UNIVERSITA' DEGLI STUDI DI PADOVA

Dipartimento di Pediatria

Scuola di Dottorato di Ricerca in Medicina dello Sviluppo e Scienze della Programmazione

Indirizzo Ematooncologia e Immunologia-Ciclo XX

FLOW CYTOMETRY APPLICATION IN HEMATOLOGICAL MALIGNANCIES OF CHILDHOOD

Direttore della Scuola: Ch.mo Prof. Giuseppe Basso

Supervisore: Ch.mo Prof. Giuseppe Basso

Dottorando: Barbara Buldini

31 gennaio 2008

A te papa',
stella del mio cammino

Table of Contents

Summary

The PhD research work was performed, for the first part (1 year) at the Pediatric Haematology-Oncology Department, Fondazione IRCCS Policlinico San Matteo, Pavia University and for the second part (2 years) at the Pediatric Haematology-Oncology Department, Padova University, two excellent setting for a specialized training in pediatric haematology-oncology. The PhD program was targeted in both a clinical and laboratory research experience in order to perform a translational research on pediatric patients affected by a wide range of hematological disorders, both malignant and non-malignant. The efforts were coordinated to study the biology and therapy of pediatric Acute Lymphoblastic Leukemia (ALL), Acute Myeloid Leukemia (AML) and Myelodysplastic Syndromes (MDS).

The first 12 months were dedicated to obtaining comprehensive clinical skills in pediatric stem cell transplantation, in a clinical setting. In this period, the PhD research took place at the Pediatric Haematology-Oncology Department, Fondazione IRCCS Policlinico San Matteo, Pavia University, the major Italian Centre for haematopoietic stem cell transplantation (HSCT) in children, with more than 100 HSCTs per year.

HSCT is currently used for the treatment of malignant and nonmalignant disorders in which replacement of HSC-derived populations of cells is mandatory. Advances in understanding the biology of SCT coupled with advances in the supportive care and clinical management of stem cell transplant recipients have made this approach an increasing effective therapy.

The research analyzed the clinical and biological advantages which characterize most tipically HSC transplantation procedures. The clinical experience took place in both inpatient and outpatient settings.

The training in blood and marrow transplantation ensured that the physician carrying out these procedure will be competent in the use of high-dose therapy for the treatment of malignancy or bone marrow failure. The specific conditioning regimens used to prepare patients affected by different diseases have been reviewed. Specific training in the following areas were required: indications for blood and marrow transplantation, identification and selection of stem cell source, including use of donor registries, implications of HLA typing, understanding of chimerism analysis, management of ABO incompatible hematopoietic progenitor cell products.

Early end aggressive use of antibiotics, antifungal agents, and antiviral agents has improved survival during the early neutropenic period and during the period of profound and persistent immunosuppression after engrafment.

In this setting, we analyzed retrospectively the safety and efficacy of caspofungin-based combination therapy in 40 children and adolescents, most of them treated for a malignant disease, who developed invasive aspergillosis (IA) between November 2002 and November 2005. The study showed that caspofungin-based combination antifungal therapy is an effective therapeutic option also for pediatric patients with IA.

Multipotent mesenchymal stromal cells (MSCs) are endowed with multilineage differentiative potential and immunomodulatory properties. Infusion of culture-expanded MSC, together with HSC transplantation, is a safe procedure and could potentially enhance marrow recovery after myeloablative treatment. The multiple immune suppressive properties of MSC provide the biological explanation of the possible efficacy of MSC in the treatment of patients with acute GvHD, and even those refractory to conventional treatment. It is still a matter of debate whether donor MSCs have a sustained engraftment in the host bone marrow (BM) after allogeneic hematopoietic stem cell transplantation (allo-HSCT). Aim of one of our study has been to analyze the donor/recipient origin of MSCs in thirty-seven pediatric patients receiving allogeneic either BM or cord blood (CB) transplantation for either a malignant or a non-malignant disorder. BM soil of pediatric patients appeared as more favorable than that of adults for sustained engraftment of transplanted MSCs and MSCs able to engraft in the host can also be transferred with CB.

In the last 2 years the PhD research work was performed in Pediatric Haematology-Oncology Department, Padova University, in both a clinical and laboratory setting.

The Haematology-Oncology Laboratory, Padova University, is the AIEOP (Associazione Italiana di EmatoOncologia Pediatrica) Reference Laboratory where bone marrow and peripheral blood samples of each Italian paediatric case of acute and chronic lympho-myeloproliferative disorders are centralized for immunophenotypic, molecular/cytogenetic analysis and DNA ploidy studies.

The main field of interest of the PhD research has been the diagnosis of childhood acute and chronic leukemias, myelodysplastic syndromes, lymphomas and the assessment of Minimal Residual Disease (MRD) in leukaemias by multiparametric flow-cytometry.

Immunophenotypic data collection using flow cytometry is a fast and relatively easily accessible technology that has already been implemented in several centers for leukemia diagnosis.

The immunophenotyping of acute leukaemias is today the preferred method to perform the identification, enumeration and characterization of blast cells at diagnosis. The commercial availability of large panels of high quality reagents facilitates the application of this analytic method in diagnosis, classification, and minimal residual disease detection. Flow cytometry allows the analysis of a large number of cells (usually

between fifteen thousand cells per sample, and three hundred thousand/one million in minimal residual disease studies) with accuracy. The simultaneous analysis of several different parameters (two related to physical properties of the cells and five to eight to the immunophenotype) contributes to increase both the specificity and sensitivity of the test. Furthermore, the results can be stored in list-mode files and analyzed by other investigators to facilitate objective interpretation.

Studies of Minimal Residual Disease are one of the most important researches in acute leukaemias because of the recent demonstration of their clinical usefulness. In the ongoing AIEOP-BFM chemotherapy protocol for treatment of childhood ALL, one of the parameters used for stratifying patients is MRD analysis by molecular methods, as is appeared an indipendent prognostic indicator with clinical relevance. Several experiences demonstrated that flow cytometric (FC) assessment of MRD in ALL, based on leukemia-associated immunophenotypes, also provides indipendent prognostic information. This approach is reliable, fast, relatively cheap, and allows to study >95% of ALL patients. Preliminary results from a multicenter standardization study of FCM-MRD began in 2000 in 4 collaborating laboratories, which comprehensively enrolled children with ALL from Austria, Germany, Italy and Switzerland *(Berlin, R.Ratei; Monza, G.Gaipa; Padova, G.Basso; Vienna, MN.Dworzak)* show that flow cytometric assessment of MRD in BM at day +15 of induction treatment, as a single time-point, is a strong prognostic indicator, independently of other biological and clinical parameters considered.

Because of its wide applicability, flow cytometry is a promising tool to monitor residual disease also in AML: FC is applicable in the vast majority of patients and it is a powerful tool to segregate patients with AML into different categories of risk. Similarly to ALL, flow cytometric detection of high level of MRD after induction and/or consolidation therapy seems to be an independent predictor of treatment outcome. Relapse remains the main cause of treatment failure in AML. Monitoring of MRD in AML seems to be useful in identifying patients at high risk of relapse from those in durable remission, providing a more rational mean of selecting therapeutic options, and a more accurate estimate of the extent of leukemic cytoreduction achieved with different therapies.

Considering that genetic markers suitable for RT-PCR studies are found in less than one-third of these patients, the PhD research was directed to study the detection of MRD in childhood AML Italian patients by FC, in order to detect the clinical value of these data. The ongoing study includes a cohort of patients with diagnosis of *de novo* AML, enrolled in the AIEOP-AML 2002 trial for treatment. We found that immunophenotypes suitable for the detection of residual disease can be identified and defined at diagnosis in all patients by four and five-colour flow cytometry and that such

immunophenotypes allow a sensitivity of detection as much as 100 times that afforded by morphological examination.

Moreover, in order to define, if flow cytometric detection of AML cells after induction or consolidation therapy is an independent predictor of treatment outcome, in line with the results obtained in adults' series, we are studying the bone marrow follow up samples of the patients, during the treatment period. Residual disease assessment with this method could be used for risk assignment in the future therapeutic trial.

Morphology represents the gold standard for the diagnosis of MDS, but in recent years several efforts to analyse MDS by flow cytometry have been reported in the adult cohort. The PhD research was involved also in a project concerning immunophenotypic evaluation of paediatric patients with MDS, based on the identification of abnormalities in the maturation pathway of the myelo-monocytic lineages, and characterization of blast phenotype*.*

Finally, the PhD work was interested on studying the correlation between genotype and antigen expression. Quantitative multivariate analysis from panels of marker proteins has previously demonstrated that marker protein expression profiles can distinguish MLLre (rearranged) from non-MLLre ALL cases and also allow to specifically distinguish MLL/AF4 cases.

In a study on 96 pediatric patients with AML, we distinguished seven subgroups of patients that are shown to be prognostically relevant, performing unsupervised analysis on antigen expression values of 35 marker proteins.

In a further study, we applied quantitative multivariate analisys (GMF values: Geometric Mean Fluorescence) to inspect the role of immunophenotyping in discriminating 41 children with Ph+ precursor-B-ALL and 99 children with Ph- precursor-B-ALL at diagnosis. Unsupervised cluster analisys was performed on the entire cohort of 140 patients to group samples according to the similarity of their antigen expression profiles. Two-dimensional clustering analisys for GMF antigen expression values separated patients into two main clusters (A and B+C) showing that childhood precursor-B-ALLs carrying the Philadelphia chromosome display a unique phenotypic profile

Compendio

L'attività di ricerca di questo dottorato si è articolata in due fasi: per la prima parte (1 anno) presso la Struttura di Oncoematologia Pediatrica, Fondazione IRCCS Policlinico San Matteo, Università di Pavia e per la seconda parte (2 anni) presso la Clinica di Oncoematologia Pediatrica del Dipartimento di Pediatria dell'Università degli studi di Padova.

Il programma del dottorato si è sviluppato in un contesto di ricerca clinica e di laboratorio ai fini dello sviluppo di una ricerca traslazionale su pazienti pediatrici affetti da patologie ematologiche.

Gli sforzi sono stati coordinati allo studio della biologia e della terapia di bambini affetti da leucemia linfoblastica acuta (LLA), leucemia mieloide acuta (LMA) e da sindromi mielodisplastiche (MDS).

Nei primi 12 mesi sono state acquisite coonoscenze sul trapianto di cellule staminali emopoietiche (TCSE) dell'età pediatrica. In questo periodo la ricerca si è svolta presso la Struttura di Oncoematologia Pediatrica, Fondazione IRCCS Policlinico San Matteo, Università di Pavia, il piu'importante centro per il trapianto di cellule staminali emopoietiche in età pediatrica, con piu' di cento procedure trapiantologiche all'anno.

Il TCSE è il trattamento di scelta per numerose patologie maligne e non maligne.

Sono stati analizzati i vantaggi clinici e biologici che caratterizzano gran parte delle procedure trapiantologiche, grazie ad un' esperienza clinica su pazienti ricoverati o in regime di Day Hospital.

Sono stati rivisti i regimi di condizionamento previsti per diverse patologie, le indicazioni al TCSE, l'identificazione e la selezione della fonte di cellule staminali emopoietiche, l'uso dei registri dei donatori, la tipizzazione HLA, la comprensione dell'analisi del chimerismo. L'utilizzo precoce ed aggressivo di terapie antibiotiche, antifungine, antivirali ad ampio spettro, ha migliorato la sopravvivenza nel periodo di neutropenia precoce e nella fase di prolungata immunosoppressione.

In questo contesto, abbiamo analizzato retrospettivamente la sicurezza e l'efficacia della terapia antifungina con caspofungin in combinazione, in 40 bambini ed adolescenti, la maggioranza dei quali trattati per patologie maligne, che hanno sviluppato aspergillosi invasiva (IA) tra novembre 2002 e novembre 2005. La ricerca ha mostrato che la terapia antifungina con caspofungin in combinazione è un'efficace opzione terapeutica per pazienti pediatrici con IA (Cap 2.1.c).

Le cellule mesenchimali sono dotate di capacità differenziative multilineari e proprietà immunomodulatorie. L'infusione di MSC espanse in coltura, associate ad un trapianto di cellule staminali emopoietiche, è una procedura sicura che potrebbe accelerare il

recupero midollare dopo terapia mieloablativa. Le multiple capacità immunosoppressive delle MSC sono una possibile spiegazione biologica dell'efficacia delle MSC nel trattamento di pazienti con GVHD acuta, anche resistenti alle terapia convenzionali.
E'oggetto di discussione se le MSC del donatore atteschiscano nel midollo osseo del ricevente dopo un trapianto allogenico di cellule staminali emopoietiche.
Scopo del nostro studio è stato quello di analizzare l'origine delle MSC (donatore/ricevente) in trentasette bambini sottoposti a trapianto di cellule staminali allogeniche, da sangue midollare o cordonale, per patologie maligne o non maligne. I dati ottenuti in questa ampia casistica pediatrica, hanno mostrato che le MSCs del donatore sono in grado di attecchire in una percentuale significativa di pazienti, anche quelli sottoposti a trapianto di cellule staminali emopoietiche da sngue cordonale (Cap.2.1.c).
Nei due anni successivi, l'attività di ricerca ha avuto sede presso la Clinica di Oncoematologia Pediatrica del Dipartimento di Pediatria dell'Università degli Studi di Padova, in un contesto clinico e di laboratorio.
Il laboratorio di Oncoematologia Pediatrica del Dipartimento di Pediatria dell'Università degli studi di Padova, è il laboratorio di riferimento dell'AIEOP (Associazione Italiana di EmatoOncologia Pediatrica) ove i prelievi di sangue midollare e di sangue periferico di ogni paziente pediatrico affetto da patologie linfo-mieloproliferative acute e croniche vengono centralizzati alla diagnosi e alla ricaduta per studi di immunofenotipo, analisi molecolare/citogenetica e di ploidia.
Il principale campo di interesse è stato la diagnosi di leucemie acute e croniche, di sindromi mielodisplastiche, di linfomi e la valutazione della malattia residua minima (MRM) nelle leucemie acute in citofluorimetria multiparametrica.
L'analisi immunofenotipica in citofluorimetria è una tecnologia rapida e relativamente accessibile. E' il metodo di scelta per una corretta identificazione, numerazione e caratterizzaione dei blasti alla diagnosi. La disponibilità commerciale di ampi pannelli di reagenti facilita l'applicazione di questo metodo analitico nella diagnosi, classificazione e valutazione della malattia residua minima. La citofluorimetria permette l'analisi di un elevato numero di cellule (generalmente tra quindicimila cellule, fino a trecentomila/un milione negli studi di malattia residua minima) con precisione.
L'analisi simultanea di differenti parametri (due legati alle caratteristiche fisiche delle cellule e da cinque a otto per l'immunofenotipo) contribuisce ad incrementare la sensibilità e la specificità del test. I risultati ottenuti, inoltre, possono essere salvati e successivamente rianalizzati da altri operatori per facilitare un'interpretazione oggettiva.
Gli studi di malattia residua minima sono uno dei principali ambiti di ricerca nelle leucemie acute. Nel protocollo terapeutico AIEOP-BFM, previsto per bambini affetti da

LLA, uno dei paramentri utilizzati nella stratificazione dei pazienti è rappresentato dal dato molecolare di MRM che in precedenti studi si è dimostrato un fattore prognostico indipendente con rilevanza clinica. Anche lo studio della MRM in citofluorimetria, basato sull'identificazione di fenotipi associati a leucemia è in grado di fornire un'informazione prognostica indipendente. Questo approccio è rapido, relativamente economico e permette lo studio di >95% dei pazienti con LLA. Risultati preliminari da uno studio collaborativo multicentrico di standardizzazione di MRM in citofluorimetria iniziato nel 2000 in 4 laboratori che complessivamente hanno arruolato pazienti con LLA da Austria, Germania, Italia e Svizzera (*Berlin, R.Ratei; Monza, G.Gaipa; Padova, G.Basso; Vienna, MN.Dworzak*) mostrano che la valutazione citofluorimetrica della malattia residua minima su sangue midollare al giorno +15 di terapia, è un forte indicatore prognostico, indipendentemente dagli altri parametri clinici e biologici.

La citofluorimetria è uno strumento promettente per monitorare la malattia residua anche nei pazienti affetti da LMA: è applicabile nella maggioranza dei casi e appare come strumento in grado di stratificare i pazienti con LMA in diverse categorie di rischio. Analogamente alle LLA, il rilievo di un elevato livello di MRM dopo la terapia di induzione o di consolidamento sembra essere un fattore prognostico indipendente. La ricaduta è la principale causa di fallimento terapeutico nei pazienti affetti da LMA. Il monitoraggio della MRM nelle LMA sembre utile nell'identificare i pazienti ad alto rischio di ricaduta, che possano beneficiare di diverse strategie terapeutiche.

Dal momento che meno di un terzo dei pazienti affetti da LMA presenta un marcatore molecolare, l'attività di ricerca è stata diretta allo studio della MRM in pazienti pediatrici affetti da LMA, in citofluorimentria nel tentativo di definire il valore clinico di tali dati. Lo studio in atto comprende una coorte di pazienti con diagnosi di *de novo* AML, arruolati nel protocollo terapeutico AIEOP-AML 2002. In tutti i pazienti, con un approccio citofluorimetrico a quattro e cinque colori sono stati identificati e definiti alla diagnosi dei profili immunofenotipici caratteristici dei blasti, in grado di riconoscere una persistenza di malattia con una sensibilità fino a 100 volte superiore quella raggiunta in morfologia.

La valutazione della MRM con tale approccio potrebbe essere usata per una corretta stratificazione dei pazienti nei futuri protocolli terapeutici. Stiamo pertanto studiando i campioni di follow-up di pazienti in terapia, al fine di definire, se l'identificazione di MRM in citofluorimetria dopo l'induzione o il consolidamento possa costituire un preciso frattore prognostico (Cap. 1.2.c).

La morfologia rappresenta il *gold* standard per la diagnosi di sindromi mielodisplastiche. All'interno del dottorato, una ricerca è stata avviata sulla valutazione immunofenotipica dei pazienti con MDS, basuta sull'identificazione di anomalie nel pathway maturativo delle lineee mielomonocitiche e la caratterizzazione del fenotipo dei blasti (Cap.1.1b).

Infine, il dottorato si è interessato nello studio sulla correlazione tra genotipo ed espressione antigenica. Un'analisi quantitativa sull'espressione di marker proteici ha precedentemente dimostrato che profili di espressione antigenica possono distinguere LLA con riarrangiamento del gene MLL.

Grazie ad un'analisi non supervisionata basata sull'espressione di 35 antigeni, in uno studio su 96 pazienti pediatrici affetti da LMA, abbiamo distinto sette sottogruppi di pazienti a diversa prognosi (Cap1.2.b).

In uno studio successivo, abbiamo valutato, mediante analisi multiparametrica quantitativa, l'immunofenotipo all'esordio di 41 pazienti pediatrici con diagnosi di LLA-B *precursor* Ph+ e di 99 pazienti pediatrici con diagnosi di LLA-B *precursor* Ph-. L'espressione dei singoli antigeni è stata quantificata tramite *Geometric Mean Fluorescence* (GMF, media geometrica dell'intensità di fluorescenza), sia nei pazienti Ph+ che in quelli Ph-. Un'analisi non-supervisionata tramite *clustering* è stata eseguita sull'intera coorte di 140 pazienti, per individuare gruppi di pazienti con simili profili di espressione antigenica (dati espressi in termini di GMF). Le LLA pediatriche B-*precursor* Ph+ presentano caratteristiche immunofenotipiche peculiari rispetto a quelle Ph-. (Cap.1.2.c)

Part 1

The Flow Cytometry: diagnostic and prognostic tool for hematological malignancies investigation

1.1.a Immunophenotipic Studies

Immunophenotyping has been a challenge in the diagnostic workup of acute leukemia for more than 20 years and has led to a more comprehensive description of leukemic cells (Catovsky D 1991; Jennings CD 1997; Bene MC 1999). The leukemic cells stained by indirect immunofluorescence or later by immunochemistry were first analysed using a microscope. These methods have beeen discharged by the fast developing flow cytometry using direct-labeled antibodies (Scwonzen M 2007).

Flow cytometric immunophenotyping, evolving from indirect immunofluorescence techniques to a complex multiparametric diagnostic tool, is now indispensable for the diagnosis of acute leukemia (AL). It allows the objective analysis of high numbers of cells in a relatively short period of time and is nowadays the preferred method for immunophenotypic identification, enumeration and characterization of blast cells at diagnosis (Basso, Buldini 2001).

With the use of multiple-color immunophenotyping, simultaneous measurement of different parameters is now routinely applied for the diagnosis of AL in most clinical laboratories. It is a powerful method for achieving a clear discrimination between normal and pathologic cells. The specific identification of leukemic cells, by immunologic gating, forms the basis for immunophenotypic diagnosis, classification as well as prognostic evaluation of patients with acute leukemias.

Furthermore, recent reports indicate that phenotypic aberrations reflect genetic abnormalities of leukemic cells and therefore their definition and identification is of clinical relevance for minimal residual disease monitoring and also for subclassyfing acute myeloid and lymphocytic leukemia.

The immunological analysis of a limited panel of surface or intra cellular antigens allows to assign most cases of acute leukemia to a specific lineage. Immunophenotyping of blast cells has thus become an elective and useful tool to characterize the myeloid or lymphoid origin of blast cell populations. The use or more extensive panels allows to identify either undifferentiated acute leukemias or acute leukemia entities expressing simultaneously several lineage antigens (Bene MC 1995; Bene MC 2001).

In lymphoid lineage-derived acute leukemia, extended immunophenotyping, moreover, allows to stratify distinct clinical and biological subsets, based on the hierarchy of antigen expression during the maturation of the normal cell counterparts of blast cells.

In acute non-lymphoblastic leukemia, several antigens (myeloperoxidase, CD13, CD33, CD117) have also been defined as hallmarks of myeloid differentiation and are specifically useful for the identification of morphologically undifferentiated AML cases or

the detection of blasts or either erythroid (Glycophorin, CD36) or megakaryoblastic lineage (CD41, CD42, CD61).

Once an initial diagnosis of hematolymphoid neoplasm is rendered, additional informations could be obtained by flow cytometric immunophenotyping (FCI). Considering that flow cytometry is more sensitive than conventional morphology for detecting disease in bone marrow and blood, FCI could be useful in staging the disease to document the extent of involvement (Duggan PR, 2000)

FCI is indicated in the detection of potential therapeutic targets (e.g. CD20, CD52). (Ginaldi L, 1998)

Furthermore, in myelodysplastic syndromes FCI could be integrative to morphologic and cytogenetic approaches: it is able to characterize the immunophenotypic features of blast cells and to define displastic signs in the myeloid compartment.

FCI is involved in the documentation of progression or relapse or disease acceleration (e.g. CML blast crisis) and in the assesment of response to therapy (including “minimal residual disease”) and persistence of FCI-detectable MRD following therapy, which is often an adverse prognostic factor. Future clinical trials to evaluate novel therapeutic regimens must consider the uselfulness of flow cytometry data and other biologic parameters. Consequently, the contribution of flow cytometry to patient care is dynamic and evolving process with important clinical applications.

References

Basso G, Buldini B, De Zen L, Orfao A: New methodologic approaches for immunophenotyping acute leukemia. Haematologica 2001; 86: 675-692.

Bene MC, Castoldi G, Knapp W, et al. Proposals for the immunological classification of acute leukemias. European Group for the Immunogical Characterization of Leukemias (EGIL). Leukemia 1995;9:1783-1786.

Bene MC, Bernier M, Castoldi et al. Impact of immunophenotyping on management of acute leukemias. Haematologica 1999;84:1024-34.

Bene MC, Bernier M, Casasnovasa RO al. Acute myeloid leukemia M0: haematological, immunophenotypic and cytogenetic characteristics and their prognostic significance: an analysis in 241 patients. Br J Haematol 2001;113:737-745.

Catovsky D, Matutes E, Buccheri V, et al. Classification of acute leukemia for the 1990s. Ann Hematol 1991; 62:16-21.

Duggan PR, Easton D, Luider J, Auer IA. Bone marrow staging of patients with non-Hodgkin lymphoma by flow cytometry: correlation with morphology. Cancer 2000; 88 (4):894-899

Ginaldi L, De Martinis M, Matutes E, Farahat N, Morilla R, Dyer M, Catovsky D. Levels of expression of CD52 in normal and leukemic B and T cells: correlation with in vivo therapeutic responses to Campath-1 H. leuk res 1998; 22 (2): 185-191.

Jennings CD, Foon KA. Recent advances in flow cytometry: application to the diagnosis of hematologic malignancy. Blood 1997; 90:2863-92.

Scwonzen M, Diehl V, Dellana M, Staib P. Immunophenotyping of surface antigens in acute myeloid leukemia by flow cytometry after red blood cell lysis. Leukemia Researc 31 (2007) 113-116.

1.1.b Pediatric myelodysplastic syndromes: can immunophenotypic characterization of blast cells be a diagnostic and prognostic tool?

Summary

The diagnosis of myelodysplastic syndromes (MDS) is mainly based on morphology and cytogenetics analysis. In recent years several efforts to analyse MDS by flow cytometry have been reported in the adults. These studies have focused on the identification of abnormalities in the maturation pathway of antigen expression of myelo-monocytic cells, and characterization of blast populations. Therefore, phenotype has been proposed as a diagnostic and prognostic criteria for adult MDS.

The current article provides data concerning the blasts phenotype in paediatric MDS. We identified a blast phenotype typically expressed in most MDS cases and a strong negative correlation between CD7 expression and outcome, from the analysis of a cohort of 36 patients with MDS, compared with 145 *de novo* paediatric acute myeloid leukaemia (AML). CD34+ compartment in MDS bone marrow was also analysed; a significant decrease of B-cell precursors was detected in MDS patients independently of age. Our data suggest that, even if paediatric MDS are different for many biological and clinical aspect from adult cases, the progenitors phenotypic features are similar and can constitute a diagnostic and prognostic tool for paediatric MDS.

Introduction

Myelodysplastic syndromes (MDS) are a heterogeneous group of clonal stem cell disorders, characterized by peripheral blood cytopenia, ineffective haematopoiesis, dysplasia in 2 or more lineages and increased risk of progression to acute myeloid leukaemia (AML) (Heaney & Golde, 1999; Steensma *et al*, 2006)**.** These disorders are rare in childhood (Hasle *et al* , 1995; Hasle *et al*, 1999), but prognosis is extremely poor and allogeneic haematopoietic stem cell transplantation (HSCT) is the only confirmed curative treatment option. The current diagnostic approach of MDS is the result of a combination of bone marrow (BM) morphology and cytogenetics; however, clonal cytogenetics abnormalities can be identified only in a minority of cases. Recently, molecular studies to detect mutations in *PTPN11*, *RAS* and *NF1* have been introduced in the diagnostic criteria of juvenile myelomonocytic leukaemia (JMML) (Tartaglia *et al*, 2003; Bollag *et al*, 1996; Miyauchi et al, 1994).

In 1982, the French-American-British (FAB) cooperative group proposed a morphological classification of MDS to differentiate five subtypes: refractory anaemia (RA), RA with ringed sideroblasts (RARS), RA with excess of blasts (RAEB), RAEB in transformation to leukaemia (RAEB-T) and chronic myelomonocytic leukaemia (CMML) (Bennett *et al*, 1982; Bennett *et al*, 1985)**.**

More recently, the World Health Organization (WHO) incorporated genetic, biologic and clinical features in a classification that updated the FAB system recognizing eight subtypes: RA, RARS, refractory cytopenia with multilineage dysplasia (RCMD), refractory cytopenia with multilineage dysplasia and ringed sideroblasts (RCMD-RS), refractory anaemia with excess blasts-1 (RAEB-1), refractory anaemia with excess blasts-2 (RAEB-2), myelodysplastic syndrome unclassified (MDS-U) and myelodysplastic syndrome associated with isolated del(5q) chromosome abnormality. The blast threshold required for the diagnosis of AML was lowered to 20% with elimination of the FAB category RAEB-T, and patients with specific recurring cytogenetics abnormalities t(8;21)(q22;q22), inv(16)(p13q22) or t(16;16)(p13;q22), and t(15;17)(q22;q12) were classified as having AML regardless of the blast percentage. Furthermore, a new category of myelodysplastic and myeloproliferative diseases (MDS/MPD) was defined, recognizing JMML as a separated disorder distinct from adult chronic myeloid or myelomonocytic leukaemia (Jaffe *et al*, 2001).

Nevertheless, both FAB and WHO systems are based on adult cohort and there are some significant differences between MDS in adult and paediatric patients that need to be highlighted. In children there is no evidence that a blast threshold of 20% could be better than 30% for the diagnosis of AML (Webb *et al*, 2002; Woods *et al*, 2002; Niemeyer *et al*, 2000), ringed sideroblasts are very rarely detected (Hasle *et al* , 1995;

Hasle *et al*, 1999; Passmore *et al*, 1995; Luna-Fineman *et al*, 1999; Sasaki *et al*, 2001), 5q- syndrome has not been described yet (Antillon F *et al*, 1998; Shikano *et al*, 1992), dyspoiesis can be detected in constitutional bone marrow failure syndromes, as Schwachman-Diamond syndrome detected (Hasle *et al* , 1995; Hasle *et al*, 1999; Passmore *et al*, 1995; Luna-Fineman *et al*, 1999; Sasaki *et al*, 2001), during drug therapy (Brichard *et al*, 1994), or in transient bone marrow hypoplasia due to viral infections (Mueller *et al*, 1996). Based on these observations, in 2003 *Hasle* and colleagues developed a classification of paediatric myelodysplastic and myeloproliferative syndromes in which three major categories of diseases are recognized: Myelodysplastic/Myeloproliferative disease (including JMML, CMML, *BCR-ABL* negative chronic myeloid leukaemia), Down syndrome disease (including transient abnormal myelopoiesis, myeloid leukaemia of Down syndrome), and Myelodysplastic Syndrome (including Refractory Cytopenia, RAEB, RAEB-T) (Hasle *et al*, 2003).

Morphology represents the gold standard for the diagnosis of myelodysplastic syndromes (Bennett *et al*, 1982; Cantù Rajnoldi *et al*, 2005), but in recent years several efforts to analyse MDS by flow cytometry have been reported, focused on the identification of abnormalities in the maturation pathway of antigen expression of myeloid and monocytic cells, or characterization of blast populations (Elghetany, 1998; Stetler-Stevenson *et al*, 2001; Ogata *et al*, 2002; Mayandie *et al*, 2002; Wells *et al*, 2003; Del Canizo *et al*, 2003; Benesch *et al*, 2004; Kussick *et al*, 2005; Malcovati *et al*, 2005; Ogata *et al*, 2005; Tavil *et al*, 2006; Pirruccello *et al*, 2006; Loken *et al*, 2008; Van de Loosdrecht *et al*, 2007). Immunophenotypic evaluation of MDS blast cells is particularly challenging, mainly due to the low amount of blasts in MDS samples, which could hamper flow cytometric analysis (FCM). Recently, *Ogata* and colleagues described immunophenotypic features of blast cells in MDS (Ogata *et al*, 2002; Ogata *et al*, 2005); in addition emerging evidence has demonstrated abnormalities in CD34+ cells in low grade MDS, with reduced B-cell precursors and aberrant antigen expression by CD34+ myeloblasts, suggesting a potential diagnostic role of analysing CD34+ compartment (Van de Loosdrecht *et al*, 2007; Ogata *et al*, 2006; Sternberg *et al*, 2005; Ribeiro *et al*, 2006). During the last year, immunophenotype has been included in the diagnostic criteria of adult MDS (Valent *et al*, 2007).

The aim of our study was to evaluate the capability of FCM to characterize immunophenotypic features of blast cells in children affected by MDS. Knowledge of immunophenotypic characteristics of MDS blast cells could provide relevant insights in understanding the biology of these disorders, and would help in the discrimination between MDS with excess of blasts and *de novo* AML, currently based on arbitrary threshold of percentage of blast cells. There we applied a multiparameter flow

cytometric approach using an extensive panel of monoclonal antibodies and considering light scatter properties for granulation assessment of various population of haematopoietic cells.

Patients and methods

Patients

Thirty-six MDS paediatric patients with more than 2% of blast cells at the morphological examination of the bone marrow (25 *de novo* MDS and 11 secondary MDS) were evaluated and compared with 145 paediatric *de novo* AML cases (M3 excluded). As control, 12 healthy age-matched donors for allogenic bone marrow transplantation (BMD) and 6 regenerating bone marrow samples, collected from children with acute lymphoblastic leukaemia (ALL) in remission at day +78 (week 12) after induction chemotherapy, were studied.

