, CSF was collected and analyzed within 24 hours by cell count, cytology and 8-color FC (precursor-B or T lineage panel). A tiny cluster of events with immunophenotype compatible with blasts at diagnosis was considered positive by FC (FC+).

Results

Eighty-seven patients with primary diagnosis of ALL were included in the study, 1050 CSF samples were analyzed. At diagnosis, there were 34 (39%) samples that were positive by FC, 5 were also CC+. FC+ patients were mainly T-ALL, with higher peripheral blast percentage and high-risk features. Relapse incidence and mortality were not different between FC+ and FC- groups at diagnosis.

During ALL treatment, other 37 samples belonging to 19 patients resulted positive by FC only. Comparison between FC+ patients during treatment and FC- did not result in significantly different outcome.

Thirteen patients affected by iBM relapsed were included and 109 CSF samples analyzed for this cohort. At relapse, 7 patients were positive by FC (53.8%), none by CC. Characteristics of FC+ patients and FC- did not differ. Mortality and relapse incidence did not show any significan difference between the two groups. During relapse treatment, other 20 samples were FC+. In total 6 relapsed patients presented ≥2 FC+ samples during therapy, this group presented a higher incidence of subsequent relapses compared to FC- patients (83.3% vs 20%, p 0.04).

Conclusion

Our data demonstrated that CNS involvement detected by FC is a frequent finding in pediatric ALL at diagnosis and at relapse. The clinical significance is probably linked to the persistent CSF positivity rather than to the single sample positivity. Actual frontline treatment protocols seem to be able to control CNS submicroscopic leukemia. In relapsed ALL patients, persistent CSF positivity may be a sign of a more resistant disease and a negative prognostic factor. A larger group of patients and a longer follow up are needed to confirm our observations.

Utilizzo della citofluorimetria a 8 colori nella determinazione della disseminazione della leucemia linfoblastica acuta al sistema nervoso centrale in pazienti pediatrici: uno studio prospettico

Riassunto

Introduzione

La leucemia linfoblastica acuta (ALL) può coinvolgere il sistema nervoso centrale (CNS) in circa il 3-6% dei pazienti pediatrici. La citologia convenzionale (CC) su liquor cefalorachidiano (CSF), insieme alla conta cellulare, è la metodica standard per definire l'infiltrazione al CNS. Sensibilità e specificità di questa tecnica si sono però dimostrate scarse. La citofluorimetria (FC) è in grado di identificare blasti in campioni che sono negativi all'analisi citologica con maggior sensibilità e specificità. Il significato clinico di questo coinvolgimento occulto del CNS nei bambini affetti da ALL non è del tutto stato chiarito.

Con questo lavoro ci prefiggiamo di valutare la frequenza del coinvolgimento CNS mediante analisi citofluorimetria del liquor dei pazienti pediatrici con ALL all'esordio e alla ricaduta. Inoltre vogliamo studiarne il significato clinico in un lavoro prospettico, paragonandolo alle metodiche standard.

Pazienti e metodi

Dal 12.09.2013 al 12.09.2016 abbiamo incluso consecutivamente tutti i pazienti di età 1-18 anni affetti da ALL (Philadelphia negativa) e ricaduta midollare isolata di ALL diagnosticati presso il nostro Centro. È stato acquisito il consenso informato

dei genitori e lo studio è stato approvato dal comitato etico locale. Le modalità di trattamento e la definizione di coinvolgimento CNS sono riportate nel protocollo BFM-AIEOP ALL 2009. I pazienti con ricaduta sono stati trattati per la maggior parte secondo il protocollo AIEOP ALL REC 2003. I campioni di liquor sono stati raccolti ad ogni punto previsto per la somministrazione della terapia intratecale e sono stata analizzati entro 24 ore tramite conta cellulare, citologia e citofluorimetria a 8 colori (con pannelli specifici di linea B o T). In presenza di una popolazione di eventi raggruppati in un cluster con caratteristiche antigeniche e fisiche sovrapponibili alla popolazione dei blasti dell'esordio, il campione di liquor è stato classificato come positivo in citofluorimetria (FC +).

Risultati

Ottantasette pazienti affetti da ALL all'esordio sono stati inclusi nello studio, 1050 campioni di liquor sono stati analizzati. Alla diagnosi 34 (39%) campioni sono risultati positivi per FC, 5 di questi lo erano anche per CC. Il gruppo FC+ comprendeva soprattutto leucemie a fenotipo T, con più alta percentuale di blasti in periferico e caratteristiche di alto rischio. Tra i pazienti FC+ e quelli FC- alla diagnosi non è stata dimostrata differenza in termini di ricadute e mortalità. Durante il trattamento altri 37 campioni appartenenti a 19 pazienti sono risultati postivi solo in FC. La prognosi dei pazienti FC+ e quelli FC- durante il trattamento non è risultata significativamente diversa.

Tredici pazienti affetti da ricaduta midollare isolata di ALL sono stati inclusi. Per questa coorte, i campioni di liquor analizzati sono stati in totale 109. Alla recidiva 7 pazienti sono risultati positivi in citofluorimetria (53.8%), nessuno alla citologia. Le caratteristiche dei pazienti del gruppo FC+ e di quello FC- sono risultate

sovrapponibili. L'incidenza di ricadute e la mortalità tra i due gruppi non sono risultate diverse. Durante il trattamento della recidiva, 6 pazienti in totale hanno presentato ≥2 campioni FC+, questo gruppo ha mostrato un'incidenza di ricadute successive statisticamente più alta rispetto al gruppo FC- (83.3% vs 20%, p 0.04), la mortalità non è risultata diversa.

Conclusioni

I nostri dati dimostrano che il coinvolgimento del sistema nervoso centrale all'analisi citofluorimetrica è un reperto frequente nelle ALL pediatriche sia alla diagnosi che alla recidiva. Il significato clinico di tale dato è probabilmente legato alla persistenza della positività del liquor in CF, piuttosto che alla positività del singolo campione. Gli attuali protocolli di prima linea appaiono in grado di controllare questa infiltrazione sub-microscopica di malattia. Nei pazienti con recidiva, la persistente positività del liquor mediante FC potrebbe essere un segno di malattia resistente al trattamento ed un fattore prognostico negativo. E' necessario uno studio su una coorte più ampia di pazienti ed un follow-up più prolungato per confermare la veridicità di tali osservazioni

Introduction

Acute lymphoblastic Leukemia (ALL) is the most common malignancy of childhood, affecting around 40 children/1.000.000 per year in Europe (1). Improved survival for children with ALL is one of the major advance of the contemporary medicine. From a survival probability around 30% in the 1960s, in the 1990s the event-free survival (EFS) at 5 years for childhood ALL generally ranged from 70 to 83% in developed countries (2). Most recent treatment protocols, like those developed at St. Jude Children's Hospital, Memphis (3), reported an EFS at 10 years of 85% (Figure 1). Similarly, ALL 2000 trial by BFM study group (Berlin Frankfurt Munster) and Aieop (Associazione Italiana di Emato Oncologia Pediatrica) resulted in EFS at 5 years of 83% and overall survival (OS) at 5 years of 90% (4). The rational use of multi–agent systemic chemotherapy over a prolonged duration (2 years) and adequate central nervous system (CNS)-directed prophylaxis and therapy as well as improved supportive treatment were responsible for the early improvements in outcome. Moreover, in the last decades, insights into the biology of ALL and the introduction of minimal residual disease (MRD) monitoring have helped to refine therapy based on risk of relapse.

Albeit uncommon, leukemia relapse is still the leading cause of treatment failure, affecting approximately 15-20% of patients. A significant percentage of children with relapsed ALL still die: survival is 30-50% with intensive chemo and radiotherapy approaches and with the use of hematopoietic stem cell transplantation (HSCT) (5,6).

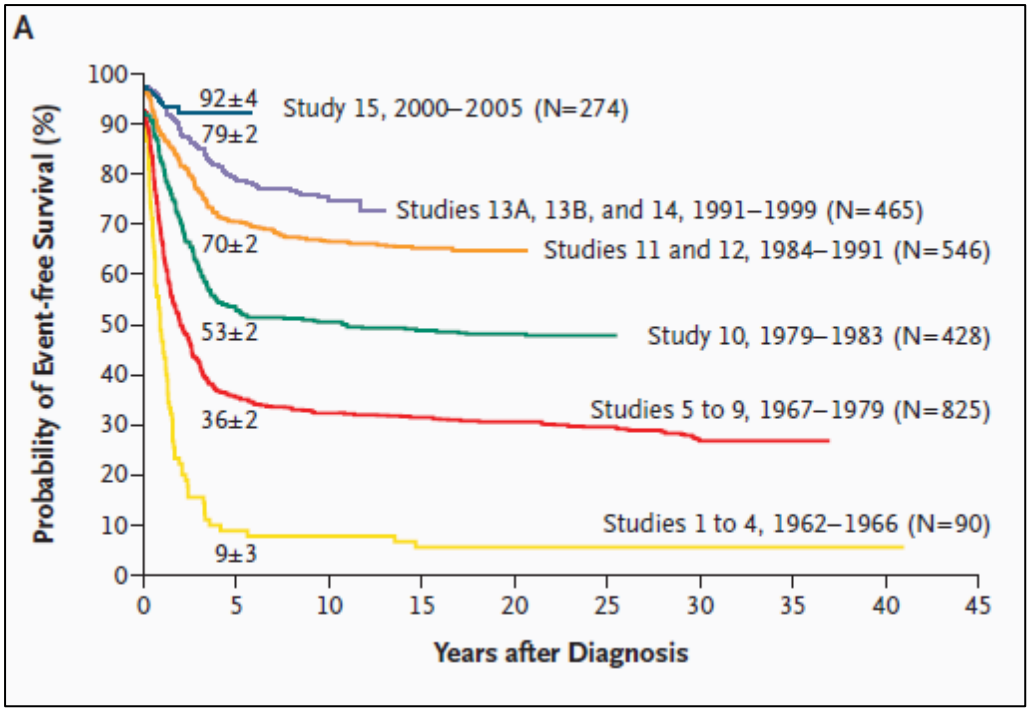

Figure 1: Event free survival at 10 years of children affected by Acute Lymphoblastic Leukemia treated with different consecutive protocols from 1962 to 2005 at St Jude Children Hospital Memphis, USA.

Leukemia relapse most frequently involves the bone marrow (BM), but it can occur in extramedullary sites, mainly the central nervous system (CNS) and the testis, either alone or in combination with BM relapse. Relapses involving the CNS account for up to 40% of all relapses, in the face of only 5% CNS involvement at diagnosis (7). Moreover, even if total number of relapses is decreased with actual frontline treatment, a minor reduction in isolated CNS relapse was observed (8). In addition, prevention of CNS dissemination with cranial radiation therapy (CRT) or intrathecal (IT) chemotherapy has improved cure rate but, at the same time, has been implicated in long term side effects like radiation induced CNS tumors and neurocognitive impairment (9-13). Current protocols aim to reduce the use of irradiation (14). Therefore, it is essential to identify patients at higher risk of CNS

relapse who may require specific intervention, as well than patients with low risk of CNS relapse who my benefit of a less toxic CNS therapy.

The standard methodology to identify blast in cerebrospinal fluid is based on cytology, but this technique has low sensitivity. Flow cytometry (FC) is widely used for ALL diagnosis and MRD monitoring, and it has been proven accurate also in identifying neoplastic cells in the CSF (15-18).

With this study, we explored the feasibility of flow cytometric analysis of CSF at each lumbar puncture during therapy in primary and relapsed ALL. Moreover, we studied prospectively its clinical significance in comparison with cytology and cell count.

Background

Acute Lymphoblastic Leukemia Diagnosis

ALL blasts are thought to derive from the clonal expansion of precursor B lymphocytes or T lymphocytes. This unrestricted growth is driven by a genetic aberration, such as chromosomal abnormality or gene translocation, amplification or mutation.

In the diagnostic pathway of a child with leukemia, information obtained by morphology, immunophenotype, cytogenetics and molecular biology are integrated with clinical data in order to define the better treatment for the type of leukemia and the individual patient.

Morphology

Acute lymphoblastic leukemia is diagnosed by the presence of lymphoblasts in the bone marrow ≥25% of total nucleated cells. Characterization of blasts on morphological appearance is part of the routine diagnostic assessment but it has nowadays no prognostic or biological meaning. Nevertheless, the definition of complete remission (CR) as presence of <5% blasts in bone marrow, it is used for therapy response assessment and requires a morphological analysis. The common classification by FAB (French American British) defines three subtypes of blasts: L1 cells, the commonest type in pediatric ALL, present small and monomorphic cells with elevated nucleus/cytoplasm ratio, L2 blasts are more heterogeneous in shape and dimension, with irregular nucleus, L3 blasts are the largest, with prominent vacuoli and nucleoli. The last subtype it is now considered the leukemic counterpart of Burkitt's Lymphoma (mature B -ALL) (19).

Immunophenotype

Flow cytometry (FC) was developed in the sixties and nowadays it is one of the most important diagnostic and research technique, especially in immunology and hematology. It is essential for ALL diagnosis and it is a well-established method for detecting minimal residual disease. The advantages of FC are: the ease of usage, the rapidity of analysis and the low cost as compared to other technique such as molecular biology.

The instrument is formed by a laser light source and a fluidic system. Cells flow into the fluidic system and pass through a narrow capillary into a single-cell line where they come in contact with the laser beam. Cells cause the light to scatter, two scatter parameters are measured by the instrument: forward scatter (FSC) and side scatter (SSC). FSC is a measure of cell size, SSC, measured at an angle of 90° to the FSC, indicates cellular granularity and nuclear complexity/lobularity. The laser light can also excite fluorochromes that, in turns, emit light at a different wavelength. Different detectors capture the light emission and convert it in digital signal. The power of flow cytometry comes from the ability to integrate the light scatter information with fluorescence information. A large number of monoclonal antibodies coupled with different fluorochrome are available against surface or intracellular antigen (called CD, cluster of differentiation). Cells are stained with antibodies and then analyzed by FC, the fluorescence intensity emitted for each single fluorochrome is proportional to the antigen expression level on the cell. The information obtained are elaborated by a software. Usually, each cell is represented by an event and events are displayed as dot plot, contour plot or

histograms. Each event is characterized by physical parameters (FSC and SSC) and by a fluorescence intensity for each antigen (20).

Blasts cells typically present aberrant immunophenotype that distinguish them from the normal cellular compartment. They may express on their surface antigens that are commonly found on normal lymphocytes together with antigens normally expressed by other cell lines or at different maturational stage, moreover they can show over-expression or under-expression of specific antigens (21). This phenotypic signature is commonly referred as LAIP (leukemic associated immunophenotype).

At diagnosis, flow cytometry is essential in lineage assessment. Precursor B (pB) blasts, the most common subtypes of pediatric ALL (85%), and T blast are distinguished by the expression of lineage specific markers (eg: CD19 for B-ALL, CD7 and CyCD3 for T-ALL). Combination of different antigens defines the maturational stage of the blasts (figure 2) as in the European Group for the Immunological Characterization of Leukemias (EGIL) classification (22).

Table 2 Classification of acute lymphoblastic leukemia (ALL)

1.	B lineage ALL[a] (CD19+ and/or CD79a+ and/or CD22+):	
	pro-B-ALL (B-I) (no expression of other differentiation B-cell antigens)	
	common all (B-II)	CD10+
	pre-B-ALL (B-III)	Cytoplasmic IgM+
	mature B-ALL (B-IV)	Cytoplasmic or surface kappa or lambda+
2.	T lineage ALL[b] (cytoplasmic/membrane CD3+):	
	pro-T-ALL (T-I)	CD7+
	pre-T-ALL (T-II)	CD2+ and/or CD5+ and/or CD8+
	cortical-T-ALL (T-III)	CD1a+
	mature-T-ALL (T-IV)	**$CD1a^{neg}$ and surface$CD3^{pos}$ ($CD3^{strong}$, or $CD3^{weak}$ and TCR^{pos})**

Figure 2: Egil classification of ALL (22)

Specific blast phenotype may be of prognostic importance. Mature B ALL (leukemic counter part of Burkitt's Lymphoma) is characterized by a high proliferation activity caused by the translocations involving *MYC* gene and it is cured with chemotherapy protocol different form pB-ALL (23). Recently, a particularly aggressive subtypes of T cell leukemia called ETP (Early T Cell Precursor) ALL has been described by St Jude's Group in collaboration with AIEOP (24). ETP blasts are defined by a specific phenotype that comprises T-lineage markers together with myeloid antigen. Children affected by ETP may benefit of a more aggressive chemotherapy approach and frontline use of HSCT.

Moreover, antigen expression on lymphoblast may also correlate, at least in some cases, with specific genetic alteration. For example, blasts with t(12;21) typically express very low or absent CD45 and CD20, high CD10 and bimodal expression of CD34 (25). The translocation t(9;22) (Philadelphia Chromosome) is frequently found in association with myeloid antigen expression, low expression of CD19 and CD38, high CD10 and CD34 (26). ALL with translocation involving MLL gene, most frequently t(4;11), are characterized by the expression of the marker NG2 (7.1), CD133, CD15 and dim expression of CD10 (27,28).

Flow cytometric analysis, based on the specific immunophenotype defined at diagnosis, can be used to detect blasts in bone marrow aspirate during therapy with a sensitivity of $1/10^{-5}$. This low amount of blasts, that is not detectable by morphological analysis is called Minimal residual disease (MRD).

Molecular aberration in ALL

Leukemia development is caused by the disruption of normal cell growth and differentiation. Conventional cyogenetich, FISH (fluorescnt in situ hybridization), PCR analysis and, recently NGS (next generation sequencing) contribute to the discover of an increasing number of genetic lesions in ALL blasts.

Pediatric B-ALL commonest translocations include t(12;21) [*ETV6–RUNX1]*(25%), t(1;19) [*TCF3–PBX1*](5%), t(9;11) [*BCR–ABL1*](3%) and translocations involving the *MLL* gene (5%) with various partner fusion genes. High–hyperdiploidy (>50 chromosomes) accounts for 25% of childhood ALL, whereas hypodiploidy (< 44 chromosomes) accounts for approximately 1% of cases. Several of these genetic changes have prognostic and therapeutic implications and are important in risk stratification schemes.

The overall survival of patients with t(12;21) or high hyperdiploid ALL is generally favorable, therefore these patients may benefit of a less aggressive treatment. Survival of children with ALL t(9;11)+ has been improved by the addition of tyrosin-kinase inhibitor to the standard chemotherapy. Hypodiploidy is still a negative prognostic factor with current chemotherapy approach. The frequency and prognostic impact of *MLL* rearrangements differ by age. Approximately 80% of infants younger than 1 year of age harbor *MLL* rearrangements and their overall outcome is generally poor (5–year survival 50%) despite very intensive therapy (29). Around 5% of children presented the translocation t(4;11) which has a negative prognostic impact.