In the MDS cohort, there were 25 males and 11 females, with a median age of 10.3 years (range: 0.17-23 years); one case, aged 23 years, previously treated for a cancer occurred in childhood, presented a secondary MDS. According to the paediatric classification proposed by Hasle (Hasle *et al*, 2003), the distribution of MDS patients was as follows: 4 RC, 12 RAEB, 9 RAEB-T, 8 JMML, 2 CMML, 1 MDS unclassifiable. The median follow-up of patients was 1.17 years.

Cytogenetics data were available for 31 (86.11%) of 36 patients: fifteen cases had clonal cytogenetics abnormalities (48.39%); of these, monosomy 7 was identified in 7 patients (46.7%), and trisomy 8 in 3 (20%); other anomalies were present in the remaining cases. Of the eight cases of JMML, mutations in PTPN11, RAS, were present in 5 and 1 patients, respectively.

Clinical data and biological characteristics of MDS patients are reported in Table I.

In the *de novo* AML group, there were 77 males and 68 females with a median age of 6.97 years (range: 0.01-17.84 years). Cytomorphological classification of AML was performed according to the FAB and WHO criteria (6 M0, 29 M1, 26 M2, 12 M4, 7 M4eo, 24 M5, 1 M6, 11 M7, 25 unclassified AML cases, for 4 cases FAB was unknown) (Bennett *et al*, 1985; Jaffe *et al*, 2001).

Twelve healthy age-matched bone marrow donors, 5 males and 7 females, with a median age of 7.35 years (range 0.58-25.8 years), and 6 regenerating bone marrow samples collected from children with ALL patients in remission at day +78 (week 12) of treatment, 4 males and 2 females, with a median age of 7.67 years (range 3.35-13.88 years), were also studied and considered as control group.

All patients had been diagnosed during 2002-2006, referred to Italian AIEOP (Italian Association of Paediatric Hematology and Hemato-Oncology) centres and centralized at the Central AIEOP Hemato-Oncology Laboratory of the University of Padua, for morphology and immunophenotyping. The study was approved by the institutional ethical committees and was done with the informed consent of the patients' parents or guardians. The diagnosis of MDS was established by morphology and cytogenetics (Bennett *et al*, 1982; Cantù Rajnoldi *et al*, 2005), and classified in accordance to the paediatric classification proposed by Hasle (Hasle *et al*, 2003); AML was diagnosed based on conventional FAB classification and immunological criteria (Bennett *et al*, 1985; Jaffe *et al*, 2001; Khalidi *et al*, 1998; Stewart, C.C. & Nicholson, J.K.A. (ed.), 2000). Flow cytometry was performed by investigators who were blinded to the clinical and morphological data.

Flow cytometric studies

Immunophenotypic analyses were performed as previously described (Basso *et al*, 2001). Briefly, a multiparametric flow cytometric 4-5 colors approach was applied, using whole blood (at least 500,000 cells/tube) incubated for 15-20 minutes in the dark at room temperature with monoclonal antibodies (MoAbs) directly conjugated with fluorochromes. The following MoAbs were used: Fluorescein isothiocyanate (FITC): CD4, CD66b, CD34 (Becton Dickinson, San Jose, CA, USA), CD15, CD64, CD10, CD7 (Beckman Coulter-Immunotech, Miami, FL, USA), CD13, CD65, MPO (Caltag Laboratories, San Francisco, CA, USA); Phycoerythrin (PE): CD8, CD11a, CD34 (Becton Dickinson, San Jose, CA, USA), CD14, CD11b, CD117, CD56 (Beckman Coulter-Immunotech, Miami, FL, USA), CD7, CD15, Lactoferrin (Caltag Laboratories, San Francisco, CA, USA), CD133 (Miltenyi Biotec, Bergisch Gladbach, Germany); Phycoerythrin-Texas-red (ECD): CD45 (Beckman Coulter-Immunotech, Miami, FL, USA); Phycoerythrin-cyanin 5 (PE-Cy5): CD20, CD38 (Becton Dickinson, San Jose, CA, USA), CD33, CD16 (Beckman Coulter-Immunotech, Miami, FL, USA), CD7, HLA-DR (Caltag Laboratories, San Francisco, CA, USA); Phycoerythrin-cyanin 7 (PE-Cy7): CD33, CD34 (Becton Dickinson, San Jose, CA, USA), CD3, CD19 (Beckman Coulter-Immunotech, Miami, FL, USA). Samples were lysed using NH_4Cl solution, then washed twice in phosphate-buffered saline (PBS), re-suspended in 1 mL of PBS and analysed by flow cytometry. All samples were processed within 24 hours after collection. Antibody labelling was measured using an Epics XL cytometer equipped with an argon laser tuned to 488 nm at the beginning of our work, and an FC500 cytometer (both from Beckmann Coulter, Inc., Miami, FL, USA) later on. Forward angle light scatter (FSC)

signals were recorded using linear amplification, whereas orthogonal light scatter (SSC) and fluorescences were collected logarithmically. The instrument set-up was optimized daily by using Calibrite™ beads (Becton Dickinson, San Jose, CA, USA), normal peripheral blood lymphocytes labelled with the anti-CD4FITC/CD8PE/CD3ECD/CD45Pe-Cy5 four-color combination or anti-CD4FITC/CD8PE/CD45ECD/CD7Pe-Cy5/CD3Pe-Cy7 five-color combination, and DAKO Fluorospheres™ (Dako, Glostrup, Denmark), with assigned molecules of equivalent fluorochrome (MEF) values for the fluorescent bead populations (De Zen *et al*, 2000). Cells not stained were used as negative controls. The immunophenotypic analysis was performed collecting 15,000-100,000 events per sample; data were acquired and collected in list-mode files with the EXPO32 software (Beckman-Coulter, Inc., Miami, FL, USA).

An extensive panel of monoclonal antibodies combinations was constructed to optimize recognition of aberrant expression of antigens on myeloid blast cells (Table SI). Blasts were isolated from various haematopoietic cells by CD45 gating strategy *vs* SSC, and then back-gated to exclude debris and non-viable cells. An example of CD45 gating *vs* SSC in normal paediatric bone marrow is shown in Fig S1. Percentage of CD34+ cells was measured on total BM viable cells according to physical parameters. CD45 and SSC were quantified by mean fluorescent intensity (MFI) measurements, expressed as mean fluorescence channel (arbitrary units scaled from 0 to 1024). Blast cells were judged to be positive for a given antigen when the intensity of expression was higher than the negative control (De Zen *et al*, 2000).

Morphology

Morphological examination of the bone marrow was performed by institutional haematologist and then centrally revised (S.F., L.S.). Morphological findings of dysplasia were assessed as previously described on MGG stained smears (Bennett *et al*, 1982; Cantù Rajnoldi *et al*, 2005). Myeloperoxidase reaction was also performed. For each bone marrow smear cellularity, presence of megakaryocytes and grade of dysplasia of the three lineages were evaluated, according to EWOG (European Working Oncology Group of Pediatric MDS) morphology criteria.

Cytogenetics

Cytogenetics analyses were performed on in vitro cultures from BM cells at diagnosis and during the follow-up. The cells were cultured in a 5% CO_2 atmosphere at 37°C in growth medium containing RPMI 1640 (GIBCO) with 20% foetal calf serum (FCS), 1%

L-glutamine, 1% penicillin/streptomycin. Cells for karyotyping were harvested after incubation with Colcemid (Irvine Scientific) 10µg/ml: 10µl for 18 hours (overnight cultured cells) and 50 µl for 1 hour (24 h cultured cells). Cells collected were treated with a hypotonic solution (KCl 0.075 M), fixed methanol and acid acetic (3:1), and dropped onto clean slides. The chromosomes were banded by QFQ method with Quinacrine mustard. The karyotypes were analysed according to ISCN nomenclature (Shaffer & Tommerup, 2005).

Statistical methods

Percentage, median and range were used as appropriate to describe continuous and categorical variables, respectively. Patient characteristics were compared using χ^2 or Fisher's exact test (as appropriate) in the case of discrete variables, or the Mann Whitney test, in the case of continuous variables.

The sensitivity and specificity were calculated according to the proportion of samples with true and false positive and negative tests. To assess the association between continuous variables Pearson's correlation and linear regression coefficient were calculated. Overall survival (OS) was calculated from the date of diagnosis to the date of death due to any cause, or to the date of last follow-up. OS was estimated by the Kaplan-Meier method with differences between groups compared by log-rank test. Standard errors (SE) were calculated according to Greenwood's formula, and all p values reported were 2-sided. The level of significance was set at $\alpha = 0.05$.

All analyses were performed using the statistical software SAS Institute, Cary, NC, Version 8.2 (Marubini & Valsecchi, 1995; Agresti, 1990).

Results

IMMUNOPHENOTYPIC FEATURES OF MDS BLAST CELLS

Bone marrow aspirates from 36 paediatric MDS who presented at least 2% of blasts at the morphological examination of the bone marrow (25 *de novo* MDS and 11 secondary MDS), and from 145 paediatric *de novo* AML (M3 excluded) were evaluated. As controls, we analysed 12 healthy age-matched donors for allogenic bone marrow transplantation and 6 regenerating bone marrow samples from paediatric ALL were analysed.

In most cases, MDS blast cells were characterized by the expression of immature myeloid antigens, CD34, CD117, CD38, HLA-DR, CD33, CD133. In detail, the percentage of positive cases among total cases examined was 91.67% for CD34

(33/36), 100% for CD117 (22/22), 92.31% for CD38 (24/26), 78.79% for HLA-DR (26/33), 100% for CD33 (36/36), 73.33% for CD133 (11/15) (Table II).

CD11b and CD11a, normally acquired at the stage of promyelocyte and metamyelocyte, respectively, were detectable in 55.17% (16/29) and 82.14% (23/28) of the cases. However, MDS blast cells only rarely expressed other myeloid maturation markers such as CD64, CD66b, CD65, CD15 (Table II).

CD16, present on NK cells but also on myeloid cells, was absent in all cases examined (0/30), whereas the NK-lineage antigen CD56 resulted positive in 2 cases out of 18 tested (11.11%). Only 1 case out of 30 expressed CD14: it was a patient affected by JMML, but the remaining 7 JMML cases had blast cells negative for this marker.

Among lymphoid-associated antigens, CD7 was expressed in 45.16% of cases (14/31), CD19 in 3.45% (1/29), CD10 in 8.33% (2/24), and no cases were CD20+, CD3+, CD4+, or CD8+ (Table II).

Combining measurements of mean fluorescence intensity values of CD45 and SSC, we found that MDS blast cells were generally restricted to the region of normal myeloid precursors, but a significant decrease in the expression of CD45 was frequent, when compared to healthy donors (p= 0.005) (Fig S2).

No differences in phenotypic features between primary and secondary MDS, and according to FAB subtype were observed (*data not shown*).

We subsequently compared MDS blast phenotype to AML cases to test whether differences in antigen expression occurred (Table III). The proportion of MDS cases whose blasts were CD34+CD117+ (*Phenotype A*), was higher than in AML patients (90.91% *vs* 50.38%), and the difference was statistically significant (p=0.0004) (Table IV). Furthermore, we found that the expression of early myeloid antigens (CD34 and CD117) together with maturation markers such as CD11a and CD11b, but not CD64, CD65, CD15, defined a phenotype (CD34+CD117+CD11a+CD11b+CD64-CD65-CD15-) (*Phenotype B*), significantly more frequent in MDS than AML cases (64.29% *vs* 6.14%, respectively, p<0.0001) (Table III). Taking into account the limited cohort of MDS patients, the two phenotypes allowed discriminating between MDS and AML cases with 91% (20/22) sensitivity and 50% (65/131) specificity for *Phenotype A;* with 64.29% (9/14) sensitivity and 93.86% (107/114) specificity for *Phenotype B.*

On the basis of data reported from adult MDS patients (Ogata *et al*, 2002; Ogata *et al*, 2005; Van de Loosdrecht *et al*, 2007; Font *et al*, 2006; Font *et al*, 2008), we attempted to evaluate the potential prognostic value of CD7 expression in our cohort of patients. CD7 was not tested in 5 out of 36 patients; for this reason, survival curves were estimated in 31 MDS cases. Because of its high prevalence, the clinical impact of CD34, as a single marker and/or in combination with CD7, was not considered. CD7 positivity

was associated with an OS of 41.67% (S.E. 17.84), whereas CD7 negative cases showed an OS of 82.54% (S.E. 11.49) (Fig 1), with a nearly significant *p* value (p= 0.09). The distribution of CD7 positivity seemed to increase according to FAB subtypes (data not shown), but the number of patients available for comparison was too low to reach significance. Multivariate analysis was not performed due to the limited sample size.

B-CELL PROGENITORS IN MDS

We evaluated CD34 compartment in MDS patients (32 cases), healthy age-matched bone marrow donors (12 specimens) and regenerating bone marrow samples (6 specimens).

The median percentage of total amount of CD34+ cells was 5.15 (range 0-27) in MDS patients, 2.86 (range 1.78-7.1) in healthy bone marrow donors, and 7 (range 3.8-11.8) in regenerating bone marrow samples. Correlation analysis between CD34+ cells and age, showed a statistically significant inverse correlation in healthy bone marrow donors (r= -0.73, regression coefficient= - 0.14, p= 0.007), as expected (McKenna *et al*, 2001); similar results were obtained in regenerating bone marrow, but the small number of patients did not permit to achieve statistical significance. Conversely, a positive correlation with age was observed in MDS patients (r= 0.36, regression coefficient= 0.37, p= 0.04), (Fig S3).

As known, (Loken *et al*, 2008; McKenna *et al*, 2001), $CD34^{+}$ cells comprise a heterogeneous population of progenitors in normal bone marrow. In healthy bone marrow donors, CD34+ cells can be divided into at least two distinct sub-populations of precursors cells, myeloid (CD34+CD33+CD19-) and B-lymphoid (CD34+CD33-CD19+), with no differences between the two groups (Fig S4).

To assess differences in CD34+ compartment among MDS patients, healthy bone marrow donors and regenerating bone marrow samples, CD34+CD33+CD19- and CD34+CD33-CD19+ sub-populations were analysed separately, based on immunophenotypic features and light scatter properties. MDS patients revealed an increase in CD34+CD33+CD19- cells but a dramatic decrease in B-cell precursors, when compared to controls (p= 0.0002 and p= <0.0001, respectively) (Fig 2, 3). Consistent with these findings, only 4 MDS cases presented two sub-populations of CD34+ cells, a predominant and aberrant CD34+CD33+CD19- population, and residual haematogones. A representative case is shown in Fig S5. Moreover, analysis of CD19+ cells showed a significant reduction in MDS patients (N=25) compared with healthy bone marrow donors (3.2% *vs* 10.4%, respectively, p=< 0.0001) (Fig S6).

Discussion

MDS are rarely observed in childhood, constituting less than 5% of all malignant hematological disorders (Hasle *et al* , 1995; Hasle *et al*, 1999). The prognosis is extremely poor in most cases, and allogeneic haematopoietic stem cell transplantation is the only curative therapy. Despite well-established morphological, cytogenetics and molecular criteria, diagnosis of MDS is particularly challenging in paediatric patients, and one of the main diagnostic dilemma is the differentiation of MDS with excess of blast from *de novo* AML, currently based on an arbitrary threshold of percentage of blast cells. In this regard, it is important to emphasize the different biology underlying these two diseases: AML is a chemo-sensitive disorder characterized by recurrent cytogenetics translocations, whereas MDS and secondary AML show poor response to chemotherapy, with numerical aberrations as the most frequent cytogenetic aberration that can be detected (Webb *et al* , 2002; Niemeyer *et al*, 2000; Hasle *et al*, 2003).

Recently, immunophenotypic characterization of blast cells in adult MDS has been reported, demonstrating a prevalence of an immature phenotype, CD34+CD38+HLA-DR+CD13+CD33+, in MDS than in *de novo* AML patients (Ogata *et al* , 2002; Ogata *et al*, 2005; Khalidi *et al* , 1998).

The purpose of our study was to characterize the immunophenotypic features of blast cells in paediatric myelodysplastic syndromes. In the majority of cases, MDS blast cells displayed an immature myeloid phenotype characterized by the expression of CD34, CD117, CD38, HLA-DR, CD33, CD133. CD13 was not informative in our experience, therefore was not considered in our study. Interestingly, we found that the coexpression of CD34 and CD117 (*Phenotype A*), was significantly more frequent in MDS than in *de novo* AML patients (90.91% *vs* 50.38%), (p=0.0004). This is in agreement with previous findings in adult series, where 97% of MDS cases expressed CD34, while *de novo* AML are CD34 positive in only 35-60% of cases (Ogata *et al* , 2002; Ogata *et al*, 2005; Khalidi *et al* , 1998).

Simultaneous expression of CD34 and CD117 with antigens that normally appear in the late stages of myeloid development, defined as maturational asynchrony, is a well-known phenomenon in AML (Stewart, C.C. & Nicholson, J.K.A. (ed.), 2000) and adult MDS (Stetler-Stevenson *et al* , 2001; Ogata *et al*, 2002; Maynadie *et al*, 2002; Wells *et al*, 2003; Ogata *et al*, 2005; Loken *et al* , 2008). In our cohort of patients, we found maturational asynchrony in many cases but, notably, CD11a and CD11b were the unique maturation markers detected, whereas lack of expression of CD64, CD65, CD15 was observed. These findings defined a phenotype CD34+CD117+CD11a+CD11b+CD64-CD65-CD15- (*Phenotype B*), significantly more frequent in MDS than AML cases (64.29% *vs* 6.14%, respectively, p<0.0001). Taking

account of the limited cohort of MDS patients, we established the discrimination power between the two tests in differentiating MDS from AML cases: *Phenotype A* reached a sensitivity of 91% and a specificity of 50%, whereas *Phenotype B* showed a sensitivity of 64.29% and a specificity of 93.86%.

CD7 expression is associated with an immature phenotype in AML (Lo Coco *et al*, 1989), and it is still a matter of debate whether CD7 positivity could indicate an adverse prognostic factor in AML (Lo Coco *et al*, 1989; Ogata *et al*, 2001; Del Poeta *et al*, 1995). Recently, prognostic relevance of CD7 expression has been reported in adult MDS patients (Ogata *et al*, 2002; Ogata *et al*, 2005; Van de Loosdrecht *et al*, 2008; Font *et al*, 2006; Font *et al*, 2008). Based on these findings, we investigated the potential prognostic impact of CD7 expression in our cohort of patients. CD7 positivity was associated with a shorter overall survival rate than CD7 negative cases (OS 41.67% vs 82.54%, respectively) (Fig 1), even if the difference was not statistical significant (p= 0.09), most likely for the limited number of MDS patients whose blasts were CD7+.

The striking positivity for CD34 observed in blast cells of paediatric MDS, prompted us to investigate the CD34+ compartment in MDS and normal bone marrow samples. As known, [37,52] in normal bone marrow donors two distinct sub-populations of CD34+ cells could be recognized: CD34+ myeloblasts (CD34+CD33+CD19-) and CD34+ B-cell progenitors (CD34+CD33-CD19+), with no significant differences between the two groups. Comparing CD34+ cells in MDS patients to normal bone marrow, we observed a significant increase in CD34+CD33+CD19- cells, due to the marrow involvement with blast cells, and a decrease in B-cell compartment, independently of age (Fig 2, 3). Evidence of involvement of B-cell compartment in MDS patients has recently been reported, implying the possibility of a B-cell progenitor defect in the pathophysiology of the disease (Van de Loosdrecht *et al*, 2008; Ogata *et al*, 2006; Sternberg *et al*, 2005; Ribeiro *et al*, 2006). Consequently, analysis of B-cell precursors has been proposed as an additional parameter to be investigate in the diagnostic approach of MDS (Loken *et al*, 2008; Van de Loosdrecht *et al*, 2008; Ogata *et al*, 2006; Sternberg *et al*, 2005; Ribeiro *et al*, 2006). However, another hypothesis to explain our finding could be also a B-cell compartment growth inhibition by MDS blast cells.

Our data suggest that MDS blast cells display an immature phenotype with maturational asynchrony in most cases, with a pattern similar to what reported in the adult cohort. This phenotype could be useful in the discrimination between MDS and *de novo* AML patients. The expression of CD7 may adversely affect the outcome of MDS patients, and we would recommend testing this marker in children affected by MDS. Moreover, exploiting B-cell compartment could represent a promising parameter to be considered

in the diagnostic evaluation of paediatric patients with MDS, as already suggested in adult patients.

In conclusion, immunophenotypic analysis of blast cells in paediatric MDS could be considered an emerging diagnostic tool, supporting morphologic and cytogenetic approaches. The data reported are promising, even though confirmation in a larger multicentric study may be needed.

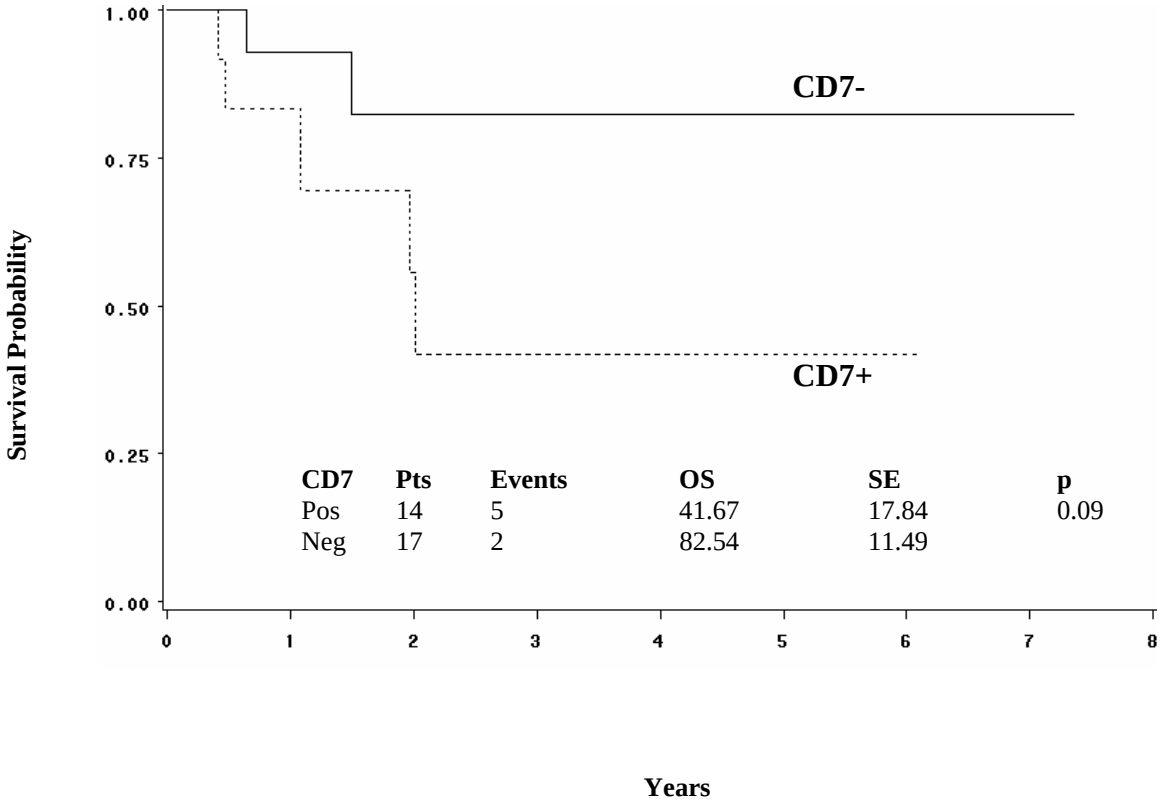


Fig 1. Overall survival in MDS patients (N=31) relating to the presence of CD7. CD7+ patients had a worst prognosis compared to CD7-, even if the difference was not statistical significant (p= 0.09).

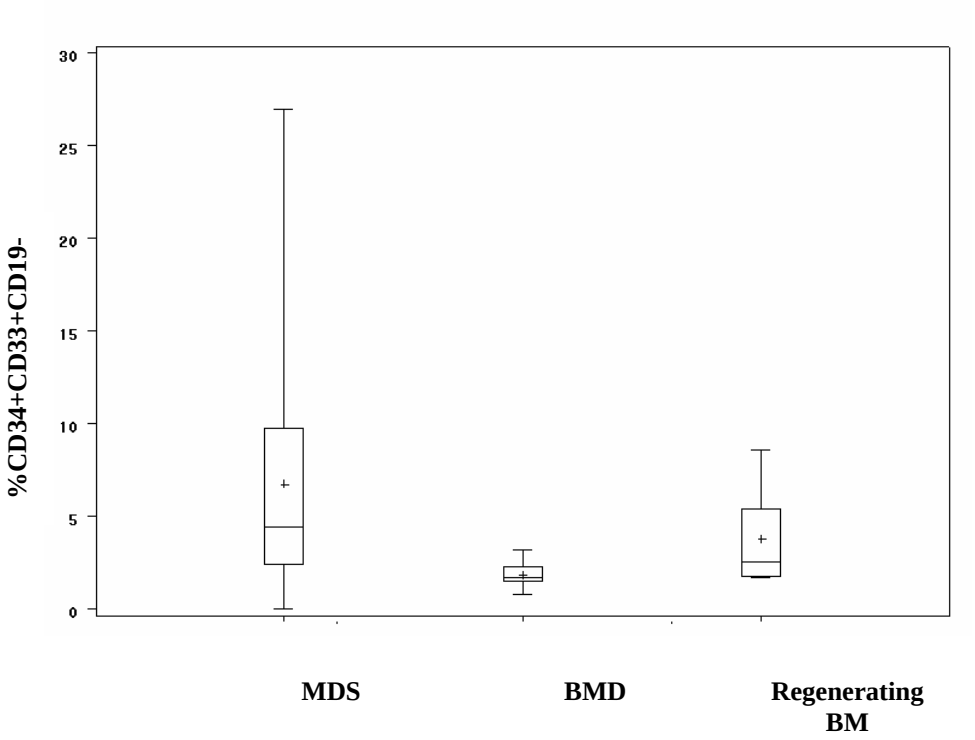


Fig 2. Distribution of CD34+CD33+CD19- cells by percentage in MDS patients (N=32), healthy bone marrow donors (N=12), regenerating bone marrow samples (N=6). MDS patients displayed a significant increase in CD34+CD33+CD19- cells in comparison to BMD (p= 0.0002).

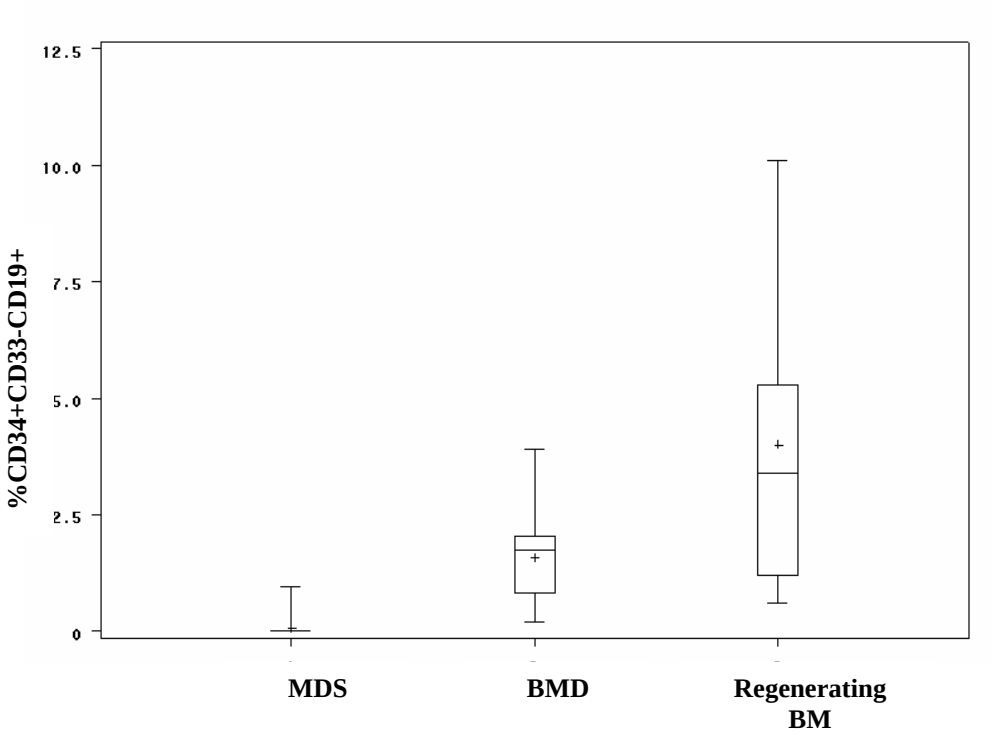


Fig 3. Distribution of haematogones (CD34+CD33-CD19+) by percentage for MDS patients (N=32), bone marrow donors (N=12), regenerating bone marrow samples (N=6). A significant decrease in haematogones in MDS patients in comparison to BMD is shown (p

REFERENCES

Agresti, A. (1990) *Categorical Data Analysis*. New York, NY, Wiley & Sons (ed.)

Antillon, F., Raimondi, S.C., Fairman, J., Liang, H., Nagarajan, L., Head, D.& Ribeiro, R.C. (1998) 5q- in a child with refractory anaemia with excess blasts: similarities to 5q- syndrome in adults. *Cancer Genet Cytogenet*, **105**, 119-122.

Bader-Meunier, B., Mielot, F., Tchemia, G., Buisine, J., Delsol, G., Duchayne, E., Lemerle, S., Leverger, G., De Lumley, L., Manel, A.M., Nathanson, M., Plantaz, D., Robert, A., Schaison, G., Sommelet, D. & Vilmer, E. (1996) Myelodysplastic syndrome in childhood: report of 49 patients from a French multicenter study. *Br J Haematol*, **92**, 344-350.

Basso, G., Buldini, B., De Zen, L. & Orfao, A. (2001) New methodologic approaches for immunophenotyping acute leukemias. *Haematologica*, **86,** 675-692.

Benesch, M., Deeg, H.J., Wells, D. & Loken, M. (2004) Flow cytometry for diagnosis and assessment of prognosis in patients with myelodysplastic syndromes. *Hematology*, **9**, 171–177.

Bennett, J.M., Catovsky, D., Daniel, M.T., Flandrin, G., Galton, D.A.G., Gralnick, H.R. & Sultan, C. (1982) Proposals for the classification of the myelodysplastic syndromes. *Br J Haematol*, **51**, 189–199.

Bennett, J.M., Catovsky, D., Daniel, M.T., Flandrin, G., Galton, D.A.G., Gralnick, H.R. & Sultan, C. (1985) Proposed revised criteria for the classification of acute myeloid leukemia. A report of the French–American–British Cooperative Group. *Ann Intern Med*, **103**, 620–625.

Bollag, G., Clapp, D.W., Shih, S., Adler, F., Zhang, Y.Y., Thompson, P., Lange, B.J., Freedman, M.H., McCormick, F., Jacks, T. & Shannon, K. (1996) Loss of NF1 results in activation of the Ras signaling pathway and leads to aberrant growth in haematopoietic cells. *Nat Genet*, **12**, 144-148.

Brichard, B., Vermylen, C., Scheiff, J.M., Ninane, J. & Cornu, G. (1994) Haematological disturbances during long-term valproate therapy. *Eur J Pediat,* **153**, 378–380.

Cantù Rajnoldi, A., Fenu, S., Kerndrup, G., van Wering, E.R., Niemayer, C.M. & Baumann, I. for the European Working Group on Myelodysplastic Syndromes in Childhood (EWOG-MDS). (2005) *Ann Hematol*, **84**, 429-433.

De Zen, L., Orfao, A., Cazzaniga, G., Masiero, L., Cocito, M.G., Spinelli, M., Rivolta, A., Biondi, A., Zanesco, L. & Basso, G. (2000) Quantitative multiparametric immunophenotyping in acute lymphoblastic leukemia: correlation with specific genotype. I. ETV6/AML1 ALLs identification. *Leukemia*, **14**, 1225-1231.

Del Canizo, M.C., Fernandez, M.E., Lopez, A., Vidriales, B., Villaron, E., Arroyo, J.L., Ortuno, F., Orfao, A. & San Miguel, J.F. (2003) Immunophenotypic analysis of myelodysplastic syndromes. *Haematologica*, **88**, 402-407.

Del Poeta, G., Stasi, R., Venditti, A., Cox, C., Aronica, G., Masi, M., Bruno, A., Simone, M.D., Buccisano, F. & Papa, G. (1995) CD7 expression in acute myeloid leukemia, *Leuk Lymphoma*, **17**, 111-119.

Elghetany, M.T. (1998) Surface marker abnormalities in myelodysplastic syndromes. *Haematologica*, **83**, 1104-1115.

Font, P., Subira, D., Mtnez-Chamorro, C., Castanon, S., Arranz, E., Ramiro, S., Gli-Fernandez, J.J., Lopez-Pascual, J., Alonso, A., Perez-Saenz, M.A., Alaez, C., Renedo, M., Blas, C., Escudero, A. & Fdez-Ranada J.M. (2006) Evaluation of CD7 and terminal deoxynucleotidyl transferase (TdT) expression in CD34+ myeloblasts from patients with myelodysplastic syndrome. *Leuk Res*, **30**, 957-963.

Font, P. & Subira, D. (2008) Expression of CD7 in myelodysplastic syndromes (MDS): Is this a truly prognostic factor? *Leuk Res*, **32**, 185-186.

Hasle, H., Niemeyer, C.M., Chessells, G.M., Baumann, I., Bennett, J.M., Kerndrup, G. & Head, D.R. (2003) A paediatric approach to the WHO classification of myelodysplastic and myeloproliferative diseases. *Leukemia*, **17**, 277–282.

Hasle, H., Kerndrup, G. & Jacobsen, B.B. (1995) Childhood myelodysplastic syndrome in Denmark: incidence and predisposing conditions. *Leukemia*, **9**, 1569-1572.

Hasle, H., Wadsworth, L.D., Massing, B.G., McBride, M. & Schultz, K.R. (1999) A population-based study of childhood myelodysplastic syndrome in British Columbia, Canada. *Br J Haematol*, **106**, 1027-1032.

Heaney, M.L. & Golde, D. (1999) Myelodysplasia. *N Engl J Med*, **21**, 1649-1660.

Jaffe, E.S., Harris, N.L., Stein, H. & Vardiman JW (ed.) (2001) *World Health Organization classification of tumours. Pathology and genetics of tumours of haematopoietic and lymphoid tissues.* IARC Press, Lyon.

Khalidi, H.S., Medeiros, L.J., Chang, K.L., Brynes, R.K., Slovak, M.L. & Arber, D.A. (1998) The immunophenotype of adult acute myeloid leukemia: high frequency of lymphoid antigen expression and comparison of immunophenotype, French-American-British classification, and karyotypic abnormalities. *Am J Clin Pathol*, **109**, 211-222.

Kussick, S.J., Fromm, J.R., Rossini, A., Li, Y., Chang, A., Norwood, T.H. & Wood, B.L. (2005) Four-color flow cytometry shows strong concordance with bone marrow morphology and cytogenetics in the evaluation for myelodysplasia. *Am J Clin Pathol*, **124**, 170–181.

Lo Coco, F., De Rossi, G., Pasqualetti, D., Lopez, M., Diverio, D., Latagliata, R., Fenu, S. & Mandelli, F. (1989) CD7 positive acute myeloid leukaemia: a subtype associated with cell immaturity. *Br J Hematol*, **73**, 480-485.

Loken, M.R., van de Loosdrecht, A., Ogata, K., Orfao, A. & Wells, D.A. (2008) Flow cytometry in myelodysplastic syndromes: report from a working conference. *Leuk Res*, **32**, 5-17.

Luna-Fineman, S., Shannon, K.M., Atwater, S.K., Davis, J., Masterson, M., Ortega, J., Sanders, J., Steinherz, P., Weinberg, V. & Lange, B.J. (1999) Myelodysplastic and myeloproliferative disorders of childhood: a study of 167 patients. *Blood*, **93**, 459-466.

Malcovati, L., Della Porta, M.G., Lunghi, M., Pascutto, C., Vanelli, L., Travaglino, E., Maffioli, M., Bernasconi, P., Lazzarino, M., Invernizzi, R. & Cazzola, M. (2005) Flow cytometry evaluation of erythroid and myeloid dysplasia in patients with Myelodysplastic syndrome. *Leukemia*, **19**, 776–783.

Marubini, E. & Valsecchi, M.G. (1995) *Analysing survival data from clinical trials and observational studies*. Chichester, John Wiley & Sons (ed.).

Maynadie, M., Picard, F., Husson, B., Chatelain, B., Cornet, Y., Le Roux, G., Campos, L., Dromelet, A., Lepelley, P., Jouault, H., Imbert, M., Rosenwadj, M., Vergé, V., Bissières, P., Raphael, M., Béné, M.C., Feuillard, J. & the Groupe d'Etude Immunologique des Leucémies (GEIL). (2002) Immunophenotypic clustering of myelodysplastic syndromes. *Blood*, **100**, 2349–2356.

McKenna, R.W., Washington, L.T., Equino, D.B., Picker, L.J. & Kroft, S.H. (2001) Immunophenotypic analysis of haematogones (B-lymphocyte precursors) in 662 consecutive bone marrow specimens by 4-color flow-cytometry. *Blood*, **98**, 2498-2507.

Miyauchi, J., Asada, M., Sasaki, M., Tsunematsu, Y., Kojima, S. & Mizutani, S. (1994) Mutations of the N-ras gene in juvenile chronic myelogenous leukemia. *Blood*, **83**, 2248-2254.

Mueller, B.U., Tannenbaum, S. & Pizzo, P.A. (1996) Bone marrow aspirates and biopsies in children with human immunodeficiency virus infection. *J Pediatr Hematol Oncol*, **18**, 266–271.

Niemeyer, C.M., Duffner, U., Bender-Götze, C., Ebell, W., Hasle, H., Klingebiel, T., Kremens, B., Nurnberger, W., Vossen, J., Zecca, M., Zintl, F. & Locatelli, F. (2000) AML-Type intensive chemotherapy prior to stem cell transplantation (SCT) does not improve survival in children and adolescents with primary myelodysplastic syndromes (MDS). *Blood*, **96**, 521a.

Ogata, K., Kishikawa, Y., Satoh, C., Tamura, H., Dan, K. & Hayashi, A. (2006) Diagnostic application of flow cytometric characteristics of CD34+ cells in low-grade myelodysplastic syndromes. *Blood*, **108**, 1037-1044.

Ogata, K., Nakamura, K., Yokose, N., Tamura, H., Tachibana, M., Taniguchi, O., Iwakiri, R., Hayashi, T., Sakamaki, H., Murai, Y., Tohyama, K., Tomoyasu, S., Nonaka, Y., Mori, M., Dan, K & Yoshida, Y. (2002) Clinical significance of phenotypic features of blasts in patients with myelodysplastic syndrome. *Blood*, **100**, 3887-3896.

Ogata, K., Yokose, N., Shioi, Y., Ishida, Y., Tomiyama, J., Hamaguchi, H., Yagasaki, F., Bessyo, M., Sakamaki, H., Dan, K. & Kuriya S. (2001) Reappraisal of the clinical significance of CD7 expression in association with cytogenetics in *de novo* acute myeloid leukaemia. *Br J Haematol*, **115**, 612-615.

Ogata, K, & Yoshida, Y. (2005) Clinical implications of blast immunophenotypes in myelodysplastic syndromes. *Leuk Lymphoma*, **46,** 1269–1274.

Passmore, S.J., Hann, I.M., Stiller, C.A., Ramani, P., Swansbury, G.J., Gibson, B., Reeves B.R. & Chessells, J.M. (1995) Paediatric myelodysplasia: a study of 68 children and a new prognostic scoring system: *Blood*, **85**, 1742-1750.

Pirruccello, S.J., Young, K.H. & Aoun, P. (2006) Myeloblast phenotypic changes in myelodysplasia. CD34 and CD117 expression abnormalities are common. *Am J Clin Pathol*, **125**, 884-894.

Ribeiro, E., Matarraz-Sudon, S., de Santiago, M., Lima, C.S., Metze, K., Giralt, M., Saad, S.T., de Matos, A.O. & Lorand-Metze, I. (2006) Maturation-associated immunophenotypic abnormalities in bone marrow B-lymphocytes in myelodysplastic syndromes. *Leuk Res,* **30,** 9-16.

Sasaki, H., Manabe, A., Kojima, S., Tsuchida, M., Hayashi, Y., Ikuta, K., Okamura, I., Koike, K., Ohara, A., Ishii, E., Komada, Y., Hibi, S. & Nakahata, T. (2001) Myelodysplastic syndrome in childhood: a retrospective study of 189 patients in Japan. *Leukemia*, **15**, 1713-1720.

Shaffer, L.G. & Tommerup, N. (ed.) (2005) *ISCN: An International System for Human Cytogenetic Nomenclature.* Karger, Basel.

Shikano, T., Ishikawa, Y. & Anakura, M. (1992) Myelodysplastic syndrome with partial deletion of the long arm of chromosome 5: first report of a case in a child. *Acta Paediatr Jpn*, **34**, 539-542.

Steensma, D.P. & Bennet, J.M. (2006) The Myelodysplastic syndromes: diagnosis and treatment. *Mayo Clin Proc*, **81**, 104-130.

Stewart, C.C. & Nicholson, J.K.A. (ed.) (2000) Steizer, G.T. & Goodpasture, L. Use of multiparametric flow cytometry and immunophenotyping for the diagnosis and classification of acute myeloid leukaemia. *Immunophenotyping*. Wiley-Liss Press, New York, NY, 215-239.

Sternberg, A., Killick, S., Littlewood, T., Hatton, C., Peniket, A., Seidl, T., Soneji, S., Leach, J., Bowen, D., Chapman, C., Standen, G., Massey, E., Robinson, L., Vadher, B., Kaczmarski, R., Jan Mohammed, R., Clipsham, K., Carr, A. & Vyas, P. (2005) Evidence for reduced B-cell progenitors in early (low-risk) myelodysplastic syndrome. *Blood*, **106,** 2982-2991.

Stetler-Stevenson, M., Arthur, D.C., Jabbour, N., Xie, X.Y., Molldrem, J., Barrett, A.J., Venzon, D. & Rick, M.E. (2001) Diagnostic utility of flow cytometric immunophenotyping in myelodysplastic syndrome. *Blood*, **98**, 979–87.

Tartaglia, M., Niemeyer, C.M., Song, X., Buechner, J., Hählen, K., Hasle, H., Jung, A., Licht, J.D. & Gelb, B.D. (2003) Somatic PTPN11 mutations in juvenile myelomonocytic leukemia, myelodysplastic syndromes and acute myeloid leukemia. *Nat Genet,* **34**, 148-150.

Tavil, B., Cetin, M. & Tuncer, M. (2006) CD34/CD117 positivity in assessment of prognosis in children with myelodysplastic syndrome. *Leuk Res*, **30,** 222-224.

Valent, P., Horny, H.P., Bennett, J.M., Fonatsch, C., Germing, U., Greenberg, P., Haferlach, T., Haase, D., Kolb, H.J., Krieger, O., Loken, M., van de Loosdrecht, A., Ogata, K., Orfao, A., Pfeilstöcker, M., Rüter, B., Sperr, W.R., Stauder, R. & Wells, D.A. (2007) Definitions and standards in the diagnosis and treatment of the myelodysplastic syndromes: Consensus statements and report from a working conference. *Leuk Res*, **31**, 727-736.

van de Loosdrecht, A.A., Westers, T.M., Westra, A.H., Drager, A.M., van der Velden, V.H.J. & Ossenkoppele, G.J. (2007) Identification of distinct prognostic subgroups in low and intermediate-1 risk myelodysplastic syndromes by flow cytometry. *Blood* [in press].

Webb, D.K.H., Passmore, S.J., Hann, I.M., Harrison, G., Wheatley, K. & Chessells, J.M. (2002) Results of treatment of children with refractory anaemia with excess blasts (RAEB) and RAEB in transformation (RAEB-t) in Great Britain 1990–99. *Br J Haematol*, **117,** 33–39.

Wells, D.A., Benesch, M., Loken, M.R., Vallejo, C., Myerson, D., Leisenring, W.M. & Deeg, J. (2003) Myeloid and monocytic dyspoiesis as determined by flow cytometric scoring in Myelodysplastic syndrome correlates with the IPSS and with outcome after hematopoietic stem cell transplantation. *Blood,* **102,** 394–403.

1.1.c Multiparameter assessment of minimal residual disease in patients with acute myeloid leukemia

Background

Acute myeloid leukemia (AML) comprises only 15-20% of the acute leukemia diagnosed in childhood. It remains a challenging disease with an inferior treatment outcome compared with paediatric acute lymphoblastic leukemia. Despite high-dose cytarabine consolidation phase and allogenic bone marrow transplantation, about 40% of the children who achieve remission, subsequently relapse, and only a minority of them are long-term survivors after further therapy (Stevens RF, Br J Haematol 1998; Perel Y, Leukemia 2005; Woods WG, Blood 2001). Slow clearance of AML cells by remission induction therapy is associated with a poor treatment outcome (Walker H, British Journal of Haematology 1999; Estey EH, Blood 2000; Kern W, Blood 2003). However, the assessment of treatment response by morphological examination of the bone marrow can be subjective and lacks sensitivity.

In chronic myeloid leukemia and acute lymphoblastic leukemia, quantification of minimal residual disease (MRD) have provided useful data in predicting risk outcome and, therefore, is now an important parameter for treatment strategies (Oehler VG, Curr Oncol 2003; Szczepanski T, Lancet Oncol, 2001). In AML, immunophenotypic and molecular based techniques for MRD assessment have recently identified patients at high-risk of relapse in few reports.

In AML, limited MRD data have been reported in adults, and few of them concerning paediatric cohorts of patients.

At a molecular level, fusion transcript genes in paediatric AML represent 31% to 45% of all cases. Among them, MLL/11q23 abnormalities represent the major part with more than 30 different partners' genes. Fusion genes associated with CBF leukemia's are rare in young patients (15-18%) (Raimondi SC, Blood 1999; Forestier E, Br J Haematol 2003). Nowadays, the frequent overexpression of the Wilms'tumore gene WT1 expression in AML is under study as a possible tool for monitoring MRD quantification: several studies have been published and results analyzed at different time points have

been conflicting (Schmid D, Leukemia 1997; Garg M, Br J Haematol 2003; Ogawa H, Blood 2003; Siehl JM, Bone Marrow Transplant 2002).

Studies of residual disease by reverse transcriptase polymerase chain reaction (RT-PCR) in adult patients have indicated that quantification of PML-RARα, AML1-ETO and CBCFB-MYH11 transcripts help to predict outcome (Grimwade, 1999; Lo Coco , 1999; Tobal, 2000; Buonamici, 2002; Guerrasio, 2002). Because of its wide applicability, flow cytometry, recognizing aberrant immunophenotypes, is a promising tool to monitor residual disease in Acute Myeloid Leukemia (AML).

Four-colour flow cytometric techniques that enabled minimal residual disease (MRD) monitoring in >95% of children with acute lymphoblastic leukemia (ALL) with 0.01% sensitivity (Campana D, Best Practice and Research Clinical Haematology 2002), has been shown to provide reliable prognostic information in a clinical setting (Coustan-Smith E, Lancet 1998; Coustan-Smith E, Blood 2000; Coustan-Smith E, Blood 2002a*;* Coustan-Smith E, Blood 2002b).

In adults with AML, three-colour flow cytometry can identify abnormal immunophenotypes at diagnosis in 65%-75% of patients; detection of residual cells with identical immunophenotypes during treatment was associated with poorer outcome. (Macedo A, Leukaemia 1005; Venditti A, Blood 2000)

Similarly, in children with AML, observation of abnormal profiles by three and four-colour flow cytometry in bone marrow samples obtained during treatment was associated with increased risk of treatment failure (Sievers EL, *Journal of the National Cancer Institute* 1996; Coustan-Smith E, *Lancet* 1998; Coustan-Smith E, *Blood* 2000; Coustan-Smith E, *Blood* 2002a; Coustan-Smith E, *Blood* 2002b; Sievers EL, *Blood* 2003).

Monitoring of MRD in AML is a powerful tool to segregate patients with AML into different risk categories. It seems to be useful in identifying patients at high risk of relapse from those in durable remission, providing a more rational mean of selecting therapeutic options, and a more accurate estimate of the extent of leukemic cytoreduction achieved with different therapies.

Considering that katyotype of the leukemic cells is considered one of the most important parameter indicating the prognosis in patients with AML, nowadays Italian therapeutic protocol stratifies AML patients according to the presence of specific genetic markers, and the morphological data after induction, differently from some American protocols where MRD assessment by FC is considereed for risk assignment.

Material and Methods

In this study, we used four and five-colour flow cytometric techniques to monitor residual disease in children with AML.

Considering that genetic markers suitable for RT-PCR studies are found in less than one-third of these patients, we are studying the detection of MRD in childhood AML Italian patients by FC, in order to detect the clinical value of these data.

In the ongoing study, we assess minimal residual disease by flow cytometry in a cohort of children with de novo AML (not M3 and not Down) to precise the impact of MRD value on survival and relapse. Between January 2003 and December 2006, 71 patients, enrolled in the AIEOP-AML 2002 trial for treatment, have been studied: one with French-American-British (FAB) subtype M0, eight M1, nine M2, five M4, two M4Eo, five M5, one M6, four M7, one unclassifiable by FAB.

To define leukemia associated immunophenotypes, we compared diagnostic AML samples with bone marrow samples from healthy individuals and regenerating bone marrow from patients with leukemia (other than AML) undergoing therapy. The discovery that leukemic cells express immunophenotypes not expressed by normal bone marrow and peripheral blood cells provide the opportunity for detecting residual disease in bone marrow and blood. Remission status, routinely evaluated by microscopy, is not straightforward: it may be particularly challenging to distinguish leukemic from regenerative blasts, especially in hypoplastic sample.

We found that immunophenotypes suitable for the detection of residual disease can be identified by flow cytometry in the vast majority of patients by four and five-colour flow cytometry and that such immunophenotypes allow a sensitivity of detection as much as 100 times that afforded by morphological examination. At least as early as during aplasia, after induction therapy, flow cytometric MRD assessment is feasible. Flow cytometry allowed the analysis of aplastic samples, not valuable by morphology, with a direct quantification of residual disease and the identification of dying cells and debris.

Furthermore, the running follow up samples allowed to advance the overt relapse in some cases: this is essential particularly in the cases for which a molecular marker was not suitable. Moreover, in order to define, if flow cytometric detection of AML cells after induction or consolidation therapy is an independent predictor of treatment outcome, in line with the results obtained in adults' series, we are studying the bone marrow follow up samples of the patients, during the treatment period. Residual disease assessment with this method could be used for risk assignment in the future therapeutic trial, providing a more accurate estimate of the extent of leukemic cytoreduction and a more rational means of selecting therapeutic options.

References

-Buonamici, S., Ottaviani, E., Testoni, N., Montefusco, V., Visani, G., Bonifazi, F., Amabile, M., Terragna, C., Ruggeri, D., Piccaluga, P.P., Isidori, A., Malagola, M., Baccarani, M., Tura, S. & Martinelli, G. (2002) Real-time quantitation of minimal residual disease in inv(16)-positive acute myeloid leukemia may indicate risk for clinical relapse and may identify patients in a curable state. *Blood*, **99**, 443– 449.
-Coustan-Smith E., Behm, F.G., Sanchez, J., Boyett, J.M., Hancock, M.L., Raimondi, S.C., Rubnitz, J.E., Rivera, G.K., Sandlund, J.T., Pui, C.H. & Campana, D. (1998) Immunological detection of minimal residual disease in children with acute lymphoblastic leukaemia. *Lancet*, **351**, 550– 554.
-Coustan-Smith E., Sancho, J., Hancock, M.L., Boyett, J.M., Behm, F.G., Raimondi, S.C., Sandlund, J.T., Rivera, G.K., Rubnitz, J.E., Ribeiro, R.C., Pui, C.H. & Campana, D. (2000) Clinical importance of minimal residual disease in childhood acute lymphoblastic leukemia. *Blood*, **96**, 2691– 2696.
-Campana D., Coustan-Smith E. (2002) Advances in the immunological monitoring of childhood acute lymphoblastic leukaemia. *Best Practice and Research Clinical Haematology*, **15**, 1– 19.
-Coustan-Smith E., Sancho, J., Behm, F.G., Hancock, M.L., Razzouk, B.I., Ribeiro, R.C., Rivera, G.K., Rubnitz, J.E., Sandlund, J.T., Pui, C.H. & Campana, D. (2002a) Prognostic importance of measuring early clearance of leukemic cells by flow cytometry in childhood acute lymphoblastic leukemia. *Blood*, **100**, 52– 58.
-Coustan-Smith E., Sancho, J., Hancock, M.L., Razzouk, B.I., Ribeiro, R.C., Rivera, G.K., Rubnitz, J.E., Sandlund, J.T., Pui, C.H. & Campana, D. (2002b) Use of peripheral blood instead of bone marrow to monitor residual disease in children with acute lymphoblastic leukemia. *Blood*, **100**, 2402.
-Estey, E.H., Shen, Y. & Thall, P.F. (2000) Effect of time to complete remission on subsequent survival and disease- free survival time in AML, RAEB-t, and RAEB. *Blood*, **95**, 72– 77.
-Forestier E, Heim S, Blennow E, et al: Cytogenetic abnormalities in childhood acute myeloid leukaemia: A Nordic series comprising all children enrolled in the NOPHO-93-AML trial between 1993 and 2001. Br J Haematol 121:566-577, 2003
-Garg M, Moore H, Tobal K, et al: Prognostic significance of quantitative analysis of WT1 gene transcripts by competitive reverse transcription polymerase chain reaction in acute leukemia. Br J Haematol 123:49-59, 2003
-Grimwade, D. (1999) The pathogenesis of acute promyelocytic leukaemia: evaluation of the role of molecular diagnosis and monitoring in the management of the disease. *British Journal of Haematology*, **106**, 591– 613
-Guerrasio, A., Pilatrino, C., De Micheli, D., Cilloni, D., Serra, A., Gottardi, E., Parziale, A., Marmont, F., Diverio, D., Divona, M., Lo, C.F. & Saglio, G. (2002) Assessment of minimal residual disease (MRD) in CBFbeta/MYH11-positive acute myeloid leukemias by qualitative and quantitative RT-PCR amplification of fusion transcripts.
-Kern, W., Haferlach, T., Schoch, C., Loffler, H., Gassmann, W., Heinecke, A., Sauerland, M.C., Berdel, W., Buchner, T. & Hiddemann, W. (2003) Early blast clearance by remission induction therapy is a major independent prognostic factor for both achievement of complete remission and long-term outcome in acute myeloid leukemia: data from the German AML Cooperative Group (AMLCG) 1992 Trial. *Blood*, **101**, 64– 70.
-Lo Coco, F., Diverio, D., Falini, B., Biondi, A., Nervi, C. & Pelicci, P.G. (1999) Genetic diagnosis and molecular monitoring in the management of acute promyelocytic leukemia. *Blood*, **94**, 12– 22.
-Macedo, A., Orfao, A., Gonzalez, M., Vidriales, M. B., Lopez-Berges, M. C. and Martinez, A. (1995) Immunological detection of blast cell subpopulations in acute myeloblastic leukemia at diagnosis: implications for minimal residual disease studies. *Leukemia* **9** , pp. 993-998.
-Oehler VG, Radich JP: Monitoring bcr-abl by polymerase chain reaction in the treatment of chronic myeloid leukemia. Curr Oncol Rep 5:426-435, 2003
-Ogawa H, Tamaki H, Ikejame K, et al: The usefulness of monitoring WT1 gene transcripts for the prediction and management of relapse following allogeneic stem cell transplantation in acute type leukemia. Blood 101:1698-1704, 2003
-Ostergaard M, Olesen LH, Hasle H, et al: WT1 gene expression: An excellent tool for monitoring minimal residual disease in 70% of acute myeloid leukaemia patients—results from a single-center study. Br J Haematol 125:590-600, 2004
-Perel Y, Auvrignon A, Leblanc T, et al: Treatment of childhood acute myeloblastic leukaemia: Dose intensification improves treatment outcome and maintenance therapy is no benefit-

multicenter studies of the French LAME (Leucémie aiguë Myéloblastique Enfant) Cooperative Group. Leukemia 19:2082-2089, 2005
-Raimondi SC, Chang MN, Ravindranath Y, et al: Chromosomal abnormalities in 478 children with acute myeloid leukemia clinical characteristics and treatment outcome in a cooperative pediatric oncology group study: POG 8821. Blood 94:3707-3716, 1999
-Schmid D, Heinze G, Linnerth B, et al: Prognostic significance of WT1 gene expression at diagnosis in adult de novo acute myeloid leukemia. Leukemia 11:639-643, 1997
-Sievers, E.L., Lange, B.J., Buckley, J.D., Smith, F.O., Wells, D.A., Daigneault-Creech, C.A., Shults, K.E., Bernstein, I.D. & Loken, M.R. (1996) Prediction of relapse of pediatric acute myeloid leukemia by use of multidimensional flow cytometry. *Journal of the National Cancer Institute*, **88**, 1483– 1488.
-Sievers EL, Lange BJ, Alonzo TA, et al: Immunophenotypic evidence of leukaemia after induction therapy predicts relapse: Results from a prospective Children's Cancer Group study of 252 patients with acute myeloid leukaemia. Blood 101:3398-3406, 2003
-Stevens RF, Hann IM, Wheatley K, et al: Marked improvements in outcome with chemotherapy alone in paediatric acute myeloid leukaemia: Results of the United Kingdom Medical Research Council's 10th AML trial. Br J Haematol 101:130-140, 1998
-Szczepanski T, Orfao A, van der Velden VH, et al: Minimal residual disease in leukaemia patients. Lancet Oncol 2:409-417, 2001
-Tobal, K., Newton, J., Macheta, M., Chang, J., Morgenstern, G., Evans, P.A., Morgan, G., Lucas, G.S. & Liu, Y.J. (2000) Molecular quantitation of minimal residual disease in acute myeloid leukemia with t(8;21) can identify patients in durable remission and predict clinical relapse. *Blood*, **95**, 815– 819.
-Venditti, A., Buccisano, F., Poeta, G Del, Maurillo, L., Tamburini, A. and Cox, C. (2000) Level of minimal residual disease after consolidation therapy predicts outcome in acute myeloid leukemia. *Blood* **96** , pp. 3948-3952.
-Walker, H. (1999) A simple, robust, validated and highly predictive index for the determination of risk-directed therapy in acute myeloid leukaemia derived from the MRC AML 10 trial. United Kingdom Medical Research Council's Adult and Childhood Leukaemia Working Parties. *British Journal of Haematology*, **107**, 69– 79.
-Woods WG, Neudorf S, Gold S, et al: A comparison of allogeneic bone marrow transplantation, autologous bone marrow transplantation, and aggressive chemotherapy in children with acute myeloid leukemia in remission: A report from the children's cancer group. Blood 97:56-62, 2001

1.2.a Data mining

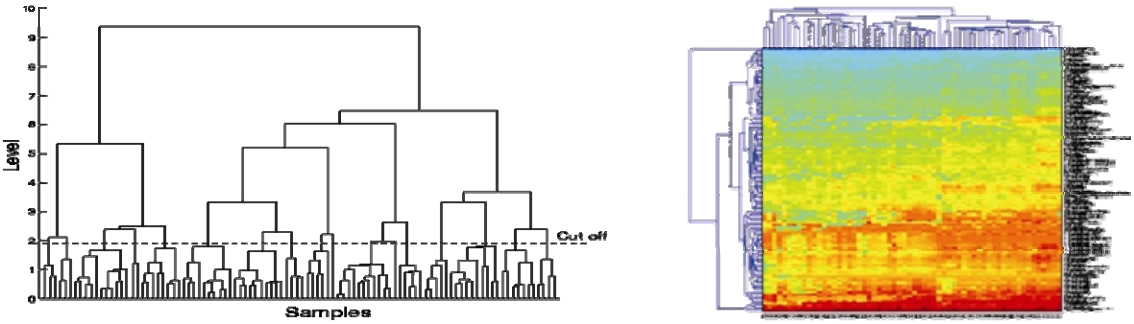
Unsupervised Methods

Unsupervised learning is a method of machine learning where a model is fit to observations gathered as a set of random variables. It is distinguished from supervised learning by the fact that there is no *a priori* output: the algorithm does not need any additional information on samples to perform the analysis. The main objective of unsupervised learning is to gather similar sample profiles in homogeneous and distinct groups. Moreover, unsupervised learning is useful for data compression: fundamentally, all data compression algorithms either explicitly or implicitly rely on a probability distribution over a set of inputs. The most used forms of unsupervised learning are *clustering* and *principal components analysis* (PCA).

Data clustering is a common technique for statistical data analysis including machine learning, data mining, pattern recognition, image analysis and bioinformatics. Clustering is the classification of similar objects into different and homogeneous groups, or more precisely, the partitioning of a data set into subsets (*clusters*), so that the data in each subset (ideally) share some common trait - often proximity according to some defined distance measure.

Data clustering algorithms can be *hierarchical* or *partitional*. Hierarchical algorithms find successive clusters using previously established clusters, whereas partitional algorithms determine all clusters at once. Hierarchical algorithms can be agglomerative (bottom-up) or divisive (top-down). Agglomerative algorithms begin with each element as a separate cluster and merge them in successively larger clusters. Divisive algorithms begin with the whole set and proceed to divide it into successively smaller clusters.

Two-way clustering, co-clustering or bi-clustering are the names for clusterings where gene / protein expressions are plotted against specimen information, i.e., if the data is represented in a data matrix the row and columns are clustered simultaneously. A clustering plot is usually represented by a *dendrogram* which graphically illustrates the arrangement of clusters in a tree-shaped diagram (Figure 1).



Figures 1. – Clustering representations: Dendrogram (left), Two-way clustering (right).

In statistics, principal components analysis (PCA) is a technique for simplifying a dataset, by reducing multidimensional datasets to lower dimensions for analysis. PCA is usually represented by a 2D or 3D model where spatial relations among samples can be easily assessed by analyzing a plot (Figure 2). We can roughly define this method as a 2D-3D handy visualization of clustering results.

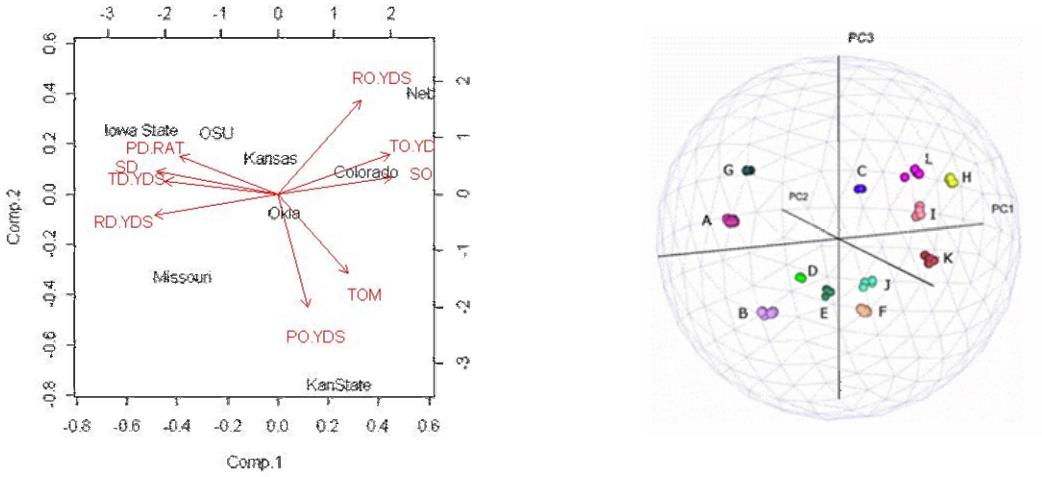


Figure 2 – 2D and 3D PCA. In the left 2D graph, samples (black names) are spatially related each others according to variable directions (red arrows); in the right 3D graph, samples (colored dots) are spatially clustered into homogeneous groups (letters) with similar features.

Supervised Learning

Supervised learning is a machine learning technique for creating a model (function) from training data. More simply, supervised learning covers techniques that are used to predict an observation class (*e.g.* a disease class), or the value of an outcome variable of interest on the basis of a set of records with known outcomes. The training data consist of pairs of input objects (typically vectors, *e.g.* patients belonging to different disease classes), and desired outputs (*e.g.* survival event, therapy protocol). The output

of the function can be a continuous value (called regression), or can predict a class label of the input object (called classification, *e.g.* 1 = dead, 0 = alive). The task of the supervised learner is to predict the value of the function for any valid input object after having seen a number of training examples (i.e. pairs of input and target output). To achieve this, the learner has to generalize from the presented data to unseen situations using training algorithms (similar to concept learning in human psychology). Most commonly, supervised learning generates a global model that maps input objects to desired outputs.

In order to solve a given problem of supervised learning (e.g. learning to classify leukemic samples according to their subtype) one has to consider various steps:

- Determine the type of training samples. Before doing anything else, the user should decide what kind of data is to be used as sample. For instance, this might be gene expression data from different leukemic samples.
- Gathering a training set. A set of input objects (*e.g.* gene expression profiles) is collected and corresponding outputs (e.g. known leukemic subtypes) are also gathered.
- Determine the input feature representation of the learned function. The accuracy of the learned function depends strongly on how the input object is represented. Typically, the input object is transformed into a feature vector, which contains a number of features that are descriptive of the object.
- Determine the structure of the learned function and corresponding learning algorithm. For example, the researcher may choose to use class comparison or class prediction algorithms.
- Complete the design. The researcher then runs the learning algorithm on the gathered training set. Parameters of the learning algorithm may be adjusted by optimizing performance on a subset (called a validation set) of the training set, or via cross-validation. After parameter adjustment and learning, the performance of the algorithm may be measured on a test set that is separate from the training set.