With actual techniques, such as genome- wide profiling of RNA and DNA and NGS, submicroscopic genetic lesions implicated in leukemogenesis are continually discovered. One of these, deletion of *IKZF1,* predicts a poor prognosis in a group of ALL termed Philadelphia like (because of a similar gene expression profile to ALL with Philadelphia chromosome) (30).

T-ALL represent 10-15% of pediatric ALL. Prognosis of T-ALL was historically poor but it has improved with current chemotherapy, even if relapsed T-ALL have still a dismal outcome. Genetic lesions in T–ALL are diverse and complex and a multitude of alterations contribute in the pathogenesis of various subtypes of T–ALL. Chromosomal translocations are present in approximately 50% of patients with T–ALL cases, but unlike B–ALL, their prognostic impact is not well defined and they are not used for risk stratification (29). Therefore, for the purpose of this thesis, they will be not discussed.

Prognostic factors

Age (infant or ≥10 years old), presenting leukocyte count (≥50.000/µl), race (Hispanic or black), male sex, and T-cell immunophenotype have been considered adverse clinical prognostic factors, although their effect is diminished by contemporary risk adapted therapy and improved supportive care. As discussed previously, presence of t(4;11), t(9;22), hypodiploidy, *IKFZ1* alterations have a negative impact on outcome (31).

Treatment response is predictive of the risk of relapse and is used to assign patients to subsequent risk-adapted therapy. Flow cytometry (detecting aberrant

immunophenotypes) and PCR (detecting immunoglobulin or T-cell receptor rearrangements) can identify blasts at levels below those detectable by microscopic morphologic assessment, allowing the measurement of minimal residual disease (MRD). MRD is currently the most powerful prognostic indicator in childhood and adult ALL: most treatment protocols stratify patients on MRD levels measured during treatment (32, 33).

Leukemia treatment

Treatment of ALL typically lasts 2–2.5 years, comprising 3 phases: remission-induction, intensification (or consolidation), and continuation (or maintenance). Most of the drugs used were developed before 1970. However, their dosage and schedule of administration in combination chemotherapy have been optimized in the following decades. Allogeneic hematopoietic stem-cell transplantation is considered for patients at very high risk.

Remission-induction treatment eradicates the initial leukemic cell burden and restores normal hematopoiesis in the majority of children. The chemotherapy agents typically include a glucocorticoid (prednisone or dexamethasone), vincristine, and asparaginase, with or without anthracycline.

Intensification (consolidation) therapy is administered after remission-induction to eradicate residual leukemic cells. This phase commonly uses high-dose methotrexate (MTX) with mercaptopurine or frequent pulses of vincristine and glucocorticoids, asparaginase. Reinduction therapy comprehends agents similar to those used during remission-induction.

Continuation therapy typically lasts 2 years or longer and comprises mainly daily

mercaptopurine and weekly methotrexate with or without pulses of vincristine and dexamethasone (2, 4, 31, 34).

Central nervous system leukemia

Leukemic blasts are able to infiltrate leptomeninges or penetrate in CSF via the choroid plexus or into the subarachnoid space via the bridging veins. Moreover, if blasts are circulating in PB, they can be introduced either by a CNS hemorrhage or iatrogenically at the time of lumbar puncture. The CNS is regarded as a therapeutic “sanctuary”, since the blood-brain barrier and blood-cerebrospinal fluid barrier prevent adequate cytotoxic level of most chemotherapeutic drugs in the CSF. Therefore, blasts can persist in the CNS escaping the effect of either chemotherapy and immunosurveillance and finally leading to relapse (35,36).

Factors associated with a higher risk of CNS relapse in ALL include: T cell immunophenotype, hyperleukocytosis, high risk translocations such as t(9;22) and t(4;11) and the presence of leukemic cells in CSF (7, 35).

Patients may present symptoms and signs suggestive of CNS involvement (cranial nerve palsy, seizure, altered mental status, headache) or spinal cord compression (weakness, paresthesias, bladder dysfunction). However, most patients are asymptomatic and CNS leukemia is discovered at the time of lumbar puncture.

Diagnosis of CNS leukemia (referred as status CNS3 in most protocols) is based on: suggestive symptoms and signs (if present) and/or a positive cerebral magnetic resonance (MR) or computed tomography (TC), and/or positive CSF analysis.

CSF evaluation comprehends usually total white blood (WBC) cell and a red blood cell (RBC) count and microscopic morphological analysis after cytospin (conventional cytology, CC). CNS3 status requires the presence in CSF of ≥5 WBC/µl and the identification of blasts by cytological analysis (16, 35). CNS3 patients, due to the high risk of subsequent CNS relapse, are generally treated with additional IT chemotherapy and/or CRT. The clinical significance of low number of blasts (<5 /µl) in the CSF (CNS2) is still debatable. Early studies reported a higher number of CNS relapses and a poor EFS for this category of patients, as well as for those who received a traumatic lumbar puncture (37,38). More recent data do not confirm this finding (39, 40). However, it is now known that the classification is relevant in the context of the treatment received, as protocols that use early CNS-directed therapy or prophylactic cranial irradiation did not find differences in CNS relapse rates for CNS1 versus CNS2 patients (41).

CNS directed therapy

CNS directed therapy includes intrathecal administration of chemotherapy and cranial irradiation. Cranial radiotherapy (CRT) is very effective in controlling CNS leukemia but its efficacy is counterbalanced by several adverse effects (35).

CRT can pose patients to an augmented risk of tumors (especially CNS neoplasms) and a reduction in neurocognitive abilities. Cumulative incidence of second neoplasms in patients who received cranial or craniospinal irradiation was found to be as high as 20% at 20 years from first diagnosis. Most of these late-onset tumors were benign or low-grade, therefore late mortality rate was not higher in irradiated patients. Nevertheless, with an extended follow up, the risk of

secondary neoplasms could even be higher (9). In addition, patients treated with CRT have an increased risk of obesity and endocrinopathy (especially growth hormone and thyroid hormone deficiency) (13).

Initial attempts were made to reduce the dose of CRT: 12 Gy were proven efficacious to prevent CNS disease in high risk patients, but the reduction in second neoplasms did not result significant (42). Most recent trials have omitted CRT from first line treatment of ALL: review of treatment outcome showed that CRT was associated with a reduced risk of relapse only in the small subgroup of patients with overt CNS disease at diagnosis, who had a significantly lower risk of isolated CNS relapse and a trend toward lower risk of any CNS relapse. Authors concluded that CRT has no impact in relapse rate of pediatric ALL treated with contemporary protocols (14).

Intrathecal (IT) administration of chemotherapy is the standard method used to deliver these drugs in the CSF compartment. Metothrexate, cytarabine and hydrocortisone are used routinely by IT route in patients with ALL. The efficacy of a single agent (MTX) versus triple IT therapy was explored in a randomized study. Authors demonstrated that IT triple therapy reduced the incidence of CNS relapse but was associated with an increase in BM and testicular relapse, leading to a poor survival rate (43). Therefore, most treatment protocols use IT MTX only.

Even IT chemotherapy approach is not without adverse events including post-dural puncture headache, CNS hemorrhage, leukoencephalopathy, chemical meningitides and spinal cord dysfunction (35). Moreover, it is reported that patients who underwent CNS prophylaxis with IT therapy only had also some neurocognitive deficit in the area of attention and memory (10,11).

Based on these premises, it is paramount to have reliable tests that can evaluate risk of CNS involvement, in order to minimizing toxicities and increasing cure rate.

Flow cytometry of CSF

The accuracy and the sensitivity of cytological analysis in defining CNS leukemia may be low due to the difficulty in distinguish normal or reactive lymphocytes, mostly of T cell lineage, and monocytes. Moreover, cells in the CSF are generally scanty and tend to deteriorate quickly (16). For almost 25 years, cell immunophenotyping by FC has been routinely used for the detection of lymphoid and myeloid malignancies in bone marrow and blood. It is an objective method for qualitative and quantitative analysis of cell suspensions and can identify small populations of malignant cells with aberrant surface marker expression (20). Since 2001, when the first report using FC in the identification of CNS leukemia was published (44) several reports analyzing both methods have appeared. The work of *Subira* and colleagues collected 168 samples from 30 patients with acute leukemia and analyzed them by both FC and CC. They concluded that FC has superior sensitivity in comparison to CC, as it is able to detect normal T lymphocytes even in sample with low cellularity and can identify blasts when CC is negative. The same conclusion derived from the paper by *Quijiano et al* in which 123 patients with newly diagnosed aggressive B-cell lymphoma were studied by FC and CC (45).

In the study by *Hegde*, 51 patients with newly diagnosed aggressive B-cell lymphoma were studied: 22% presented CNS involvement by FC but not by CC

(46). FC+ group, compared to FC-, had similar characteristics with the exception of the involvement of more extra-nodal sites in patients FC+. Other groups (47,48) confirmed that FC is able to identify blasts in 10-16% of patients affected by aggressive B cell lymphoma.

Less pediatric studies have been published on this topic: *Sayed* and colleagues described a group of 24 newly diagnosed and 9 relapsed ALL in whom CC, FC and molecular biology of CSF were used in order to improve sensitivity in detections of CSF involvement (49). *Ranta et al* studied retrospectively a cohort of 214 pediatric ALL patients and found 8% of them positive by FC only at diagnosis, this group showed a higher rate of marrow relapse without statistical significance. FC+ patients of this cohort were mostly HR and T-ALL (50).

Established that FC is feasible and can give additional information on CNS involvement in leukemia and lymphoma, other groups investigated the prognostic impact of this finding in prospective studies. Most studies concerned adult patients affected by non-Hodgkin Lymphoma (NHL). In the work by *Sancho* and collaborators 105 patients with aggressive lymphoma were studied at diagnosis: 14% resulted positive by FC only, these patients showed a higher risk of CNS relapse (51). Two other prospective studies (52, 53) came to the same conclusion: patients with high risk NHL who had a positive CSF by FC at diagnosis (6-16%) showed an increased risk of CNS relapses and a worst OS. Finally, *Del Principe et al* demonstrated in a prospective work that, among 38 adult patients with ALL or lymphoblastic lymphoma (LL), those who were FC+ at diagnosis (24%) had inferior overall survival (54).

Studies regarding children with ALL are less uniform. One hundred and eight pediatric patients with ALL were described by *Martinez-Laperche* and co-authors. They performed FC analysis at each time point during treatment, finding that at diagnosis FC+ patients were mostly T-ALL with hyperleukocytosis and high risk features. CSF positivity by FC during treatment, but not at diagnosis, was associated to a higher mortality in this cohort (55).

A Chinese study randomized FC+ patients to receive or not enhance IT treatment. They found that children who received the standard treatment showed a higher rate of CNS relapse and higher mortality (56). The authors stated in the discussion that in China chemotherapy protocols and CNS prophylaxis are less intensive than in Western countries, thus explaining the high rate of CNS relapses. The third study that analyzed prospectively pediatric ALL was published last year: among 300 patients with ALL, 29% had CSF involvement by FC at diagnosis, these patients were more frequently T-ALL, younger and with high WBC count on peripheral blood. 10% of them were still positive at day 15, subsequent samples were not analyzed by FC. In this study 9 relapsed patients were studied by FC, 56% of them were positive, but CC results were note reported nor clinical characteristics and outcome. The clinical significance of these findings could not be ruled out by this study (57).

FC is able to identify blast even in sample with low cellularity and it is more sensitive than CC. Moreover, it helps in discriminating cases that are doubtful by cytology. In adults with aggressive non-Hodgkin Lymphoma, it appears to identify patients at higher risk of CNS relapse and with adverse prognosis. This may be the same for adult ALL/LL although only one study with limited number of patients have been

published. Regarding children affected by ALL, the only three papers that studied prospectively the prognostic value of FC CSF positivity, all published after the initiation of this thesis work, did not reach concordant results. Moreover, almost no data are published about the use of CSF FC in pediatric patients with relapsed ALL. Therefore, to further address this issue, we conducted the study described in the following chapters.

Aim of the study

With this work, we aim to explore the frequency of cerebrospinal involvement by FC analysis of CSF at diagnosis and at each lumbar puncture during therapy in primary and relapsed ALL. Moreover, we want to study prospectively its clinical significance in comparison with cytology and cell count.

Methods

Patients

We included all consecutive pediatric patients (age ≥1 year and <18 years) affected by primary ALL and by isolate bone marrow (iBM) relapse of ALL diagnosed at our Institution between 12.09.2013 and 12.09.2016. Informed consent was acquired from parents or legal guardians and the study was approved by the local ethics committee.

ALL patients were treated according to AIEOP-BFM ALL 2009 Protocol (EudraCT Number: 2007-004270-43), patients with presence of t(9;22) are not included in this protocol and were therefore excluded from the study. Relapsed patients were treated according to AIEOP ALL-REC 2003 protocol, to BFM-IntReALL 2010 protocol (Eudra-CT Number: 2012-000793-30) or to other treatment strategies.

CSF samples were collected at each time point of intrathecal therapy administration during frontline and relapse treatment. Sample collection and analysis were carried out until 31.12.2016, while clinical follow up was updated until 20.04.2017.

AIEOP-BFM ALL 2009 protocol definitions

Definitions of CNS involvement in the AIEOP-BFM ALL 2009 protocol are as follow.

CNS3 status requires the presence of at least one of the following criteria:

- Clinical signs or symptoms of CNS involvement (such as cranial nerve palsy)
- Radiological signs detected by cerebral imaging (CT or MR)
- Presence of blasts in the CSF detected by cytology, with >5 WBC/µl

(exception applies for patients classified as CNS2c, see further)

Patients with no CNS involvement are defined CNS1, they must present CSF WBC≤5/µl and absence of blast by cytological analysis of CSF.

CNS2 status refers to those patients who present blasts by CC, with WBC count ≤5/µl. A particular CNS2c status is assigned to patients with positive cytology and more than 5 WBC/µl, if the following formula is satisfied: CSF WBC count/ CSF RBC count < 2 x peripheral blood (PB) WBC count/PB RBC count.

The following definitions are applied to therapy response assessment by morphology:

- Prednisone good responder (PGR): patients who present in PB blasts <1000/µl at day 8
- Prednisone poor responder (PPR): patients who present in PB blasts ≥1000/µl at day 8
- Complete remission (CR): morphological detection of bone marrow blasts<5% at the marrow aspirate performed at day 33 or after, no blast in CSF by cytology, no evidence of leukemia infiltrate in any other organ by clinical examination or by radiological tests

The following definitions are applied to MRD assessment by flow cytometry at day 15 bone marrow aspirate:

- Standard risk (SR) FC-MRD if blasts are <0.1%
- Medium risk (MR) FC-MRD if blasts are ≥0.1% and <10%
- High risk (HR) FC-MRD if blasts are ≥10%

The following definitions are applied to MRD assessment by polymerase chain reaction (PCR) at day 33 (time point 1, TP1) and day 78 (time point 2, TP2) bone marrow aspirates:

- SR PCR-MRD: negative PCR-MRD at both time points
- MR PCR-MRD: positive MRD at TP1 $<10^{-3}$ and positive MRD at TP2$<10^{-3}$
- Slow early responder (SER) PCR-MRD: positive MRD at TP1 $\geq 10^{-3}$ and positive MRD at TP2$<10^{-3}$
- HR PCR-MRD: positive MRD at TP2 $\geq 10^{-3}$

In the AIEOP-BFM ALL 2009 protocol, information regarding biological features and therapy response assessment by morphology and MRD are incorporated in order to stratify patients into three different risk categories, defined as follow.

High risk, if at least one of the following situation occurs:

- Presence of t(4;11) translocation
- Hypodiploidy (DNA index <0.8 or ≤44 chromosomes by standard karyotype)
- PPR
- HR FC-MRD at day 15
- No RC at day 33
- HR o SER PCR-MRD

Medium risk:

- Absence of high risk criteria and MR PCR-MRD

Standard risk:

- Absence of high risk criteria and SR PCR-MRD

<u>AIEOP-BFM ALL 2009 protocol treatment</u>

Chemotherapy administration is divided into 4 treatment phases: Induction, Consolidation, Re-Induction, Maintenance (if HSCT is not indicated, see further), outlined in Figure 3.

Induction

All patients receive induction treatment consisting in:

- Phase IA: prednisone, 4 doses Vincristine (VCR), 4 doses Daunorubicin (DNM), 3 IT methotrexate administrations (IT MTX) (or 5 for CNS3 and CNS2 patients). Patients with T-ALL and PGR, after day 8, receive dexamethasone instead of prednisone. Patients with T-ALL and PPR continue with prednisone and receive a dose of Cyclophosphamide (CPM, 1000 mg/m^2).
- Phase IB: containing oral 6-mercaptopurine (6-MP), 16 doses cytarabine (Ara-C) at 75 mg/m^2, 2 doses CPM (1000 mg/m^2), 2 IT MTX

Consolidation

SR or MR patients receive 4 chemotherapy blocks of high dose intravenous MTX (HD-MTX 5 gr/m^2), each one containing 1 IT MTX.

HR patients receive 3 high risk blocks as follow:

- HR-1 block containing oral dexamethasone, 2 doses VCR, 5 doses CPM (200 mg/m^2), HD-MTX, 2 high doses Ara-C (2 gr/m^2), 1 dose Peg-asp, 1 IT MTX
- HR-2 block containing oral dexamethasone, 2 doses vindesine, DNM (30 mg/m^2), HD-MTX, ifosfamide, 1 dose Peg-asp, 1 IT MTX (or 2 if CNS3)
- HR-3 block containing oral dexamethasone, 5 doses etoposide, 4 high doses Ara-C (2 gr/m^2), 1 dose Peg-asp, 1 IT MTX

Re-induction

- For patient SR or MR, a single Reinduction protocol (Protocol II) is administered, containing oral dexamethasone, 4 doses VCR, 4 doses Doxorubicin, 1 dose Peg-asp, 1 dose CPM (1000 mg/m^2), 8 doses Ara-C

(75 mg/m^2), oral thioguanine, 2 IT MTX (4 if CNS3)

- For patient HR, three Reinduction protocols (Protocol III) are administered, after the first one and the second one an ad-interim Maintenance phase (orally 6-MP and MTX) is provided. Protocol III contains oral dexamethasone, 2 doses VCR, 2 doses Doxorubicin, 1 dose Peg-asp, 1 dose CPM (500 mg/m2), 8 doses Ara-C (75 mg/m^2), oral thioguanine, 2 IT MTX (3 if CNS3)

Maintenance

Patients who are not candidate to HSCT receive oral 6-MP and MTX until 24 months from the start of therapy. Some particular categories (see further) receive IT MTX during this phase.

Randomizations

In addition, the protocol presents some randomizations:

- Random 1: patients who present t(12;21) translocation or SR FC-MRD at day 15 can be randomized to 2 doses of DNM instead of 4 during Induction IA
- Random HR: patients who present high risk features can be randomized to receive adjunctive 4 doses of Peg-Asparaginase (Peg-Asp) during Induction IB
- Random 2: MR patients can be randomized to receive adjunctive 9 doses of Peg-Asparaginase during Protocol II and Maintenance

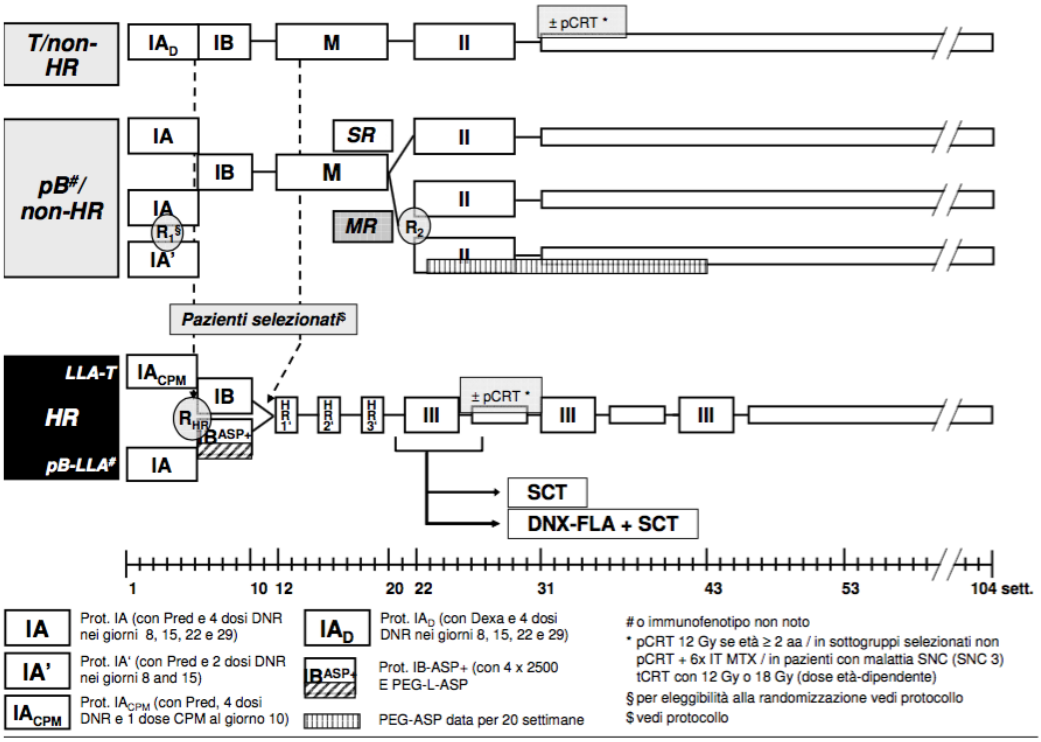

Figure 3: Treatment outline according to AIEOP-BFM ALL 2009 protocol.

CNS directed therapy in the AIEOP-BFM ALL 2009 protocol

CNS directed therapy/prophylaxis comprehends intrathecal administration of methotrexate and cranial radiotherapy (CRT). Methotrexate is administered intrathecally at a dose depending on patient age: 8 mg to children ≥1 year and <2 years old, 10 mg to children ≥2 years and <3 years old, 12 mg to children ≥3 years old. CRT is administered at 12 Gy or 18 Gy.

The following patient categories are defined in the protocol, each one receives different CNS directed therapy:

1. CNS1, pB-ALL patients, with MR or SR features, receive 11 IT administrations of Methotrexate (standard IT therapy), none during Maintenance
2. CNS1, pB-ALL patients, with HR features, receive 14 IT administrations of

Methotrexate during treatment and additional 6 during Maintenance (if they are not candidate to HSCT)

3. CNS1, T-ALL patients, <2 years of age or with PB WBC<100.000/µl at diagnosis and no HR features, receive standard IT therapy and additional 6 IT MTX during Maintenance
4. CNS1, T-ALL patients, ≥2 years of age, with PB WBC >100.000/µl at diagnosis or HR features, receive standard IT therapy and CRT (12 Gy) during interim Maintenance (if they are not candidate to HSCT)
5. CNS2 patients receive 2 additional IT MTX during Induction IA, subsequent CNS treatment is administered on the same criteria of CNS1 patients
6. CNS3 patients receive additional 4 IT MTX during therapy if SR or MR, or 6 if HR; moreover, they receive CRT at 12 Gy if <2 years of age, 18 Gy if ≥2 years of age
7. Patients with HR features, for whom allogeneic HSCT is indicated, do not receive CRT. They are treated with TBI as part of the conditioning regimen, plus a cranial boost if CNS3.

<u>Hematopoietic stem cell transplantation criteria in AIEOP-BFM ALL 2009</u>

Allogeneic HSCT should be offered to patients presenting at least one of the following HR criteria:

- no CR at day 33
- HR PCR-MRD
- t(4;11) translocation + MR, SER or HR PCR-MRD
- hypodiploidy + MR, SER or HR PCR-MRD
- T-ALL, PPR + HR PCR-MRD or no MRD results

Relapse definition

ALL relapse is defined as the recurrence of leukemia after CR achievement. It may involve bone marrow, CNS or extramedullary sites. The following definitions were applied in our study:

- Isolated BM (iBM) relapse: ≥25% of blasts by morphological examination of bone marrow smear, with no CSF blasts by cytology, no evidence of leukemia infiltrates in any other organ
- Isolated CNS (iCNS) relapse: CSF with blasts detected by cytology and >5 WBC/µl or clinical signs or symptoms of CNS involvement (such as cranial nerve palsy) or radiological signs detected by cerebral imaging
- Combined Relapse: ≥5% BM blasts together with another leukemia localization

Depending on time between the onset of relapse and primary diagnosis, the following terms are used:

- very early relapse, if it occurs less than 18 months from primary diagnosis,
- early relapse if it occurs later than 18 months from diagnosis and less than 6 months from treatment discontinuation
- late relapse if it occurs more than 6 months from treatment discontinuation

In the AIEOP ALL 2003 protocols, patients were stratified according to 3 prognostic factors (site of relapse, time to relapse and phenotype) into 4 risk categories:

- S1 late extra-medullary relapses
- S2 early or very early extra-medullary relapses, early pB-ALL combined relapses, late pB-ALL iBM relapses

- S3 early pB-ALL iBM relapses
- S4 T-ALL iBM relapses, very early pB-ALL iBM or combined relapses

Treatment of relapse

Therapy outline according to Aieop ALL REC 2003 protocol is reported in figure 4. Patients with iBM relapse were treated according to S2 or S3-S4 arm. Therapy for S2 patients comprehends randomization between induction with two blocks (F1 and F2, arm A) or a continuous therapy (protocol IA-IDA, Arm B).

- Block F1

  Prednisone for three days, followed by dexamethasone, 2 doses VCR, ev MTX (1 gr/m^2), 1 dose L-Asparaginase, 2 IT MTX

- Block F2

  Dexamethasone, 2 doses VCR, 4 doses of HD ARA-C (3 gr/m2), 1 dose L-Asparaginase, 1 IT MTX

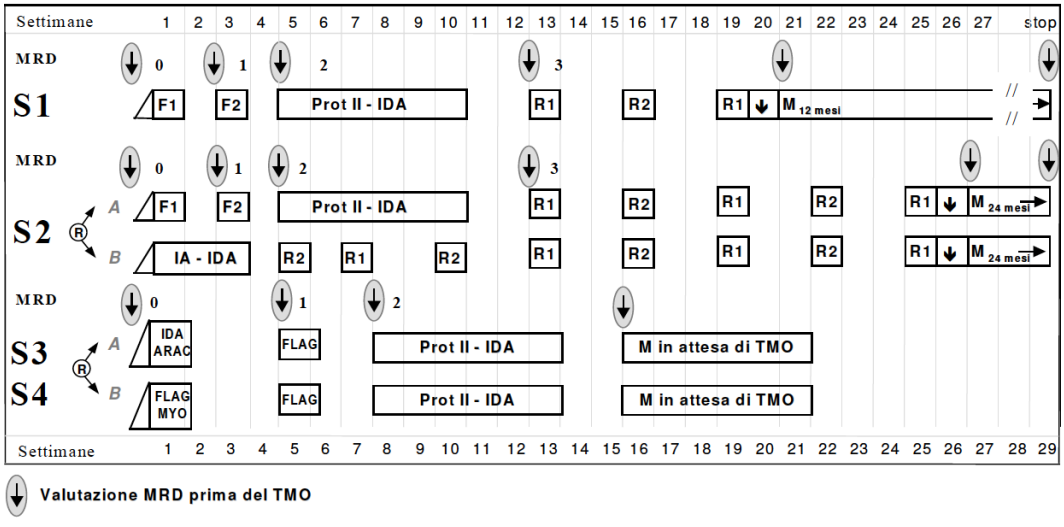

Figure 4: outline of AIEOP ALL REC 2003 protocol treatment

- Protocol II- IDA

  Dexametasone, 4 doses L-Asparaginase, 4 doses VCR, 4 doses idarubicin, 1 dose CPM (1000 mg/m^2), 8 doses Ara-C (75 mg/m^2), oral 6-MP, 4 IT MTX

- Protocol IA-IDA

  Prednisone, 4 doses VCR, 4 doses idarubicin, 8 doses L-Asparaginase, 3 IT MTX

- Block R1

  Dexamethasone, oral 6-MP, 2 doses VCR, ev MTX (1 gr/m^2), 2 doses of HD ARA-C (2 gr/m^2), 1 dose L-Asparaginase, 1 IT MTX

- Block R2

  Dexamethasone, oral thioguanine, 1 dose vindesine, ev MTX (1 gr/m^2), 5 doses of ifofamide, 1 dose L-Asparaginase, 1 dose daunomicine, 1 IT MTX

Patients stratified as S3-S4 were randomly assigned to arm A (IDA-AraC followed by FLAG) or Arm B (FLAG-Myocet followed by FLAG)

- IDA-Ara C

  Oral prednisone, 1 dose idarubicine, 5 doses HD ARA-C (3 gr/m^2), 2 IT MTX

- FLAG (+/- Myocet)

  Fludarabine, 5 doses HD ARA-C (3 gr/m^2), 1 dose Myocet (liposomal doxorubicine), 2 IT MTX G-CSF (granulocyte- colony forming unit)

For S3-S4 patients, HSCT is indicated from matched familiar donor (MFD), matched unrelated donor (MUD) or mismatched donor (MMD). For patients with S2 relapse, HSCT is indicated in case of MFD availability or from MUD if relapse occurred within 48 months from diagnosis. Fore relapses occurring >48 moths from primary diagnosis, the decision to proceed to MUD or MMD is based on

MRD levels.

CSF Samples

At each point of intrathecal therapy, patients underwent lumbar puncture under deep sedation. Before MTX administration, CSF was collected by clinicians in two vials, containing approximately 1 ml of CSF each. The first vial was send to the central laboratory of our hospital (Laboratorio Centrale, Azienda Ospedaliera di Padova), the second one to our laboratory in the Pediatric Onco-hematology Unit. All samples were analyzed within 24 hours by cell count, cytology and 8-color flow cytometry. Operators were blinded to the results of the 2 other techniques. Clinicians were blinded to FC results.

Cell count was performed in the central laboratory by the automatic cell counter XE5000 (Sysmex Europe, Gmbh, Germany) that is specifically dedicated to CSF samples. Results were expressed as number of WBC and RBC/µl.

In our laboratory, each CSF sample was divided in two aliquots: one was used for cytology, one for flow cytometry. Slides were prepared after cytospin centrifugation by Shandon Cytospin 4 centrifuge (Thermo Electron Corporation). First, the filter cards were wet with Hanks' Salt solution (Biochrom Gmbh). Each sample was loaded into the cytospin centrifuge and a filter card and a glass slide were added in the position provided. Samples were centrifuged at 450 rpm for 10 minutes.

After cytocentrifugation, slides were dried and stained with May-Grunwald Giemsa stain by an automated instrument ADVIA S60 (Bayer). Cytological analysis with Leitz Wezlar optic microscope was performed by an expert morphologist.

For flow cytometry, each CSF sample was centrifuged at 1200 rpmi for 5 minutes. The sample was resuspended in 100µl of PBS (+1% bovine albumin) and

incubated with a mixture of fluorescence-conjugated antibodies for 10 minutes in the dark. An antibody combination was used for B-lineage ALL, and another one for T-ALL (Figure 5). Antibodies were purchased from Beckton Dickinson Biosciences (BD) and Beckman Coulter.

A

FITC	PE	PC5	PC7	APC	APC Cy7	Horizon V450	Horizon V500
CD58	CD10	CD34	CD19	CD3	CD20	CD38	CD45

B

FITC	PE	PC5	PC7	APC	APC Cy7	Horizon V450	Horizon V500
CD99	CD5	CD34	CD38	CD33	CD3	CD7	CD3

Figure 5: B-lineage ALL (A) or T-lineage ALL (B) antibody combination and the corresponding conjugated fluorochrome used for FC CSF analysis

After incubation, 3 ml of PBS+1% BSA were added and the sample was then centrifuged at 1200 rpmi for 10 minutes. The supernatant was discarded and the pellet resuspended in 300µl of PBS+1% BSA. Sample were run until exhaustion on FACSCanto II flow cytometer (Becton Dickinson Biosciences, BD, San José, CA, USA) and analyzed with FACS Diva software (BD).

For analysis, dead cells and debris were excluded in FSC/SSC dot plots (primary gate). For pB-ALL a second gate was set on CD19 positive events, blasts were

identified on CD45/CD10/CD58/CD34/CD20/CD38 expression, normal T cells were identified based on CD3/CD45 expression, monocytes on CD58/CD45 expression, normal B cells on CD45/CD19/CD34/CD20. For T-ALL a second gate was set on CD7 positive events, blasts were identified on CD45/CD3/CD5/CD99/CD38 expression, normal T cells were identified based on CD3/CD5/CD7/CD45 expression, monocytes on CD33/CD45 expression (58).

A tiny cluster of events with immunophenotype compatible with blasts at diagnosis was considered positive by FC (FC+). If a cluster of events with aberrant phenotype, but very different from blasts at diagnosis was found, the sample was defined as uncertain (FC+/-). Total number of events, number of events identified as blasts, normal T-lymphocytes, monocytes, normal B-lymphocytes were reported. Sample volume was also recorded.

Statistical analysis

Categorical variables were compared using Chi-square test (Pearson) performed using in-silico.net. Continuous variables were compared using Mann-Whitney-Wilcoxon test for unpaired data performed using www.astatsa.com. Two-sided *p* values <0.05 were regarded as significant.

Results

CSF from patients with *de-novo* ALL

Characteristics of patients

A total of 87 patients with *de-novo* ALL were recruited in this study.

Patient characteristics were the following: 54 (62.0%) male, 33 (38.0%) female, median age at diagnosis was 4.7 years (range 1.4-17.7 years). Leukemia immunophenotype was precursor B (pB) in 86.2% of cases, T in 13.8%. The translocation t(12;21) was found in 19 (21.8%) cases, t(1;19) in 2 (2.3%) cases, the rest were negative for each of the following: t(12;21), t(9;22), t(4;11), t(1;19).

At the time of lumbar puncture, 14.9% of patients had hyperleukocytosis (white blood cells in peripheral blood >50.000/µl) and median blast percentage in PB was 38% (0-93%). Six children (6.9%) showed blasts at CSF cytological analysis, 80 were negative and 1 was diagnosed as CNS3 in another center. Flow cytometric analysis of CSF resulted positive in a total of 34 (39.1%) children at diagnosis.

Five patients (5.8%) were classified as CNS3 at diagnosis: 3 cases for CC positivity, 1 case for cranial nerve palsy (CC was negative) and 1 case was diagnosed as CNS3 in another center (CSF was not available for analysis at diagnosis). Seven patients (8.3%) were classified as CNS2: of those 3 presented positive CC and low cell count (<5/µl) as per protocol CNS2 definition, 4 patients were negative by laboratory analysis but treated as CNS2 for clinicians' decision. A total of 75 children resulted CNS1. Patients characteristics, grouped by CNS status, are shown in Table 1.

Risk stratification as per protocol resulted in 25 (28.7%) HR, 44 (50.6%) MR and 18 (20.7%) SR.

Median follow up was 24.8 months (range 7.2-43.9 months). Two patients experienced an isolated bone marrow (iBM) relapse, no CNS or combined relapses occurred. Among relapsed patients: one was a pB-ALL, CNS1, FC+ at diagnosis, who was classified HR for molecular MRD; he presented a very early iBM relapse and subsequently died of disease progression. The other one was T-ALL, CNS1, FC+ at diagnosis and at following time points, HR for not CR at the end of induction; he presented a very early iBM relapse and subsequently died of a septic shock during a neutropenic phase. Other two patients died for treatment related complications: 1 patient (pB-ALL, CNS1, FC-, HR for molecular MRD) died of a complicated pneumonia, the other one (T-ALL, CNS3, FC+ at various time points, HR for not CR at the end of induction) underwent HSCT from a matched unrelated donor and died soon after transplantation of multiorgan failure.

Statistical analysis revealed that CNS3 status was associated, in our cohort, with T immunophenotype, positive CSF cytology and high risk features (Table 2). CNS2 patients showed higher frequency of positive CSF cytology, CNS1 patients were mainly pB-ALL with negative CSF cytology. Gender, age, presence of t(12;21), hyperleukocytosis, peripheral blast percentage and FC at diagnosis did not differ among CNS3, CNS2 and CNS1 patients. Number of relapses and deaths, as well, were not differently distributed.

A total of 1072 CSF samples were collected and analyzed by cell count and cytology, FC was not performed in 22 samples for inadequate material. 1050 samples were included in the analysis: 84 were collected at diagnosis, 966 at other time points. Median volume was 400 µl (range 50-1500 µl).

Peripheral blood contamination was determined by the presence of RBC by cell count and/or cytology: a total of 323 out of 1050 samples (30.7%) were positive for RBC.

Using flow cytometry, we observed that the large majority (90.1%) of samples contained T lymphocytes, a small proportion showed also monocytes (17.5%) or mature B lymphocytes (1.7%). After exclusion of samples with RBC contamination, these normal cell populations were still evident (89.7% of samples contained T lymphocytes, 17.5% monocytes and 0.9% mature B lymphocytes).

CSF evaluation by flow cytometry at diagnosis

Eighty-four CSF samples had available FC analysis at diagnosis (Table 3).

Flow cytometry showed blasts in 34 samples (39.0%) at diagnosis: 5 were positive by CC (3 CNS3 and 2 CNS2), 29 negative (28 CNS1, 1 patient defined CNS2 for clinical reason but with negative CC). Fifty (57.5%) patients resulted FC negative, they were all negative by CC with one exception. The latter showed blasts at CC analysis, low cell count (<5/µl) and was thus classified CNS2, but FC revealed only mature T and B lymphocytes.

FC+ samples were most frequently CC+ as compared to FC- samples (14.7% vs 2%, p 0.03). RBC contamination and median CSF WBC count were significantly higher in specimens that were FC+ in comparison with FC- (44.2% vs 6.0%, p<0.0001 and 1/µl vs 0/µl, p 0.04, respectively). Median volume between FC+ and FC- samples did not differ (450 µl vs 400 µl).