For our researches, *class comparison* and *class prediction* supervised algorithms have been largely performed on gene expression data to find differentially expressed genes among two or more leukemia subclasses. The algorithms listed below are world-wide used to investigate clinical data using supervised learning and are commonly accepted as standard methods in microarray research field:

- Significance Analysis of Microarrays (SAM), class comparison algorithm
- Prediction Analysis of Microarrays (PAM), class prediction algorithm
- Nearest neighbours, both class comparison and prediction algorithm
- Multivariate ANalysis Of Variance (MANOVA)

1.2.b Immunophenotype Signature as a Tool to Define Prognostic Subgroups in Childhood Acute Myeloid Leukemia

ABSTRACT

Purpose

Multiple techniques are involved in diagnosis and classification of Acute Myeloid Leukemias (AML). We provide further insights into the role of immunophenotyping as a relevant tool in AML diagnosis and prognosis. We apply computational analysis on antigen expression data to identify homogeneous immunophenotypes with prognostic relevance.

Patients and Methods

A total of 96 *de novo* pediatric AML patients (59 males; 37 females) are included in the study (median age 11; range 1-18 years). Morphological, immunophenotypic, cytogenetic and biomolecular analyses have been performed in the central AIEOP laboratory in Padua. Sample groups and their associated *signature markers* are highlighted using hierarchical agglomerative clustering.

Results

Initial unsupervised analysis involved 42 specimens and the expression levels of 35 antigens. Patients clustered into two main subgroups according to their antigen expression levels. Secondary analysis was then performed on the entire patient cohort applying the same computational method. Two-dimensional clustering analysis for mean antigen expression values separated patients into prognostically relevant clusters.

Conclusions

96 pediatric patients with AML are separated using antigen expression profiles drawn on quantitative flow cytometry (FC) data. Patients sharing different morphological subtypes but homogeneous genotypes are aggregated according to their antigen expression profiles. Subgroups identified as presenting these antigenic profiles contribute, together with morphological and genotypic analyses, to define prognostically relevant AML

INTRODUCTION

Acute Myeloid Leukemia (AML) is a heterogeneous disease group morphologically classified, based on the French-American-British (FAB) classification, into 8 main subgroups defined as subtypes M0 to M7. Besides morphologic differences, some genetic abnormalities have been recognized; cytogenetics and molecular analyses are also being used to identify subgroups of AML with different prognosis. For instance, translocations t(8;21), t(15;17) and inversion of chromosome 16 have favorable prognoses, while the presence of a complex karyotype, monosomy of chromosome 5, deletions of the long arm of chromosome 5, monosomy of chromosome 7, t(8;21) with del(9q), inversion of the long arm of chromosome 3, 11q23 abnormalities, t(6;9) and t(9;22) translocations, deletion of the long arm of chromosome 9 or aberration of the short arm of chromosome 17 results in a relatively poor prognosis[1,2,3]. Biologic and genetic features, including internal tandem duplication of the *FLT3* gene[4,5,6], aberrant expression of drug-resistance transporter genes[7,8] and of *BCL2* family genes[9,10] as well as *NMP1*[11] and *PTPN11*[12] gene mutations, have been recently reported to be associated with outcome and are useful prognostic factors. However, in spite of many available prognostic factors, accurate prediction of risk for treatment failure or relapse is still difficult. In order to improve risk assignment and develop new therapeutic strategies, gene expression profiling and proteomic analysis seem to offer important improvements in leukemia classification.

Recently, gene expression profiling in adult and pediatric patients has provided a framework for the global molecular analysis of AML patients. Indeed, differentially expressed and discriminating genes have been identified for each of the major prognostic subtypes of pediatric AML[13,14]. Additionally, gene expression profiling analysis has distinguished new AML subgroups with favorable or unfavorable prognoses previously unrecognized by other methods[15,16,17].

Immunophenotypic data collection using flow cytometry (FC) is a fast and relatively easily accessible technology that has already been implemented in most centers for leukemia diagnosis; the translation into quantitative expression data sets is a matter of flow cytometer settings and output calibration. Quantitative multivariate analysis from panels of marker proteins has demonstrated that marker protein expression profiles can distinguish specific ALL subtypes and MDS[18,20]. Potentially, these quantitative expression analyses can be used in clinical diagnosis. Here we consider the application of immunophenotyping by FC to analyze AML samples using panels of antibodies to characterize specific blast cell populations. The present study is aimed at providing further insights into the role of immunophenotyping as a relevant tool in AML

management. To this end, a cohort of 96 pediatric, AML patients harboring all known AML subtypes were investigated using multi-parametric FC and analyzed by computational methods normally applied in high-throughput gene expression profiling to identify new homogeneous subgroups with clinical relevance.

MATERIALS and METHODS

Patients and samples

A total of 96 de novo pediatric AML patients (59 males; 37 females) were included in the study (median age 11; range 1-18 years). In all cases, AML diagnosis was performed by morphology, cytochemistry, cytogenetics, immunophenotype and molecular genetics. The patients were enrolled in the AIEOP AML-2002[11] protocol according to diagnostic guidelines. Briefly, a 2ml sample of bone marrow of each enrolled patient was sent by courier within 24h to the Laboratory of Hemato-Oncology, Department of Pediatrics, University of Padua where diagnosis was made for each patient (**Table 1**).

Pazient ID	Sex	Genotype	Karyotype
M0.1	M	neg	46,XY,t(6;9)(p23;q34)
M0.2	M	neg	NA
M0.3	M	neg	NA
M0.4	M	neg	NA
M0.5	F	neg	NA
M1.1	F	neg	46,XX
M1.2	F	neg	NA
M1.3	F	neg	46,XX
M1.4	F	neg	NA
M1.5	F	neg	46,XX
M1.6	F	neg	45,XX,-7
M1.7	F	AML1-ETO + - t(8:21)	45,X,-X,t(8;21)(q22;q22)
M1.8	F	neg	NA
M1.9	F	neg	NA
M1.10	M	neg	NA
M1.11	M	neg	46,XY
M1.12	F	neg	NA
M1.13	M	AML1-ETO + - t(8:21)	46,XY,t(8;21)(q22;q22),del(9)(q13q22)[14]
M1.14	F	neg	NA
M1.15	F	neg	NA
M1.16	M	neg	46,XY,t(6;11)(q27;q23),inv(9)(p11q13)[13]/46,idem,dup(2)(q23q33)[7]/46,XY,inv(9)(p11q13)[2]
M1.18	M	neg	NA
M2.1	M	AML1-ETO + - t(8:21)	NA
M2.2	M	neg	46,XY,t(1;11)(q21;q25)[21]
M2.3	F	neg	NA
M2.4	M	neg	NA
M2.5	F	AML1-ETO + - t(8:21)	NA
M2.6	M	AML1-ETO + - t(8:21)	46,XY,t(8;21)(q22;q22)[5]/45,X,-Y,t(8;21)(q22;q22)[8]/46,XY[1]
M2.7	M	AML1-ETO + - t(8:21)	46,XY,t(8;21)(q22;q22)[1]/45,idem,-Y[6]/47,idem,+4[4]/83,XXY,-Y,-1,-2,-4,-5,-8,t(8;21)(q22;q22)x2,-11,?-D,-16[4]/46,XY[7]
M2.8	M	AML1-ETO + - t(8:21)	45,X,-Y,t(8;21)(q22;q22)22ps+
M2.9	F	AML1-ETO + - t(8:21)	NA
M2.10	M	AML1-ETO + - t(8:21)	NA
M2.11	M	AML1-ETO + - t(8:21)	NA
M2.12	M	neg	NA
M3.1	F	PML-RARa + - t(15;17)	NA
M3.2	F	PML-RARa + - t(15;17)	NA
M3.3	M	PML-RARa + - t(15;17)	46,XY,t(15;17)(q22;q21)
M3.4	M	PML-RARa + - t(15;17)	46,XY,t(15;17)(q22;q21)
M3.5	F	PML-RARa + - t(15;17)	NA
M3.6	F	PML-RARa + - t(15;17)	NA

M3.7	M	PML-RARa + - t(15;17)	46,XY,t(15;17)(q22;q21)
M3.8	M	PML-RARa + - t(15;17)	NA
M3.9	M	PML-RARa + - t(15;17)	NA
M3.10	M	PML-RARa + - t(15;17)	NA
M3.12	M	PML-RARa + - t(15;17)	NA
M3.13	F	PML-RARa + - t(15;17)	46,XX,fish ins (15;17)(q22;q21)(PML+.RARA+)
M3.14	M	PML-RARa + - t(15;17)	NA
M3.15	M	PML-RARa + - t(15;17)	47,XY,t+X,(15;17)(q22;q21)
M3.16	M	PML-RARa + - t(15;17)	47,XY,t(6;11)(q23;p15),t(15;17)(q22;q12),-9,+21,+mar(90%)/46,XY(10%)
M3.18	F	PML-RARa + - t(15;17)	NA
M3.19	M	PML-RARa + - t(15;17)	NA
M3.20	M	PML-RARa + - t(15;17)	NA
M3v.1	M	PML-RARa + - t(15;17)	46,XY,t(15;17)(q22;q21)
M3v.3	M	PML-RARa + - t(15;17)	NA
M3v.4	M	PML-RARa + - t(15;17)	46,XY,t(15;17)(q22;q21)
M3v.6	M	PML-RARa + - t(15;17)	NA
M3v.7	M	PML-RARa + - t(15;17)	46,XY,t(15;17)(q22;q21)
M3v.8	F	PML-RARa + - t(15;17)	NA
M3v.9	M	PML-RARa + - t(15;17)	NA
M4.2	M	neg	47,XY,+8[1]/47,XY,add(4)(q35),+8,add(16)(q24)[23]
M4.3	M	CBFb-MYH11 + - inv	NA
M4.4	F	neg	NA
M4.5	F	CBFb-MYH11 + - inv	47,XX,+22[7]/46,XX[31]
M4.6	F	neg	46,XX
M4.7	M	CBFb-MYH11 + - inv	NA
M4.8	F	CBFb-MYH11 + - inv	NA
M4.9	M	AML1-ETO + - t(8:21)	45,X,-Y,t(8;21)(q22;q22)[23]/46,XY[1]
M4.11	F	CBFb-MYH11 + - inv	46,XX,inv(16)(p13q22)[14]/46,XX[3]
M4.12	M	CBFb-MYH11 + - inv	NA
M4.13	M	neg	NA
M4.14	M	neg	NA
M4.15	M	neg	NA
M5.1	F	neg	NA
M5.2	M	neg	46,XY,t(11;17)(q23;q12)
M5.3	M	neg	NA
M5.4	F	neg	NA
M5.5	M	neg	NA
M5.6	F	neg	NA
M5.7	M	neg	47,XY,?+r[2]/46,XY[20]
M5.8	M	neg	46,XY,add(11)(?q21)[16]/46,XY,add(8)(p?)[2]/46,XY,add(8)(p?),add(22)(p?)[2]/46,XY,add(22)(p?)[1]/46,XY[2]
M5.9	M	neg	NA
M5.10	M	neg	46,XY,inv(10)(p13q24)del(15)(q14q22)[11]/46,XY[6]
M5.11	M	neg	46,XY,t(7;10;11)(q22;p15;q21)
M5.12	M	neg	NA
M5.13	M	neg	46,XX,t(9;11)(p21;q23)[27]46,XY[1]
M5.14	F	neg	NA
M5.16	F	neg	NA
M6.1	F	neg	46,XX,der(9)(9::9q22-9q13::9q11-9p22::9q22-9qter),del(13)(q12q22)[5]/46,XX[17]
M6.2	M	neg	46,XY
M7.1	M	neg	NA
M7.2	F	neg	NA
M7.3	F	neg	NA
M7.5	F	neg	46,XX (der(8)t(8p;21q)
M7.6	M	neg	NA
M7.7	M	neg	NA
M7.8	M	neg	NA

Table 1 - FAB classification of the 96 pediatric AML patients enrolled in our study. “neg” defines patients negative for considered genotypes, NA is not assessable.

Morphological classification

Three independent investigators performed the morphological classification and the conclusive diagnosis for every case was reported according to the FAB criteria. In cases where consensus was not obtained, the three investigators re-analyzed the slides together in order to obtain consensus as to final diagnosis.

Immunophenotypic studies

Immunophenotypic analyses were performed by multi-parameter four-color FC using an Epics XL cytometer (Beckmann Coulter, Inc., Miami, FL, USA). Data were acquired with the EXPO32 software (Beckmann-Coulter). AML immunophenotypic analysis was made using the panel of fluorochrome-conjugated monoclonal antibodies (MoAbs) routinely employed by the AIEOP Reference Laboratory for Immunophenotypic Studies, of the University of Padua. Briefly, at least 0.5 x 10^6 cells in a final volume of 100 µl of BM were used in each analysis and MoAbs, directly conjugated with fluorescein isothiocyanate (FITC), phycoerythrin (PE), and both PE-Texas red (ECD) and PE-cyanine 5 (PECy5) fluorochrome tandem conjugates, were combined in each tube according to previously reported methods[19,20]. A specific isotypic control was used.

The following panel of monoclonal antibodies was monitored: CD2-FITC, CD7-FITC, CD10-FITC, CD11a-PE, CD11b-PE, CD13-FITC, CD14-PE, CD16-FITC, CD19-FITC, CD20-PE/Cy5, CD33-PE, CD38-PE/Cy5, CD56-PE, CD61-FITC, CD64-FITC, CD66b-FITC, CD66c-FITC, CD80-FITC, CD83-PE/Cy5, CD86-PE, CD114-PE, CD116-PE, CD117-PE, CD135-PE, GLICOA-PE, CD7.1-PE (Beckman Coulter-Immunotech, Miami, FL, USA), CD44-PE, CD45-PE/Cy5, CD52-FITC, CD65-FITC, HLADR-PE/Cy5 (Caltag Laboratories, San Francisco, CA, USA), MPO-FITC (DAKO Cytomation, Glostrup, Denmark), CD15-FITC, CD34-PE (Becton Dickinson, San Jose, CA, USA), AC133-PE (Mylteny, Bergisch Gladbach, Germany).

FC analyses were performed using an immunological gate (CD45 vs Side Scatter) to identify all myeloid cells including immature blast cells. Once the myeloid population was selected, the expression level of each monitored MoAb was analyzed in a fixed set of MoAbs/fluorochrome combinations in order to avoid possible bias related to use of different fluorochromes for the same antibody[18,19,20]. Among the panel for non-lymphoblastic acute leukemia, we used 35 well-characterized leukemia antigens.

We quantified antigen expression by (M)ean (F)luorescence (I)ntensity (MFI) using WinMDI (v2.8) software[18].

Cytogenetic analysis

Samples were processed and cytogenetic studies were performed using the Q-banding technique. About 15-20 metaphases for each sample were acquired (with CASTI System) and analyzed in order to avoid clone loss. Chromosomes were identified and assigned according to the International System for Human Cytogenetic Nomenclature[21]. These analyses were later confirmed by the FISH technique.

Biomolecular analysis

The AIEOP AML protocol, which screens for the fusion gene transcripts *AML1-ETO*, *PML-RARα* and *CBFβ-MYH11*, was performed as stated in the previously reported method; briefly, total RNA was isolated using the RNAzol-B reagent (Duotech srl Milan, Italy) following manufacturer's instructions. One microgram of total RNA from each specimen was reverse transcribed using the Superscript reverse transcriptase (Life Technologies Milan, Italy) and random hexamers. PCR amplification was performed using AmpliTaq polymerase (Applied Biosystems) according to the BIOMED-1 protocols[22]. An independent PCR reaction was performed with shift primers for confirmation of each positive result. The ABL housekeeping gene expression was assessed to determine the presence of amplifiable RNA and the efficacy of reverse transcriptions. After electrophoresis, the PCR reaction products were stained with ethidium bromide[23].

Computational analysis

Sample groups and their associated *signature markers* were highlighted using hierarchical agglomerative clustering. Before clustering, all antigen expression values (MFI) were log-transformed and median centered. These centered values were used to calculate correlations between antigens (35) and samples (96) and serve as the basis for merging nodes. Uncentered Pearson correlation coefficient between the standardized expression values and the centroid-linkage were performed as distance and linkage methods, using Cluster and TreeView software[24].

We used unsupervised hierarchical clustering analysis to group patients and antigens; patients are clustered according to the similarity of antigen expression profiles. Initially we tested the ability of the antigen profile analysis to distinguish between the major AML genetic translocations (*PML-RARα*, *AML1-ETO* and *CBFβ-MYH11*). Then, we applied the same unsupervised clustering method (without taking into account hematologic, cytogenetic or other external information) to identify patient clusters in the entire cohort. Probabilities of overall survival (OS) analysis were estimated according to the Kaplan-Meier method using R software[25,26].

RESULTS

Unsupervised hierarchical clustering

The study cohort, a total of 96 patients of which 42 were positive to the screened genetic abnormalities (**Table 1**), is a representative series of Italian pediatric AML

patients. Indeed, the frequency of genetic abnormalities is similar to a previously reported Italian study[23]. Furthermore, the morphologic and genotypic characterizations confirm the correlation between FAB subgroups and the major genetic aberrations. The *PML-RARα* fusion gene was present in all M3 and M3v cases (Acute Promyelocytic Leukemia, APL); *AML1-ETO* was present in M2 patients (8 of 12); 6 of 13 *CBFβ-MYH11* cases were classified as M4. Patients with M0, M5, M6 and M7 FAB subtypes resulted negative for the analyzed cytogenetic abnormalities[23].

Initial analysis involved the expression levels of 35 antigens in the bone marrow of 42 patients positive for screened translocations. In this two-dimensional cluster analysis of antigen expression levels, patients cluster into two main branches: an upper branch with *PML-RARα* samples (23 of 25 M3 samples) and a lower branch with the other two translocations (*AML1-ETO* and *CBFβ-MYH11*) clearly separated from each other (**Figure 1**). The correlation value between the two branches is negative (i.e., -0.100641) indicating a clear distinction of *PML-RARα* from *AML1-ETO* and *CBFβ-MYH11* (specificity of 92%).

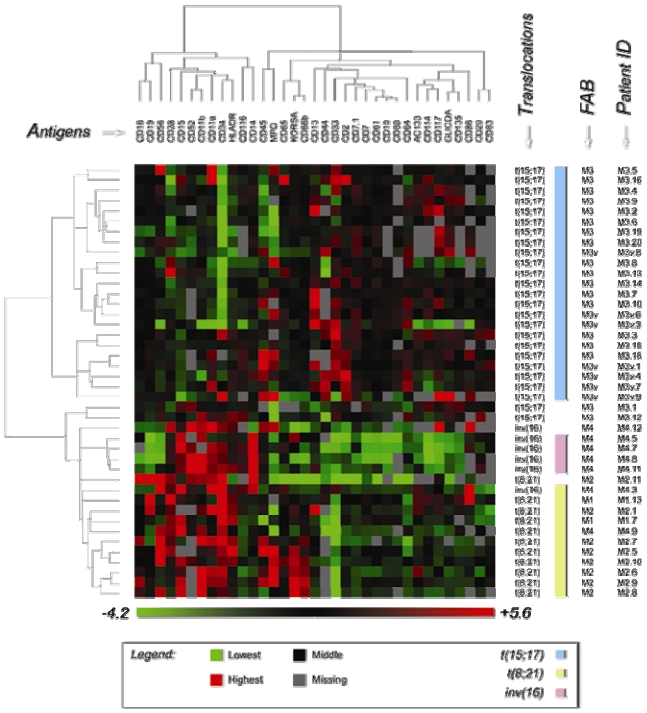


Figure 1 - Hierarchical Clustering on 42 patients using 35 antigen expression levels. Each column represents an antigen; each row is a patient. Antigen expression levels (MFI) are log-transformed, median centered and color depicted (red- values higher than the median level; green- values lower than the median, black- values comparable to the median, gray- missing values).

Secondary analysis was performed on the entire cohort of 96 AML patients applying the same computational method. Two-dimensional clustering analysis (**Figure 2**) for mean antigen expression values separates patients into two main branches (A and B, Pearson correlation value = -0.062729). The dendrogram on the left in figure 2 shows that patients are clearly grouped in two main clusters: a smaller but homogeneous branch (A), is separated from a larger heterogeneous branch (B) which further divides into six sub-branches (B1-B6). Mann-Whitney test has been performed on logarithmic expression values of all markers (mean values) to identify significantly expressed antigens for each cluster group.

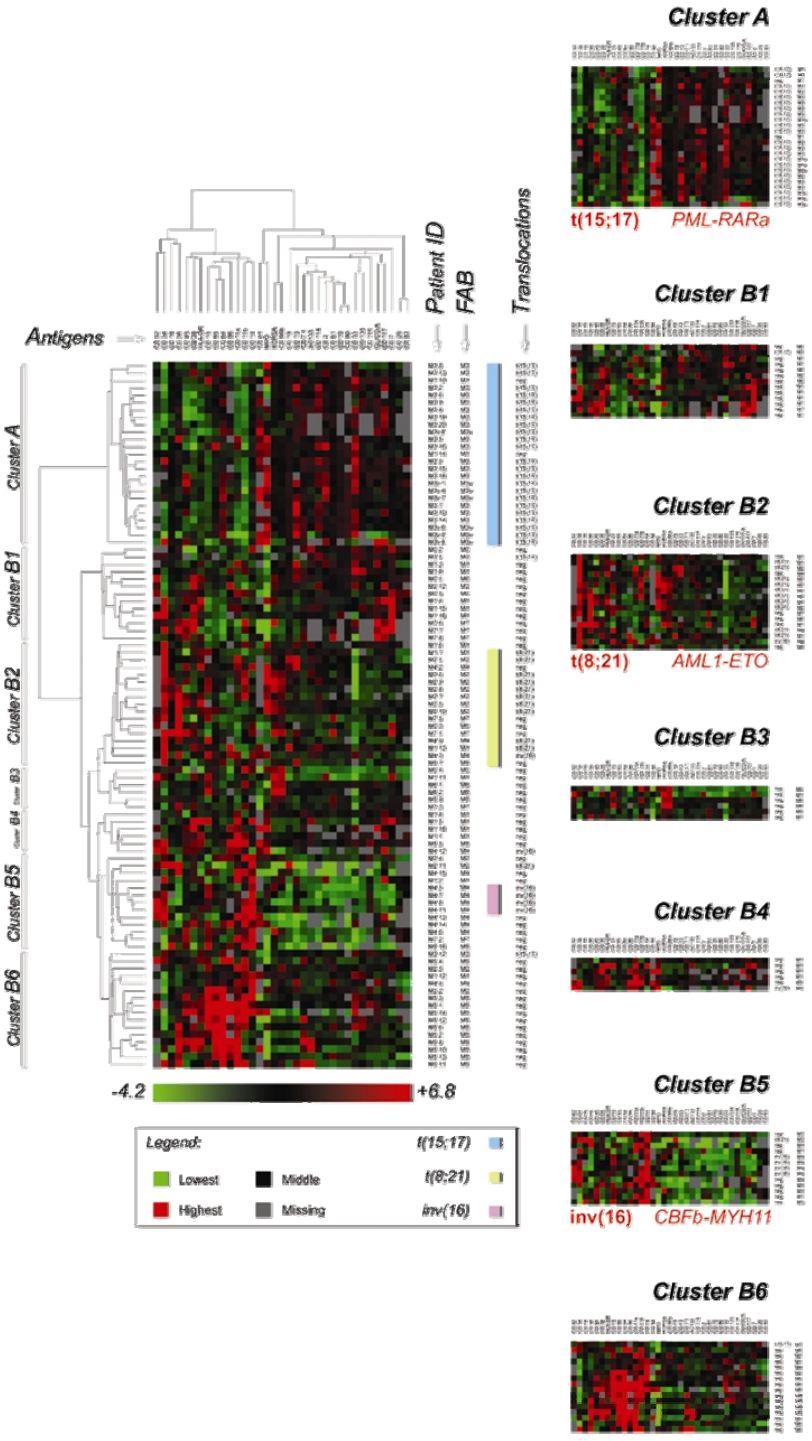


Figure 2 - Hierarchical Clustering on patients (96) and antigens (35) for AML panel (mean values). Each column represents an antigen; each row is a patient. Light blue bar represents the cluster of patients with t(15;17) or *PML-RAR a*, yellow t(8;21) or *AML1-ETO*, pink inv(16) or *CBFβ-MYH11*. Negative or positive are relative to the mean.

Cluster A is mainly composed of M3 and M3v subtypes (APL) with all patients positive for *PML-RARα*; it has the highest specificity both for morphological and genotypic information. A characteristic antigen expression signature for this cluster (e.g. higher expression of MPO and CD33 and lower expression of CD11a and CD11b) is reported in table 2C. M3v is distinct from M3 in its low expression of CD114. Two M1 patients, negative for *PML-RARα*, and without characteristic clinical features were included in this cluster.

A closer inspection of the major B branch reveals that the B1 cluster groups patients belonging to different morphologic subgroups and all samples but one (M3.1 - *PML-RARα*) are negative for the translocations. M7 cases were grouped at the bottom of cluster B1 which is characterized by blast cells expressing CD7, CD38, CD117 and negative for CD44, MPO, CD15, and CD64.

Cluster B2 is basically composed of 17 patients, 10 with *AML1-ETO*, 1 with *CBFβ-MYH11* and 6 negative for all translocations; 7 of 12 morphologically M2 samples were included in this cluster. The most relevant antigen information of cluster B2 was represented by the lower expression levels of CD33, CD44, CD135 and the higher values of CD34 and CD56. A higher expression level of CD56 in *AML1-ETO* samples confirmed the previously reported results[27,28].

Cluster B3 and B4 can be distinguished by their divergent expression of CD66b, CD66c (positive in cluster B3), and CD44 (positive in cluster B4). However, since both of these clusters comprise only 6 patients, no further interpretation as to its significance is considered here.

B5 and B6 branches, considering a lower value of Pearson correlation, form a super cluster in which mainly monocyte leukemias are included with some differences. Although the clusters have some features in common (also confirmed by the dendrogram), they differ in some peculiar characteristics. Specifically, cluster B5 is composed of eight M4 subtypes and five non-M4 cases (**Table 2A**). Blast cells highly express CD14, CD11b, CD11a and CD52 while generally being negative for CD114 and CD61. Instead, cluster B6 includes 11 of 15 M5 subtype patients and 5 non-M5. The antigen profiling highlights a higher expression level in CD64, CD11a, CD15, CD86 and lower values for MPO, CD34 and CD114. Higher levels of CD64, CD11a and CD86 consistently characterize M5 cases. In particular, CD86 antigen was a marker of dendritic/monocytic lineage in AML patients showing positive in 9/12 M5 patients[29].

Tab. 2A	*A*	*B1*	*B2*	*B3*	*B4*	*B5*	*B6*	**ToT**
neg	2	12	6	6	5	8	15	**54**
t(8;21)			10			1		**11**
inv(16)			1		1	4		**6**
t(15;17)	23	1					1	**25**
ToT	**25**	**13**	**17**	**6**	**6**	**13**	**16**	**96**

Tab. 2B	*A*	*B1*	*B2*	*B3*	*B4*	*B5*	*B6*	**ToT**
M0		3	1	1				**5**
M1	2	5	3	1	4	1	1	**17**
M2		1	7			2	2	**12**
M3	16	1					1	**18**
M3v	7							**7**
M4			3		1	8	1	**13**
M5			1	1	1	1	11	**15**
M6				2				**2**
M7		3	2	1		1		**7**
ToT	**25**	**13**	**17**	**6**	**6**	**13**	**16**	**96**

Table 2C	*Lower antigens*		*Higher antigens*		*Representative FAB*	*Considered Genotype*	*Prognosis*
	5%	**10%**	**90%**	**95%**			
Cluster ***A***	CD11a CD11b	CD34 CD38 CD11a CD11b	CD44 MPO CD13 CD33	MPO CD33	M3 M3v	t(15 ;17)	Good
Cluster ***B1***	CD64 MPO	CD15 CD64 CD44 MPO	CD38 CD117 CD7	CD7	M0 M1 M7	Neg	Poor
Cluster ***B2***	CD44 CD33	CD44 CD33 CD135	CD34 CD56 MPO CD66c	CD34 CD56	M2	t(8;21)	Good
Cluster ***B3***	CD44 CD33	CD34 CD45 CD44 CD33	CD15 CD11b CD66c CD66b	CD66c CD66b	M6	Neg	NA
Cluster ***B4***	CD66c CD66b	CD16 CD66c CD66b CD2	CD52 CD11a CD44 MPO	CD11a CD44	M1	Neg	NA
Cluster ***B5***	CD114 CD80	CD7.1 CD114 CD61	CD52 CD11a CD11b CD14	CD11b CD14	M4	inv(16)	Poor
Cluster ***B6***	MPO CD83	CD34 MPO CD114	CD15 CD64 CD86 CD11a	CD64 CD11a	M5	Neg	Intermediate

Table 2A-B-C (Previous page)

A, B - Patients partitioning according to seven clusters (as shown in figure 2), compared to A. major genotypic aberrations and B. FAB groups.

C - Logarithmic expression values for all antigens have been retrieved from GeneCluster analysis. Within each cluster, antigens have been selected at the 5th, 10th, 90th and 95th percentile cutoffs. The last two columns report representative FABs and considered genotypes for each cluster. NA is not assessable.

Survival analysis

The overall survival rates were calculated on previously identified clusters to evaluate the prognostic significance for patient clusters with more than 10 samples (i.e. clusters A, B1, B2, B5 and B6) (**Figure 3**). The results of the Kaplan-Meier plot were significant in distinguishing the clusters considered. Cluster A (involving *PML-RARa*) and cluster B2 (involving *AML1-ETO*) contain specimens with a genetic relatively favorable prognosis and patients without genetic good prognostic signature. Cluster B1 and B6, composed of patients without major translocations and with very heterogeneous morphological classification, present indeed a high variability in overall survival. In particular, B1 patients are characterized by a poor prognosis while B6 cases do not have a poor survival rate. Cluster B5 (mainly composed of 4 cases with *CBFβ-MYH11* and other M4 subtypes) results instead in a poor prognosis. We then reanalyzed the FC clustered data excluding patients with t(15;17), t(8;21) and inv(16) abnormalities. Survival analysis on the remaining 43 clustered patients with normal karyotype reproduced the same trend as the previous comprehensive analysis confirming patient stratification in good and poor prognosis clusters (data not shown).

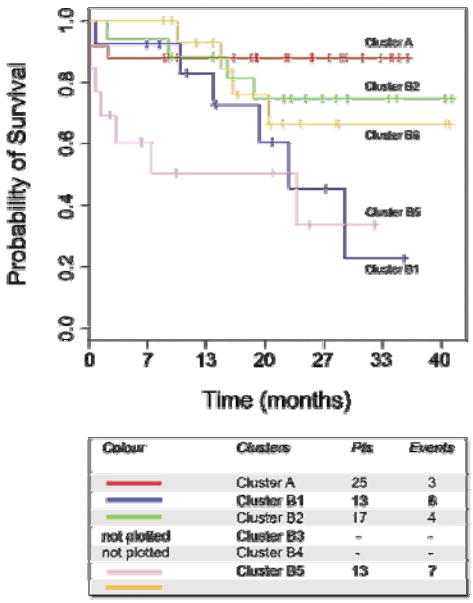


Figure 3 - Survival analysis for AML study (OS). Kaplan-Meier plot shows survival analysis on groups generated by clustering (p=0.0179).

DISCUSSION

Acute Myeloid Leukemia (AML) is a heterogeneous disease with variable prognosis. Many useful prognostic factors have been studied and validated[1,2,3] but attempts to accurately predict risk of treatment failure remain unsatisfactory. Recently comprehensive gene expression profiling analyses have demonstrated to be a new powerful tool for identifying prognostically relevant patient subgroups in AML[15,16,17]. Patient sub grouping partly coincides with known prognostic categories but new prognostic relevant groups have been also identified. The use of computational analysis performed on gene expression data in medicine strongly suggests that this method can be applied in various diagnostic approaches[30,31].