Among FC+, median number of events regarded as blasts by FC was 54 (range 4-3284). In most of CSF samples, blast immunophenotype was similar to the one of blasts detected in bone marrow. In 9/24 (37.5%) children affected by pB-ALL, CSF

blasts showed CD34 downregulation in comparison to marrow blasts (data not shown).

Clinical characteristics of patients who showed FC+ CSF at diagnosis in comparison with FC- are listed in Table 4. FC+ patients (total number 34) had more frequently T-ALL immunophenotype (23.5% vs 6.0%, p 0.02), and a higher percentage of peripheral blast (median 63% vs 25%, p 0.0003). Moreover, FC+ patients, compared to FC-, were mostly stratified as HR (41.2% vs 20%, p 0.03) and SR (32.3% vs 14%, p 0.04). The two groups did not differ for male/female ratio, median age, presence of t(12;21), WBC count in peripheral blood (>50.000/µl or <50.000/µl) and CNS status. Among FC+ patients, there were 3 CNS3 (for positive CC), 3 CNS2 (2 for CC+, 1 CC- but classified CNS2 for clinical reasons) and 28 CNS1. Among FC- patients, 1 patient was CNS3 for cranial nerve palsy (CC was negative) and 4 patients were treated as CNS2 by clinicians but were negative by cytology. Relapses and deaths of any cause were more frequent in the FC+ group of patients (5.8% vs 0% and 8.8% vs 2%, respectively) but the difference did not result statistically significant. The number of deaths due to disease progression or to treatment were not different between the two groups.

Twenty-eight patients (32.2%) were classified as CNS1 but showed occult CNS involvement by flow cytometry at diagnosis (CNS1 FC+), while 45 (60.0%) were totally negative for CNS involvement (CNS1 FC-). Characteristics of samples and patients of the two groups (CNS1 FC+ vs CNS1 FC-) are listed in table 5 and 6. The proportion of samples containing RBC was higher in the group FC+ compared to FC- (32.1% vs 4.4%, p 0.001). Median volume and median WBC count/µl did not differ between the two groups. Median number of events identified as blast by FC were 50 (range 4-2687).

CNS1 FC+ patients had more frequently T-ALL phenotype (17.9% vs 2.2%, p 0.04) and a higher peripheral blast percentage (median 60% vs 27%, p 0.001). Moreover, frequency of SR patients was higher in FC+ group as compared to FC- (32.3% vs 11.1%, p 0.02) and MR patients were more frequent in the FC- group (68.9% vs 28.6%, p 0.0008). The two groups did not differ in gender, median age, presence of t(12;21), peripheral blood WBC count (>50.000/µl or <50.000/µl) and HR features. Two patients, both CNS1 FC+ at diagnosis, experienced an iBM relapse and subsequently died. However, relapse incidence and mortality did not result significantly different between CNS1 FC+ and CNS1 FC- patients.

CSF evaluation by flow cytometry during treatment

During treatment, a total of 966 CSF samples from 87 ALL patients were analyzed by flow cytometry. CSF data are presented in Table 7.

No sample resulted positive by cytology, while 37 (3.8%) were positive by flow cytometry, and 11 (1.1%) were defined as “uncertain” by flow cytometry (FC +/-). The frequency of samples contaminated by RBC was similar in each group (FC+ 32.4%, FC+/- 27.3% and FC- 31.3%), as was the median CSF volume. FC+ samples had higher median number of CSF WBC/µl than FC- (1/µl vs 0/µl, p 0.03). Median number of WBC/µl and median number of events did not differ between FC+ and FC+/-.

CSF specimens that were positive by flow cytometry were distributed during treatment as follow: 6 at day 15, 3 at day 33, other 2 during Induction IA (both adjunctive lumbar punctures for CNS3 patients), 8 during Induction IB, 12 during Consolidation phase and 6 in Reinduction. No FC+ sample was found during Maintenance. Uncertain sample by FC were distributed as follow: 2 in Induction, 4

in Consolidation, 3 in Reinduction, 1 at the end of therapy, 1 in other treatment phases.

CSF specimens that resulted FC+ during treatment referred to 19 patients. Characteristics of children with FC+, FC+/- or FC- samples during treatment are listed in Table 8. Compared to patients with FC- samples, those with FC+ samples were mostly affected by T-ALL (47.4% vs 3.2%, p <0.0001), CNS3 status at diagnosis (26.3% vs 0%, p<0.0001) and HR leukemia (57.9% vs 20.6%, p 0.002). Moreover, FC+ patients during treatment were more frequently FC+ at diagnosis (57.8% vs 33.3%, p 0.02). Gender, median age and presence of t(12;21) translocation did not differ between patients FC+ and FC- during treatment. Characteristics of patients who presented at least one FC+/- sample during treatment did not differ to those of FC- patients (not shown).

Relapses were almost uniformly distributed: 1 (5.3%) occurred in the group of patients with FC+ during treatment, the other one (1.5%) in the group of patients FC- (this boy was FC+ only at diagnosis). Number of deaths did not differ, 2 were in the FC+ group (10.5%) and 2 in the FC- (3.0%). No relapses nor deaths occurred among FC+/- patients.

We subsequently grouped patients on the basis of the number of samples FC+ or FC+/- from diagnosis during the entire treatment course. One group included patients with 2 or more FC+ (or FC+/-) samples (15 pt, 17.2%), one group patients with only one FC+ (or FC+/-) sample (28 pt, 32.2%) and one group patients who were always negative by FC (44 pt, 50.6%). Characteristics of patients of those three groups are listed in Table 9, statistical analysis in Table 10. Patients with more than 2 samples positive by FC, compared to those with only 1 FC+ or those FC-, had mostly T phenotype (60% vs 7.1% and 2.3%, respectively, p 0.0002 for

both), absence of t(12;21) translocation (0% vs 28.6% and 25.0%, respectively), CNS3 status (26.7% vs 3.6% and 0%, respectively), HR features (80.0% vs 14.3% and 20.5%, respectively, p<0.0001 for both) and a low number of MR patients (6.7% vs 50% and 65.9%, respectively). Moreover, as expected, in comparison with FC- group, patients with more than two FC+ samples showed higher rate of FC+ at diagnosis (86% vs 0%, p<0.0001) and lower rate of CNS1 patients (60% vs 95.5%, p 0.0005). Gender and median age did not differ. Patients with only 1 FC+ sample had clinical characteristics comparable to FC- patients with the exception of a higher frequency of FC+ at diagnosis (75% vs 0%, p< 0.0001) and of SR patients (35.7% vs 13.6%, p 0.03).

There were 1 relapse in the group with more than 2 FC+ samples (6.7%), one in the group with 1 FC+ (3.6%) and no relapses in the FC- group; however, the difference was not statistically significant. Number of deaths did not differ significantly among the three groups.

Children with more than 2 CSF samples positive by flow cytometry showed positivity along all the treatment phases (Table 11), 26 FC+ samples were evident during Induction, other 26 in other treatment phases.

Relapsed ALL

Characteristics of patients

Thirteen patients with isolated BM relapse met the inclusion criteria.

Characteristics of patients are described in Table 12: 8 males, 5 females, 12 pB-ALL, 1 T-ALL. Median age at relapse was 9.5 years (range 2.7- 12.37). Regarding molecular characterization, 1 patient presented t(9,22), 1 t(12;21) and one T-ALL

patient had FLT3-ITD mutation. Six were late relapses, 1 early relapse, 4 very early relapses and 2 occurred after HSCT. Patients were mostly treated according to AIEOP ALL- REC2003 protocol (61.5%), 30.8% received other therapy, only one patient was treated with the recent IntReALL-SR protocol. HSCT was performed from a matched family donor in 3 patients, from a matched unrelated donor in 4, from a partially matched (haploidentical) family donor in 4. Two patients died of disease progression before undergoing HSCT.

Median follow up was 18 months (range 4.8-82.3 months). During this period 7 patients presented a subsequent relapse: 5 isolated BM relapses, 2 isolated CNS relapses. In total 7 patients died at a median follow up of 11.5 months from first relapse (range 4.8-82.3 months). Six out of 7 patients with a second relapse died: 2 of toxicity, 4 of disease progression. One patient died of transplant related mortality (TRM) in second complete remission. The only patient who is alive after the second relapse (iCNS), is affected by pB-ALL with t(9;22)+: she had a third iBM relapse, after this she remained in CR for a long period with tyrosine kinase inhibitor therapy, unfortunately she recently developed a fourth (iCNS) relapse.

CSF samples included in the analysis were 109, 13 samples at relapse onset, 96 during relapse treatment.

RBC contamination was evident either by cytology or by cell count in 31 samples (27.9%). By flow cytometry we observed that most samples contained T lymphocytes (82.8%), few samples showed also monocytes (16.2%), no mature B lymphocytes were detectable. Normal blood cell populations were evident also in samples not contaminated by RBC: 80.0% of samples contained T lymphocytes, 15.0% contained monocytes.

CSF evaluation by flow cytometry at relapse

At relapse, 7 samples were positive by flow cytometric analysis (53.8%), 6 were negative (46.2%) (Table 13). All samples resulted negative by cytology. Contamination with RBC was similarly present among FC+ samples and FC- (28.6% vs 33.3%). Median sample volume and median number of WBC/µl were comparable between FC+ and FC- samples. FC+ specimens showed a median number of events identified as blasts by flow cytometry of 92 (10-579).

Clinical characteristics of patients who showed FC+ CSF at relapse in comparison with FC- are listed in Table 14. The two groups did not differ in relation to gender, age, blast phenotype, time to relapse, treatment protocol, HSCT donor. Among 7 patients who were FC+ at relapse, 5 (71.4%) presented a subsequent relapse (3 iBM, 2 iCNS), among 6 FC- patients 2 experienced a second relapse (iBM). This difference did not result statistically significant. There were 4 deaths in the FC+ group (3 disease related, 1 treatment related) and 3 deaths in the FC- group (1 disease related, 2 treatment related), difference was not statistically significant.

CSF evaluation by flow cytometry during relapse treatment

Samples collected during relapse treatment were 96 in total. CSF specimen features are listed in Table 15. Twenty samples (20.8%) were positive by FC, 75 samples (78.1%) were negative, 1 sample resulted “uncertain”. Three FC+ samples showed blasts by cytology, these belonged to 2 patients who had a subsequent iCNS relapse. Contamination with RBC was not significantly different between FC+ and FC- (40% vs 26.7%) nor median CSF volume (400 µl vs 400 µl). Median CSF WBC count was higher in the FC+ group as compared to FC- (2/µl vs 0/µl, p 0.002). Median number of event identified as blasts among FC+ samples

was 100 (range 5-32300).

FC+ samples during treatment referred to 6 patients (5 already FC+ at relapse, 1 FC- at relapse).

We grouped patients on the basis of the number of samples FC+ or FC+/- from relapse diagnosis during the entire treatment course (Table 16). One group comprised patients with 2 or more FC+ (or FC+/-) samples (6 pts, 46.1%), one group patients with only one FC+ (or FC+/-) sample (2 pts, 15.4%) and one patients who were always negative by FC (5 pts, 38.5%). Statistical comparison was done for the two larger groups (≥2 FC+ vs FC-): these two groups did not differ in relation to gender, age, blast phenotype, time to relapse, treatment protocol, HSCT donor. The group with ≥2 FC+ showed a higher frequency of patients who were FC+ at relapse (83.3% vs 0%, p 0.006). Total number of subsequent relapses was higher among patients with more than 2 FC+ samples compared to FC- (5 vs 1, p 0.04). In particular, isolated CNS relapses were more frequent in the group with ≥2 FC+ compared to FC- (33.3% vs 0%) but the difference was not significant. Overall mortality and disease related mortality were also higher in the group ≥2 FC+ compared to FC- (66.7% and 40.0%, 50% vs 0%, respectively) but numbers were too low to demonstrate a statistical association.

Tables

Patient Characteristics	*CNS3*	*CNS2*	*CNS1*	*Total*
Number of patients	**5 (5.7%)**	**7 (8.1%)**	**75 (86.2%)**	**87 (100%)**
Gender				
Male	3 (60.0%)	5 (71.4%)	46 (61.3%)	54 (62.0%)
Female	2 (40.0%)	2 (28.6%)	29 (38.7%)	33 (38.0%)
Median age, range	9.9 (4.7-14.6)	5.0 (2.4-11.4)	4.5 (1.4-17.7)	4.7 (1.4-17.7)
Immunophenotype				
pB	0	6 (85.7%)	69 (92.0%)	75 (86.2%)
T	5 (100.0%)	1 (14.3%)	6 (8.0%)	12 (13.8%)
Translocation				
t(12;21)	0	1 (14.3%)	18 (24.0%)	19 (21.8%)
t(1;19)	0	0	2 (2.7%)	2 (2.3%)
Negative*	5 (100.0%)	6 (85.7%)	55 (73.3%)	66 (75.8%)
PB WBC at lumbar puncture				
WBC >50.000/µl	2 (40.0%)	1 (14.3%)	10 (13.3%)	13 (14.9%)
WBC <50.000/ µl	2 (40.0%)	6 (85.7%)	65 (86.7%)	73 (83.9%)
Not known	1 (20.0%)	0	0	1 (1.2%)
PB blast percentage (median, range)	42 (6-85)	64 (0-84)	38 (0-93)	38 (0-93)
CSF CC at diagnosis				
CC+	3 (60.0%)	3 (42.9%)	0	6 (6.9%)
CC-	1 (20.0%)	4 (57.1%)	75 (100.0%)	80 (91.9%)
Not available	1 (20.0%)	0	0	1 (1.2%)
CSF FC at diagnosis				
FC+	3 (60.0%)	3 (42.9%)	28 (37.3%)	34 (39.1%)
FC-	1 (20.0%)	4 (57.1%)	45 (60.0%)	50 (57.5%)
Not available	1 (20.0%)	0	2 (2.7%)	3 (3.4%)
Risk group				
HR	4 (80.0%)	1 (14.3%)	20 (26.7%)	25 (28.7%)
MR	1 (20.0%)	2 (28.6%)	41 (54.7%)	44 (50.6%)
SR	0	4 (57.2%)	14 (18.6%)	18 (20.7%)
Outcome				
Relapse (total)	0	0	2 (2.7%)	2 (2.3%)
Isolated BM	0	0	2 (2.7%)	2 (2.3%)
Isolated CNS	0	0	0	0
Combined	0	0	0	0
Deaths (total)	1 (20%)	0	3 (4.0%)	4 (4.5%)
Disease related	0	0	1 (1.3%)	1 (1.1%)
Treatment related	1 (20.0%)	0	2 (2.7%)	3 (3.4%)

Table 1: Patient characteristics grouped by CNS status at diagnosis. *negative for t(4;11), t(9;22), t(12;21), t(1;19).

Abbreviations: CNS central nervous system, PB peripheral blood, WBC white blood cells, CSF cerebrospinal fluid, CC conventional cytology, FC flow cytometry, HR high risk, MR medium risk, SR standard risk, BM bone marrow.

Patient Characteristics	p value (CNS3 vs CNS2+1)	p value (CNS2 vs CNS3+1)	p value (CNS1 vs CNS 2+3)
Gender (M vs F)	ns	ns	ns
Median age	ns	ns	ns
Immunophenotype (pB vs T)	**<0.0001**	ns	**<0.0001**
t(12;21) vs no translocation	ns	ns	ns
PB WBC (>50.000/µl vs <50.000/ µl)	ns	ns	ns
PB blast percentage (median)	ns	ns	ns
CSF CC at diagnosis (CC+ vs CC-)	**<0.0001**	**0.0001**	**<0.0001**
CSF FC at diagnosis (FC+ vs FC-)	ns	ns	ns
Risk group			
HR vs MR+SR	**0.03**	ns	ns
MR vs HR+SR	ns	ns	ns
SR vs MR+HR	ns	**0.01**	ns
Relapse (n° relapse vs not relapsed)	ns	ns	ns
Deaths (n° total dead vs alive)	ns	ns	ns

Table 2: Statistical analysis of patient characteristics at diagnosis. Frequency were compare with two tailed Chi square test, median of continuous variables were compared with Mann Whitney Wilcoxon test.

Abbreviations: CNS central nervous system, PB peripheral blood, WBC white blood cells, CSF cerebrospinal fluid, CC conventional cytology, FC flow cytometry, HR high risk, MR medium risk, SR standard risk, BM bone marrow, ns not significant

ALL at diagnosis	*FC+*	*FC-*	*FC not available*	*P value (FC+ vs FC-)*	*Total*
Number of samples	**34 (39.0%)**	**50 (57.5%)**	**3 (3.4%)**	-	**87 (100%)**
Cytology					
CC+	5 (14.7%)	1 (2.0%)	0	**0.03**	6 (6.9%)
CC-	29 (85.3%)	49 (98.0%)	2 (66.6%)		80 (92.0%)
Not available	0	0	1 (33.3%)		1 (1.1%)
RBC Contamination					
RBC+	15 (44.2%)	3 (6.0%)	0	**<0.0001**	18 (20.7%)
RBC-	19 (55.8%)	47 (94.0%)	2 (66.6%)		68 (78.2%)
Not available	0	0	1 (33.3%)		1 (1.1%)
Median n° of CSF WBC/µl by cell count (range)	1 (0-50)	0 (0-15)	NA	**0.04**	0 (0-50)
Median volume (µl), range	450 (100-1000)	400 (100-1500)	NA	ns	400 (100-1500)
Median events identified as blasts in FC (n, range)	54 (4-3284)	0	NA	-	0 (0-3284)

Table 3: Characteristics of CSF samples at diagnosis grouped by result of flow cytometric analysis (positive FC+ vs negative FC-).

Abbreviations: ALL acute lymphoblastic leukemia, FC flow cytometry, RBC red blood cells, CC conventional cytology, WBC white blood cells, ns not significant

ALL at diagnosis	*FC+*	*FC–*	*FC not available*	*p value (FC+ vs FC-)*	*Total*
Number of patients	**34 (39.0%)**	**50 (57.5%)**	**3 (3.4%)**		**87 (100%)**
Gender					
Male	24 (70.6%)	28 (56.0%)	2 (66.7%)	ns	54 (62.0%)
Female	10 (29.4%)	22 (44.0%)	1 (33.3%)		33 (38.0%)
Median age, range	4.1 (1.4-17.3)	4.9 (1.8-17.6)	5.9 (2.7-17.7)	ns	4.7 (1.4-17.7)
Immunophenotype				**0.02**	
pB	26 (76.4%)	47 (94.0%)	2 (66.7%)		75 (86.2%)
T	8 (23.5%)	3 (6.0%)	1 (33.3%)		12 (13.8%)
Translocation					
t(12;21)	5 (14.7%)	14 (28.0%)	0	ns	19 (21.8%)
t(1;19)	1 (2.9%)	1 (2.0%)	0		2 (2.3%)
Negative*	28 (82.4%)	35 (70.0%)	3 (100.0%)		66 (75.8%)
PB WBC at lumbar puncture				ns	
WBC >50.000/µl	8 (23.5%)	6 (12.0%)	0		53 (60.9%)
WBC <50.000/ µl	26 (76.5%)	44 (88.0%)	2(66.7%)		33 (38.0%)
Not known	0	0	1 (33.3%)		1 (1.1%)
PB blast percentage (median, range)	63 (4-93)	25 (0-87)	13.5 (2-25)	**0.0003**	43 (0-93)
CNS status					
CNS1	28 (82.4%)	45 (90%)	2 (66.7%)	ns	75 (86.2%)
CNS2	3 (8.8%)	4 (8.0%)	0	ns	7 (8.0%)
CNS3	3 (8.8%)	1 (2.0%)	1 (33.3%)	ns	5 (5.8%)
Risk group					
HR	14 (41.2%)	10 (20.0%)	1 (33.3%)	**0.03**	25 (28.7%)
MR	9 (26.5%)	33 (66.0%)	2 (66.7%)	**0.0004**	44 (50.6%)
SR	11 (32.3%)	7 (14.0%)	0	**0.04**	18 (20.7%)
Outcome					
Relapse (total)	**2 (5.8%)**	**0**	**0**	**ns (0.08)**	**2 (2.3%)**
Isolated BM	2 (5.8%)	0	0		2 (2.3%)
Isolated CNS	0	0	0		0
Combined	0	0	0		0
Deaths (total)	**3 (8.8%)**	**1 (2.0%)**	**1 (33.3%)**	**ns**	**4 (4.5%)**
Disease related	1 (2.9%)	0	0	ns	1 (1.1%)
Treatment related	1 (2.9%)	1 (2.0%)	1(33.3%)	ns	3 (3.4%)

Table 4: Characteristics of patients who presented positive CSF (FC+) or negative (FC-) by flow cytometry at diagnosis. *negative for t(4;11), t(9;22), t(12;21), t(1;19)

Abbreviations: ALL acute lymphoblastic leukemia, FC flow cytometry, PB peripheral blood, WBC white blood cells, CNS central nervous system, HR high risk, MR medium risk, SR standard risk, BM bone marrow, ns not significant.

CNS1 at diagnosis	*FC+*	*FC–*	*p value (FC+ vs FC-)*	*FC not available*	*TOTAL CNS1*
Number of samples	**28 (37.4 %)**	**45 (60.0%)**		**2 (2.7%)**	**75 (100%)**
RBC Contamination			**0.001**		
RBC+	9 (32.1%)	2 (4.4%)		0	11
RBC-	19 (67.9%)	43 (97.7%)		2 (100.0%)	64
Median volume (µl), range	400 (100-850)	400 (100-1500)	ns	NA	400 (100-1500)
Median n° of CSF WBC/µl by cell count (range)	0 (0-14)	0 (0-2)	ns		0 (0-14)
Median events identified as blasts (n, range)	50 (4-2687)	0	-	NA	0 (0-2687)

Table 5: Characteristics of samples of CNS1 patients grouped by flow cytometry at diagnosis.

Abbreviations: CNS central nervous system, FC flow cytometry, RBC red blood cells, CSF cerebrospinal fluid, WBC white blood cells, ns not significant.

CNS1 at diagnosis	*FC+*	*FC–*	*P value (FC+ vs FC-)*	*FC not available*	*TOTAL CNS1*
Number of patients	**28 (37.4 %)**	**45 (60.0%)**		**2 (2.7%)**	**75 (100%)**
Gender			ns		
Male	20 (71.4%)	24 (53.3%)		2 (100.0%)	46 (61.3%)
Female	8 (28.6%)	21 (46.7%)		0	29 (38.7%)
Median age, range	4.1 (1.4-17.3)	4.7 (1.8-17.6)	ns	11.5 (2.7-17.7)	4.5 (1.4-17.7)
Immunophenotype			**0.04**		
pB	23 (82.1%)	44 (97.8%)		2 (100.0%)	69 (92.0%)
T	5 (17.9%)	1 (2.2%)		0	6 (8.0%)
Translocation					
t(12;21)	5 (17.8%)	13 (28.9%)	ns	0	18 (24.0%)
t(1;19)	1 (3.6%)	1 (2.2%)		0	2 (2.7%)
Negative*	22 (78.6%)	31 (68.9%)		2 (100.0%)	55 (73.3%)
PB WBC at lumbar puncture			ns		
WBC >50.000/µl	6 (21.4%)	4 (8.9%)		0	10
WBC <50.000/ µl	22 (78.6%)	41 (91.1%)		2 (100.0%)	65
PB blast percentage (median, range)	60 (4-93)	27 (0-87)	**0.001**		
Risk group					
HR	11 (39.3%)	9 (20.0%)	ns	0	20 (26.7%)
MR	8 (28.6%)	31 (68.9%)	**0.0008**	2 (100.0%)	41 (54.7%)
SR	9 (32.1%)	5 (11.1%)	**0.02**	0	14 (18.6%)
Outcome					
Relapse (total)	2 (7,1%)	0	ns (0.07)	0	2 (2.7%)
Isolated BM	2 (7.1%)	0		0	2 (2.7%)
Isolated CNS	0	0		0	0
Combined	0	0		0	0
Deaths (total)	2 (7.1%)	1 (2.0%)	ns	0	3 (4.0%)
Disease related	1 (3.6%)	0		0	1 (1.3%)
Treatment related	1 (3.6%)	1 (2.0%)		0	2 (2.7%)

Table 6: Characteristics of CNS1 patients who presented positive CSF (FC+) or negative (FC-) by flow cytometry at diagnosis. *negative for t(4;11), t(9;22), t(12;21), t(1;19)

Abbreviations: ALL acute lymphoblastic leukemia, FC flow cytometry, PB peripheral blood, WBC white blood cells, CNS central nervous system, HR high risk, MR medium risk, SR standard risk, BM bone marrow, ns not significan

During treatment	*FC+*	*FC–*	*P value (FC+ vs FC-)*	*FC+/-*	*P value (FC+ vs FC+/-)*	*Total*
Number of samples	**37 (3.8%)**	**918 (95.1%)**		**11 (1.1%)**		**966 (100%)**
RBC contamination						
RBC+	12 (32.4%)	287 (31.3%)	ns	3 (27.3%)	ns	302 (31.3%)
RBC-	25 (67.6%)	631 (68.7%)		8 (72.7%)		664 (68.7%)
Cytology						
CC+	0	0	ns	0		0
CC-	37	918		11		966
Median volume, range (µl)	450 (50-1500)	400 (50-1500)	ns	450 (120-800)	ns	400 (50-1500)
Median n° of CSF WBC/µl by cell count (range)	1 (0-20)	0 (0-50)	0.03	1 (0-4)	ns	0 (0-50)
Median events identified as blasts (n, range)	17 (5-419)	0	-	7 (4-50)	ns	0 (4—419)

Table 7: Characteristics of positive (FC+), negative (FC-) or uncertain samples (FC+/-) by flow cytometry during treatment

Abbreviations: FC flow cytometry, RBC red blood cells, CSF cerebrospinal fluid, WBC white blood cells, ns not significant.

During treatment	*At least one FC+*	*At least one FC +/-*	*FC-*	*P value (Fc+ vs FC-)*	*Total*
Number of patients	**19 (21.8%)**	**5 (5.7%)**	**63 (72.4%)**		**87 (100%)**
Gender					
Male	14 (73.7%)	2 (40.0%)	38 (60.3%)	ns	54 (62.0%)
Female	5 (26.3%)	3 (60.0%)	25 (39.7%)		33 (38.0%)
Median age, range	5.9 (2.1-17.7)	4.6 (1.4-9.8)	4.5 (1.7-17.6)	ns	4.7 (1.4-17.7)
Immunophenotype					
pB	10 (52.6%)	4 (80.0%)	61 (96.8%)	**<0.0001**	75 (86.2%)
T	9 (47.4%)	1 (20.0%)	2 (3.2%)		12 (13.8%)
Translocation					
t(12;21)	3 (15.8%)	1 (20.0%)	15 (23.8%)	ns	19 (21.8%)
t(1;19)	0	0	2 (3.2%)		2 (2.3%)
Negative*	16 (84.2%)	4 (80.0%)	46 (73.0%)		66 (75.8%)
CNS status at diagnosis					
CNS1	11 (57.9%)	5 (100.0%)	59 (93.7%)	**0.0001**	75 (86.2%)
CNS2	3 (15.8%)	0	4 (6.3%)	ns	7 (8.0%)
CNS3	5 (26.3%)	0	0	**<0.0001**	5 (5.8%)
FC at diagnosis					
FC+	11 (57.8%)	2 (40.0%)	21 (33.3%)	**0.02**	34 (40.2%)
FC-	6 (31.6%)	3 (60.0%)	41 (65.1%)		50 (56.3%)
Not known	2 (10.5%)	0	1 (1.6%)		3 (3.4%)
Risk group					
HR	11 (57.9%)	1 (20.0%)	13 (20.6%)	**0.002**	25 (28.7%)
MR	5 (26.3%)	4 (80.0%)	35 (55.6%)	**0.03**	44 (50.6%)
SR	3 (15.8%)	0	15 (23.8%)	ns	18 (20.7%)
Outcome					
Relapses (total)	**1 (5.3%)**	**0**	**1 (1.5%)**	**ns**	**2 (2.3%)**
Isolated BM	1 (5.3%)	0	1 (1.5%)		2 (2.3%)
Isolated CNS	0	0	0		0
Combined	0	0	0		0
Deaths (total)	**2 (10.5%)**	**0**	**2 (3.0%)**	**ns**	**4 (4.5%)**
Disease related	0	0	1 (1.5%)	ns	1 (1.1%)
Treatment related	2 (10.5%)	0	1 (1.5%)	ns	3 (3.4%)

Table 8: Characteristics of patients who presented positive (FC+), negative (FC-) or or uncertain samples (FC+/-) by flow cytometry during treatment

Abbreviations: FC flow cytometry, CNS central nervous system, HR high risk, MR medium risk, SR standard risk, BM bone marrow, ns not significant.

ALL at diagnosis and during treatment	*≥2 FC+ or FC+/- samples*	*Only 1 FC+ or FC+/- sample*	*FC-*	*Total*
Number of patients	**15 (17.2%)**	**28 (32.2%)**	**44 (50.6%)**	**87 (100%)**
Gender				
Male	12 (80.0%)	17 (60.7%)	25 (56.8%)	54 (62.0%)
Female	3 (20.0%)	11 (39.3%)	19 (43.2%)	33 (38.0%)
Median age, range	5.9 (1.4-14.6)	4.2 (1.7-17.7)	4.7 (1.8-17.6)	4.7 (1.4-17.7)
Immunophenotype				
pB	6 (40.0%)	26 (92.9%)	43 (97.7%)	75 (86.2%)
T	9 (60.0%)	2 (7.1%)	1 (2.3%)	12 (13.8%)
Translocations				
t(12;21)	0	8 (28.6%)	11 (25.0%)	19 (21.8%)
t(1;19)	0	1 (3.6%)	1 (2.3%)	2 (2.3%)
Negative	15 (100%)	19 (67.8%)	32 (72.7%)	66 (75.8%)
CNS status				
CNS1	9 (60.0%)	24 (85.7%)	42 (95.5%)	75 (86.2%)
CNS2	2 (13.3%)	3 (10.7%)	2 (4.5%)	7 (8.0%)
CNS3	4 (26.7%)	1 (3.6%)	0	5 (5.8%)
Fc at diagnosis				
FC+	13 (86.6%)	21 (75.0%)	0	33 (37.9%)
FC-	1 (6.7%)	6 (21.4%)	43 (97.7%)	51 (58.6%)
Not known	1 (6.7%)	1 (3.6%)	1 (2.3%)	3 (3.4%)
Risk group				
HR	12 (80.0%)	4 (14.3%)	9 (20.5%)	25 (28.7%)
MR	1 (6.7%)	14 (50.0%)	29 (65.9%)	44 (50.6%)
SR	2 (13.3%)	10 (35.7%)	6 (13.6%)	18 (20.7%)
Outcome				
Relapses	**1 (6.7%)**	**1 (3.6%)**	**0**	**2 (2.3%)**
Isolated BM	1 (6.7%)	1 (3.6%)	0	2 (2.3%)
Isolated CNS	0	0	0	0
Combined	0	0	0	0
Deaths	**2 (13.3%)**	**1 (3.6%)**	**1 (2.3%)**	**4 (4.5%)**
Disease related	0	1 (3.6%)	0	1 (1.1%)
Treatment related	2 (13.3%)	0	1 (2.3%)	3 (3.4%)

Table 9: Characteristics of patients grouped by number of FC+ or FC+/- samples at diagnosis and during treatment.

Abbreviations: FC flow cytometry, CNS central nervous system, HR high risk, MR medium risk, SR standard risk, BM bone marrow, ns not significant

FC CSF at diagnosis and during treatment	*p value (≥2 FC+ vs 1FC+)*	*p value (≥2 FC+ vs FC-)*	*p value (1 FC+ vs FC-)*
Gender (M vs F)	ns	ns	ns
Median age	ns	ns	ns
Immunophenotype (pB vs T)	**0.0002**	**0.0002**	ns
t(12;21) vs no translocation	**0.02**	**0.002**	ns
CNS 1 vs CNS2+3	ns	**0.0005**	ns
CNS2 vs CNS1+3	ns	ns	ns
CNS3 vs CNS1+2	**0.02**	**0.0004**	ns
FC at diagnosis (FC+ vs FC-)	ns	**<0.0001**	**<0.0001**
HR vs MR+SR	**<0.0001**	**<0.0001**	ns
MR vs HR+SR	**0.005**	**<0.0001**	ns
SR vs MR+HR	ns	ns	**0.03**
Relapses (n° relapses vs not relapsed)	ns	ns (0.08)	ns
Deaths (n° total dead vs alive)	ns	ns (0.07)	ns

Table 10: Statistical analysis (Chi-square or Mann-Whitneytest) of patient characteristics grouped by number of FC+ or FC+/- samples at diagnosis and during treatment.

Abbreviations: FC flow cytometry, CNS central nervous system, HR high risk, MR medium risk, SR standard risk, BM bone marrow, ns not significant

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
Pt	**CNS status**	**IF**	**risk**	**CRT**	**HSCT**	**outcome**	**diagn**	**d15**	**(IA)**	**(IA)**	**d33**	**IB**	**IB**	**cons**	**reind**	**other**
1	CNS1	T	H	TBI	MUD	Alive CR	+	+	nd	nd	+	-	-	-	-	
2	CNS2	pB	S	no	No	Alive CR	+	+	-	-	-	-	-	+(1)	+/- (1)	
3	CNS1	pB	H	no	No	Alive CR	+	-	nd	nd	-	-	-	+/- (1)	-	
4	CNS1	pB	S	no	No	Alive CR	+	-	nd	nd	-	-	+	-	-	
5	CNS3	T	H	18 Gy	No	Alive CR	+	+	-	-	-	-	-	+ (1)	+	
6	CNS3	T	H	18 Gy	No	Alive CR	+	+	+	-	-	-	-	-	-	
7	CNS3	T	H	18 Gy	No	Alive CR	+	+	+	+/-	-	+	-	-	+ (4)	
8	CNS1	pB	H	no	No	Alive CR	+	-	nd	nd	-	+	-	-	-	
9	CNS1	pB	H	no	No	Alive CR	+	-	nd	nd	-	-	-	+ (1)	-	
10	CNS1	pB	H	no	No	Alive CR	+	-	nd	nd	-	-	-	+ (1)	-	
11	CNS1	pB	H	TBI	MUD	Alive CR	+	+	nd	nd	-	-	-	+/- (1)	-	
12	CNS2	T	H	TBI	MUD	Alive CR	-	-	-	-	+	-	-	+ (1)	-	+/-
13	CNS1	T	M	no	No	Alive CR	+	-	nd	nd	+/-	-	-	-	-	
14	CNS1	T	H	No (dead before)	No	Rel, TR	+	-	nd	nd	-	+	+	+ (3)	-	
15	CNS3	T	H	TBI+ CNS boost	MUD	Rel, TR	NA	NA	NA	NA	+	-	+	+ (2)	-	

Table 11: Patients with more than 2 FC+ samples are listed. First 7 columns refer to patient characteristics. From column 8, flow cytometric results on CSF samples are reported at different treatment time points. Column 10 and 11 refer to the adjunctive lumbar puncture during IA for CNS3 patients. Results of lumbar puncture performed during consolidation and reinduction are grouped in one column (15 and 16) and the number of positive samples is reported in brackets.

Abbreviations: Pt patient, CNS central nervous system, IF immunophenotyped, CRT cranial radiotherapy, HSCT hematopoietic stem cell transplantation, diagn diagnosis, d15 day 15, IA induction IA, d 33 day 33, IB Induction IB, cons consolidation therapy, Reind Reinduction therapy, H high (risk), M medium, S standard, TBI total body irradiation, MUD matched unrelated donor, CR complete remission, Rel relapse, TR treatment related, nd not done (it was part of the treatment protocol only for CNS2 and CNS3), NA sample not available (patient treated in another center)

ID	gender	Age at relapse (years)	IF	Time to relapse	Genetic	Second line treatment	HSCT	FC at relapse	Other FC+ (n)	rec	outcome
1	M	11,33	pB	very early	no	REC 2003	haplo	+	5	iCNS	Dead, TR
2	F	9,50	pB	late	no	REC 2003	MUD	+	3	iBM	Dead Progression
3	M	9,70	pB	late	no	REC 2003	MSD	+	1	no	Alive CR
4	M	6,05	pB	late	t(12;21)	REC 2003	MSD	+	4	iBM	Dead Progression
5	F	4,64	pB	very early	no	REC 2003	MUD	-	0	no	Dead TR
6	F	4,85	pB	late	no	REC 2003	MUD	-	0	no	Alive CR
7	F	10,82	pB	post HSCT	t(9;22)	personalized	MSD	+	5	iCNS	Alive Relapse
8	M	7,00	pB	late	no	REC 2003	haplo	-	0	iBM	Dead TR
9	M	10,45	T	early	FLT3ITD	REC 2003	MUD	+	0	iBM	Alive CR
10	M	2,74	pB	very early	no	personalized	no	+	0	no	Dead Progression
11	M	5,12	pB	very early	no	personalized	no	-	2	iBM	Dead Progression
12	M	12,37	pB	post HSCT	no	personalized	haplo	-	0	no	Alive CR
13	F	10,24	pB	late	no	IntReAll	haplo	-	0	no	Alive CR

Table 12: Characteristics of patients with isolated bone marrow relapse.

Abbreviations:IF immunophenotyped, HSCT hematopoietic stem cell transplantation, M male, F female, MUD matched unrelated donor, MSD matched sibling donor, haplo haploidentical donor, FC flow cytometry (of CSF), iCNS isolated central nervous system, iBM isolated bone marrow, CR complete remission, TR treatment related.