Here 96 pediatric patients with AML are separated using antigen expression profiles drawn on quantitative FC data. At the same time, patients sharing different morphological phenotypes but homogeneous genotypes are aggregated according to their antigen expression profiles. We demonstrate that these antigenic profiles are relevant in clinical diagnosis and prognosis; additionally, they support morphological and genotypic analyses in defining prognostically relevant AML subgroups. Antigen expression profiling, in comparison to gene expression profiling, is a cheap and relatively fast technique: it uses a limited number of parameters to perform consolidated and routinely applied leukemia diagnoses. Performing unsupervised analysis on antigen expression values, we distinguished AML patients in seven subgroups that are shown to be prognostically relevant. Patients in clusters A, B2 and B6 have a relatively favorable prognosis, while patients in cluster B1 and B5 have a relatively poor prognosis. We are able to identify in cluster A and B2 almost all patients with specific prognostic relevant genotypes (*PML-RAR*α and *AML1-ETO*, respectively). Cluster A highly matches with APL and shows the highest probability of survival among all groups, as expected. Cluster B2 includes 10/17 patients with the *AML1-ETO* translocation and it predicts a good survival prognosis; the other 7 patients included in cluster B2, but without this specific prognostic translocation, share similar phenotype and relative good prognosis.

Patients in cluster B1 have relatively poor prognosis; all specimens but one (M3) are negative for principal aberrations and are morphologically classified as FAB M0, M1 and M7. In fact, M0 and M7 specimens usually show a poor prognosis and M1 specimens included in this cluster have a similar clinical behavior (3/4 death). Once again, the antigen expression clustering is able to distinguish different prognostic groups also among M1 FAB patients. Cluster B5 brings together specimens with a poor prognosis. The presence of 4 patients with *CBF*β*-MYH11* in this cluster with poor prognosis is only

apparently contradictory because not all studies identified this translocation as a good prognosis factor[3].

Considering a lower grade of Pearson correlation, B5 and B6 branches form a single cluster in which mainly M4 and M5 samples are included. M4 and M5 FAB subtypes share many features as confirmed by the results of the dendrogram; acute monocytic leukemia (AML-M5) differs from acute myelomonocytic leukemia (AML-M4) mainly when monocytes count is greater than 80% without myeloid component. B5 and B6 clusters identify patients with M4 and M5, respectively. Cluster B6 does not have a poor prognosis and includes patients (except one with t(15;17)) without the considered genotype aberrations. Interestingly, patients in this cluster generally show stronger expression of CD64, CD11a and 11 of 16 patients are classified as FAB M5, confirming the variable risk related to this subtype[32].

Notably, our method places AML patients with a normal karyotype into clusters with different prognosis: B2 and B6 (low and variable risk) versus B1 and B5 (high risk). In fact, the clustering approach helps us to associate patients without prognostically relevant features to patients with known survival risk according to immunophenotype information.

Moreover cluster analysis on normal karyotype may support the identification of distinct subgroups involving molecular pathogenesis of AML[16]. Indeed, the use of antigen expression profiling like gene expression profiling helps us to separate clinically relevant subgroups.

Previously, a number of studies using FC immunophenotyping have attempted to identify single marker antigens with prognostic relevance for AML patients. In a recent study on adult AML patients[33], a similar approach was used considering the expression of a limited set of 7 antigens. They identified five subsets based on the qualitative differences in antigen expression considering as positives those cases with a 20% cutoff of blast cells at least. The strength of our clustering analysis is ascribed to the quantitative analysis of a large number of antigens that allow us to distinguish clusters of AML patients with clinical impact. In fact, our first analysis shows a high specificity for clustering of the three major genotypes among *PML-RARα*, *AML1-ETO* and *CBFβ-MYH11* specimens and rests on the comprehensive profiling of 35 antigen expression data. Clustering of patients with *PML-RARα*, *AML1-ETO* and *CBFβ-MYH11* confirmed the potency of antigen expression pattern analysis to distinguish genotypically homogeneous profiles of acute leukemias as reported earlier[18,20,34]. The second cluster analysis applied to the full cohort of patients separates not only genotypic homogenous clusters but also subgroups without genotypic aberrations. Both clustering of specimens

with known aberrations and clustering of the full cohort of patients separate specimens with *PML-RARα* with the highest probability (cluster A). Within the cluster of *PML-RARα* specimens, [13] separated M3 and M3v subtypes using computational analyses on 82 genes. They found that *FLT3* is mutated more frequently in AML M3v than in AML M3 (67% vs. 19%, P = 0.001). We have verified a higher expression of CD2 and CD34 in M3v subtype[35] and observed a lack of CD114 as a possible powerful marker to separate M3v from M3 subtype. The differential expression of CD114 (G-CSF receptor, involved in granulocytes growth) could indicate a possible different sensitivity of blast cells to G-CSF and its potential clinical utilization. Thus, the clustering method is a fast and powerful tool in recognizing not only known features but also in finding new correlations among patients.

The computational analysis performed on gene and protein expression is a powerful approach for delineating profiles for diagnosis and prognosis. The main difficulty remains the high heterogeneity characterizing many diseases like AML that does not allow us to assign patients to well-defined clinical groups. The analysis of combined immunophenotypic, genotypic and biomolecular data is the right way to face this problem. This kind of analysis may gain further relevance in aiding us to include patients with unidentified genotype to low-risk group.

REFERENCES

1. Lowenberg B, Downing JR, Burnett A.: Acute myeloid leukemia. N Engl J Med 341:1051-1062, 1999
2. Grimwade D, Howe K, Langabeer S, et al: Establishing the presence of the t(15;17) in suspected acute promyelocytic leukaemia: cytogenetic, molecular and PML immunofluorescence assessment of patients entered into the M.R.C. ATRA trial. M.R.C. Adult Leukaemia Working Party. Br J Haematol 94:557-573, 1996
3. Kalwinsky DK, Raimondi SC, Schell MJ, et al: Prognostic importance of cytogenetic subgroups in de novo pediatric acute nonlymphocytic leukemia. J Clin Oncol 8:75-83, 1990
4. Kiyoi H, Naoe T, Nakano Y, et al: Prognostic implication of FLT3 and N-RAS gene mutations in acute myeloid leukemia. Blood 93:3074-3080, 1999
5. Kottaridis PD, Milligan DW, Chopra R, et al: In vivo CAMPATH-1H prevents graft-versus-host disease following nonmyeloablative stem cell transplantation. Blood 96:2419-2425, 2000
6. Thiede C, Steudel C, Mohr B, et al: Analysis of FLT3-activating mutations in 979 patients with acute myelogenous leukemia: association with FAB subtypes and identification of subgroups with poor prognosis. Blood 99:4326-4335, 2002
7. Leith CP, Kopecky KJ, Chen IM, et al: Frequency and clinical significance of the expression of the multidrug resistance proteins MDR1/P-glycoprotein, MRP1, and LRP in acute myeloid leukemia: a Southwest Oncology Group Study. Blood 94:1086-1099, 1999
8. Steinbach D, Sell W, Voigt A, et al: BCRP gene expression is associated with a poor response to remission induction therapy in childhood acute myeloid leukemia. Leukemia 16:1443-1447, 2002
9. Campos L, Rouault JP, Sabido O, et al: High expression of bcl-2 protein in acute myeloid leukemia cells is associated with poor response to chemotherapy. Blood 81:3091-3096, 1993

10. Kohler T, Schill C, Deininger MW, et al: High Bad and Bax mRNA expression correlate with negative outcome in acute myeloid leukemia (AML). Leuk. 16:22-29, 2002
11. Cazzaniga G, Dell'oro MG, Mecucci C, et al: Nucleophosmin mutations in childhood acute myelogenous leukemia with normal karyotype. Blood (in press)
12. Tartaglia M, Martinelli S, Iavarone I, et al: Somatic PTPN11 mutations in childhood acute myeloid leukaemia. Br J Haematol 129:333-339, 2005
13. Schoch C, Kohlmann A, Schnittger S, et al: Acute myeloid leukemias with reciprocal rearrangements can be distinguished by specific gene expression profiles. Proc Natl Acad Sci U S A 99:10008-10013, 2002
14. Ross ME, Mahfouz R, Onciu M, et al: Gene expression profiling of pediatric acute myelogenous leukemia. Blood 104:3679-3687, 2004
15. Yagi T, Morimoto A, Eguchi M, et al: Identification of a gene expression signature associated with pediatric AML prognosis. Blood 102:1849-1856, 2003
16. Bullinger L, Dohner K, Bair E, et al: Use of gene-expression profiling to identify prognostic subclasses in adult acute myeloid leukemia. NEJ Med 350:1605-1616, 2004
17. Valk PJ, Verhaak RG, Beijen MA, et al: Prognostically useful gene-expression profiles in acute myeloid leukemia. N Engl J Med 350:1617-1628, 2004
18. De Zen L, Bicciato S, te Kronnie G, Basso G.: Computational analysis of flow-cytometry antigen expression profiles in childhood acute lymphoblastic leukemia: an MLL/AF4 identification. Leukemia 17:1557-1565, 2003
19. Basso G, Buldini B, De Zen L, Orfao A.: New methodologic approaches for immunophenotyping acute leukemias. Haematologica 86:675-692, 2001
20. De Zen L, Orfao A, Cazzaniga G, et al: Quantitative multiparametric immunophenotyping in acute lymphoblastic leukemia: correlation with specific genotype. I. ETV6/AML1 ALLs identification. Leukemia 14:1225-1231, 2000
21. Mitelman F.: Chromosomes, genes, and cancer. CA Cancer J Clin 44:133-135, 1994
22. van Dongen JJ, Macintyre EA, Gabert JA, et al: Standardized RT-PCR analysis of fusion gene transcripts from chromosome aberrations in acute leukemia for detection of minimal residual disease. Report of the BIOMED-1 Concerted Action: investigation of minimal residual disease in acute leukemia. Leukemia 13:1901-1928, 1999
23. Frascella E, Rondelli R, Pigazzi M, et al: Clinical features of childhood acute myeloid leukaemia with specific gene rearrangements. Leukemia 18:1427-1429, 2004
24. Eisen MB, Spellman PT, Brown PO, Botstein D.: Cluster analysis and display of genome-wide expression patterns. Proc Natl Acad Sci USA 95:14863-14868, 1998
25. The R project for statistical computing [http://www.R-project.org]
26. Kaplan EL, Meier P.: Non-parametric estimation from incomplete observations. J Am Stat Assoc 53:457-481, 1958
27. Hurwitz CA, Raimondi SC, Head D, et al: Distinctive immunophenotypic features of t(8;21)(q22;q22) acute myeloblastic leukemia in children. Blood 80:3182-3188, 1992
28. Ferrara F, Di Noto R, Annunziata M, et al: Immunophenotypic analysis enables the correct prediction of t(8;21) in acute myeloid leukaemia. Br J Haematol 102:444-448, 1998
29. Re F, Arpinati M, Testoni N, et al: Expression of CD86 in acute myelogenous leukemia is a marker of dendritic/monocytic lineage. Exp Hematol 30:126-134, 2002
30. Ramaswamy S, Tamayo P, Rifkin R, et al: Multiclass cancer diagnosis using tumor gene expression signatures. Proc Natl Acad Sci USA 98:15149-15154, 2001
31. Ross DT, Scherf U, Eisen MB, et al: Systematic variation in gene expression patterns in human cancer cell lines. Nat Genet 24:227-235, 2000
32. Tallman MS, Kim HT, Paietta E, et al: Acute monocytic leukemia (French-American-British classification M5) does not have a worse prognosis than other subtypes of acute myeloid leukemia: a report from the Eastern Cooperative Oncology Group. J Clin Oncol 22:1276-1286, 2004
33. Casasnovas RO, Slimane FK, Garand R, et al: Immunological classification of acute myeloblastic leukemias: relevance to patient outcome. Leukemia 17:515-527, 2003
34. Hrusak O, Porwit-MacDonald A.: Antigen expression patterns reflecting genotype of acute leukemias. Leukemia 16:1233-1258, 2002
35. Paietta E, Goloubeva O, Neuberg D, et al: A surrogate marker profile for PML/RAR alpha expressing acute promyelocytic leukemia and the association of immunophenotypic markers with morphologic and molecular subtypes. Cytometry B Clin Cytom 59:1-9, 2004
36. Philip A. Pizzo, David G. Poplack: Principles and Practice of Pediatric Oncology (ed. 4) Lippincott Williams & Wilkins, 2005

1.2.c Identification of immunophenotipic signatures by clustering analysis in pediatric patients with Philadelphia chromosome-positive acute lymphoblastic leukemia.

Introduction

The Philadelphia (Ph) chromosome is a shortened chromosome 22 resulting from the reciprocal translocation of the long arm of the chromosome 9 with the long arm of chromosome 22, t(9;22)(q34;q11). At molecular level, this translocation causes parts of the BCR gene from chromosome 22 to be placed in juxtaposition with downstream tyrosine kinase domains of the ABL gene from chromosome 9. The translocation is present in a heterogeneous group of leukemias: it is detectable in more than 95% of chronic myelogenous leukemia (CML) and 15-20% adult B-lineage acute lymphoblastic leukemia (B-ALL) (Westbrook 1992). In children, the Ph chromosome has been reported in literature to occur in 1-5% ALL cases (Radich 2001) and in some AML cases.

Whereas the breaks in c-ABL occur in the same region, three different breakpoint regions affect the BCR gene (major-M-, minor-m-, micro-μ-), generating three main BCR-ABL chimeric transcripts (p190, p210, p230) (Melo 1994; Ravandi 1999). The p190 is frequently associated with cases of ALL while the p210 occurs predominantly in CML (Deininger 2000) and occasionally in ALL (Radich 2001). More than 90% of children with Ph+ ALL show a break in the “minor” breakpoint cluster region between the BCR exons e1 and e2 (Suryanarayan 1991).

The chimeric BCR/ABL transcript can be easily detected by reverse transcription-polymerase chain reaction (RT-PCR) (Maurer 1991; Van Dongen 1999), but a rapid screening method for identification of BCR/ABL+ precursor B ALL is not currently available.

In both children and adults with ALL, the t(9;22) presence is associated with high risk for treatment failure (Aricò 2000; Radich 2001). All samples from newly diagnosed ALL patients need to be screened for the presence of the t(9;22) translocation for prognostic stratification; treatment decision making can include allogenic stem cell transplantation and STI-571, a specific inhibitor of the BCR-ABL tyrosine kinase.

Recent studies identified specific gene signatures for genetic subclasses performing microarray analyses on childhood and adult ALL cases (Yeoh EJ 2002; Hofmann 2002; Armstrong 2002). Advances in identification of leukemia subtypes have been recently achieved using also flow cytometry (FC) techniques: several leukemia classes could be distinguished on the basis of peculiar expression profiles. Phenotypic aberrations have shown to be associated with specific cytogenetic abnormalities such as t(1;19) (Deveraj 1995; Pui 1994; Borowitz 1993), t(12;21) (Borowitz 1998; Borowitz 1993; De Zen 2000) and 11q23 abnormalities (Tritz 1995; Pui 1992; Pui 1994; De Zen 2003).

BCR-ABL positive leukemias have undergone an immunophenotypic characterization, in a cohort of 82 adult patients with *precursor* ALL (pB-ALL), including 12 Ph+: Tabernero et al. described a unique immunophenotypic profile based on the pattern of CD10, CD34, CD13 and CD38 (Tabernero 2001).

The aim of the present study is the phenotypic characterization of paediatric Ph+ pB-ALL using an objective flow cytometric approach. We performed quantitative multivariate analysis on immunophenotypic data in a cohort of 41 children with Ph+ pB-ALL and 99 children with Ph- pB-ALL at diagnosis.

The expression level of 16 marker proteins have been monitored by four or five color flow cytometry and quantified in terms of Geometric Mean Fluorescence (GMF), in both Ph+ and Ph- patients. Unsupervised cluster analysis was performed on the entire cohort of 140 patients to group samples according to the similarity of antigen expression profiles.

Materials and methods

Patients

A retrospective analysis has been performed on 140 patients affected by pB-ALL. This study includes 41 patients with pB-ALL Ph+, diagnosed between March 2000 and January 2007. The control group included 99 pB-ALL patients negative for Philadelphia chromosome, diagnosed between January and April 2006. For immunophenotypic and molecular/cytogenetic analysis, bone marrow and/or peripheral blood samples were centralized to the AIEOP (Associazione Italiana di EmatoOncologia Pediatrica) Reference Laboratory, Padua University.

Among the 41 Philadelphia positive patients, thirteen were females and 28 males, with median age at diagnosis 9 years (range 1-16 years). The control group included 46 females and 53 males, with median age at diagnosis 5 years (range 1-16 years); 2 Ph- patients were affected by Down syndrome (trisomy 21).

All patients had an unequivocal diagnosis of *de novo* pB-ALL based on morphological, cytochemical, and immunophenotypic criteria (EGIL, Leukemia 1995). The diagnosis of pB-ALL Ph+ was based on morphological, cytochemical, immunophenotypic and molecular analysis.

All patients have been treated according to the ongoing AIEOP ALL protocol. ALL children positive for the BCR/ABL fusion transcript were enrolled in the high-risk group of the AIEOP-ALL protocol.

BM investigations and treatment protocol were generally approved by the institutional ethical committees, and carried out after an informed consent.

Immunophenotypic studies

Immunophenotypic studies have been performed at diagnosis on whole BM or PB samples by flow cytometry, using a direct immunofluorescence technique with four or five color combinations of monoclonal antibodies (MoAbs) (Tab. 1 and 2). Blood samples arrived in the centralized laboratory within 24-36 hours. We used erythrocyte-lysed whole samples, instead of Ficoll separated sample, to allow a real picture of the whole bone marrow population without selective loss or enrichment.

Immunophenotypic and genetic studies have been performed on BM samples in 33 pB-ALL Ph+ and 97 pB-ALL Ph- at diagnosis. PB samples have been used for immunophenotypic and genetic analysis on the remaining 8 Ph+ and 2 Ph- patients.

All samples were processed within 24-36h after collection, and analyses were performed as previously described (Basso, Buldini, De Zen, 2001).

Briefly, in each analysis, $0.5x10^6$ cells in a final volume of 100 µl were incubated for 20 min at dark and at room temperature with the appropriate combinations of monoclonal antibodies (MoAbs) directly conjugated with fluorescein isothiocyanate (FITC), phycoerythrin (PE), tandem PE-Texas red (ECD), tandem PE-cyanine 5 (PECy5) and tandem PE-cyanine 7 (PECy7). Samples were subsequently lysed using 3 ml of NH_4Cl, then washed in phosphate-buffered saline (PBS) and resuspended in 0.5 ml of PBS. Intracellular staining (Tdt, cyIg) was performed by two step fixation and permeabilization procedure using a commercially kit (Fix&Perm^{TM}, Caltag Laboratories, Hamburg, Germany) according to manufacturer's instructions.

The 4 and 5 color protocols (Tab. 1 and 2) are routinely employed for diagnosis of B-lineage acute leukemias at the AIEOP Reference Laboratory for Immunophenotypic Studies, Padua University. Five color protocol (FITC, PE, ECD, PECy5, PECy7) analyses were performed on all the pB-ALL Ph- and 25 pB- ALL Ph+ samples, while 17 pB- ALL Ph+ were monitored using four color protocol (FITC, PE, ECD, PECy5).

The source of each monoclonal antibody reagent used in the present study was as follows: Fluorescein isothiocyanate (FITC): CD4, CD34, CD2 (Becton Dickinson, San Jose, CA, USA), KOR-SA (Becton Dickinson Pharmigen, San Jose, CA, USA) CD15, CD10, CD7, CD58, CD16 CD61, Tdt, k (Beckman Coulter-Immunotech, Miami, FL, USA), CD7, CD65, CD52 (Caltag Laboratories, San Francisco, CA, USA), Smig (Dako); Phycoerythrin (PE): CD8, CD11a, CD34, λ (Becton Dickinson, San Jose, CA, USA), CD14, CD56, CD15 7.1, CD33, CD135, CD19 (Beckman Coulter-Immunotech, Miami, FL, USA), CD133 (Miltenyi Biotec, Bergisch Gladbach, Germany), CD44, CD22 (Caltag

Laboratories, San Francisco, CA, USA), cyIg (Dako); Phycoerythrin-Texas-red (ECD): CD45, CD3, CD19, CD34 (Beckman Coulter-Immunotech, Miami, FL, USA); Phycoerythrin-cyanin 5 (PE-Cy5): CD20, CD38, CD34, (Becton Dickinson, San Jose, CA, USA), CD33 CD24, CD13, CD22 (Beckman Coulter-Immunotech, Miami, FL, USA), CD45, CD7, HLA-DR (Caltag Laboratories, San Francisco, CA, USA); Phycoerythrin-cyanin 7 (PE-Cy7): CD3, CD19, CD20 (Beckman Coulter-Immunotech, Miami, FL, USA).

Cell acquisition was performed using a flow cytometer Beckman Coulter FC500, equipped with a 488 nm argon laser, and the software program EXPO 32 (both from Beckmann Coulter, Inc., Miami, FL, USA). The immunophenotypic diagnoses were performed collecting 15,000 events for each sample. Instrument set-up was routinely optimized by analyzing the expression of normal peripheral blood T lymphocytes labeled with the anti-CD4FITC/CD8PE/CD3ECD/CD45Pe-Cy5 four-color combination or anti-CD4FITC/CD8PE/CD45ECD/CD7Pe-Cy5/CD3Pe-Cy7 five-color combination, as previously reported (Basso, Buldini, De Zen, 2001). Cells not stained were used as negative control.

Staining with the cell-permeant, live-cell acid fluorochrome SYTO 16 (Molecular Probes, Leiden, The Netherlands) was used to exclude residual non-nucleated erythroid cells, thrombocytes or debris. In all combinations, leukemic cells were identified using an immunological gate based on CD19 expression associated with a physical parameter (90° Scatter, SSC). CD19 antigen was chosen for immunological gating instead of the more commonly applied CD45, (Braylan 1997) whose expression may be low or negative in a large percentage of B-lineage ALL. The normal CD19 positive cells can be discriminated from blast cells on the basis of normal expression of CD45, negative expression of CD34 and low CD58 (De Zen 2000; Basso, Buldini 2001; Veltroni 2003). Once the blast population was selected, the intensity of each antigen expression was analyzed in association with SSC using logarithmic scale. Fluorescence signals were quantified using Geometric Mean Fluorescence (GMF) values for further comparison of antigen expression.

Genetic studies

All samples from newly diagnosed ALL patients enrolled in the AIEOP-ALL study were screened for the presence of the t(9;22) translocation. Fresh bone marrow aspirates retrieved at diagnosis were centralized to the AIEOP Reference Laboratory, University of Padova.

Mononuclear cells were obtained after centrifugation on a Ficoll-Hypaque gradient and cryopreserved in 4 mol/l guanidium isothiocyanate.
Subsequently, samples were sent to the laboratory in charge for analysis, where RNA was recovered by a standard extraction procedure (Chomczynski and Sacchi, 1987). Total RNA was isolated using RNAzol-B reagent (TEL-TEST, Inc., Duotech, Milan, Italy), according to manufacturer's protocols. An independent PCR reaction was performed with shifted primers for confirmation of each positive result. The ABL expression was assessed to determine the quality of cDNA and the efficacy of reverse transcription. PCR reaction products were analyzed by electrophoresis (2% agarose gels) and stained with ethidium bromide.
The t(9;22) translocation was diagnosed by single-round RT-PCR, with a sensitivity of 10^{-3}.

Statistical Analysis

Antigen expression values were calculated for the sixteen markers using Geometric Mean Fluorescence (GMF) measures; data have been log transformed and mean centered for unsupervised analysis. Hierarchical clustering was performed on the entire cohort of 140 patients to group samples and antigens according to the similarity of antigen expression profiles.
We used unsupervised analysis results to calculate the accuracy of the clustering approach in identifying Ph+ samples. Specificity, sensitivity and predictive values have been retrieved for the cluster group that included Ph+ samples.
Supervised analysis has been applied to the same clustered groups using class comparison statistics. SAM algorithm has been performed on antigen expression values to identify significantly differentially expressed markers between Ph+ and Ph- groups.

RESULTS

From the 140 pB-ALL included in the study, 41 cases displayed the BCR/ABL translocation RT-PCR. The reverse-transcriptase polymerase chain reaction (RT-PCR) analysis detected a minor BCR/ABL chimeric gene in 29 cases and major BCR/ABL chimeric gene in 12 cases. From the 41 pB-ALL Ph+ patients, 29 showed common ALL phenotype, 7 Pre-B, 4 Pre-pre B/Common and 1 Pre-B/B. From the control group of 99 pB-ALL Ph- patients, 69 were diagnosed as common ALL, 21 Pre-B, 7 Pre-pre B/Common, 1 Pre-B/B and 1 Pre-B/Common.

The marker protein expression levels in leukemic cells from patients diagnosed with pB-ALL bearing the BCR/ABL translocation have been compared to those of individuals diagnosed with pB-ALL not involving this genetic aberration.

Leukemic cells were identified using the immunological gate based on CD19 expression associated with Side Scatter; each antigen expression was quantified by Geometric Mean Fluorescence (GMF) values.

For analysis purpose, we restricted the number of monitored antigens according to the presence of the data in an adequate number of patients (at least 24) and considering the immunophenotypic characteristics of pB-ALL Ph+ previously reported in adult patients (Tabernero 2001; Primo 2005). For each MoAb, we used only one fluorochrome to avoid possible bias related to utilization of different MoAb combinations or different fluorochromes for the same antibody (Basso, Buldini 2001). According to these criteria, the antigen and fluorochromes taken into account in cluster analysis are: CD10-PE, CD38-PECy5, CD24-Pecy5, CD22-PECy5, CD45-ECD, HLADR-PECy5, CD19-PECy7, CD33-PECy5, CD56-PE, TdT-FITC, CD52-FITC, CD58-FITC, CD34-PE, KOR-FITC, CD11a-PE, CD44-PE.

Unsupervised cluster analysis was performed on the entire cohort of 140 patients to group samples and antigens according to the similarity of antigen expression profiles (GMF). Two-dimensional clustering analysis (Fig 1) for GMF values separated patients into two main groups (A and B+C): a smaller and homogeneous branch (A) is separated from a larger and heterogeneous branch (B+C), which further divides into two sub-branches (B and C). Cluster A included the majority of pB-ALL Ph+ (35/41) and 13/99 pB-ALL Ph-. Cluster B+C gathered 86/99 pB-ALL Ph- and 6/41 pB-ALL Ph+ (Cluster B: 4 Ph+ and 35 Ph-; Cluster C: 2 Ph+ and 51 Ph-) (Tab.3)

We calculated the sensitivity, specificity and predictive values of the immunophenotypic clustering criteria in identifying cells carrying the BCR/ABL+ gene rearrangements. Thirthy-five out of 41 (85.37%) and 86/88 (86.87%) were correctly predicted as true positive (Ph+) and true negative (Ph-), respectively.

The accuracy measure, a comprehensive value reflecting the proportion of correctly predicted Ph(+) and Ph(-) patients, indicates that our method identified a total of 121/140 (86.43%) cases that showed concordant results in predicting Ph(+) presence in pB-ALL patients (Table 4).

We confirmed here the previous studies that have shown a clear association between the immunophenotype of blast cells and the presence of underlying BCR/ABL gene rearrangements in adult BCP-ALL (Tabernero 2001, Primo 2005).

Subsequently, we compared cluster A and B+C samples which have been previously separated by unsupervised method. Supervised analysis was performed on GMF data

to find differentially expressed antigens with statistical relevance between Ph(+) and Ph(-) clusters. Six and five antigens were found to be statistically relevant (*q*-value=0) in discriminating Ph(+) and Ph(-) groups, respectively, using SAM algorithm as class comparison approach. The over-expression of CD52, TdT, CD45, CD34, HLA-DR and CD33 antigens characterizes Ph(+) group, while the same markers are down-regulated in Ph(-) group. A higher expression of CD38, CD24, CD58, CD22 and CD19 antigens depicts the Ph(-) profile, while Ph(+) group shows low expression values for the same markers. (Tab.5).

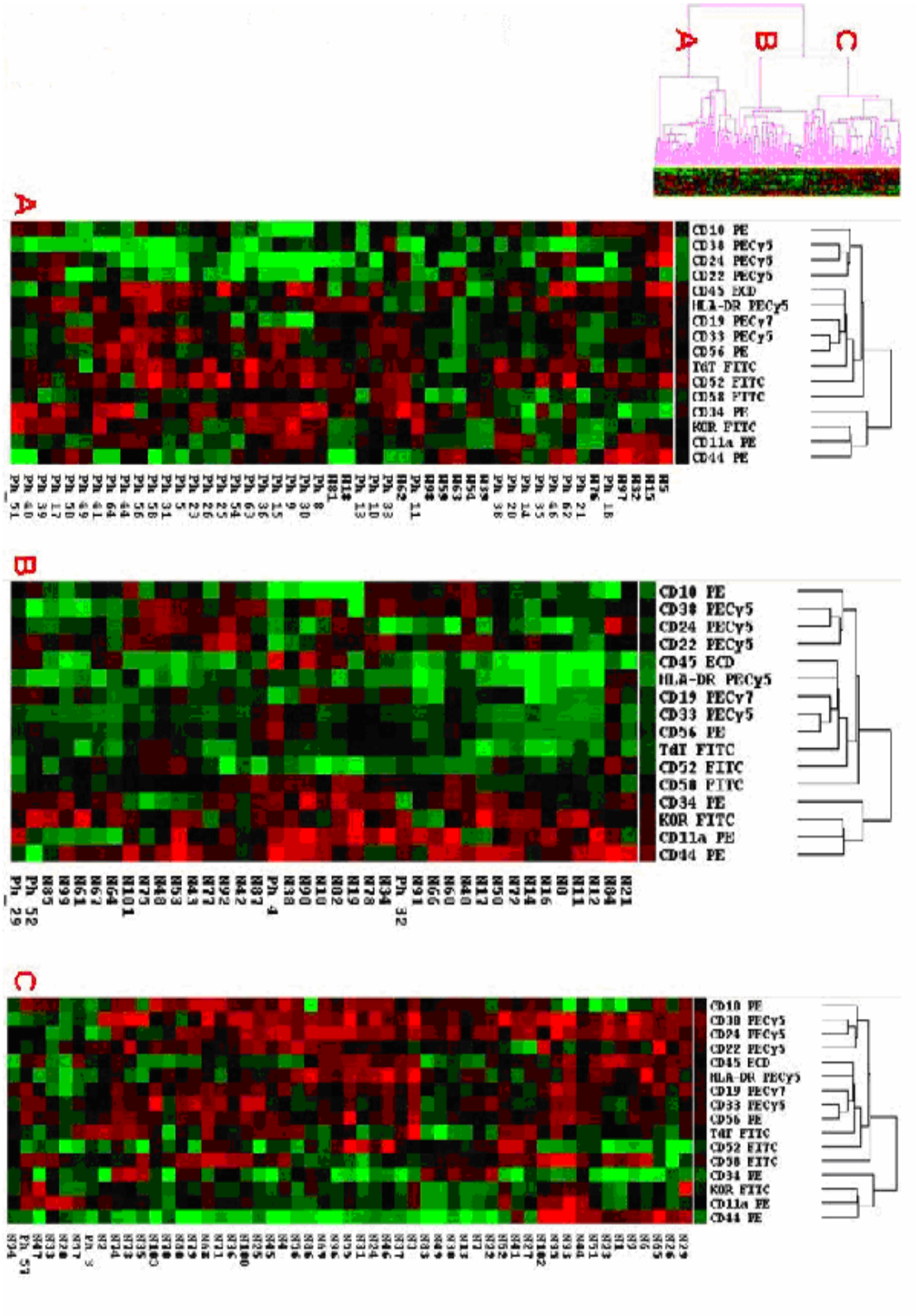


Fig. 1 *Cluster analysis of expressions of 16 surface membrane antigens in 140 childhood pBCP ALL.*

FITC	PE	ECD	PE-Cy5
CD4	CD8	CD3	CD45
CD7	CD34	CD19	CD45
KOR-SA	7.1	CD34	CD45
CD52	CD44	CD45	CD34
CD58	CD10	CD19	CD34
CD10	CD11a	CD19	CD20
CD16	CD56	CD19	CD24
CD2	CD22	CD19	CD38
CD65	CD33	CD19	CD13
CD34	AC133	CD19	CD38
CD10	CD34	CD19	CD20
CD15	CD14	CD19	HLA-DR
CD61	CD135	CD19	CD45
TdT	cylgμ	CD19	CD22
SMIG	cylgμ	CD19	CD20
SYTO16	CD19	CD45	7AAD
K	Λ	CD19	CD20

Table 1. Monoclonal antibody panel used for immunophenotypic evaluation of pB-ALL patients (4 colors)

FITC	PE	ECD	PE-Cy5	PE-Cy7
CD4	CD8	CD45	CD7	CD3
CD7	CD34	CD45	CD33	CD19
KOR-SA	7.1	CD45	CD25	CD19
CD52	CD44	CD45	CD7	CD19
CD58	CD10	CD45	CD34	CD19
CD10	CD11a	CD45	CD20	CD19
CD16	CD56	CD3	CD24	CD19
CD2	CD135	CD34	CD38	CD19
CD65	CD15	CD14	HLA-DR	CD19
CD34	AC133	CD45	CD38	CD19
TdT	cylgμ	CD45	CD22	CD20
SMIG	cylgμ	CD45	CD20	CD19
SYTO16	CD19	CD45	7AAD	CD20
K	Λ	CD45	CD20	CD19

Table 2. Monoclonal antibody panel used for immunophenotypic evaluation of pB-ALL patients (5 colors)

	Cluster A	Cluster B+C
Philadelphia positive	35 / 41	6 / 41
Philadelphia negative	13 / 99	86 / 99
Total	48 / 140	92 / 140

Table 3. Patients' distribution in the two main clusters

	Cluster A	%
True Positive	35 / 41	85.37
True Negative	86 / 99	86.87
False Positive	13 / 41	
False Negative	6 / 99	
Concordant	121 / 140	86.43
Discordant	19 / 140	13.57
Sensitivity	0.85	85.37
Specificity	0.87	86.87
Positive Predictive Value	0.73	72.92
Negative Predictive Value	0.93	93.48
Positive Diagnostic Likelihood Ratios	6.50	
Negative Diagnostic Likelihood Ratios	0.17	
Accuracy	0.86	86.43

Table 4 - The accuracy measure

	Antigen	Score(d)	Fold Change	q-value(%)
Cluster A	**CD52** FITC	-5.356745064	0.405494617	0
	TdT FITC	-4.089794121	0.472972722	0
	CD45 ECD	-3.909778445	0.448427797	0
	CD34 PE	-2.861839363	0.592043108	0
	HLA-DR PECy5	-2.591477664	0.599744388	0
	CD33 PECy5	-1.812571989	0.599265409	0
Cluster B+C	**CD38** PECy5	6.437362232	3.265153392	0
	CD24 PECy5	3.713070618	1.903207926	0
	CD58 FITC	3.572164712	1.642109373	0
	CD22 PECy5	3.23235248	2.051537025	0
	CD19 PECy7	1.719973617	1.172227881	0

Table 5. Eleven antigens extracted by SAM analysis comparing Ph(+) and Ph(-) groups. Antigens are ranked according to their statistic significance (Score) imposing *q*-value=0 as selecting criterion.

CONCLUSIONS

Childhood precursor-B-ALL carrying the Philadelphia chromosome display a unique phenotypic profile. The translocation t(9;22) is associated with upregulation of CD52, TdT, CD45, CD34, HLA-DR, CD33 and downregulation of CD38, CD24, CD58, CD22, CD19.

Immunophenotypic characterization seems to provide a rapid and effectual screening method for the identification of Ph+ precursor-B-ALL.

References

- Basso G, Buldini B, De Zen L, Orfao A: New methodologic approaches for immunophenotyping acute leukemia. Haematologica 2001; 86: 675-692.
- Primo D, Tabernero MD, Perez JJ, Rasillo A, Sayagues JM, Espinosa AB, Lopez-Berges MC, Garcia-Sanz R, Gutierrez NC, Hernandez JM, Romero M, Osuna CS, Giralt M, Barbon M, San Miguel JF, Orfao A: Genetic heterogeneity of BCR/ABL+ adult B-cell precursor acute lymphoblastic leukemia: impact on the clinical, biological and immunophenotypical disease characteristics. Leukemia 2005; 19: 713-720.
- Tabernero MD, Bortoluci AM, Alaejos I, Lopez-Berges MC, Rasillo A, Garcia-Sanz R, Garcia M, Sayagues JM, Gonzalez M, Mateo G, San Miguel JF, Orfao A: Adult precursor B-ALL with BCR-ABL gene rearrangements displays a unique immunophenotype based on the pattern of CD10, CD34 CD13 and CD38 expression. Leukemia 2001; 15: 406-414.
-Braylan RC, Borowitz MJ, Davis BH, Stelzer GT, Stewart CC. US-Canadian consensus on the immunophenotypic analysis of hematologic neoplasia by flow cytometry. Cytometry 1997; 30:213.
- De Zen L, Orfao A, Cazzaniga G, Masiero L, Cocito MG, Spinelli M, Rivolta A, Biondi A, Zanesco L, Basso G: Quantitative multiparametric imunophenotyping in acute lymphoblastic leukemia: correlation with specific genotype. I. ETV6/AML1 ALLs identification. Leukemia 2000; 14: 1225-1231.
-Benè MC, Castoldi G, Knapp W, Ludwig WD, Matutes E, Orfao A, van't Veer MB. Proposals for the immunological classification of acute leukemias. European Group for the immunological characterization of leukemias (EGIL). Leukemia 1995; 9:1783-1786.
-Schrappe M, Aricò M, Harbott J: Philadelphia chromosome positive childhood acute lymphoblastic leukemia: good initial steroid response allows early prediction of a favourable treatment outcome. Blood 1998; 92: 2730-2741.
-Cazzaniga G, Lanciotti M, Rossi V, Di Martino D, Aricò M, Valsecchi MG: Prospective molecular monitoring of BCR/ABL transcripts in children with Ph+ acute lymphoblastic leukemia unravels differences in treatment response. Br J Haematol 2002; 19: 445-453.
-Secker-Walker LM, Prentice HG, Durrant J, Richards S, Hall E, Harrison G. Cytogenetics adds independent prognostic information in adults with acute lymphoblastic leukemia on MRC trial UKALL XA. MRC Adult Leukaemia Worting Party. Br J Haematol 1997; 96:601-610.
-Radich JP. Philadelphia chromosome-positive acute lymphocytic leukemia. Hematol Oncol Clin North Am 2001;15:21-36
-Aricò M, Valsecchi MG, Camitta B, Schrappe M, Chessels J, Baruchel A, Gaynon P, Silverman L, Janka-Shaub G, Kamps W, Pui CH, Masera G: Outcome of treatment in children with Philadelphia chromosome-positive acute lymphoblastic leukemia. N Eng J Med 2000; 342: 998-1006.
-Yeoh EJ, Ross ME, Shurtleff SA: Classification, subtype discovery, and prediction of outcome in pediatric acute lymphoblastic leukaemia by gene expression profiling. Cancer Cell 2002; 1: 133.
-Hofmann WK, de Vos S, Elashoff D, Gschaimeier H, Hoelzer D, Koeffler HP et al. Relation between resistance of Philadelphia-chromosome-positive acute lymphoblastic leukaemia to the tyrosine kinase inhibitor STI571 and gene-expression profiles study. Lancet 2002; 359: 481-486.

-Westbrook CA, Hooberman AL, Spino C, Dodge RK, Larson RA, Davey F, Wurster-Hill DH, Sobol RE, Schiffer C, Bloomfield CD. Clinical significance of the BCR-ABL fusion gene in adult acute lymphoblastic leukemia: a Cancer and Leukemia Group B Study (8762). Blood 1992; 80:2983-90
-Melo JV, Myint H, Galton DA, Golman JM. P190BCR-ABL chronic myeloid leukaemia: the missing link with chronic myelomonocytic leukaemia? Leukemia 1994; 8:208-211
-Ravandi F, Cortes J, Albitar M et al. Chronic myelogenous leukaemia with p185BCR-ABL expression: characteristics and clinical significance. Br J Haematol. 1999; 107:581-586.
- Suryanarayan K, Hunger SP, Kohler S, Carroll AJ, Crist W, Link MP, Cleary ML. Consistentt involvement of the bcr gene by 9;22 breakpoins in pediatric acute leukemias. Blood 1991; 77, 324-330
- Deininger MW, Goldman JM, Melo JV. The molecular biology of chronic myeloid leukemia. Blood 2000; 96, 3343-3356
- Maurer J, Janssen JW, Thiel E, van Denderen J, Ludwig WD, Aydemir U, Heinze B, Fonatsch C, Harbott J, Reiter A, Riehm H, Hoelzer D, Bartram CR. Detection of chimeric BCR/ABL genes in acute lymphoblastic leukaemia by the polymerase chain reaction. Lancet 1991; 337, 1055-1059
-van Dongen JJM, Macintyre EA, Gabert JA et al: Standardized RT-PCR analysis of fusion gene transcripts from chromosome aberrations in acute leukemia for detection of minimal residual disease. Report of BIOMED-1 Concerted Action: investigation of minimal residual disease in acute leukemia. Leukemia 1999; 13: 1901-1928.
-Borowitz MJ, Rubnitz J, Nash M, Pullen DJ, Camitta B. Surface antigen phenotype can predict TEL-AML1 rearrangement in childhood B-precursor ALL: a Pediatric Oncology Group. Leukemia 1998; 12:1764-1770
-Borowitz MJ, Hunger SP, Carroll AJ, Shuster JJ, Pullen DJ, Steuber CP, Clearly ML. Predictability of the t(1;19) (q23;p13) from surface antigen phenotype: implications for screening cases of childhood acute lymphoblastic leukemia for molecular analysis. A Pediatric Oncology Group study. Blood 1993; 82:1086-1091
-Tritz D, Pettigrew A, Talkington S, Smith L, jennings CD. Association of Cd15 expression and 11q23 translocation in acute leukemias. Mol Pathol 1995; 8:122-129.
-Pui CH. Acute lymphoblastic leukemias with the t (4;11)(q21;q23). Leuk Lymphoma 1992;7:173-179
-Pui CH, Raimondi SC, Head DR, Schell MJ, Rivera GK, Mirro J jr, Crist WM, Behm FG. Characterization of childhood acute lymphoblastic leukemia with multiple myeloid and lymphoid markers at diagnosis and at relapse. Blood 1991; 78:1327-1337
- Armstrong SA, Staunton SE, Silverman LB, Pieters R, den Boer ML, Minden MD: MLL translocation specify a distinct gene expression profile that distinguishes a unique leukemia. Nat Genet 2002; 30: 41-47.
-De Zen L, Bicciato S, te Kronnie G, Basso G: Computational analysis of flow-cytometry antigen expression profiles in childhood acute lymphoblastic leukemia: an MLL/AF4 identification. Leukemia 2003; 17: 1557-1565.
-Veltroni M, De Zen L, Sanzari MC, Maglia O, Dworzak MN, Ratei R, Biondi A, Basso G, Gaipa G: Expression of CD58 in normal, regenerating and leukemic bone marrow B cells: inplications for the detection of minimal residual disease in acute lymphocitic leukemia. Leukemia 2003; 88(11): 1245-1252
-Nachman JB, Heerema NA, Sather H, Camitta B, Forestier E, Harrison CJ, Dastugue N, Schrappe M, Pui CH, Basso G, Silverman LB, Janka-Schaub GE: Outcome of treatment in children with hypodiploid acute lymphoblastic leukemia. Blood 2007; 110: 1112-1115.

Part 2

Hematopoietic stem cell transplantation

2.1.a Hemopoietic stem cells transplantation in childhood: overview

Allogenic hematopoietic stem cell transplantation (HSCT) is largely employed for treating children affected by many hereditary and/or hematological conditions, of both malignant and non-malignant origin (Copelan EA, *N Engl J Med* 2006). It involves the infusion of HSCs from a related or unrelated donor (UD), while reinfusion of a patient's own HSCs after administration of high dose chemotherapy, radiation therapy, or both is referred to as autologous SCT.

The year 1968 (Gatti RA, Lancet 1968; Bach FH, Lancet 1968), thus introduces the fascinating, and in some respects dramatic, scenario of a therapy which is one of the major triumphs of clinical medicine and tipically has the goal of producing *biological chimeras*, an expression which has beeen effectively borrowed from Homeric mythology to designate an individual in whom the individual's own cell lines (the *self*, therefore, in the original meaning of this term according to Burnet) (Burnet M, 1970) live alongside foreign cell lines that are *non-self* (and are so by definition in the case of transplantation from an allogenic donor). (Locatelli F, Burgio GR. Haematologica 1998)

Over the past decade, relevant improvements and refinements have significantly changed the indications, technique and results obtained with allogenic transplantation of hematopoietic stem cell (HSC) in childhood.

The range of diseases for which HSC transplantation had become the therapy of choice (or the only feasible therapy) had expanded rapidly with the respect to the indication given by the initial historical experiences. A listing by the category of the diseases that could be treated by allogenic transplantation, compiled 10 years ago (Burgio GR. Haematologica 1988), would have to be significantly rewritten in view of the progress made in the hematological, oncological and immunological field, both in terms of the benefits that can be achieved by allogenic transplant of HSC and in terms of the risks linked to this procedure.

The creation of a biological chimera began by using bone marrow from an HLA-compatible related donor. Considering that only 25-30% of individuals requiring BMT have an HLA-identical family donor, inevitably, the pool of donors possibly suitable for transplantation need to be expanded. Information have to be gradually acquired regarding the importance of the exact match of HLA system antigens between the donor and the recipient in terms of the risk of rejection and the development of graft versus host disease (GVHD). (Anasetti C, N Engl J Med 1989; Anasetti C, Transf Sci 1994)

A fundamental turning point in the history of allogenic transplantation of HSC occurred in 1988, when transplant of umbilical cord blood cells (UCBC) was used for the first time.

(Gluckman E, N Engl J med 1989). It is beyond doubt that in pediatrics, the most significant alternative to BMT is still UCBC transplantation.

The haematopoietic stem cell source has expanded progressively, also with a more frequent use of peripheral blood cells both in the autologous and in the allogenic setting.

Autologous circulating progenitor cells are increasingly being used following high-dose therapy for malignant disease, because of the ease of collection and the markedly faster kinetics of engrafment in comparison with bone marrow.

Moreover, the pool of donors has expanded with increasing use of alternative HSCTs from mismatched family members that is performed in selected cases in childhood, considering the genetic disparity between the donor and the recipient, and the procedures of T-cell depletion.

Before 1996, majority of the allografts (70%) were performed from human leukocyte antigen-matched sibling donors (MSD). During the last 10 years, the number of alternative HSCTs, including volunteer unrelated donor (VUD), partially matched family donor, and cord blood (CB) HSCT increased significantly and reached 61% of the allografts carried out between 1999 and 2002 (Miano M, Bone Marrow Transpl 2007).

Early complications of SCT are due to profound pancytopenia, regimen-related toxicity, immunological reaction of the graft against tissues (acute GVHD), and protracted immune incompetence. Late complications of SCT are due to chronic end organ damage from drug and immune insults, ongoing or *de novo* manifestations of immune dysregulation such as poor immune function or chronic GVHD, and disease recurrence.

The evolution of SCT into a practical and curative therapy has depended on a number of important advances. These include improved knowledge of the human histocompatibility system and developmant of more exact methods to establish the degree of histocompatibility between the donor and recipient. Ability to deliver the specific conditioning regimens used to prepare patients affected by different diseases preparative regimen with greater accuracy (improvements in TBI dosimetry and strategy to monitor busulfan pharmacocinetics) has significantly decreased regimen-related toxicity.

Early end aggressive use of antibiotics, antifungal agents, and antiviral agents has improved survival during the early neutropenic period and during the period of profound and persistent immunosuppression after engrafment.

REFERENCES

-Copelan EA. Hematopoietic stem cell transplantation. *N Engl J Med* 2006; **354**: 1813–1826.

-Gatti RA, Meuwissen HJ, Allen HD, Hong R, Good RA. Immunological reconstitution of sex linked lymphpenicc immunological deficency. Lancet 1968; ii:1366-9

-Bach FH, Albertini RJ, Joo P, Anderson JL, Bortin MM. Bone-marrow tranplantation in a patient with the Wiskott-Aldrich syndrome. Lancet 1968; ii 1364-6

-Burnet M. Self and not-self. Melbourne: University Press: 1970.

-Locatelli F, Burgio GR. Transplant of hematopoietic stem cells in chilhood: where we are and where we are going. Haematologica 1998 Jun; 83 (6): 550-63

-Anasetti C, Amos D, Beatty PG, et al. Effect of the HLA compatibility on engrafment of bone marrow transplants in patients with leucemia or lymphoma. N Engl J Med 1989; 320:197-204

-Anasetti C, Hansen J. Effect of the HLA incompatibility in marrow transplantation from unrelated HLA-mismatched related donors. Transf Sci 1994; 14:221-30.

-Burgio GR, Nespoli L, Porta F, Bonett F. Bone marrow transplantation in children: 20 years after its first successful application. Haematologica 1988; 73:437-43

-Gluckman E, Broxmeyer HA, Auerbach AD, et al. Hematopoietic reconstitution in a patient with Fanconi's anemia by means of umbilical-cord blood from an HLA-identical sibling. N Engl J med 1989; 321: 1174-8.

-Miano M, Labopin M, Hartmann O, Angelucci E, Cornish J, Gluckman E, Locatelli F, Fischer A, Egeler RM, Or R, Peters C, Ortega J, Veys P, Bordigoni P, Iori AP, Niethammer D, Rocha V, Dini G; Paediatric Diseases Working Party of the European Group for Blood and Marrow Transplantation. Haematopoietic stem cell transplantation trends in children over the last three decades: a survey by the paediatric diseases working party of the European Group for Blood and Marrow Transplantation. Bone Marrow Transplant. 2007 Jan;39(2):89-99.

2.1.a Donor Multipotent Mesenchymal Stromal Cells May Engraft in Pediatric Patients Given Either Cord Blood or Bone Marrow Transplantation

Abstract

Introduction Multipotent mesenchymal stromal cells (MSCs) are endowed with multilineage differentiative potential and immunomodulatory properties. It is still a matter of debate whether donor MSCs have a sustained engraftment in the host bone marrow (BM) after allogeneic hematopoietic stem cell transplantation (allo-HSCT). Aim of the study: to analyze the donor/recipient origin of MSCs in pediatric patients receiving allogeneic either BM or cord blood (CB) transplantation.

Patients and Methods Thirty-seven pediatric patients undergoing allo-HSCT for either a malignant or a non-malignant disorder were enrolled in the study; 19 received CB and 18 BM transplantation. Results were compared with those obtained in 11 adults given allo-HSCT for either malignant or non-malignant disorders. MSCs were grown from BM aspirates obtained 1-17 and 2-192 months after allo-HSCT in pediatric and adult patients, respectively. MSC samples at the third-fourth passage were phenotipically characterized. Donor/recipient origin of MSCs was assessed by amelogenin assay and microsatellite analysis.

Results MSCs could be grown from 30 of 37 pediatric patients; at the third-fourth passage MSCs resulted positive for CD73, CD105, CD106, CD29, CD13, CD44 (≥99%) and negative for CD34, CD45, CD14 (≤1%). A mixed-chimerism with donor cells was observed in 4 BM and 5 CB transplantation recipients, respectively; while a full-recipient-chimerism was detected in the remaining children. A full-recipient chimerism was observed in all assessable (9/11) adult patients.

Conclusions BM soil of pediatric patients might be more favorable than that of adults for sustained engraftment of transplanted MSCs and MSCs able to engraft in the host can also be transferred with CB.

Introduction

Multipotent mesenchymal stromal cells (MSCs) have the ability to differentiate into several mesenchymal lineages and have been reported to display immunomodulatory properties (1-7). Thanks to their relatively easy isolation from bone marrow (BM) and to their extensive capacity for *in vitro* expansion, MSCs have been considered for approaches of cell therapy and tissue engineering (8-10). A number of clinical trials are ongoing to explore the effect of MSCs *in vivo* in several contexts, such as facilitation of hematopoietic recovery after hematopoietic stem cell transplantation (HSCT; 11,12), prevention and treatment of graft versus host disease (GvHD; 13-15), treatment of *Osteogenesis Imperfecta* (*O.I.*; 16-18) and metabolic disorders (19).

Even though in animal models it has been shown that MSCs can engraft and distribute to a number of tissues after systemic infusion (20-21), the transplantability of MSCs remains controversial in humans. Several studies have reported that human-MSCs remain of host origin years after a successful allogeneic HSCT (allo-HSCT; 22-27), whereas *Horwitz et al.* have demonstrated that BM-derived MSCs can engraft in patients with O.I. contributing to the formation of new dense bone tissue (28, 16) and *Cilloni et al* have documented a limited, but detectable capacity of donor BM-derived MSCs to engraft in recipients of T-cell depleted allo-HSCT (24).

MSCs have been isolated from various tissue sources, including cord blood (CB; 29, 30). Although the frequency of MSCs in CB is much lower than in BM (31, 32), CB-derived MSCs have been shown to display broader differentiation ability and to possess a huge expansion potential, up to 10^{15} cells (33, 34). The fate of MSCs transferred with CB transplantation (CBT) has not been studied so far and few data are available on the origin of MSCs in children receiving BM transplantation (BMT), as well.

Aim of this study was to analyze the donor/recipient origin of MSCs in children undergoing either BMT or CBT and to compare the results with those obtained in a control group of adult patients receiving BMT. Our results demonstrate that MSCs remain host-derived after successful BMT in adults, while in some pediatric patients donor MSCs can be detected in the BM after both BMT and CBT. In addition, our findings document, for the first time, that MSCs able to engraft can be transferred with cryopreserved CB cells.

Patients and methods

Patients

Thirty-seven pediatric patients undergoing allogeneic HSCT were enrolled in the study. Children were affected by malignant diseases in 25 cases and non-malignant disorders in the remaining 12 cases. Nineteen patients received CBT (13 from a related and 6 from an unrelated donor) and 18 patients BMT (7 from a related and 11 from unrelated donor). Results were compared with those obtained in 11 adults transplanted because of malignant diseases in 7 cases and severe aplastic anemia in the remaining 4 cases. All transplants were performed from an HLA-identical or HLA-matched donor, except two pediatric patients who received an HLA-mismatched CBT (5 out of 6 *loci* matched). Patients characteristics and conditioning regimens employed are listed in Table 1A and 1B. Preparative regimens employed were myeloablative in all cases. In patients undergoing BMT, graft-versus-host disease (GvHD) prophylaxis consisted of Cyclosporin-A (Cs-A) alone when the donor was a sibling, while a combination of Cs-A and short-term methotrexate was administered in patients transplanted from an unrelated volunteer. In children receiving CBT from a sibling donor, GvHD prophylaxis consisted of Cs-A alone, whereas in children given an unrelated donor CBT a combination of Cs-A and steroids was employed.

At time of *in vitro* MSC cultures and chimerism analysis, all patients included in the study were in clinical remission of the original disorder. With respect to the pediatric group, MSCs were grown, after having obtained an informed consent from patients' parents, from BM aspirates taken 1-17 months (median value: 5,7) after HSCT performed at the Unit of Oncoematologia Pediatrica, IRCCS Policlinico San Matteo, Pavia, Italy. Control samples were obtained, after informed consent, from 11 adult patients, where BM was collected from 2 to 192 months (median value: 32,4) after HSCT at Divisione di Ematologia e Trapianto, Ospedale San Martino, Genova, Italy.

Isolation and culture of BM-derived MSCs

Mononuclear cells were isolated from BM samples (5-10 ml) by density gradient centrifugation (Ficoll 1.077 g/ml; Lymphoprep, Nycomed Pharma, Oslo) and plated in non-coated 25-75 cm^2 polystyrene culture flasks (Corning Costar, Celbio, Milan, Italy) at a density of 160.000/cm^2 in culture medium (Mesencult, StemCell Technologies, Vancouver, Canada) supplemented with 10 % fetal calf serum (Mesenchymal Stem Cell Stimulatory Supplements, StemCell Technologies, Vancouver, Canada) and Gentamycin (Gibco-BRL, Life Technologies, Paisely, UK). Cultures were maintained at 37°C in a humidified atmosphere containing 5% CO_2. MSCs were allowed to adhere for

48 hours, followed by medium changes every 3-4 days. When grown to confluence, adherent cells were harvested with Trypsin (Sigma, Taufkirchen, Germany) and re-plated for expansion. Confluent MSC layers were trypsinized 3 to 4 times to achieve a substantial depletion of monocytes-macrophage contamination of the cultures. MSC samples at the third-fourth passage were tested for flow cytometry and chimerism analysis.

CFU-F assay.

The assay for colony-forming unit-fibroblast (CFU-F) was performed as described before (35, 36). Briefly, 1 x 10^6 BM mononuclear cells obtained after density gradient centrifugation and resuspended in complete MSC culture medium, were plated in 60 mm Petri dishes. CFU-F formation was examined after incubation for 14 days in a humidified atmosphere (37°C, 5% CO_2); clonogenic efficiency was calculated as number of colonies per 10^6 BM mononuclear cells seeded.

Flow cytometry

To phenotipically characterize MSCs and to define their purity, fluorescein isothiocyanate (FITC) or phycoerithrin (PE)-conjugated monoclonal antibodies specific for CD45, CD34, CD14, CD73, CD13 (BD Pharmigen Biosciences, San Diego, CA, USA), CD105, CD29 (Serotec, Kidlington, Oxford, UK), CD106 (Southern Biotechnology, Birmingham, AL, USA), and CD44 (Immunotech, Marseille, France) were used. Appropriate, isotype matched, non-reactive fluorocrome-conjugated antibodies were employed as controls. Analysis of cell populations was performed by means of direct immunofluorescence on a FACScalibur flow cytometer (BD Biosciences) and data calculated using CellQuest software (BD Biosciences).

Chimerism analysis

DNA was prepared from 1x10^5 to 1x10^6 MSCs, peripheral blood mononuclear cells (PBMCs) and BM cells using Genomic DNA purification Kit (Gentra, USA), according to manifacturer's instructions. The concentration and purity of each DNA sample was evaluated by means of measurement of optical density (OD) at 260 and 280 nm with a UV spectrophotometer (GeneQuantPro, Amersham). Donor/recipient origin of cells was assessed by amelogenin assay (in case of male recipient/female donor) and Short Tandem Repeats (STR) analysis. DNA was amplified using a GeneAmp 2400 PCR System (Applied Biosystems, Foster City, CA).

Amelogenin assay

Amelogenin gene amplification was allowed by a single step PCR reaction as described by Pugatsch *et al.* (37). The female cells were detected by a single amplification product of 977 bp (AMGX) and male cells gave rise to two products of 977 bp (AMGX) and 788 bp (AMGY), respectively. Amplification products were analyzed in 2% agarose gel in TBE1X buffer with 0.5 ug/ml ethidium bromide.

STR analysis

A STR locus was defined as being informative for a given donor/recipient pair if there was at least one allele of the recipient's genotype that could be distinguished from donor alleles. In the initial screening process to identify the most informative STR locus in a given donor/recipient pair, a set of 5 paired primers, namely HumFGA (GenBank accession no.G33478), HumvWA (GenBank accession n. M25716), HumTH01 (GenBank accession n. D00269), HumLPL (GenBank accession n. D83550), and HumCD4 (GenBank accession n. M86525) was used. After screening, PCR analysis was carried out using at least two of the most informative STR loci for each donor/recipient pair. Following amplification, PCR products were separated by an 8% polyacrylamide gel electrophoresis and analysed in visible light, after silver staining, and compared with sequenced allelic ladders (38).

Results

Mononuclear cells were isolated from BM aspirates of 37 pediatric and 11 adult patients receiving HSCT. MSC growth was achieved from samples of 30/37 pediatric patients; in 7 samples (3 obtained from children given BMT and 4 from children receiving CBT) a confluent or subconfluent layer of cells did not grow, leading to an insufficient quantity of MSCs to be tested. With respect to adults, in 2 out of 11 samples an adequate number of cells were not harvested for the analysis.

BM samples from pediatric and adult patients were assayed for CFU-F frequency after 14-day culture. The pediatric group showed a median value of 24,6 CFU-Fs per 10^6 mononuclear cells plated (range 0-65), whereas the median value of adult patients was 27,5 CFU-Fs (range 0-58) (Table 2A and 2B). No correlation between CFU-F frequency and type of disease, time from HSCT, conditioning regimen or GVHD prophylaxis was observed in groups of patients given either BMT or CBT.

MSCs were trypsinized when they reached 70-80% confluence and expanded until passage 3-4, before being analyzed for immunephenotype and chimerism. By the third-fourth passage, contamination with hematopoietic cells was not detected in MSC cultures by flow cytometry. In particular CD45, CD34 and CD14 resulted equal or less

than 1% in all cultures and greater than 99% of cells expressed MSC surface markers, namely CD73, CD105, CD106, CD29, CD13 and CD44 (Figure 1).

MSCs at the same passage were evaluated for donor/recipient origin. DNA was extracted and amelogenin assay was performed in case of male recipient/female donor, while STR analysis was performed on all samples. Results obtained by the amelogenin assay were always confirmed by STR-analysis. Molecular analysis showed a full recipient MSC chimerism in 21/30 (70%) of the assessable pediatric patients, in particular in 11/15 and 10/15 children receiving either BMT or CBT, respectively. A mixed MSC chimerism with donor cells was detected in 4 patients undergoing BMT and in 5 given CBT, representing 30% of the total number of children evaluated. Chimerism analysis performed at the same time-point on both PBMCs and BM mononuclear cells showed full donor chimerism in all children given BMT except one, whereas a mixed chimerism was detected in 6 out of 19 children given CBT (Table 2A). Within the group of the 9 pediatric patients showing a mixed MSC chimerism, 5 children were affected by a non-malignant disorder (4 Thalassemia Major, 1 Kostmann Syndrome), and the remaining 4 by a malignant disease. Interestingly, the 4 Thalassemia patients with a mixed MSC chimerism were all transplanted with CB cells collected from an HLA-identical sibling. With respect to malignant disorders, 3 out of 4 patients displaying donor MSCs received a HLA-identical BMT. In 2 cases stem cells were from an unrelated donor, while the remaining one was transplanted from a sibling. All pediatric patients with mixed MSC chimerism showed a full donor chimerism in BM and PBMCs; whereas none of the children with a mixed PBMC/BM chimerism displayed presence of donor MSCs. The age of pediatric patients showing donor MSCs ranged between 0,8 and 15,4 years, while the age of their HSCT donors varied between 0 (in case of CBT) and 14 years, with the exception of 2 children given an unrelated BMT from an adult donor. The median number of cells infused per kilogram/body weight (Kg/BW) was 0,34 x 10^8 (range: 0,19-0,45) in case of CBT and 6,62 x 10^8 (range: 2,9-12) for patients receiving BMT.

With respect to adult patients, all assessable MSC samples (9/9) resulted host-derived, whereas chimerism analysis on both PBMCs and BM mononuclear cells showed a full donor hematopoiesis (Table 2B).

Discussion

Results of this study demonstrate that MSCs of donor origin may engraft in pediatric patients undergoing allo-HSCT. In particular, our findings show, for the first time, that MSCs able to sustainly engraft can be transferred with CBT.

A number of previously published studies have documented that marrow stroma remains of host origin after allo-HSCT in adults (26, 39, 40), while only in a few patients, a small number of MSCs of donor origin was detected (24, 27). Moreover, *Koc et al*. reported full recipient stroma 14 years after transplantation in pediatric patients with metabolic disorders (25). These data suggest that some technical aspects need to be carefully evaluated when investigating donor/recipient origin of MSCs following allo-HSCT. In our study, MSC samples from pediatric and adult patients were analyzed after repeated trypsinizations, with the purpose to eliminate monocytes-macrophages contaminating the cultures. MSCs were detached when approaching 70-80% confluence and 3 to 4 passages in culture resulted in a MSC population containing percentages of CD45/CD14+ cells equal to or less than 1%.

Thirty percent of our pediatric population showed a mixed donor/recipient MSC chimerism, in the presence of a full donor hematopoiesis. All children showing donor MSCs, received a myeloablative conditioning regimen and were transplanted from an HLA-matched donor, who was a sibling in 7 out of 9 cases. No correlation was found between the presence of donor MSCs and diagnosis, patient or donor age at HSCT and number of cell infused per Kg/BW. Even though numbers of cells infused in CBT and BMT are not directly comparable, a broad variation was present within the two groups, especially in case of BMT. All MSC samples from adult patients showed a full recipient chimerism, irrespectively of type of disease and transplant, source of HSC and number of infused cells. PBMC/BM cells harvested at the same time point resulted donor-derived.

MSC content of CB has been object of debate in the near past (31, 41); however, a number of recent studies reported that MSCs are present in CB, although in low frequency, and exhibit an intrinsic pluripotent differentiation potential (33, 34). Our data, showing that donor MSCs engraft in a consistent percentage of the pediatric population, including CBT-recipients, suggest that BM soil of children might be more favorable than that of adults for the engraftment of allogeneic MSCs. The reason why BM microenvironment of pediatric patients might be more prone to the engraftment of donor MSCs remains unclear. Moreover, detection of donor MSCs in CBT recipients indicates that, despite their low frequency in CB units, MSCs contained in placental blood display a remarkable engraftment potential, which is not abrogated by the procedures of cryopreservation and thawing.

Table 1.

Patient characteristics of pediatric patients.