ALL at iBM relapse	***FC+***	***FC–***	***P value (FC+ vs FC-)***	***Total***
Number of samples	**7 (53.8%)**	**6 (46.2%)**		**13 (100%)**
RBC contamination				
RBC+	2 (28.6%)	2 (33.3%)	ns	4 (30.8%)
RBC-	5 (71.4%)	4 (66.7%)		9 (69.2%)
Cytology				
CC+	0	0	ns	0
CC-	7 (100%)	6 (100%)		13 (100%)
Median volume, range (µl)	500 (200-800)	700 (300-1000)	ns	700 (200-1000)
Median n° of CSF WBC/µl by cell count (range)	1(0-4)	1.5 (0-5)	ns	1 (0-5)
Median events identified as blasts (n, range)	92 (10-579)	0	-	10 (0-579)

Table 13: Characteristics of positive (FC+), negative (FC-) or uncertain samples (FC+/-) by flow cytometry at iBM relapse

Abbreviations: ALL acute lymphoblastic leukemia, iBM isolated bone marrow relapse, FC flow cytometry, RBC red blood cells, CSF cerebrospinal fluid, WBC white blood cells, ns not significant.

ALL at iBM relapse	*FC+*	*FC–*	*P value (FC+ vs FC-)*	*Total*
Number of patients	**7 (53.8%)**	**6 (46.2%)**		**13 (100%)**
Gender				
Male	5 (71.4%)	3 (50.0%)	ns	8 (61.5%)
Female	2 (28.6%)	3 (50.0%)		5 (38.5%)
Age median, range	9.7 (2.7-11.3)	6 (4.6-10.2)	ns	9.5 (2.74-12.37)
Immunophenotype				
pB	6 (85.7%)	6 (100.0%)	ns	12 (92.3%)
T	1 (14.3%)	0		1 (7.7%)
Time to relapse				
Late	3 (42.8%)	3 (50.0%)	ns	6 (46.2%)
early	1 (14.3%)	0	ns	1 (7.7%)
Very early	2 (28.6%)	2 (33.3%)	ns	4 (30.8%)
Post HSCT	1 (14.3%)	1 (16.7%)	ns	2 (15.4%)
Protocol of CT				
ALL-REC 2003	5 (71.4%)	3 (50.0%)	ns	8 (61.5%)
IntReALL-SR	0	1 (16.7%)	ns	1 (7.7%)
personalized	2 (28.6%)	2 (33.3%)	ns	4 (30.8%)
HSCT				
MFD	3 (42.8%)	0	ns	3 (23.1%)
MUD	2 (28.6%)	2 (33.3%)	ns	4 (30.8%)
Haplo	1 (14.3%)	3 (50.0%)	ns	4 (30.8%)
No HSCT	1 (14.3%)	1 (16.7%)	ns	2 (15.3%)
Outcome				
Second Relapse (Total)	**5 (71.4%)**	**2 (33.3%)**	**ns**	**7 53.8%)**
iBM	3 (42.8%)	2 (33.3%)	ns	5 (38.5%)
iCNS	2 (28.6%)	0	ns	2 (15.4%)
combined	0	0		
Deaths (total)	4 (57.2%)	3 (50.0%)	ns	7 (53.8%)
Disease related	3 (42.8%)	1 (16.7%)	ns	4 (30.8%)
Treatment related	1 (14.3%)	2 (33.3%)	ns	3 (23.1%)

Table 14: Characteristics of patients who presented positive CSF (FC+) or negative (FC-) by flow cytometry at isolated bone marrow relapse

Abbreviations: M male, F female, CT chemotheraoy, HSCT hematopoietic stem cell transplantation, MUD matched unrelated donor, MSD matched sibling donor, haplo haploidentical donor, FC flow cytometry (of CSF), iCNS isolated central nervous system, iBM isolated bone marrow, CR complete remission, TR treatment related, ns not significant.

CSF during relapse treatment	*FC+*	*FC–*	*p value (FC+ vs FC-)*	*FC+/-*	*Total*
Number of samples	**20 (20.8%)**	**75 (78.1%)**		**1 (1%)**	**96 (100%)**
Cytology					
CC+	3 (15.0%)	0	**0.0007**	0	3 (3.1%)
CC-	17 (85.0%)	75 (100%)		1 (100%)	93 (96.9%)
RBC contamination					
RBC+	8 (40.0%)	20 (26.7%)	ns	0	28 (28.6%)
RBC-	12 (60.0%)	55 (73.3%)		1 (100%)	68 (71.4%)
Median volume, range (µl)	400 (100-1200)	400 (100-1500)	ns	500	400 (100-1500)
Median n° of WBC/µl by cell count (range)	2 (0-214)	0 (0-14)	**0.002**	0 (0-1)	1 (0-214)
Median events identified as blasts (n, range)	100 (5-32300)	0	-	10 (21-7)	0 (0-32300)

Table 15: Characteristics of positive (FC+), negative (FC-) or uncertain samples (FC+/-) by flow cytometry during treatment of iBM ALL relapse.

Abbreviations: ALL acute lymphoblastic leukemia, iBM isolated bone marrow relapse, FC flow cytometry, RBC red blood cells, CSF cerebrospinal fluid, WBC white blood cells, ns not significant

CSF at relapse and during treatment	*≥2 FC+ or FC+/-*	*Only 1 FC+ or FC+/-*	*FC–*	*P value (>2 FC+ vs FC-)*	*Total*
Number of patients	**6 (46.1%)**	**2 (15.4%)**	**5 (38.5%)**		**13 (100%)**
Gender					
Male	4 (66.7%)	2 (100.0%)	2 (40.0%)	ns	8 (61.5%)
Female	2 (33.3%)	0	3 (60.0%)		5 (38.5%)
Median age, range	9.6 (5.1-11.3)	6.5 (2.7-10.4)	7.0 (4.6-12.3)	ns	9.5 (2.74-12.37)
Immunophenotype					
pB	6 (100.0%)	1 (50.0%)	5 (100.0%)	ns	12 (92.3%)
T	0	1 (50.0%)	0		1 (7.7%)
FC at relapse					
FC+	5 (83.3%)	1 (50.0%)	0	**0.006**	6 (46.2%)
FC-	1 (16.7%)	1 (50.0%)	5 (100%)		7 (53.8%)
Time to relapse					
Late	3 (50.0%)	0	3 (60.0%)	ns	6 (46.2%)
Early	0	1 (50.%)	0	ns	1 (7.7%)
Very early	2 (33.3%)	1 (50.0%)	1 (20.0%)	ns	4 (30.8%)
Post HSCT	1 (16.7%)	0	1 (20.0%)	ns	2 (15.4%)
Protocol of CT					
ALL-REC 2003	4 (66.6%)	0	3 (60.0%)	ns	7 (53.8%)
IntReALL-SR	0	0	1 (20.0%)	ns	1 (7.7%)
personalized	2 (33.3%)	2 (100.0%)	1 (20.0%)	ns	5 (38.5%)
HSCT					
MFD	3 (50.0%)	0	0	ns	3 (23.1%)
MUD	1 (16.7%)	1 (50.0%)	3 (60.0%)		5 (38.5%)
Haplo	1 (16.7%)	0	2 (40.0%)		3 (23.0%)
No HSCT	1 (16.7%)	1 (50.0%)	0		2 (15.4%)
Outcome					
Relapses (total)	**5 (83.3%)**	**1 (50%)**	**1(20.0%)**	**0.04**	**7 (53.8%)**
iBM	3 (50.0%)	1 (50.0%)	1(20.0%)	ns	5 (38.5%)
iCNS	2 (33.3%)	0	0	ns	2 (15.4%)
combined	0	0	0		0
Deaths (total)	**4 (66.7%)**	**1 (50%)**	**2 (40.0%)**	**ns**	**7 (53.8%)**
Disease related	3 (50.0%)	1 (50.0%)	0	ns	4 (30.8%)
Treatment related	1 (16.7%)	0	2 (40.0%)	ns	3 (23.1%)

Table 16: Characteristics of patients grouped by number of FC+ or FC+/- samples at relapse and during relapse treatment.

Abbreviations: M male, F female, FC flow cytometry, CT chemotherapy, HSCT hematopoietic stem cell transplantation, MUD matched unrelated donor, MSD matched sibling donor, haplo haploidentical donor, FC flow cytometry (of CSF), iCNS isolated central nervous system, iBM isolated bone marrow, CR complete remission, TR treatment related, ns not significant.

Discussion

Despite the great advances in the cure of ALL, relapses still occur in approximately 15-20% of patients. Relapses involving the CNS account for up to 40% of all relapses, as apposite to 5% CNS involvement at diagnosis (59). Factors associated with augmented risk of CNS relapse are: the presence of blasts in the cerebrospinal fluid at diagnosis, T-ALL phenotype, hyperleukocytosis and high risk translocations such as t(9;22) and t(4;11) (35).

Prophylaxis and therapy directed to the CNS include intrathecal administration of chemotherapy and cranial irradiation. In case of HSCT, total body irradiation (TBI) combined to a radiotherapy boost to cranium and, in some cases, spine, are used to completely eradicate meningeal leukemia in patients at risk. CRT increases the risk of secondary neoplasms (especially CNS neoplasms), neurocognitive and endocrine dysfunctions and growth impairment (35). Therefore, most recent trials have omitted radiotherapy form first line therapy of ALL (14).

Even an intrathecal chemotherapy approach is not without adverse events including post-dural puncture headache, CNS hemorrhage, leukoencephalopathy, chemical meningitides and spinal cord dysfunction (35). Moreover, it is reported that patients who underwent CNS prophylaxis with IT therapy only had also some neurocognitive deficit in the area of attention and memory (10,11).

In order to define which patients may benefit of a less toxic treatment or, on the contrary, may have a high risk of CNS relapse therefore requiring specific intervention, it is essential to identify reliable risk factors.

Current definition of CNS involvement in asymptomatic patients requires a positive cytology with cell count over a certain threshold and/or a positive neuroimaging. Sensitivity and, in some cases, also specificity of cytology has been proven low. Limitations to cytological diagnosis include: the small sample volume and the paucity of cellular material, the need to process the sample quickly to prevent cell degeneration and the possible difficulty in distinguish blasts from normal lymphocytes or monocytes. Flow cytometry has been used to analyze CSF samples, showing high sensitivity even in samples with low cellularity. Moreover, it can help in identifying blasts from normal cells, thus improving CC specificity (16,17). Cell degradation can be prevented by the use of fixative before FC analysis (18,45,55).

Studies on adult patients with lymphoma or leukemia reported a higher prevalence of CSF positivity by FC in comparison to cytology (44, 45, 47, 48). Moreover, prospective studies on patients affected by aggressive B non-Hodgkin lymphoma (NHL) reported an inferior outcome and a higher frequency of CNS relapses in patients who presented blasts in CSF by flow cytometry (46-48).

In pediatric ALL, CNS involvement detected by FC but not by standard CC has been found in around 30% of children at primary diagnosis (49, 55, 57). Clinical significance in children with ALL is less clear: four papers until now had addressed this topic.

A retrospective study published in 2015 reported the results in a group of 302 children with ALL diagnosed from 2000 to 2012. CSF samples were studied at diagnosis by FC (from three to eight colors). Treatment protocols and risk stratification were heterogeneous: patients were treated according to NOPHO ALL1992, ALL2000, ALL2008, Interfant99 or Interfant06. FC positivity was found

in 4% of children, in this group a higher frequency of high risk patients was observed. Patients with FC+ sample at diagnosis had a higher incidence of relapse compared to FC- but the small numbers did not allow a statistical comparison (50).
In a Spanish multicenter study, 108 children (0-15 years) were included and CSF was analyzed prospectively at diagnosis and during treatment (in total 990 samples, traumatic lumbar punctures were excluded). Patients were treated according to two chemotherapy protocol (SHOP or PETHEMA), median follow up was 35 months. Five colors-FC resulted positive in 27.8% samples a diagnosis and in 7.2% during treatment. The presence of FC+ was associated with other poor prognostic features such as high WBC counts, HR classification, T-ALL, MLL rearrangement and t(9;22) translocation. CSF positivity by FC during treatment, but not at diagnosis, was associated to a higher incidence of relapses and a higher mortality. Multivariable analysis, however, was not performed in order to rule out the impact of high risk factors on the inferior outcome of FC+ patients (55).
A Chinese prospective single center's study analyzed 313 children (2-14 years) with ALL treated uniformly with a chemotherapy protocol not including CRT. Infant cases and children with t(9;22) were included. Seventy-nine patients presented FC+ samples by 3 colors-FC (13 at presentation and 66 during therapy), they were randomly allocated to standard treatment or to enhanced IT therapy. The latter group showed a better OS (80% vs 50%) and a lower incidence of CNS relapse (30% vs 10%). A multivariable analysis was not performed. However, these results can be difficultly compared to other works, as treatment protocol, access to care, and subsequently, relapse and mortality rates are very different from that area in China to Western countries (56).

Finally, a paper published last year by NOPHO (Nordic Society of Pediatric Hematology and Oncology) reported results obtained by 6-7 colors-FC on a cohort of 300 children affected by ALL and 9 relapsed cases. Patients were treated according to Interfant-06 (if younger than 1 year), EsPhALL (if Philadelphia positive) or NOPHO ALL2008. FC found blasts at diagnosis in 29% of children, these patients were more frequently T-ALL, younger and with high WBC count on peripheral blood. Among 9 relapsed patients 56% were FC+, but nor CC results, nor clinical characteristics or outcome were reported for this group. Among FC+ patients at diagnosis, 10% of them were still positive at day 15, subsequent samples were not analyzed. The clinical significance of these findings could not be ruled out by this study (57).

Given that the clinical significance of CSF involvement detected by flow cytometry is not clear in children with ALL and relapsed ALL, we performed a prospective single-center study from 2013 to 2016. One thousand and one hundred sixty-one CSF samples, at each lumbar puncture during therapy in primary and relapsed ALL, were analyzed by FC and compared to cytology and cell count; prognostic value of FC analysis was studied.

A number of aspects made this study different from the previous ones. Collected CSF samples were processed directly for FC without the addiction of any preservative or fixative, to exclude the possibility that this could alter final results. This was possible because the work was carried out at a single institution and the samples could be analyzed within 24 hours. It is important, as we did, that all the samples were handled in the same way. Notably, in our hand, samples were still evaluable by FC at least until 24 hours from collection.

For this study we used 8-colors flow cytometry, thus allowing a complete analysis in a single tube with less CSF volume requirement. Moreover, the higher number of antigen analyzed (as compared to other pediatric studies) improves the specificity of the analysis. Differently to other studies (45, 52, 54), that used 10 to 20 events as an arbitrary cut off value, we did not set a threshold to define the positivity of a sample by FC, because we choose not to exclude any finding from the analysis based on arbitrary rules. For the same reason, we decided not to exclude samples possibly contaminated by peripheral blood. Nevertheless, the percentage of FC+ at diagnosis in our cohort was comparable to another published report (55).

RBC contamination was higher in the FC+ group at diagnosis compared to FC- (44% vs 6%, $p<0.0001$), suggesting that samples resulted positive by FC because of PB contamination. Nevertheless, 56% of FC+ samples were negative for RBC. Moreover, even if blasts detected by FC could have been of PB origin, we thought that their presence in CSF could be a potential risk factor for subsequent CNS relapse. Therefore, we decided not to exclude those samples from the analysis. During treatment, there was no difference in terms of RBC contamination between FC+ and FC- samples. Results were not influenced by sample volume, as no difference in median volume was evident between FC+ and FC- specimens at diagnosis and during treatment.

Both PB contaminated or no-contaminated samples contained T lymphocytes, and a small proportion monocytes and B lymphocytes. This finding is not reported by studies on pediatric ALL, but it is in line with adult reports (15, 45).

Cytological analysis resulted less sensible than flow cytometry in detecting blasts, as reported by other authors (16-18,45): in total 29/87 (33.3%) samples were

negative by CC but positive by FC. This frequency is comparable to the study by *Martinez-Laperche et al* (55) who found 25% of samples positive by FC only. One case resulted CC+ but FC-: flow cytometry showed normal B and T lymphocytes that were possibly interpreted as blasts by morphologist.

Regarding clinical characteristics, patients with FC+ at diagnosis had mainly T-ALL, higher peripheral blast percentage and high-risk features. The same was also observed by the works of *Martinez-Laperche* and *Levinsen* (55,57), with the exception that they found a higher peripheral WBC count among FC+ patients. In our study, in the CNS1 group, significant differences between FC+ and FC- at diagnosis remained phenotype and PB blast percentage.

FC+ patients at diagnosis showed a trend towards an inferior outcome: more BM relapses and deaths were found in FC+ group compared to FC-, but the difference did not result statistically significant. Moreover, due to the low numbers of relapsed patients, a survival analysis nor a multivariable analysis were performed. These results are in line with the work by *Ranta et al* (50), that, retrospectively, found a higher number of relapses among FC+ patients at diagnosis without statistical significance, and with *Martinez-Laperche et al* (55) who did not find any difference in outcome based on FC results at diagnosis. Our data are in contrast with those reported by *Liang et al* (56), who found more CNS relapses among FC+ patients. Since the incidence of relapses is relatively low in pediatric ALL, the total number of patients and the length of follow up of our work may have been too small to detect any statistical difference between FC+ and FC- patients. As a demonstration, when children were treated with less intensive protocols, as in the Chinese study, FC detection of blasts in CSF was predictive of CNS relapse.

Moreover, our cohort did not include patients with t(9;22) translocation nor infant ALL patients, both categories having a higher risk of CNS relapse (35).

We then analyzed separately CSF samples obtained during ALL treatment. As mentioned in the results, RBC contamination and volume were homogenously distribute between FC+ and FC- samples, CSF WBC count was higher among FC+. Like at diagnosis, FC+ patients during treatment were mainly T-ALL, CNS3 and HR. The same characteristics were reported by *Martinez-Laperche et al* when they analyzed the group with FC+ during treatment (55). Differently to their work, our study did not find any outcome difference for this subgroup of patients. Even considering patients with more than 2 FC+ samples at diagnosis and during treatment, we could not demonstrate an augmented risk of relapse or death.

Looking at Table 11, where characteristics of patients having more than 2 FC+ samples are reported, it appears that patients having T-ALL, CNS3 and/or HR features are prone to have occult CNS involvement at diagnosis and at different time points during treatment. Nevertheless, they do not exhibit overt CNS leukemia probably thanks to intensive treatment that it is already administered to these categories, including RCT in CNS3 and T-HR patients.

Regarding patients with iBM ALL relapse, FC found blasts in CSF at presentation in 53.8% of patients, similarly to that reported by *Levinsen et al* (57).

In our cohort of relapsed ALL, FC+ samples were not associated to higher RBC contamination, CSF WBC count or sample volume compared to FC-. Patient characteristics and outcome did not differ in relation to FC results at relapse presentation.