Patient ID n.	Age at HSCT (years)	Diagnosis	HSCT source	Type of donor	Compatibility	Donor age (years)	Conditioning regimen	N. cells infused (× 10^8/kg)	PMN recovery (day)	PLT recovery (day)
3460	4.2	ALL	CB	RD	HLA-identical	0	TBI + TT + Fl	0.32	26	32
2764	4.8	ALL	CB	MUD	HLA-identical	0	TBI + TT + Fl + ATG	0.38	15	19
4149	6.2	Thal	CB	RD	HLA-identical	0	BU + TT + Fl	0.33	35	72
2647	1.3	SCID	CB	MUD	HLA-identical	0	TT + Fl + ATG	1.80	14	32
4326	10.0	ALL	BM	RD	HLA-identical	2	TBI + TT + Fl	4.70	18	25
3999	8.0	SCD	BM	RD	HLA-identical	2	BU + TT + Fl	4	12	23
3304	2.5	AML	BM	RD	HLA-identical	14	TBI + TT + L-PAM	6	9	21
2866	12.6	CML	BM	MUD	HLA-identical	51	TBI + TT + CTX + ATG	2.90	15	20
2796	5.1	ALL	CB	RD	HLA-identical	0	TBI + TT + Fl	0.45	26	107
3458	8.9	Thal	CB	RD	HLA-identical	0	BU + TT + Fl	0.36	14	21
3394	15.4	Thal	CB	RD	HLA-identical	0	BU + TT + Fl	0.19	15	31
3946	3.3	MDS	BM	MUD	HLA-identical	32	TT + Fl + L-PAM + ATG	12	31	59
3204	6.7	CML	BM	MUD	HLA-identical	46	TBI + TT + CTX + ATG	7.50	14	26
4955	1.2	AML	BM	MUD	HLA-identical	35	BU + CTX + L-PAM + ATG	8	13	18
4956	3.2	ALL	BM	RD	HLA-identical	16	TBI + TT + CTX	8.70	38	44
4999	6.3	Kostmann Syndrome	BM	RD	HLA-identical	14	TT + Fl + L-PAM	6.50	15	21
5426	4.9	ALL	BM	RD	HLA-identical	1	TBI + TT + Fl	3	19	27
5602	5.9	Neuroblastoma	CB	RD	HLA-identical	0	BU + L-PAM	0.72	13	24
1369	3.5	MDS	CB	RD	HLA-identical	0	BU + CTX + L-PAM	0.57	27	21
2074	0.8	Thal	CB	RD	HLA-identical	0	BU + TT + Fl	0.57	23	28
3055	0.9	Thal	CB	RD	HLA-	0	BU + TT +	0.41	24	35

Patient ID n.	Age at HSCT (years)	Diagnosis	HSCT source	Type of donor	Compatibility	Donor age (years)	Conditioning regimen	N. cells infused (× 10^8/kg)	PMN recovery (day)	PLT recovery (day)
					identical		FI			
3189	1.9	AML	BM	RD	HLA-identical	3	BU + CTX + L-PAM	8	13	21
3567	9.6	Fanconi Anemia	BM	RD	HLA-identical	7	FI + CTX	5.80	12	21
2580	3.3	Thal	CB	RD	HLA-identical	0	BU + TT + FI	0.40	18	35
6556	4.2	Thal	CB	RD	HLA-identical	0	BU + TT + FI	0.36	21	32
8253	6.6	SCD	CB	RD	HLA-identical	0	BU + TT + FI	0.27	26	46
9346	7.8	MDS	CB	MUD	HLA-identical	0	TT + FI + L-PAM + ATG	0.25	39	59
9687	3.8	AML	CB	MUD	HLA-identical	0	BU + CTX + L-PAM + ATG	0.62	15	34
9802	9.9	ALL	CB	MUD	1 Ag-mismatched	0	TBI + TT + CTX + ATG	0.31	23	43
9901	7.5	ALL	CB	MUD	1 Ag-mismatched	0	TBI + TT + CTX + ATG	0.22	44	62
8363	5.0	AML	CB	RD	HLA-identical	0	TBI + TT + CTX	0.32	20	39
8695	3.0	ALL	BM	RD	HLA-identical	12	TBI + TT + FI	7.80	12	23
9202	13.0	AML	BM	MUD	HLA-identical	40	BU + CTX + L-PAM + ATG	3.70	17	21
8550	15.7	MDS	BM	MUD	HLA-identical	31	BU + CTX + L-PAM + ATG	5.30	19	21
7180	5.2	ALL	BM	MUD	HLA-identical	31	TBI + TT + L-PAM + ATG	4.20	16	20
9907	0.8	Hurler Syndrome	BM	RD	HLA-identical	12	BU + TT + FI	6	15	22
7815	2.0	AML	BM	MUD	HLA-identical	48	BU + CTX + L-PAM + ATG	1.10	18	22
Mean	5.81					10.72		3.08	20.18	33.16
Median	5					0		1.1	18	27
SD	3.94					16.27		3.26	8.42	18.43
Range	0.8–15.7					0–51		0.19–12	9–44	18–107

ALL indicates Acute Lymphoblastic Leukemia; AML, Acute Myeloid Leukemia; CML, Chronic Myeloid Leukemia; MDS, Myelodysplastic Syndrome; Thal, Thalassemia Major; SCD, Sickle Cell Disease; SAA, Severe Aplastic Anemia. RD indicates a related donor; MUD, matched unrelated

donor; CTX, cyclophosphamide; TT, Thiotepa; TBI, Total Body Irradiation; BU, Busulfan; Fl, Fludarabine; L-PAM, Melphalan, ATG, Antitymocyte Globulin.
Polymorphonucleae cell (PMN) recovery is defined by the first of three consecutive days with an absolute neutrophil count > 0,5 x 10^9/l in peripheral blood; platelet (PLT) recovery is defined as the first of three consecutive days with an unsupported platelet count > 20 x 10^9/l in peripheral blood.

Table 2.

Patient characteristics of adult patients

Patient ID n.	Age at HSCT (years)	Diagnosis	HSCT source	Type of donor	Compatibility	Donor Age (years)	Conditioning regimen	N. cells infused(× 10^8/kg)	PMN recovery (day)	PLT recovery (day)
1417	19	SAA	BM	RD	HLA-identical	18	CTX 200	5	24	14
1436	43	SAA	BM	RD	HLA-identical	46	TT + CTX	6.9	22	18
378	30	SAA	BM	RD	HLA-identical	21	CTX 200	2.7	12	18
1416	25	AML	BM	RD	HLA-identical	22	CTX 120 + TBI	4.5	18	22
1326	43	SAA	BM	RD	HLA-identical	63	CTX 200	1.7	14	15
1526	32	AML	BM	RD	HLA-identical	37	CTX 120 + TBI	4.5	21	20
1511	34	AML	BM	RD	HLA-identical	57	CTX 120 + TBI	3.9	16	19
961	42	CML	BM	MUD	HLA-identical	37	TT + CTX + ATG	3	18	22
1361	39	CML	BM	RD	HLA-identical	26	CTX 120 + TBI	3.6	16	17
1302	35	CML	BM	RD	HLA-identical	42	CTX 120 + TBI	5.6	27	30
1524	38	AML	BM	RD	HLA-identical	32	CTX 120 + TBI	2.6	18	25
1528	39	CML	BM	RD	HLA-identical	32	CTX 120 + TBI	4.2	15	23
1562	28	AML	BM	MUD	HLA-identical	42	CTX 120 + TBI	2.9	17	24
1530	41	SAA	BM	RD	HLA-identical	37	CTX 200	3.4	21	26
Mean	34.85					36.57		3.89	18.50	20.92
Median	36.5					37		3.75	18	21
SD	7.30					13.06		1.35	4.09	4.48
Range	19–43					18–63		1.7–6.9	12–27	14–30

AML, acute myeloid leukemia; CML, chronic myeloid leukemia; SAA, severe aplastic anemia; RD, related donor; MUD, matched unrelated donor; CTX, cyclophosphamide; TT, thiotepa; TBI, total-body irradiation; ATG, anti-thymocyte globulin; SD, standard deviation.

Polymorphonuclear cell (PMN) recovery is defined as the first of three consecutive days with an absolute neutrophil count ≥ 0.5 × 10^9/L in peripheral blood; platelet (PLT) recovery is defined as the first of three consecutive days with an unsupported platelet count ≥ 20 × 10^9/L in peripheral blood.

Table 3.

CFU-F assay and chimerism analysis, pediatric patients

				Chimerism analysis on MSCs		Chimerism analysis on PBMC/BM	
Patient ID n.	**HSCT source**	**Time of sample collection after HSCT (months)**	**CFU-F per 1 × 10^6 MNCs**	**Amelogenin assay**	**STR analysis**	**Amelogenin assay**	**STR analysis**
3460	CB	3	2	ND	ND	full donor	full donor
2764	CB	2	0	ND	ND	mixed chimerism	mixed chimerism
4149	CB	3	25	mixed chimerism	mixed chimerism	full donor	full donor
2647	CB	2	0	ND	ND	full donor	full donor
4326	BM	2	2	ND	ND	full donor	full donor
3999	BM	5	63	full recipient	full recipient	full donor	full donor
3304	BM	3	4	NA	mixed chimerism	full donor	full donor
2866	BM	17	4	mixed chimerism	mixed chimerism	full donor	full donor
2796	CB	17	31	mixed chimerism	mixed chimerism	full donor	full donor
3458	CB	11	11	mixed chimerism	mixed chimerism	full donor	full donor
3394	CB	12	56	mixed chimerism	mixed chimerism	full donor	full donor
3946	BM	6	30	NA	mixed chimerism	NA	full donor
3204	BM	13	34	NA	full recipient	NA	full donor
4955	BM	5	3	full recipient	full recipient	full donor	full donor
4956	BM	4	3	full recipient	full recipient	full donor	full donor
4999	BM	3	39	mixed chimerism	mixed chimerism	full donor	full donor

				Chimerism analysis on MSCs		Chimerism analysis on PBMC/BM	
Patient ID n.	**HSCT source**	**Time of sample collection after HSCT (months)**	**CFU-F per 1×10^6 MNCs**	**Amelogenin assay**	**STR analysis**	**Amelogenin assay**	**STR analysis**
5426	BM	6	14	NA	full recipient	NA	full donor
5602	CB	4	65	NA	full recipient	NA	mixed chimerism
1369	CB	7	6	full recipient	full recipient	mixed chimerism	mixed chimerism
2074	CB	4	4	full recipient	full recipient	full donor	full donor
3055	CB	4	0	ND	ND	full donor	full donor
3189	BM	4	0	ND	ND	mixed chimerism	mixed chimerism
3567	BM	3	0	ND	ND	full donor	full donor
2580	CB	3	22	NA	mixed chimerism	NA	full donor
6556	CB	13	25	full recipient	full recipient	mixed chimerism	mixed chimerism
8253	CB	3	39	NA	full recipient	NA	mixed chimerism
9346	CB	2	7	full recipient	full recipient	full donor	full donor
9687	CB	1	5	NA	full recipient	NA	full donor
9802	CB	2	28	NA	full recipient	NA	full donor
9901	CB	2	9	NA	full recipient	NA	full donor
8363	CB	7	32	full recipient	full recipient	full donor	full donor
8695	BM	5	44	NA	full recipient	NA	full donor
9202	BM	3	31	NA	full recipient	NA	full donor
8550	BM	6	23	NA	full recipient	NA	full donor
7180	BM	12	26	full recipient	full recipient	full donor	full donor
9907	BM	1	17	NA	full recipient	NA	mixed chimerism
7815	BM	10	35	NA	full recipient	NA	full donor
Mean		5.67	19.97				
Median		4	17				

				Chimerism analysis on MSCs		Chimerism analysis on PBMC/BM	
Patient ID n.	**HSCT source**	**Time of sample collection after HSCT (months)**	**CFU-F per 1×10^6 MNCs**	**Amelogenin assay**	**STR analysis**	**Amelogenin assay**	**STR analysis**
SD		4.38	18.53				
Range		1-17	0-65				

ND, not determined; NA, not applicable; SD, standard deviation.

Table 4.

CFU-F assay and chimerism analysis, adult patients

				Chimerism analysis on MSCs		Chimerism analysis on PBMC/BM	
Patient ID N.	**HSCT source**	**Time of sample collection after HSCT (months)**	**CFU-F per 1×10^6 MNCs**	**Amelogenin assay**	**STR analysis**	**Amelogenin assay**	**STR analysis**
1417	BM	7	58	full recipient	full recipient	full donor	full donor
1436	BM	4	45	full recipient	full recipient	full donor	full donor
378	BM	192	0	ND	ND	full donor	full donor
1416	BM	7	23	full recipient	full recipient	full donor	full donor
1326	BM	16	25	NA	full recipient	NA	full donor
1526	BM	2	18	full recipient	full recipient	full donor	full donor
1511	BM	5	20	full recipient	full recipient	full donor	full donor
961	BM	84	35	NA	full recipient	NA	full donor
1361	BM	12	42	full recipient	full recipient	full donor	full donor
1302	BM	24	37	full recipient	full recipient	full donor	full donor
1524	BM	3	0	ND	ND	full donor	full donor
1528	BM	15	19	full recipient	full recipient	full donor	full donor
1562	BM	8	27	NA	full recipient	NA	full donor

				Chimerism analysis on MSCs		Chimerism analysis on PBMC/BM	
Patient ID N.	**HSCT source**	**Time of sample collection after HSCT (months)**	**CFU-F per 1×10^6 MNCs**	**Amelogenin assay**	**STR analysis**	**Amelogenin assay**	**STR analysis**
1530	BM	11	32	full recipient	full recipient	full donor	full donor
Mean		27.85	27.21				
Median		9.5	26				
SD		51.58	16.10				
Range		2–192	0–58				

ND, not determined; NA, not applicable; SD, standard deviation.

REFERENCES

1. Caplan AI. The mesengenic process. Clin.Plast.Surg. **1994**; 21:429-435.
2. Prockop DJ. Marrow stromal cells as a stem cells from nonhematopoietic tissues. Science **1997**;276:71-74.
3. Pittenger MF, Mackay AM, Beck SC, et al. Multilineage potential of adult human mesenchymal stem cells. Science **1999**;284:143-147.
4. Bianco P, Gehron Robey P. Marrow stromal stem cells. J Clin Invest **2000**;105:1663-1668.
5. Bartolomew A, Sturgeon C, Siatskas M, et al. Mesenchymal stem cells suppress lymphocyte proliferation in vitro and prolong skin graft survival in vivo. Exp Hematol **2002**; 30: 42-48.
6. Tse WT, Pendleton JD, Beyer WM, et al. Suppression of allogeneic T-cell proliferation by human marrow stromal cells: implications in transplantation. Transplantation **2003**; 75: 389-397.
7. Maccario R, Podestà M, Moretta A, et al. Interaction of human mesenchymal stem cells with cells involved in alloantigen-specific immune response favours the differentiation of CD4+ T-cell subsets expressing regulatory/suppressive phenotype. Haematologica **2005**; 90 (4): 516-525.
8. Deans RJ, Moseley AB. Mesenchymal stem cells: biology and potential clinical use. Exp Hermat **2000**; 28: 875-884.
9. Barry FP, Murphy JM. Mesenchymal stem cells: clinical applications and biological characterization. Int J Bioch & Cell Biol **2004**; 36: 568-584.
10. Jorgensen C, Gordeladze J, Noel D. Tissue engineering through autologous mesenchymal stem cells. Curr Opin Biotechnol **2004**; 15: 406-410.
11. Koc ON, Gerson SL, Cooper BM et al. Rapid hematopoietic recovery after co-infusion of autologous-blood stem cells and culture-expanded marrow mesenchymal stem cells in advanced breast cancer patients receiving high-dose chemotherapy. J Clin Oncol **2000**; 18:307-316.
12. Lazarus HM, Koc ON, Devine SM et al. Cotransplantation of HLA-identical sibling culture-expanded mesenchymal stem cells and hematopoietic stem cells in hematologic malignancy patients. Biol Blood Marrow Transpl **2005**; 11: 389-398.

13. Le Blanc K, Rasmusson I, Sundberg B et al. Treatment of severe graft-versus-host disease with third party haploidentical mesenchymal stem cells. The Lancet **2004**; 363: 1439-1441.
14. Lazarus HM, Koc ON, Devine SM, et al. Cotransplantation of HLA-identical sibling culture-expanded mesenchymal stem cells and hematopoietic stem cells in hematologic malignancy patients. *Biol Blood Marrow Transplant*. **2005**;11:389-39.
15. Frassoni F, Labopin M, Bacigalupo A, et al. Expanded mesenchymal stem cells (MSC), co-infused with HLA-identical hematopoietic stem cell transplant, reduce acute and chronic graft versus host disease: a matched pair analysis [abstract]. *Bone Marrow Transplant.* **2002**; 29 (Suppl 2):75.
16. Horwitz EM, Prockop DJ, Fitzpatrick LA, et al. Transplantability and therapeutic effects of bone marrow-derived mesenchymal cells in children with osteogenesis imperfecta. Nat Med **1999**;5:309-313.
17. Horwitz EM, Gordon PL, Koo WKK, et al. Isolated allogeneic bone marrow derived mesenchymal stem cells engraft and stimulate growth in children with osteogenesis imperfecta: implications for cell therapy of bone. PNAS **2002**; 99: 8932-8937.
18. Le Blanc K, Gotherstrom C, Ringden O, et al. Fetal mesenchymal stem-cell engraftment in bone after in utero transplantation in a patient with severe osteogenesis imperfecta. Transplantation **2005**; 79:1607-1614.
19. Koc ON, Day J, Nieder M, Gerson SL, Lazarus HM and Krivit W. Allogeneic mesenchymal stem cell infusion for treatment of metachromatic leukodystrophy and Hurler syndrome. Bone Marrow Transpl **2002**; 30: 215.222.
20. Almeida-Porada G, Flake AW, Glimp HA and Zanjani ED. Cotransplantation of stroma results in enhancement of engraftment and early expression of donor hematopoietic stem cells in utero. Exp Hematol. **1999**;27:1569-1575.
21. Devine SM, Cobbs C, Jenning M et al. Mesenchymal stem cells distribute to a wide range of tissues following systemic infusion into non-human primates. Blood **2003**; 101: 2999-3001.
22. Simmons PJ, Przepiorka D, Thomas ED and Torok-Storb B. Host origin of marrow stromal cells following allogeneic bone marrow transplantation. Nature **1987**;328 (6129):429-432.Devine SM, Hoffman R. Role of mesenchymal stem cells in hematopoietic stem cell transplantation. Curr Opinion Hemat **2000**;7:358-363.
23. Cilloni D, Carlo-Stella C, Falzetti F, et al. Limited engraftment capacity of bone marrow-derived mesenchymal cells following T-cell-depleted hematopoietic stem cell transplantation. Blood **2000**;96:3637-3643.
24. Koc ON, Peters C, Aubourg P, et al. Bone marrow-derived mesenchymal stem cells remain host-derived despite successful hematopoietic engraftment after allogeneic transplantation in patients with lysosomal and peroxisomal storage diseases. Exp Hematol **1999**;27:1675-1681.
25. Stute N, Fehse B, Schroder J, et al. Human mesenchymal stem cells are not of donor origin in patients with severe aplastic anemia who underwent sex-mismatched allogeneic bone marrow transplant. J Hematother Stem Cell Res **2002**;11:977-984.
26. Dickhut A, Schwerdtfeger R, Kuklick L et al. Mesenchymal stem cells obtained after bone marrow transplantation or peripheral blood stem cell transplantation originate from host tissue. Ann Hematol **2005**; 84 (11): 722-727.
27. Horwitz EM, Prockop DJ, Gordon PL, et al. Clinical responses to bone marrow transplantation in children with severe osteogenesis imperfecta. Blood **2001**;97:1227-1231.
28. Erices A, Conget P, Minguell JJ. Mesenchymal progenitor cells in human umbilical cord blood. Br J Haematol **2000**;109:235-242.
29. Bieback K, Kern S, Kluter H, Eichler H. Critical parameters for the isolation of mesenchymal stem cells from umbilical cord blood. Stem Cells **2004**; 22 (4): 625-634.
30. Wexler SA, Donaldson C, Dennung-Kendall P, et al. Adult bone marrow is a rich source of human mesenchymal stem cells but umbilical cord and mobilized adult blood are not. Br J Haematol **2003**;12:368-374.
31. Kang TJ, Yeom JE, Lee HJ, et al. Growth kinetics of human mesenchymal stem cells from bone marrow and umbilical cord blood. Acta Haematol **2004**;112:230-233.
32. Kogler G, Sensken S, Airey JA, et al. A new human somatic stem cell from placental cord blood with intrinsic pluripotent differentiation potential. J Exp Med **2004**; 200 (2): 123-135.
33. Lee OK, Kuo TK, Chen W-M, et al. Isolation of multipotent mesenchymal stem cells from umbilical cord blood. Blood **2004**; 103: 1669-1675.

34. Friedenstein AJ, Deriglazova UF, Kulagina NN, et al. Precursors for fibroblasts in different populations of hematopoietic cells as detected by the in vitro colony assay method. Exp Hematol **1974**;2:83-92.
35. Castro-Malaspina H, Gay RE, Resnick G, et al. Characterization of human bone marrow fibroblast colony-forming cells (CFU-F) and their progeny. Blood **1980**;56:289-301.
36. Pugatsch T, Oppenheim A, Slavin S. Improved single-step PCR assay for sex identification post-allogeneic sex-mismatched BMT. Bone Marrow Transplant. **1996**;17:273-275.
37. Allen RC, Graves G, Budowle B. Polymerase chain reaction amplification products separated on rehydratable polyacrylamide gels and stained with silver. Biotechniques **1989**;7:736-744.
38. Awaya N, Rupert K, Bryant E and Torok-Storb B. Failure of adult marrow-derived stem cells to generate marrow stroma after successful hematopoietic stem cell transplantation. Exp Hematol **2002**;30:937-942.
39. Rieger K, Marinets O, Fietz T, et al. Mesenchymal stem cells remain of host origin even a long time after allogeneic peripheral blood stem cell or bone marrow transplantation. Exp Hematol **2005**;33:605-611.
40. Minjun Y, Xiao Z, Shen L and Li L. Mid-trimester fetal blood-derived adherent cells share characteristics similar to mesenchymal stem cells but full-term umbilical cord blood does not. British J Hematol **2003**; 124: 666-675.

2.1.c Safety and efficacy of a caspofungin-based combination therapy for treatment of proven or probable aspergillosis in pediatric hematological patients

Abstract

Background. Fungal infections are diagnosed increasingly often in patients affected by hematological diseases and their mortality has remained high. The recent development of new antifungal drugs gives the clinician the possibility to assess the combination of antifungal drugs with *in-vitro* or in animal-model synergistic effect.

Methods. We analyzed retrospectively the safety and efficacy of caspofungin-based combination therapy for in 40 children and adolescents, most of them were being treated for a malignant disease, who developed invasive aspergillosis (IA) between November 2002 and November 2005.

Results: Thirteen (32.5%) patients developed IA after hematopoietic stem cell transplantation (HSCT), 13 after primary diagnosis, usually during remission-induction chemotherapy, and 14 after relapse of disease. Severe neutropenia was present in 31 (78%) out of the 40 patients. IA was classified as probable in 20 (50%) and documented in 20 (50%) patients, respectively. A favorable response to antifungal therapy was obtained in 21 patients (53%) and the probability of 100-day survival was 70%. Different, though not significant, 100-day survival was observed according to the timing of diagnosis of IA: 51.9% after HSCT; 71.4% after relapse; and 84.6% after diagnosis of underlying disease, p 0.2. After a median follow-up of 0.7 years, 20 patients are alive (50%). Overall, the combination therapy was well tolerated. In multivariate analysis, the

factors that were significantly associated to a better overall survival were favorable response to antifungal therapy, p 0.003, and the timing of IA in the patient course of underlying disease, p 0.04.

Conclusions. This study showed that caspofungin-based combination antifungal therapy is an effective therapeutic option also for pediatric patients with IA. These data need to be

Background

Fungal infections, especially those caused by *Aspergillus spp*. or by other filamentous fungi, are diagnosed increasingly often in patients affected by hematological diseases.[1,2] Despite the introduction of liposomal and lipid formulations of amphotericin B during the 1990's, infection-related mortality of invasive mycoses has remained high.[3,4] The recent development of new antifungal drugs, such as voriconazole and caspofungin gives the clinician more therapeutic options both for first-line and for salvage therapy of invasive mycoses.[5,6] Notably, caspofungin has a different target of action with respect to the polyenes and triazoles, i.e. it inhibits the synthesis of a component of the fungal cell wall, namely beta-1,3-D-glucan; and data obtained either *in-vitro* or in animal models have shown that the combination of caspofungin with either amphotericin B or voriconazole may exert a synergistic effect.[7-9] On the basis of these premises, several authors have explored the use of caspofungin in combination with either liposomal amphotericin B, itraconazole or voriconazole in patients with invasive mycoses refractory to first-line treatment, with reported response rates ranging between 42 and 60% in the largest series. [10-14] Despite that published pediatric data on the use of caspofungin in combination with other systemic antifungal drugs are limited to single-center experience [15-17], recent multicenter studies showed that, as in adults, this therapeutic strategy is being increasingly adopted by pediatric centers. [18-19]

In this study, we report the data collected among centers belonging to the Italian Association of Pediatric Hematology Oncology (AIEOP) to investigate the safety and efficacy of caspofungin in combination with other systemic antifungal drugs.

Methods

From January 2002 to December 2003, the AIEOP centres performed a prospective surveillance study aimed at assessing the incidence and outcome of invasive fungal infection in children and adolescents affected by hematological and oncological diseases. [19] During the first year of study, it was noted that caspofungin was often used in combination with other antifungal drugs both as front-line and rescue treatment for invasive aspergillosis (IA). Therefore, a registry was established starting in November 2002 in order to collect prospectively the data on the antifungal combination therapy for IA in children. Each investigator sent to the principal investigator (S.C.) the main clinical and microbiological data of the patients developing IA and treated within 30 days from diagnosis with combination antifungal therapy. Informed consent was obtained from parents or patient's legal representatives. Recruitment of patients was closed on November 2005 and follow-up data are as 31st January 2006.

The eligibility criteria were as follows: pediatric hematological or oncological patients treated with a caspofungin-based combination antifungal therapy for proven or probable IA diagnosed whilst on chemotherapy or after hematopoietic stem cell transplantation (HSCT). Since this was a retrospective study, the main objectives of the study were the definition of a favorable response rate, 100-day survival and overall survival (OS) of patients treated with a caspofungin-based combination therapy, as well as the safety and toxicity of the combination regimen.

Management of febrile patients: neutropenic and HSCT patients were nursed in reverse-isolation or high-efficiency particulate-filtered air (HEPA) rooms, respectively. Published recommendations were used for diagnosis and treatment of febrile episodes [20, 21] , i.e a) ensuring prompt clinical and microbiological evaluation of patients with a search for clinical foci of infection by physical examination, chest X-ray, abdominal ultrasound (if appropriate), cultures of peripheral and central venous catheter (CVC) blood, and, if indicated, mouth and CVC exit-site swabs, stool and urine cultures; b) intravenous

administration of broad-spectrum antibiotic therapy for at least 72-96 hours and, in case of persistence of fever, c) a thorough re-assessment of the patient and introduction of empiric antifungal therapy based on either amphotericin B or a lipid/liposomal derivative.[22,23] In recent years, these guidelines have been implemented in patients considered at higher risk of invasive fungal infection, through the use of chest computed tomography (CT) scanning early in the course of the febrile episode for patients not responding to broad spectrum antibiotic therapy and the determination of serum galactomannan. [24-26] The following groups of patients underwent this more thorough diagnostic work-up at the participating AIEOP centers: patients with acute myeloid leukemia; patients with *de novo* or relapsed acute lymphoblastic leukemia while on induction or re-induction therapy; patients undergoing HSCT; patients with prolonged severe neutropenia or on steroid therapy.

For the purposes of the study, the caspofungin-based combination therapy was considered as introduced early (group A) or late (group B) if started within or after 7 days from diagnosis of IA, respectively.

Definitions: according to the timing of diagnosis of IA, we distinguished 3 groups of IA: a) those occurring after HSCT, if the patient have been transplanted before IA and, in case of malignant disease, did not experience a subsequent relapse; b) those diagnosed after relapse, if the patient had relapsed prior to the diagnosis of IA; and c) those developing IA during remission-induction chemotherapy for newly diagnosed malignancy, or in case of non-malignant disease, after diagnosis of underlying disease. Regarding the status of the underlying malignant disease at the time of diagnosis of IA, we distinguished 2 groups of patients: those in complete remission, and those without an adequate control of disease, i.e. other remission status.

Severe neutropenia was defined by an absolute neutrophil count < 0.5 x 10^9/l. Drug-related side effects, organ toxicity and complications after HSCT were defined according to standard criteria.[27]

Proven and probable IA were defined according to internationally accepted criteria. [28] For the purposes of this study, the caspofungin-based combination therapy was considered as primary therapy if the patients were not receiving any mould-active antifungal drug or were on prophylaxis with fluconazole or itraconazole; and as salvage therapy if the patients were receiving empirically or therapeutically any mould-active antifungal monotherapy, at diagnosis of IA, respectively.

The response to antifungal treatment was defined on the basis of the Denning criteria [29], as follows: complete response (CR) was the resolution of all clinical signs and symptoms attributable to IA, together with complete or very nearly complete radiographic resolution ($\geq$ 90%); partial response (PR) was a major improvement or resolution of the attributable clinical signs and symptoms together with at least a 50% improvement in radiological signs; stable response (SR) was consistent with some but less than 50% radiological improvement; and Failure (F) was progression of, or death from, IA. Favorable (or major) response comprised both CR and PR.

Statistical analysis

Demographic, clinical and microbiological characteristics of patients and infectious episodes were collected through specific case-report forms filled-in by the investigators; data were stored on an Access 97 data base (Microsoft, Seattle, WA, USA). Analysis used January 31st 2006 as the reference date, i.e., the day at which all centers locked data on patient outcomes. Where appropriate, the characteristics of the patients and of infectious episodes were compared using chi-square or Fisher's exact test for categorical variables.

The end points of the study were: rate of favorable response, i.e. complete and partial response; 100-day survival, OS, safety and toxicity of the antifungal combination therapy.

One-hundred-day survival and OS were calculated from the date of diagnosis of invasive fungal infection (IFI) to 100 days after diagnosis of IFI, or to the date of death due to any cause or to the date of last follow-up, respectively, by the Kaplan-Meier method. Differences between patients who received a caspofungin-based combination therapy as first line (group A) or rescue therapy (group B) were compared by the log-rank test.

The following variables were included in the analysis of prognostic factors predicting favorable response, 100-day survival and OS: patient gender (M vs. F); median age at diagnosis of IA; occurrence of IA after diagnosis, after relapse or after HSCT, respectively; presence of severe neutropenia at time of diagnosis of IA; single organ vs multiple organ involvement; type of IA (documented vs. probable); surgical treatment of IA; early introduction of caspofungin, i.e. group A vs. group B; and caspofungin-based combination therapy given as primary vs. salvage treatment. Moreover, the rate of favorable response to antifungal therapy was included in the analysis of prognostic factors for 100-day survival and OS. The variables proving significant in univariate analysis were included in a multivariate analysis: favorable response and 100-day survival were assessed by a stepwise logistical regression analysis, whilst OS was determined by a Cox regression analysis. All reported p values are 2-sided, and a significance level of α= 0.05 was used. The statistical analysis was performed using the SAS statistical program (SAS Institute, Cary, NC, Version 8.2).

Results

The main demographic and clinical characteristics of the patients are shown in table 1. During the study period, 40 patients, 21 males and 19 females, were recruited, median age at diagnosis being 11.1 years (range 1.2-17.9 years).

Thirteen (32.5%) patients developed IA after allogeneic unrelated (10) or related (3) HSCT. The median time from HSCT to IA was 14 days (range 1-308). In the month

before the diagnosis of IA, 10 and 2 patients had been treated with steroids at median dose of 2 mg/kg/day for acute GVHD (grade I-II, 5; grade III-IV, 5) and for extended chronic GVHD, respectively.

In 13 patients (32.5%), IA occurred after a median time of 74 days (range 13-318) from the diagnosis of underlying disease whilst in 14 patients (35%) IA occurred after a median time of 74 days (range 7-261) from relapse.

Characteristics of episodes of fungal infections: Table 2 summarizes the main data on episodes of IA. They were classified as probable in 20 (50%) and proven in 20 (50%) patients, respectively. In proven IA, the species identification obtained by culture was as follows: *Aspergillus fumigatus*, 7; *Aspergillus flavus*, 3; in the remaining 10 episodes of proven IA, histopathology on tissue sample showed the presence of filamentous fungi consistent with *Aspergillus spp*.

Table 1. Main demographic and clinical characteristics of patients included in this study.