Five out of six patients who presented FC+ at relapse, had other FC+ samples during relapse treatment. One patient was negative at relapse but showed other FC+ samples during the course of therapy. Five out of 6 patients with more than 2 FC+ samples presented a subsequent relapse and 4/5 died (one is still alive but not in CR). Relapse incidence resulted higher for this group of children compared to FC-, mortality showed the same trend but without statistical significance. The 2 CNS relapses were confined to the group of patients FC+, but we could not demonstrate a statistical association. Notably, phenotype, time to relapse, type of treatment and HSCT did not differ between the two groups (≥2 FC+ vs FC-). Due to the low number of patients, it was not possible to perform a survival curve or a multivariable analysis. Nevertheless, we think that these results are important for several reasons. First, because this is the first report that prospectively evaluated FC analysis on CSF of children with relapsed ALL. Second, our observation reinforces the concept, suggested by the study of *Hagedorn* who found submicroscopic BM involvement in the majority of isolated extramedullary relapse (60), that most ALL relapses have a systemic rather than an isolated recurrence. The persistent presence of blasts in CSF may reflect either an occult CNS involvement not sufficiently treated (because not apparent by CC) or a resistant BM disease that constantly spreads to CNS. The two patients who developed a CNS relapse probably had an occult CNS involvement that was not adequately treated by systemic chemotherapy. Persistent CSF positivity may also be a signal of a more aggressive disease.

Our study was limited by the small patient sample size, especially of relapsed ALL, and by the relatively short follow up. A multicenter study and a longer follow up

may overcome these limitations. However, the single-center approach permitted us to analyze specimens without fixation and to collect precise clinical data. Moreover, the number of CSF samples we analyzed was larger than published, reaching the total of 1159 samples.

Another possible limitation is that we did not establish a threshold to define CSF positivity by FC, neither we were able to calculate blast load in CSF samples. Given the fact that CSF is usually hypocellular, we think that is not correct to report blast quantity as a percentage, as it is usually done on marrow and blood samples. Quantification of leukemic cells in CSF should be done precisely with counting beads, a method already reported on CSF samples (15, 45). As current available literature is based only on a qualitative assessment of CSF by FC, our results are at least comparable to those published.

In conclusion, our data demonstrated that FC analysis of ALL CSF samples is feasible; CNS involvement by FC is a frequent finding in pediatric ALL at diagnosis and, even more, at relapse. The clinical significance is probably linked to the persistence of CSF positivity rather than to the single sample analysis. Actual frontline treatment protocols seem to be able to control CNS submicroscopic leukemia, although a longer follow up is needed to confirm this finding. In relapsed ALL patients, persistent CSF positivity may be a sign of a more resistant disease and a prognostic negative factor. A larger group of patients has to be studied in order to verify our observations.

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Hematopoietic Stem Cell Transplantation for Isolated Extramedullary Relapse of Acute Lymphoblastic Leukemia in Children

Abstract

Relapse of Acute Lymphoblastic Leukemia (ALL) may occur in extramedullary sites, mainly the central nervous system (CNS) and the testis. Optimal post-remissional treatment for isolated extramedullary relapse (IEMR) is still controversial. To address this issue, we collected data of patients treated with hematopoietic stem cell transplantation (HSCT) for ALL IEMR. From 1990 to 2015, 281 children underwent HSCT for ALL IEMR in Italy. Pre-transplant treatment protocols were based on Berlin-Frankfurt-Münster (BFM) Study Group backbone. HSCT was performed in second complete remission (CR2) or subsequent remission (CR>2); also patients transplanted with active disease were included in the analysis. HSCT from an HLA-matched donor was performed whenever a matched family (MFD) or a matched unrelated donor (MUD) was available; otherwise, the single center chose to perform either autologous HSCT (auto HSCT) or HLA-haploidentical HSCT (haplo HSCT).

Of the 281 patients (203 male, 78 female) 167 presented relapse confined to CNS, 73 to testis, 14 to mediastinum, 11 to CNS and other sites and 18 to other organs. Ninety-seven patients underwent auto HSCT, 79 MFD HSCT, 75 MUD HSCT and 30 haplo HSCT. At transplantation, 72.6% of children were in CR2, 21.0% in CR>2 and 6.4% were not in remission. Overall survival (OS) for the entire cohort was 56% at 10 years and was not influenced by gender, ALL blast

immune-phenotype, age, site of relapse, duration of first remission, type of HSCT (auto *v*s MFD *vs* MUD *vs* haplo). In multivariable analysis, the only factors influencing outcome were disease status at time of HSCT and year of transplantation. Patients in CR>2 had a risk of death 2.3 times greater than those in CR2. Children treated after 2000 had half the risk of death than those treated before that year.

Our results suggest that both autologous and allogeneic HSCT are a suitable treatment for ALL IEMR.

Introduction

Acute lymphoblastic leukemia (ALL) is the most common malignancy of childhood. Although current treatments are able to cure up to 85% of children, relapse is the leading cause of treatment failure, affecting approximately 15-20% of patients. Leukemia relapse most frequently involves the bone marrow (BM), but it can occur in extramedullary sites, mainly the central nervous system (CNS) and the testis, either alone or in combination with BM relapse [1].

Site of relapse and duration of first remission are the most important prognostic factors, early and isolated BM relapse predicting the worst outcome [2, 3]. While the benefit of allogeneic hematopoietic stem cell transplantation (allo HSCT) has been demonstrated for high-risk relapsed patients, optimal post-remissional treatment for low-risk relapsed patients is still controversial [4-8]. Our previous works [9, 10] demonstrated that autologous HSCT (auto HSCT) may be a good curative option for children experiencing isolated extramedullary relapse (IEMR).

For further addressing this issue, we collected and analyzed data of a large cohort of patients with first or subsequent ALL IEMR: 281 children with IEMR were treated with either auto HSCT or allo HSCT over a 25 years period (1990-2015) in Italy. To the best of our knowledge, this is the largest study that uniformly analyzes the outcome of this subgroup of patients.

Patients and Methods

This is a retrospective multicenter study involving 20 Italian centers, members of the Italian Pediatric Onco-Hematology Association (AIEOP) network.

Data were extracted from the AIEOP-Stem Cell Transplantation (AIEOP-SCT) Registry. We included children (age 1-18 years) with IEMR of ALL who underwent HSCT between 1st of January 1990 and 31st December 2015. Written informed consent was obtained from parents or legal guardians.

Patients' characteristics are shown in Table 1. IEMR was defined as presence of leukemia blasts in extramedullary sites with <5% blasts in BM. CNS relapse was defined as presence of >5 cell/μL in the cerebrospinal fluid (CSF) and detection of lymphoblasts by examination of CSF cytocentrifugate. Alternatively, clinical signs of cranial nerves involvement or radiologic signs at cerebral magnetic resonance imaging/ computed tomography (MRI/CT) were considered diagnostic of CNS relapse. Relapse involving testis or other organs was confirmed by biopsy.

"Very early" relapse was defined as disease recurrence occurring less than 18 months from primary diagnosis, "early" relapse if it occurred later than 18 months from diagnosis and less than 6 months from treatment discontinuation, "late" relapse if it occurred more than 6 months from treatment discontinuation [3].

At diagnosis and relapse, patients were treated according to national protocols available at that time, based on Berlin-Frankfurt-Münster (BFM) Study Group backbone.

HSCT was mainly performed in second or subsequent complete remission (CR); patients transplanted with active disease were also included in the study. If a HLA-matched family donor (MFD) or a matched unrelated (MUD) donor was available, allo HSCT was performed; if not, the decision to proceed with either auto HSCT or haploidentical HSCT (haplo HSCT) was taken by the single center.

Conditioning regimen mainly included 12 Gy total body irradiation (TBI), details on conditioning regimens are listed in Table 2.

Statistical analysis

Data were extracted from the AIEOP-SCT Registry on the 28th of February, 2016. Overall Survival (OS) was defined as the time from transplantation to last follow-up or death due to any cause. Disease free survival (DFS) was defined as the time from transplantation to disease recurrence or death from any case. Relapse free survival (RFS) was defined as the time from transplantation to documented relapse of ALL for patients transplanted in CR, for the purpose of this study RFS was calculated in the group of patient with CR2CI of treatment-related mortality (TRM) was defined as the time from transplantation to death from causes other than disease recurrence/progression, taking into account relapse as competing event.
OS, DFS, and RFS were calculated at 10 years using the Kaplan-Meier method with 95% confidence interval. Difference in survival was estimated with the log-rank test.
Cumulative incidence of TRM was evaluated at 100 days, 6 months, 1 year and 10 years from transplantation. Incidence curves were compared with Gray's test. In

multivariable Cox regression analysis, factors with a p-value <0.2 were included. The risk of death was expressed as the hazard ratio (HR) with 95% confidence interval. Differences in the distribution of various parameters were compared using chi-square or Fisher exact test as appropriate. A p-value <0.05 was considered to be statistically significant.

Analysis was performed with SAS software (SASPC, version 9.3, SAS Institute, Cary, NC).

Results

Patient characteristics

Two hundred and ninety-two patients underwent HSCT for IEMR from 1st January 1990 to 31st December 2015. Two hundred and eighty-one (203 male, 78 female) patients were included in this study, 11 patients were excluded because of insufficient data. Median age at diagnosis was 5.5 years (range 0.3 – 18), while median age at relapse was 7.7 years (range 1.1 – 20.5).

Relapse was confined to CNS in 167 patients (59.4%), to testis in 73 patients (26.0%), to mediastinum in 14 patients (5.0%), to CNS and other sites in 11 patients (3.9%) and to other sites in 16 patients (5.7%). These sites comprised: eye in 4 patients, lymph nodes in 3 patients and still others (eye and skin, skin, abdomen, retroperitoneum, kidney, liver, pelvis, ovary and soft tissue) in 1 patient each. Characteristics of patients are listed in Table I.

TBI was part of the preparative regimen 93.2% of cases (details are given in Table II). Data regarding additional radiotherapy are known only for 93 patients: 10 out of 93 received a radiotherapy boost to the site of relapse at time of TBI.

Gender, age, site of relapse and TBI containing regimens were uniformly distributed among HSCT types ($p>0.05$). ALL blast immune-phenotype, remission status at transplantation, duration of first CR, source of stem cells and year of transplantation were not uniformly distributed; in particular, patients with T-ALL were treated more frequently with either MUD or haplo HSCT (p 0.0028). Likewise, very early relapses were mainly treated with MUD HSCT (p 0.0043) and patients

with CR greater than 2nd were more frequent in the haplo HSCT group (p 0.0031) (Table I).

Mean follow up from transplantation was 5.9 years (range 0.01-26 years, median 2.8 years). During this period, 81 patients experienced a second relapse or disease progression at a median time of 176 days (range 15-2345) from HSCT: 48 marrow isolated, 16 extramedullary isolated, 7 combined, 10 unknown site. One hundred and fourteen patients died at a median time of 241.5 days (range 12-6623) from HSCT: 61 for relapse, 46 for treatment-related complications, 4 for a second tumor, 3 for an unknown event. In particular, mortality for a second malignancy occurred at a median time of 3117 days (range 88-6623). Acute GVHD (aGVHD) (grade II-IV) occurred in 79 (42.9%) of 184 patients who received an allograft, while chronic GVHD (cGVHD) was diagnosed in 32 out of 151 patients alive at days +100 (21.2%).

Overall survival

The overall survival for the entire cohort was 56% at 10 years (SE ± 3%) and was not influenced by gender, ALL blast immune-phenotype (B-cell precursor, Bcp-, *vs* T-ALL), age (≤10 years *vs* >10 years). As per site of relapse, OS was slightly better for patients with isolated testicular relapse (OS 66% at 10 years, SE ± 6%) as compared to patients with CNS relapse alone (OS 53%, SE ± 4%), CNS and other sites (OS 55% SE ± 15%), mediastinum (OS 39% SE ± 14%) and other organs (OS 62% SE ± 13%), but this difference is not statistically significant (p 0.22).

The length of first CR was not associated with a better survival, as the 10-year OS for very early, early and late IEMR was 52%± 6%, 53% ± 5%, and 61%± 6% respectively (p 0.39).

Comparison between auto and allo HSCT did not show any difference in terms of OS at 10 years (57% ± 5% vs 56% ± 4%, p 0.53). No statistically significant difference was also observed if different type of HSCT were compared: OS for auto HSCT, MFD, MUD and haplo HSCT was 57% ± 5, 56% ± 6%, 62% ± 6%, and 46% ±10%, respectively (p 0.09) (Figure I). The source of stem cells (peripheral blood, bone marrow or cord blood) did not affect OS, as well.

TBI-containing regimen yielded a better OS (59%) compared with conditioning without TBI (40%) but this difference was not statistically significant (p 0.069).

In univariable analysis, the only prognostic factors associated with OS in our cohort were: remission status at transplantation (CR2, CR>2 or presence of disease) and the year in which patients were treated (either before or after the year 2000). Patients transplanted in CR2 had the better OS at 10 years (64%± 4%); those given HSCT in subsequent CR showed an OS of 44%± 7%, while patients transplanted with active disease had an OS of only 11%± 7% (p<0.0001) (Figure II). For HSCT performed before the year 2000 the OS was 45% ± 5% at 10 years, while for those performed after 2000 was it was 63% ± 4% (p=0.0009).

Disease free survival

Global DFS was 54% at 10 years (SE ±3%); DFS did not differ in relation to gender, ALL blast immune-phenotype (pB- *vs* T-ALL), age (≤10 years *vs* >10 years).

Regarding the site of relapse, DFS at 10 years was 65% (± 6%) for testicular relapse, 49% (± 4%) for CNS relapse, 55% (± 15%) for CNS relapse together with other sites, 40% (± 14%) for mediastinal relapse and 65% (± 13%) for other sites involvement (p 0.22). As for OS, in our cohort DFS was not influenced by the duration of first CR: at 10 years, it was 52% (± 6%) for very early relapse, 53% (± 5%) for early relapse and 58% (± 6%) for late relapse (p 0.55).

Auto and allo HSCT had very similar DFS at 10 years (54% ±5% *vs* 55% ±,4% p 0.66), DFS was 55% ± 6% for MFD, 59% ± 6% for MUD, 47% ± 10% for haplo HSCT. The source of stem cells (peripheral blood, bone marrow or cord blood) did not affect RFS. The only factors statistically significant for DFS were: presence of TBI in conditioning regimen, remission status at HSCT and year of transplantation.

TBI-containing regimens were associated with a better DFS at 10 years as compared to non-TBI containing regimens (58% ± 4% vs 37% ± 8%, p 0.008).

Remission status at HSCT strongly correlated with DFS: patients transplanted in CR2 had a better DFS (63%±4%) in comparison to those transplanted in CR>2 (39% ±7%) or not in remission (11%±7%) (p<0.0001).

DFS for patients transplanted before and after 2000 was 45%± 5% and 61%± 4%, respectively (p 0.0008).

Transplant-Related Mortality

TRM for the entire cohort was 10% ± 2% at 100 days, 11% ± 2% at 6 months and 1 year and 16% ± 2% at 10 years. TRM for auto HSCT was 4% ± 2%, 6% v 2%,

6% ± 2%, and 11% ± 3%, while TRM for allo HSCT (MUD, MFD and haplo HSCT) was 13% ± 2%, 14% ± 3%, 14% ± 3%, and 18% ± 3% at 100 days, 6 months, 1 year and 10 years, respectively.

Subgroup analysis and multivariable analysis

As length of CR1 is one of the most important prognostic factors in relapsed ALL, we performed separate analyses for patients with very early, early and late IEMR.

Regarding very early relapse (n= 87) curves of DFS and OS at 10 years showed a trend in favor of allogeneic HSCT *versus* autologous HSCT (58% ± 6% *vs* 44% ± 12% and 59% ± 6% *vs* 44% ± 12%, p 0.28 and 0.29 respectively). In early relapsed patients (n=97), DFS and OS at 10 years were comparable irrespectively whether patients were treated with either auto or allo HSCT (55% ± 9% vs 50 ± 7%, p 0.88 and 54% ± 9% *vs* 52%, ± 7% p 0.87). If only patients with late relapse were considered (n= 87), DFS and OS at 10 years showed slightly better values with auto HSCT in comparison with allo HSCT (MFD, MUD and haplo combined): 65% ± 8% vs 48% ± 9% and 68% ± 7% *vs* 52% ± 9%, respectively, the differences were not statistically significant (p 0.13 and p 0.12). Curves of OS are shown in Figure III.

Remission status at transplantation is well known to influence outcome, so we conducted a separate analysis for patients in CR2 at time of HSCT (n=204). RFS and OS for the entire cohort were 74% ± 3% and 64% ± 3%, respectively; outcome of patients given either autologous HSCT or allogeneic HSCT was similar (RFS for

auto HSCT 70% ± 5% *vs* 78% ± 4% for allo HSCT p 0.37; OS for auto HSCT 67% ± 5% *vs* 64% ± 5% for allo HSCT p 0.84).

Multivariable analysis was conducted after adjustment for remission status: patients with active disease at transplantation were excluded from the analysis due to the high incidence of treatment failure in this group. As shown in Table III, in multivariable analysis the only factors influencing OS in patients with IEMR treated with HSCT were number of relapses and year of transplantation. Patients experiencing more than one relapse have a risk of death 2.3 times greater than patient transplanted in CR2; children treated with HSCT after 2000 have half the risk of death than those treated from 1995 to 2000.

	AUTO-HSCT (n=97)	MFD-HSCT (n=79)	MUD-HSCT (n=75)	Haplo-HSCT (n=30)	total of patients (n=281)	
Gender						**p 0.8308**
Male	67 (69.1%)	58 (73.4%)	55 (73.3%)	23 (76.7%)	203 (72.3%)	
Female	30 (30.9%)	21 (26.6%)	20 (26.7%)	7 (23.3%)	78 (27.7%)	
Mean age at relapse (years)	6.0	6.8	6.3	5.8		p 0.55
Lineage #						**p 0.0028 ***
pB	82 (84.5%)	59 (74.7%)	55 (73.3%)	15 (50.0%)	211 (75.1%)	
T	7 (7.2%)	10 (12.6%)	15 (20.0%)	9 (30.0%)	41 (14.6%)	
Other	2 (2.1%)	1 (1.3%)	0	0	3 (1.1%)	
Not known	6 (6.2%)	9 (11.4%)	5 (6.7%)	6 (20.0%)	26 (9.2%)	
Site of relapse #						**p 0.2304**
CNS	57 (58.8%)	51 (64.5%)	44 (58.7%)	15 (50.0%)	167 (59.4%)	
Testis	34 (35.0%)	17 (21.5%)	14 (18.7%)	8 (26.7%)	73 (26.0%)	
Mediastinum	1 (1.0%)	2 (2.6%)	8 (10.7%)	3 (10.0%)	15 (5.0%)	
CNS+other	2 (2.1%)	3 (3.8%)	5 (6.6%)	1 (3.3%)	11 (3.9%)	
CNS+ cerebral parenchima	*1*	*0*	*2*	*1*	*4*	
CNS+testis	*0*	*2*	*1*	*0*	*3*	
CNS+mediastinum	*0*	*0*	*2*	*0*	*2*	
CNS+eye	*0*	*1*	*0*	*0*	*1*	
Eye	*0*	*3*	*0*	*1*	3	
Lymph-nodes	*1*	*1*	*0*	*1*	3	
Other sites (liver, ovary, kidney, skin..)	*2*	*2*	*4*	*1*	9	
Other	3 (3.1%)	6 (7.6%)	4 (5.3%)	3 (10.0%)	16 (5.7%)	
Time to relapse #						**p 0.0043***
Very early	16 (16.5%)	27 (34.2%)	33 (44.0%)	11 (36.7%)	87 (31.0%)	
Early	33 (34.0%)	28 (35.4%)	26 (34.7%)	10 (33.3%)	97 (34.5%)	
Late	42 (43.3%)	21 (26.6%)	16 (21.3%)	8 (26.7%)	87 (31.0%)	
not known	6 (6.2%)	3 (3.8%)	0	1 (3.3%)	10 (3.5%)	
Remission status at HSCT						**p 0.0031***
CR2	78 (80.4%)	58 (73.4%)	56 (74.7%)	12 (40.0%)	204 (72.6%)	
CR>2	13 (13.4%)	16 (20.3%)	15 (20.0%)	15 (50.0%)	59 (21.0%)	
no remission	6 (6.2%)	5 (6.3%)	4 (5.3%)	3 (10.0%)	18 (6.4%)	
TBI-based conditioning #						**p 0.056**
Yes	82 (84.5%)	71 (89.9%)	55 (73.3%)	27 (90.0%)	235 (83.6%)	
No	14 (14.5%)	7 (8.9%)	18 (24.0%)	3 (10.0%)	42 (15.0%)	
not known	1 (1.0%)	1 (1.2%)	2 (2.7%)	0	4 (1.4%)	
Source of PBSC #						**p<0.0001***
BM	60 (61.9%)	71 (89.9%)	52 (69.4%)	7 (23.3%)	190 (67.6%)	
CB	0	2 (2.5%)	17 (22.6%)	1 (3.3%)	20 (7.1%)	
PBSC	36 (37.1%)	3 (3.8%)	6 (8.0%)	22 (73.4%)	67 (23.9%)	
BM+other	1 (1.0%)	3 (3.8%)	0	0	1 (0.4%)	
Year of HSCT						**p<0.0001***
1990-2000	57 (58.8%)	37 (46.8%)	7 (9.3%)	6 (20%)	107 (38.1%)	
2000-2015	40 (41.2%)	42 (53.2%)	68 (90.7%)	24 (80%)	174 (61.9%)	

Table I: Characteristics of 281 children who underwent HSCT for an isolated extramedullary relapse of ALL from 1990 to 2015 in Italy. * statistically significant (p<0.05). # analysis of significance performed on the most representative groups: Lineage (T , B), Site of relapse (CNS, Testits), Time to relapse (Very early, Early, late), TBI-based conditioning (Yes, No), source of cell (BM, CB, PBSC)

Abbreviations; CNS central nervous system, HSCT hematopoietic stem cell transplantation, CR complete remission, TBI total body irradiation, PBSC peripheral blood stem cell, BM bone marrow, PB peripheral blood

Conditioning Regimen	number of patients
Cyclo+Thiotepa+ TBI	50 (17.8%)
Ara-c +TBI	45 (16%)
Thiotepa+ Cyclo+ATG+TBI	24 (8.5%)
Ethoposide+TBI	18 (6.4%)
Vincristine+Cyclo+TBI	18 (6.4%)
Ethoposide+Cyclo+TBI	14 (5%)
Thiotepa+ Fludara+TBI	11 (3.9%)
Cyclo+TBI	10 (3.5%)
Thiotepa+L-Pham+TBI	10 (3.5%)
others + TBI	62 (22%)
NON TBI	19 (6.8%)
Bus+Thiotepa+Cyclo	10
Bus+Cyclo	5
Bus+Thiotepa+Fludara	4

Table II: Conditioning Regimens.

Abbreviatios: Cyclo Cyclophosphamide, TBI total body irradiation, Ara-C Cytarabine, ATG anti-thymocyte globulin, Fludara Fludarabine, L-pham Melphalan, Bus Busulphan

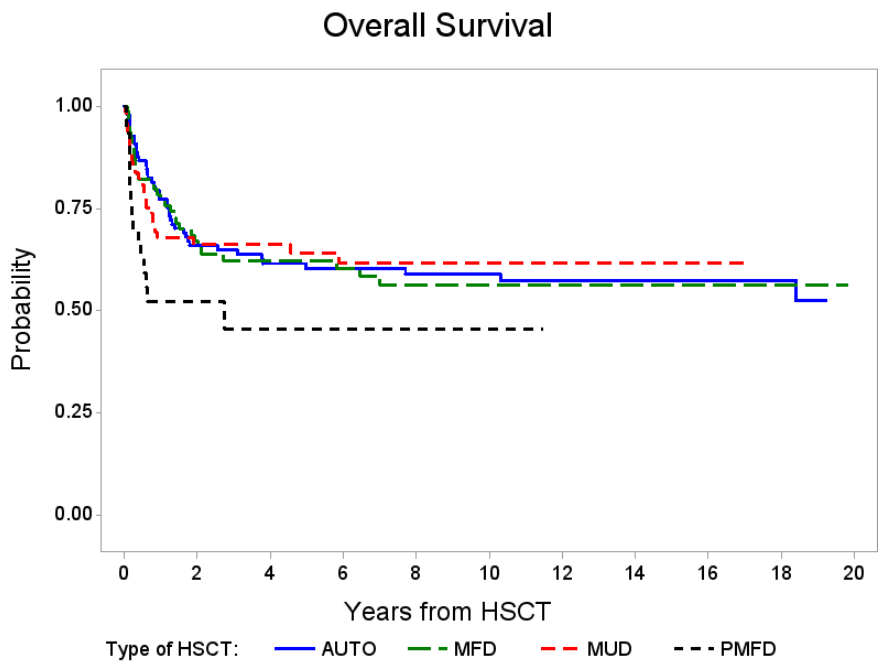


Figure I: Overall survival of patients transplanted for extramedullary relapse of ALL: stratification per HSCT donor

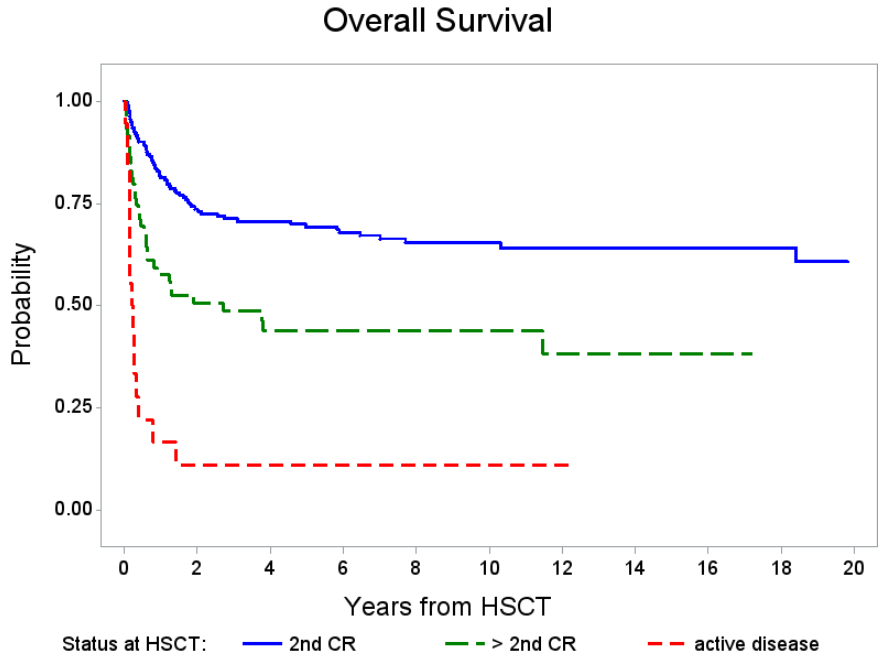
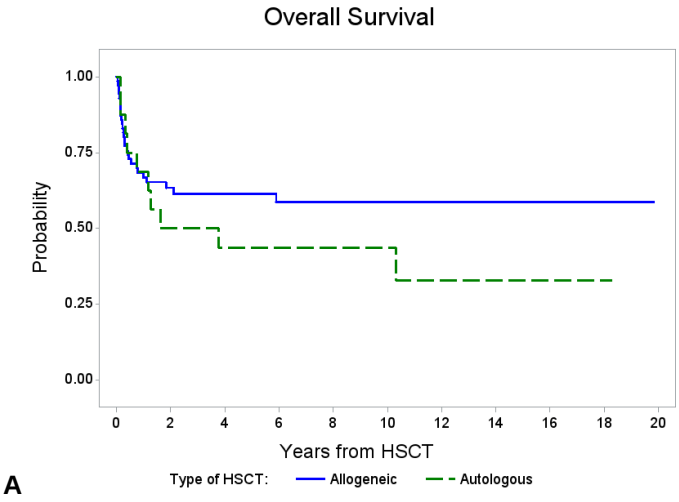
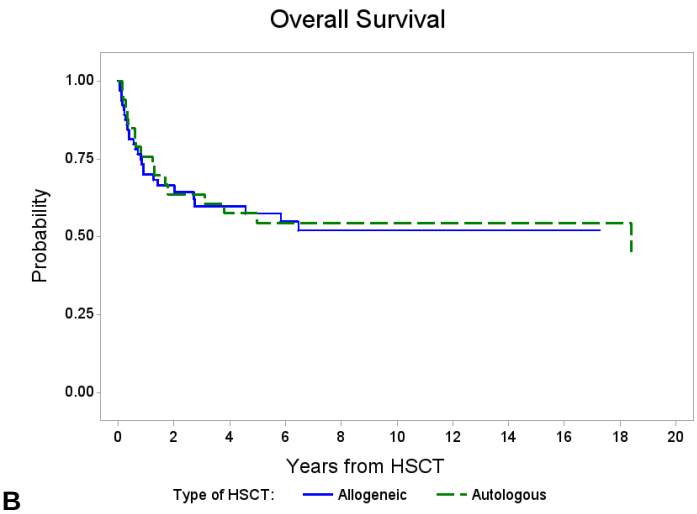


Figure II: Overall survival of patients transplanted for extramedullary relapse of ALL: stratification per remission status at HSCT



A



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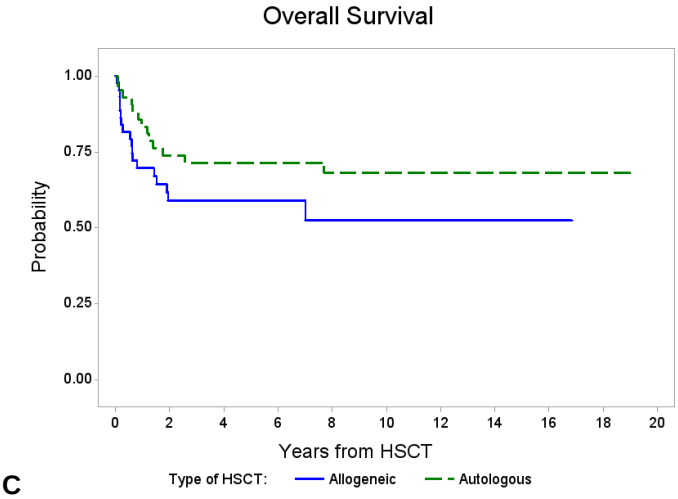

C

Figure III: Overall survival for patients with very early (A), early (B) and late (C) relapse: auto HSCT *versus* allo HSCT

Characteristics	Categories	# Pts	Events	10-y OS % (SE%)	Univariable P-value	Multivariable P-value	Hazard Ratio (95% CI)
Age	≤ 10 yrs	215	82	59 (4)	0.50	-	
	> 10 yrs	48	16	66 (7)			
Gender	Female	73	25	60 (6)	0.58	-	
	Male	190	73	60 (4)			
Lineage	B	203	76	61 (4)	0.42	-	
	T	33	9	68 (9)			
Relapse site	testis	72	24	67 (6)	0.23	0.56	
	CNS	154	60	58 (4)			
TBI conditioning regimen	No	36	17	43 (10)	0.21	0.86	
	Yes	224	80	60 (4)			
Years of HSCT	Before 2000	94	49	51 (5)	0.0064	0.0035	0.5 (0.3-0.8)
	After 2000	169	49	65 (4)			
HSCT type	Autologous	91	36	62 (5)	0.63	-	
	Allogeneic	172	62	60 (4)			
Status at HSCT	Second CR	204	65	65 (4)	<0.0001	0.0005	2.3 (1.4-30.7)
	Other CR	59	33	44 (7)			

Table III: Multivariable analysis of factors influencing outcome in patients with IEMR.

Discussion

Although the vast majority of children affected by ALL are cured with current protocols, relapses still occur and pose remarkable challenges to pediatric hematologists. Allo HSCT is currently used to treat patients in CR2 with high-risk features (early and isolated marrow relapse, recurrence of T lineage ALL [1,11]), and is now considered in those patients who present MRD positivity above certain thresholds at the end of induction therapy [12,13]. Treatment of extramedullary relapse is less well established. The absence of BM involvement is traditionally considered a favorable prognostic feature [14], and these patients are commonly treated with intensive systemic and intrathecal chemotherapy (CT) followed by either cranio-spinal or cranial irradiation (RT) [5, 7, 15, 16]. EFS with this approach ranges from 45% [3, 7, 17, 18] to 70% [15]. Despite the high curative rates obtained in the two Children Oncology Group trials [15,19], with global EFS at 5 years approaching 70%, for particular subgroups of IEMR, prognosis is still dismal. Very early and early IEMR or ICNS relapses show survival probability of only 20-30% in most studies; in this regard, Tallen [3] reported 33% EFS for very early ICNS relapse. Similarly, in other published papers, RFS and EFS ranged from 20% to 35% for very early/early IEMR [7,17,18].

HSCT has been used for treatment of IEMR, but published data are conflicting and limited to small numbers of patients [6, 20-22]. Our previous work [9] analyzed the outcome of 69 patients with early (<30 months from diagnosis) ICNS relapse treated with either auto HSCT or CT/RT: EFS with auto HSCT was clearly superior

to CT/RT (56% *vs* 12%). Moreover, in another report, we demonstrated that auto HSCT performed in CR2 offers a better chance of cure than when it is employed in subsequent CR [10]. More recent papers compared allogeneic HSCT with CT/RT in patients with ICNS relapse of ALL. Eapen and colleagues [5] reported a comparable outcome in 209 patients treated with CT/RT or MFD HSCT: EFS were 66% and 58% respectively. Similarly, other studies [2, 7] did not find any statistical difference when patients with IEMR in CR2 were treated with either HSCT or chemotherapy, but numbers of patients treated with HSCT were very small.

In this study, we present the largest cohort of patients with IEMR of ALL, morphologically defined, and the largest number of HSCT ever performed for this indication, with a long follow up (up to 26 years from HSCT).

OS and DFS at 10 years were the same for autologous, MFD and MUD HSCT, being around 60% and 70%. Even if a control group of patients treated with CT/RT was not included in the study, our results are comparable with those reported in previous studies, since published OS with CT/RT is 45-70% [3, 5, 7, 15-17, 19]. Moreover, if only patients in second CR are considered, as in all published series, the OS of 64% at 10 years is in line with the most favorable reports [15,19].

Notably, in our study, the use of HSCT seemed to abrogate the impact of some “classical” prognostic factors like site of IEMR, duration of first remission and ALL blast immune-phenotype. If the site of relapse was considered, OS was only slightly better for testicular relapse. Neither duration of CR1 nor ALL immune-phenotype affected the outcome in univariable and multivariable analysis; in fact, the only factors influencing outcome resulted to be year of HSCT and remission status at transplantation. Regarding the prognostic impact of year of

transplantation, this likely reflects the improvement in supportive therapy and donor selection. About the prognostic significance of remission status before HSCT, these data confirm what we reported previously [10], namely that survival for children transplanted in CR2, morphologically defined, is significantly better than for patients transplanted in subsequent remission. This observation emphasizes the importance of identifying those patients at higher risk of further relapse, who, thus, may benefit of HSCT soon after achievement of CR2. Very early and early IEMR or ICNS relapses treated with CT/RT have been previously shown to have a survival probability around 20-30% [3, 7, 17, 18], while the use of HSCT in this study resulted into an OS probability of 53% for early relapses and 52% for very early relapses. This result is even more striking considering that patients with third or subsequent CR and even with active disease were included in this analysis. Therefore, given the high risk of further relapse of very early/early iIEMR treated with CT/RT and the poor outcome with HSCT performed after a second relapse, our data support the choice of HSCT in patients experiencing very early/early IEMR once that a second CR is achieved.

The 44% OS and 39% DFS at 10 years obtained in patients transplanted in third or subsequent CR (59 cases) are remarkable, these results comparing favorably with those previously published by other goups with shorter follow up. In fact, in this subgroup of patients, EFS of 35% at 3 years after MUD HSCT was reported (21) whileother studies [23-25] reported EFS ranging from 20 to 48% for children with ALL transplanted from MFD or MUD in CR3.

Furthermore, no difference in outcome was observed regarding the type of HSCT. This is in line with our previous observation in a smaller group of patients in which

auto HSCT had the same chance to cure children with ICNS relapsed ALL than allogeneic (MFD) HSCT [9]. The present data strengthen this observation, widening the comparison with inclusion of MUD HSCT and (even if with a low number of cases) with haplo HSCT. It is possible that in extra medullary sites, the GvL effect be less relevant [26] and that the good results obtained with HSCT may be due to the inclusion of TBI in the conditioning regimen. In fact, TBI-containing regimens showed better survival rates compared with non-TBI regimens. Surprisingly, TRM at 10 years was no different between auto, MFD and MUD HSCT (11%, 16% and 16% respectively).

This study shows that both auto and allo HSCT are effective treatment for IEMR of ALL. Auto HSCT can cure children with late IEMR with efficacy at least comparable to that of CT/RT. Despite the possibility of long-term complications, auto HSCT, compared to a second complete course of chemo-radiotherapy, may allow a shorter duration of treatment, this resulting into a better quality of life for patients and their families. Furthermore, our results indicate that particularly children with very early and early IEMR may benefit from HSCT, either autologous or allogeneic. Notably, autologous stem cell use may significantly reduce the time patients wait before transplantation, the risk of GVHD, of infection and graft failure associated with allogeneic HSCT. Although the retrospective nature of this study is an important limitation, the large number of patients with iEMR and the very long follow up strengthen our results Data from contemporary treatment protocols, which include MRD assessment for stratifying patients experiencing disease recurrence, will further clarify the role of HSCT in the treatment of extramedullary relapse of pediatric ALL.

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