	Group A	Group B	Total
Number of patients	22	18	40
Gender			
Male	14	7	21
Female	8	11	19
Age at IA diagnosis			
Median (years)	11.94	8.64	11.05
Range	(1.3-17.2)	(1.18-17.9)	(1.18-17.9)
Underlying disease			
ALL-AML	15	13	28
CML	2	0	2
NHL+HD	2	2	4
MDS	2	1	3
Non-malignant diseases	1	2	3
IA after relapse of underlying disease	9	5	14
Interval from relapse to IA (days)			
Median	69	105	74
Range	(18-174)	(7-261)	(7-261)
IA after Allogeneic **HSCT**	7	6	13
Source of SC			
BM	7	4	11
PB	/	1	1
CB	/	1	1
Interval from HSCT to IA (days)			
Median	14	15	14
Range	(4-308)	(0-54)	(0-308)
IA after diagnosis of underlying disease	6	7	13
Interval from diagnosis to IA (days)			
Median	115	46	74
Range	(15-318)	(13-76)	(13-318)
Remission status underlying of disease *	21	16	37
Complete remission	6	7	13
Other status	15	9	24

Group A comprises patients given caspofungin-based combination therapy starting within 7 days from diagnosis of IA; Group B consists of patients given caspofungin-based combination therapy starting after 7 days from diagnosis of IA.
IA, invasive aspergillosis; ALL, acute lymphoblastic leukemia; AML, acute myeloid leukemia; NHL, non-Hodgkin Lymphoma; HD, Hodgkin Lymphoma; MDS, myelodysplastic syndrome; HSCT, hematopoietic stem cell transplantation; BM, bone marrow; PB, peripheral blood; CB, cord blood; Other status: refractory or progressive disease; *only for the 37 children with malignant diseases. (Previous page)

Severe neutropenia was present at diagnosis of IA in 31 patients (78%) and lasted for a median time of 29 days, range 2-251. The median lymphocyte count at time of diagnosis of IA was 170 x 10^9/L, range 0-5,710.

In 27 (68%) episodes, the patient was on steroid therapy at diagnosis of IA, whilst in 14 (35%) episodes the patient was receiving either cyclosporine-A or tacrolimus.

The lungs were the most frequent organ affected, pulmonary IA being involved in 39 (98%) out of the 40 children enrolled in this analysis. Most patients, 29 (73%), had involvement of a single site: lung, 28; skin, 1; whilst 11 patients (27%) had 2 or more organs involved. In this last group, central nervous system involvement was present in 4 patients.

Thirty-three of 40 patients (82.5 %) were on antifungal treatment before starting the combination therapy, as follows: 13 patients were on prophylaxis with fluconazole (12 patients) or itraconazole (1 patient) for a median time of 28 days (range 10-50); 6 patients were on liposomal amphotericin-B as empiric treatment of fever and neutropenia for a median time of 10 days (range 3-43); and 14 patients were on liposomal amphotericin-B (10 patients) or caspofungin (3 patients) or voriconazole (1 patient) as first-line treatment of IA for a median time of 8 days (range 3-56). The reasons for switching to combination antifungal therapy in the patient on antifungal therapy were progression of disease or failure to improve clinically or radiographically. The remaining 7 patients were started on combination therapy as first-line treatment of IA.

Fourteen patients (35%) underwent elective surgical resection of the fungal lesions, from lungs 11 (27.5%) or from other organs 3 (7.5%), after a median time of 26 days (range 0-218 days) from diagnosis of IA.

Response to therapy and survival: the median duration of any combination of antifungal therapy was 29 days (range 3-382 days). Considering the 36 patients who received at least seven days of combination therapy, 18 patients (50%) received the combination of caspofungin and liposomal amphotericin B for a median time of 26 days (range 7-90 days), 9 patients (25%) received the combination of caspofungin and voriconazole for a median of 38 days (range 12-94 days) and the remaining 9 patients (25%) received sequentially both combinations, for a median of 19 days (range 7-84 days). The dosages used were as follows: caspofungin, 70 mg/m^2 the first day, followed by 50 mg/m^2/day; voriconazole, 2 x 6 mg/kg the first day, followed by 2 x 4 mg/kg/day; and liposomal amphotericin B, 3-5 mg/kg/day. No difference was found in terms of outcome at 100 days from diagnosis of IA in these 3 groups of patients, a favorable response being observed in 39%, 56% and 56%, respectively, (p NS).

At 100-days from diagnosis of IA, complete response was observed in 12 patients (30%), partial response in 9 patients (22.5%), stable response in 4 patients (10%) and failure in 2 (5%) patients. Noteworthy, 2 of 4 patients with CNS involvement had significant clinical improvement and were classified as partial responder. Overall, a favourable response was documented in 21 of 40 patients (53%). One-hundred-day survival was 70%, confidence interval (C.I.) being 55-84. Figure 1a shows that no difference was observed for the 100-day survival according to the timing of adoption of a caspofungin-based combination therapy: group A, 70.4%, C.I. 56-85; vs group B, 74.1%, C.I. 58-91, p 0.8. Moreover, 100-day survival resulted different, though not significant, according to the timing of diagnosis of IA: 84.6% (C.I. 65-100) for IA occurring after diagnosis of underlying disease; 71.4% (47.8-95.1) for IA occurring after relapse of underlying disease; and 51.9% (C.I. 23.9-80) for IA occurring after HSCT, p 0.2 (figure 1b).

After a median follow-up of 0.7 years (range 100 days – 3.1 years), 20 patients (50%) are alive. The probability of OS for the whole group of patients was 44%, C.I. 27-61. Figure 1c shows that OS probability was significantly better in patients who developed

IA after diagnosis of underlying disease, 48 % (C.I. 7-89), as compared with those experiencing IA either after relapse, 20.9%, (C.I. 2-40), or after HSCT, 32.4% (C.I. 9-56), respectively, p 0.007.

Sixteen of 20 patients (80%) who died had active aspergillosis before death on the basis of clinical, radiological and microbiological investigations; 8 of these 16 patients (50%) were either not in remission of their underlying disease or had clinical and/or hematological signs of progression of their primary disease. The other causes of death were not related to IA but to progression of the primary malignant disease, 1 (5%), graft-versus-host disease, 2 (10%) and septic shock, 1 (5%).

Analysis of factors predictive of favorable response, 100-survival and OS: table 3 shows the results of analysis on potentially prognostic factors. No factor was found to predict a favourable response in univariate and multivariate analysis.

Factors significantly associated in univariate analysis with a better 100-day survival and OS were type of IA, surgical treatment, and the achievement of a favourable response rate.

In multivariate analysis, no factor was predictive for 100-day survival whilst the favorable response to antifungal therapy and the occurrence of IA after diagnosis of underlying disease vs. after HSCT or relapse resulted significantly associated to better OS, p 0.03 and p 0.04, respectively.

Safety and toxicity: the antifungal combination therapy was generally well tolerated and no severe renal or liver impairment (grade II-IV WHO toxicity) attributable to the antifungal drugs was observed. Two patients were withdrawn whilst on voriconazole, for bradi-arrhythmia, 1; and diarrhea and bone pain, 1, respectively. One patient developed a transient skin rash while on caspofungin therapy; this side effect was controlled with symptomatic drugs and did not require withdrawal of caspofungin.

Discussion

Despite recent advances in diagnosis and treatment, IA still represent an important cause of mortality in immune-compromised hosts.[2-5] In this last few years, a new strategy of antifungal therapy, using a combination of antifungal drugs, has been an important subject of investigation, and as a whole, the available experimental and clinical results suggest that combination antifungal therapy may improve patient outcome. [30] The recent introduction of new molecules with broad spectrum of activity, low toxicity, and novel mechanisms of action such echinocandin is certainly favoring this strategy. There are several arguments that justify the strategy of combining antifungal drugs to optimize therapy such as the *in vitro* data showing a potential for a synergistic effect, broader spectrum of activity and a decreased risk of emergence of resistant strains; and the absence of a negative or harmful effect when an azole is combined with a polyene or an echinocandin in animal models of IA. [30-34]

Recent reports have suggested that combination therapy with the new antifungal drugs may be more effective that antifungal mono-therapy, raising the hope that new standards for the treatment of invasive mycoses may become available in the near future. [10-15]

Our study represents the largest survey on the use of combination antifungal therapy for IA in children and adolescents. The first observation deriving from this study is that the use of combination therapy is definitely increasing compared with past practice. According to published data, only 249 cases of combination antifungal therapy were reported among 6,281 total cases of IA diagnosed in the period from 1966 to 2001. [30] In contrast, we collected 40 episodes of combination therapy in the 36-month duration of this study (1.1 episodes/month). A recent prospective multicenter surveillance survey from 2001 to 2002 among the AIEOP centers showed that 49% of episodes of fungemia, or proven, probable and possible mycoses have been treated with combination therapy [19].

Differently from other authors [10-11], we excluded from analysis patients with a lower certainty of IA, namely children fulfilling the criteria of possible IA, as we reasoned that these cases should be considered not sufficiently reliable to assess the efficacy of an antifungal drug. As a whole, only 17 patients with diagnosis of possible IA were treated with combination therapy during the study period (data not shown).

Overall, the favorable response rate and 100-day survival were 53% and 70% respectively, which are noteworthy if one considers our high-risk population where 31 patients (78 %) had severe neutropenia; 27 patients (67.5%) had IA after relapse of underlying disease or after HSCT; and 20 patients (50%) were refractoriness to empiric or first-line antifungal therapy. Despite this, we obtained a response rate and 100-survival superimposable to that observed in two recent randomized studies where voriconazole and liposomal amphotericin-B (3 mg/kg) were used as first-line treatment for IA i.e. the favorable response rate was 52.8% and 50%; and the 12-week survival 70.8% and 72%, respectively. [5,35] Moreover, combination antifungal therapy was associated to a superior successful outcome than previous studies with newer agents used as salvage mono-therapy: amphotericin B-lipid complex, 42% [36]; voriconazole 39% [29]; caspofungin, 45%-44%. [37, 38] *Walsh et al.* reported a favorable response rate of 33% and 38% in children with either hematological malignancy or given HSCT who received voriconazole as rescue therapy for IA.[39] A recent prospective, open-label, non-comparative study on 53 adults reported the results of caspofungin given with other antifungals as salvage therapy for IA refractory (87%) or intolerant (13%) to first-line antifungal therapy. Favorable response at the end of combination therapy was 55% whilst 12-week favorable response and survival rate was 49% and 55%, respectively [40]

The overall survival of 44% of our patients compared well with that observed retrospectively by Abbasi et al. in 66 pediatric cancer patients cancer where 1-year mortality was 85%; moreover, in that study lung involvement was associated with poorer outcome.[41]

In patients given allogeneic HSCT, IA is still associated to lower response to antifungal drugs and survival. This is confirmed by recent study on adult HSCT patients, who had been diagnosed with proven or probable IA in 2002, that showed a discouraging 4-month survival of 34%. [42]

We found a response rate and a 100-day survival of 44% and 63% in patient with IA after HSCT or relapse that compared well with the results observed for the same subgroup of patients in the studies with voriconazole or caspofungin as salvage mono-therapy [29;37]. Our results are in line with those of Marr et al. who showed that combination therapy with caspofungin and voriconazole in HSCT patients gave a better 3-month survival than that observed in a historical control group treated with voriconazole alone.[13] However, the retrospective design of this study did not allow to ascertain clearly if the combination caspofungin-based combination therapy was significantly better than any other monotherapy without caspofungin.

Neither the type of combination antifungal therapy nor the early introduction of caspofungin-based combination therapy had a significant effect on the response rate and 100-day survival. In the multivariate analysis, the only factors that resulted significantly associated to better overall survival were the achievement of a favourable response to antifungal treatment and the occurrence of IA after the diagnosis of underlying disease.

Conclusions

This study has shown that the combination of caspofungin with other antifungal drugs is effective and well tolerated also in pediatric patients with IA. Despite the inclusion of patients with high-risk characteristics of poor outcome, favorable response and 100-day survival were not inferior to those reported for antifungal mono-therapy with new antifungal drugs used as first-line or rescue treatment for IA. The potential benefit of combination antifungal therapy over mono-therapy needs to be investigated by prospective controlled studies.

Table 2. Main data on invasive mycosis.

	Group A	Group B	Total
Type of mycosis	13	7	20
Probable	9	11	20
Documented			
Severe neutropenia at diagnosis of IFI	17	14	31
Lymphocyte count at diagnosis of IFI			
Median (x 10^9/L)	100	200	170
Range	(0-2230)	(0-5710)	(0-5710)
IS therapy at IFI			
Steroids	14	13	27
CSA or FK 506 *	8	6	14
Number of organ involved			
1	17	12	29
2	3	4	7
$\geq$ 3	2	2	4
Type of organ involvement			
Lung	22	17	39
CNS	3	1	4
Skin	1	2	3
URA	2	0	2
Heart	0	2	2
Other	1	6	7
Surgery	7	7	14
Site			
Lung	6	5	11
other	1	2	3
Time from diagnosis of IA to surgery			
Median (days)	27	20	26
Range	(16-218)	(0-81)	(0-218)

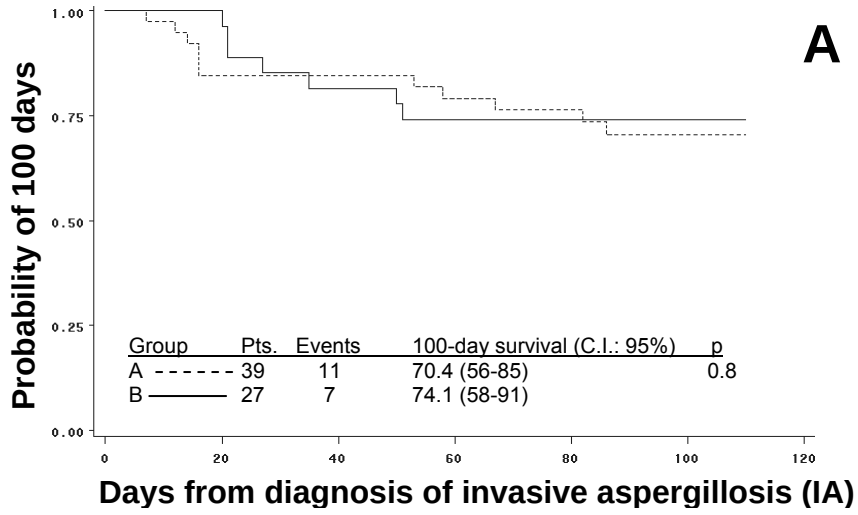
Group A comprises patients given caspofungin-based combination therapy starting within 7 days from diagnosis of IA; Group B consists of patients given caspofungin-based combination therapy starting after 7 days from diagnosis of IA. IS, immunosuppressive drugs; IFI, invasive fungal infection; CNS, central nervous system; URA, upper respiratory airways; *with or without other IS drugs; CSA, cyclosporine-A, FK 506, tacrolimus.

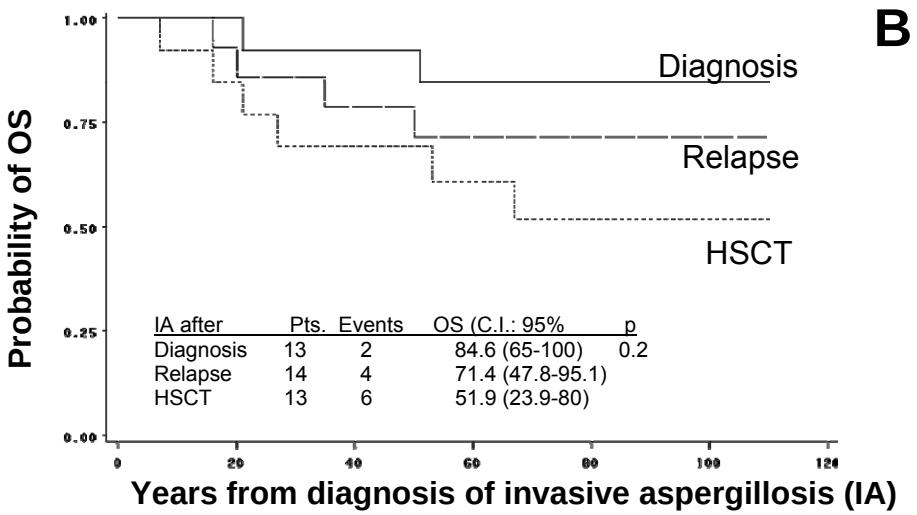
Table 3. Univariate and multivariate analysis of the factors associated with favorable response to antifungal therapy, 100-day survival and overall survival rate

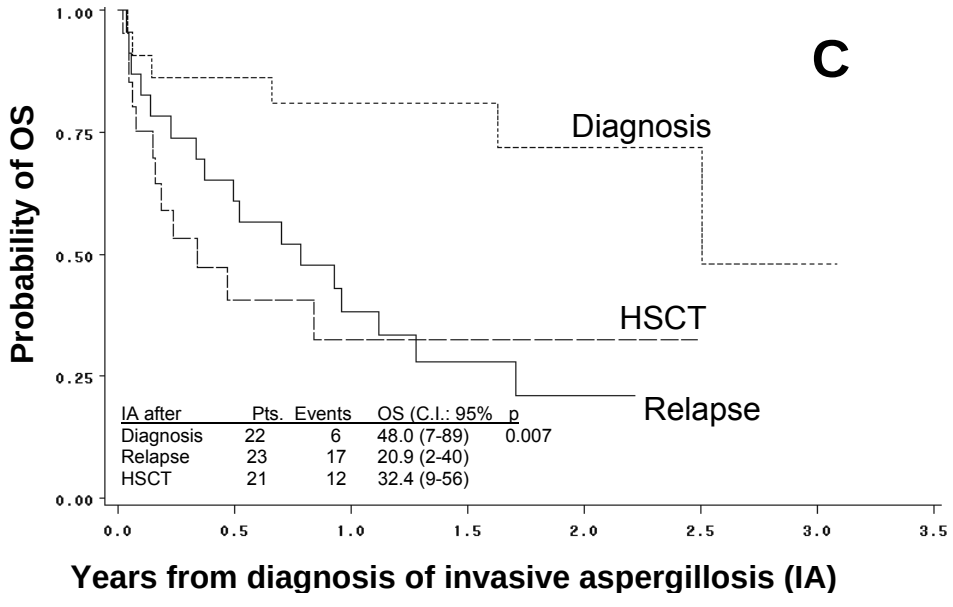
Factors	Favorable response rate	Univ P	Multiv P	100-day-survival rate	Univ P	Multiv P	OS rate	Univar P	Multiv P
Gender M F	 11/21=52% 10/19=53%	1		 15/21=71% 13/19=68%	0.8		 9/21=43% 11/19=58%	0.3	
Age (years) at IA < the median ≥ the median	 10/20=50% 11/20=55%	0.8		 15/20=75% 13/20=65%	0.5		 12/20=60% 8/20=40%	0.2	
IA after: HSCT / Relapse Diagnosis	 12/27=44% 9/13=69%	0.1		 17/27=63% 11/13=85%	0.3		 10/27=37% 10/13=77%	0.02	0.048
ANC < 0.5 x 10^9/l yes not	 16/31=52% 5/9=56%	1		 21/31=68% 7/9=78%	0.7		 14/31=45% 6/9=67%	0.5	
Number of organ affected: Single organ Multiple organs	 14/29=48% 7/11=64%	0.4		 20/29=69% 8/11=73%	1		 12/29=41% 8/11=73%	0.08	
Type of IA Probable IA Documented IA	 8/20=40% 13/20=65%	0.1		 11/20=55% 17/20=85%	0.04	-	 6/20=30% 14/20=70%	0.01	
Surgical treatment Yes No	 9/14=64% 12/26=46%	0.3		 13/14=93% 15/26=58%	0.03	-	 10/14=71% 10/26=38%	0.047	
Timing of caspofungin-based combination therapy < 7 days from IA ≥ 7 days from IA	 13/22= 59% 8/18= 44%	0.4		 17/22= 77% 11/18= 61%	0.3		 10/22= 45% 10/18=56%	0.5	
Combination therapy as : Primary or Salvage therapy	 12/20= 60% 9/20= 45%	0.3		 14/20= 70% 14/20= 70%	1		 10/20= 50% 10/20= 50%	1	
Favorable response yes no	 NE	NE	NE	 21/21=100% 7/19=37%	<0.001	-	 16/21=76% 4/19=21%	<0.001	0.003

Univ.: univariate analysis; Multiv.: multivariate analysis; OS: overall Survival; M: Male; F: Female; IA: Invasive aspergillosis; ANC: absolute neutrophil count; NE.: not evaluated;

Figures I


A
Probability of 100 days
1.00
0.75
0.50
0.25
0.00
0
20
40
60
80
100
120
Group Pts. Events 100-day survival (C.I.: 95%) p
A - - - - - - 39 11 70.4 (56-85) 0.8
B ——— 27 7 74.1 (58-91)
Days from diagnosis of invasive aspergillosis (IA)


B
Probability of OS
1.00
0.75
0.50
0.25
0.00
0
20
40
60
80
100
120
Diagnosis
Relapse
HSCT
IA after Pts. Events OS (C.I.: 95% p
Diagnosis 13 2 84.6 (65-100) 0.2
Relapse 14 4 71.4 (47.8-95.1)
HSCT 13 6 51.9 (23.9-80)
Years from diagnosis of invasive aspergillosis (IA)


C
Probability of OS
1.00
0.75
0.50
0.25
0.00
0.0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
Diagnosis
HSCT
Relapse
IA after Pts. Events OS (C.I.: 95% p
Diagnosis 22 6 48.0 (7-89) 0.007
Relapse 23 17 20.9 (2-40)
HSCT 21 12 32.4 (9-56)
Years from diagnosis of invasive aspergillosis (IA)

A) No difference was found in the 100-day survival between the patients of group A, who were started on a caspofungin-based combination therapy within < 7 days from diagnosis of IA vs the patients of group B, who were started on a caspofungin-based combination therapy after 7 days from diagnosis of IA. **B**) and **C**) Kaplan-Meier estimate of 100-day survival and OS according to the timing of diagnosis of IA: IA after diagnosis of underlying.

REFERENCES

1) McNeil MM, Nash SL, Hajjeh RA, Phelan MA, Conn LA, Plikaytis BD, Warnock DW: Trends in mortality due to invasive mycotic diseases in the United States, 1980-1997. *Clin Infect Dis* 2001, 33: 641-647.

2) Marr K, Carter R, Crippa F, Wald A, Corey L: Epidemiology and outcome of mould infections in hematopoietic stem cell transplant recipients. *Clin Infect Dis* 2002, 34: 909-907.

3) Morgan J, Wannemuehler KA, Marr KA, Hadley S, Kontoyiannis DP, Walsh TJ, Fridkin SK, Pappas PG, Warnock DW : Incidence of invasive aspergillosis following hematopoietic stem cell and solid organ transplantation: interim results of a prospective multicenter surveillance program. *Med Mycol* 2005, 43(S1):S49-S58.

4) Lin SJ, Schranz J, Teusch SM: Aspergillosis case-fatality rate: systematic review of the literature. *Clin Infect Dis* 2001, 32: 358-366.

5) Herbrecht R, Denning DW, Patterson TF, Bennett JE, Greene RE, Oestmann JW, Kern WV, Marr KA, Ribaud P, Lortholary O, Sylvester R, Rubin RH, Wingard JR, Stark P, Durand C, Caillot D, Thiel E, Chandrasekar PH, Hodges MR, Schlamm HT, Troke PF, de Pauw B; Invasive Fungal Infections Group of the European Organisation for Research and Treatment of Cancer and the Global Aspergillus Study Group: Voriconazole versus amphotericin B for primary therapy of invasive aspergillosis. *N Engl J Med* 2002, 347: 408-415.

6) Maertens J, Raad I, Petrikkos G, Boogaerts M, Selleslag D, Petersen FB, Sable CA, Kartsonis NA, Ngai A, Taylor A, Patterson TF, Denning DW, Walsh TJ; Caspofungin Salvage Aspergillosis Study Group: Efficacy and safety of caspofungin for treatment of invasive aspergillosis in patients refractory to or intolerant of conventional antifungal therapy. *Clin Infect Dis* 2004, 39: 1563-1571.

7) Arikan S, Lozano-Chiu M, Paetznick V: In vitro synergy of caspofungin and amphotericin B against *Aspergillus* and *Fusarium* spp. *Antimicrob Agents Chemother* 2002, 46: 245-247.

8) Kirkpatrick WR, Perea S, Coco BJ, Patterson TF: Efficacy of caspofungin alone and in combination with voriconazole in a Guinea pig model of invasive aspergillosis. *Antimicrob Agents Chemother* 2002, 46: 2564-2568 .

9) Petraitis V, Petraitiene R, Sarafandi AA, Kelaher AM, Lyman CA, Casler HE, Sein T, Groll AH, Bacher J, Avila NA, Walsh TJ: Combination therapy in treatment of experimental pulmonary

aspergillosis: synergistic interaction between an antifungal triazole and an echinocandin. *J Infect Dis* 2003, 187: 1834-1843.

10) Aliff TB, Maslak PG, Jurcic JG, Heaney ML, Cathcart KN, Sepkowitz KA, Weiss MA: Refractory Aspergillus pneumonia in patients with acute leukemia: successful therapy with combination caspofungin and liposomal amphotericin. *Cancer* 2003, 97: 1025-1032.

11) Kontoyiannis DP, Hachem R, Lewis RE, Rivero GA, Torres HA, Thornby J, Champlin R, Kantarjian H, Bodey GP, Raad II :Efficacy and toxicity of caspofungin in combination with liposomal amphotericin B as primary or salvage treatment of invasive aspergillosis in patients with hematologic malignancies. *Cancer* 2003, 98: 292-299.

12) Rubin MA, Carroll KC, Cahill BC: Caspofungin in combination with itraconazole for the treatment of invasive aspergillosis in humans. *Clin Infect Dis* 2002, 34: 1160.

13) Marr KA, Boeckh M, Carter RA, Kim HW, Corey L: Combination antifungal therapy for invasive aspergillosis. *Clin Infect Dis* 2004, 39: 797-802.

14) Dinser R, Grgic A, Kim Y-J, Pfreundschuh M, Schubert J: Successful treatment of disseminated aspergillosis with the combination of voriconazole, caspofungin, granulocyte transfusions, and surgery followed by allogeneic blood stem cell transplantation in a patient with primary failure of an autologous stem cell graft. *Eur J Haematol* 2005, 74: 438-441.

15) Cesaro S, Toffolutti T, Messina C, Calore E, Alaggio R, Cusinato R, Pillon M, Zanesco L: Safety and efficacy of caspofungin and liposomal amphotericin B, followed by voriconazole in young patients affected by refractory invasive mycosis. *Eur J Haematol* 2004, 73: 50-55.

16) Elanjikal Z, Sorensen J, Schmidt H, Dupuis W, Tintelnot K, Jautzke G, Klingebiel T, Lehrnbecher T: Combination therapy with caspofungin and liposomal amphotericin B for invasive aspergillosis. *Pediatr Infect Dis* 2003, 22: 653-656.

17) Pancham S, Hemmaway C, New H, Albert E, Dokal I, Roberts IA, McCloy M: Caspofungin for invasive fungal infections:combination treatment with liposomal amphotericin B in children undergoing hematopoietic stem cell transplantation. *Pediatr Transplantation* 2005, 9: 254-257.

18) Groll AH, Attarbaschi A, Schuster FR, Herzog N, Grigull L, Dworzak MN, Beutel K, Laws HJ, Lehrnbecher T: Treatment with caspofungin in immunocompromised paediatric patients: a multicentre survey. *J Antimicrob Chemother* 2006, 57: 527-535.

19) Castagnola E, Cesaro S, Giacchino M, Livadiotti S, Tucci F, Zanazzo G, Caselli D, Caviglia I, Parodi S, Rondelli R, Cornelli PE, Mura R, Santoro N, Russo G, De Santis R, Buffardi S, Viscoli C, Haupt R, Rossi MR: Fungal infections in children with cancer: a prospective, multicenter surveillance study. *Pediatr Infect Dis J* 2006, 25: 634-639.

20) Viscoli C, Castagnola E, Caniggia M, De Sio L, Garaventa A, Giacchino M, Indolfi P, Izzi GC, Manzoni P, Rossi MR, Santoro N, Zanazzo GA, Masera G: Italian guidelines for the management of infectious complications in pediatric oncology: empirical antimicrobial therapy of febrile neutropenia. *Oncology* 1998, 55: 489-500.

21) Hughes WT, Armstrong D, Bodey GP, Bow EJ, Brown AE, Calandra T, Feld R, Pizzo PA, Rolston KV, Shenep JL, Young LS: 2002 guidelines for the use of antimicrobial agents in neutropenic patients with cancer. *Clin Infect Dis* 2002, 34: 730-751.

22) Walsh TJ, Finberg RW, Arndt C, Hiemenz J, Schwartz C, Bodensteiner D, Pappas P, Seibel N, Greenberg RN, Dummer S, Schuster M, Holcenberg JS: Liposomal amphotericin B for empirical therapy in patients with persistent fever and neutropenia. National Institute of Allergy and Infectious Diseases Mycoses Study Group. *N Engl J Med* 1999, 340: 764-771.

23) Prentice HG, Hann IM, Herbrecht R, Aoun M, Kvaloy S, Catovsky D, Pinkerton CR, Schey SA, Jacobs F, Oakhill A, Stevens RF, Darbyshire PJ, Gibson BE: A randomized comparison of liposomal versus conventional amphotericin B for treatment of pyrexia of unknown origin in neutropenic patients. *Br J Haematol* 1997, 98: 711-718.

24) Caillot D, Couaillier JF, Bernard A, Casasnovas O, Denning DW, Mannone L, Lopez J, Couillault G, Piard F, Vagner O, Guy H: Increasing volume and changing characteristics of invasive pulmonary aspergillosis on sequential thoracic computed tomography scans in patients with neutropenia. *J Clin Oncol* 2001, 19: 253-259.

25) Maertens J, Verhaegen J, Lagrou K, Van Eldere J, Boogaerts M: Screening for circulating galactomannan as a non-invasive diagnostic tool for invasive aspergillosis in prolonged neutropenic patients and stem cell transplantation recipients: a prospective validation. *Blood* 2001, 97: 1604-1610.

26) Marr KA, Balajee A, McLaughlin L, Tabouret M, Bentsen C, Walsh TJ: Detection of galactomannan antigenemia by enzyme immunoassay for the diagnosis of invasive aspergillosis: variables that affect the performance. *J Infect Dis* 2004, 190: 641-649.

27) The EBMT Handbook. Haemopoietic stem cell transplantation. European Group for Blood and Marrow Transplantation and European School of Haematology. Edited by Apperley J, Carreras E, Gluckman E, Gratwohl A, Masszi T. Genova: *Forum Service Editore*, 2004.

28) Ascioglu S, Rex JH, de Pauw B, Bennett JE, Bille J, Crokaert F, Denning DW, Donnelly JP, Edwards JE, Erjavec Z, Fiere D, Lortholary O, Maertens J, Meis JF, Patterson TF, Ritter J, Selleslag D, Shah PM, Stevens DA, Walsh TJ; Invasive Fungal Infections Cooperative Group of the European Organization for Research and Treatment of Cancer; Mycoses Study Group of the National Institute of Allergy and Infectious Diseases: Defining opportunistic invasive fungal infections in immunocompromised patients with cancer and hematopoietic stem cell transplants: an international consensus. *Clin Infect Dis* 2002, 34: 7-14.

29) Denning DW, Ribaud P, Milpied N, Caillot D, Herbrecht R, Thiel E, Haas A, Ruhnke M, Lode H: Efficacy and safety of voriconazole in the treatment of acute invasive aspergillosis. *Clin Infect Dis* 2002, 34: 563-571.

30) Steinbach WJ: Combination antifungal therapy for invasive aspergillosis: utilizing new targeting strategies. *Curr Drug Targets Infect Disord* 2005, 5: 203-210.

31) Chandrasekar PH, Cutright JL, Manavathu EK: Efficacy of voriconazole plus amphotericin B or micafungin in a guinea-pig model of invasive pulmonary aspergillosis. *Clin Microbiol Infect Dis* 2004, 10: 922-950.

32) Baddley JW, Pappas PG: Antifungal combination therapy: clinical potential. *Drugs* 2005, 65: 1461-1480.

33) Kontoyiannis DP, Lewis RE: Toward more effective antifungal therapy: the prospects of combination therapy. *Br J Haematol* 2004, 126: 165-175.

34) Marr K. Combination antifungal therapy: where are we now, and where are we going? *Oncology* 2004, 18(S7): 24-29 .
35) Cornelly AO, Maertens J, Bresnik M, Herbrecht R: Liposomal amphotericin B (L-AMB) as initial therapy for filamentous fungal infections (IFFI): a randomized, prospective trial of a high loading regimen vs standard dosing (Ambiload trial). *Blood* 2005, 106(II):abs3222.
36) Walsh TJ, Hiemenz JW, Seibel NL, Perfect JR, Horwith G, Lee L, Silber JL, DiNubile MJ, Reboli A, Bow E, Lister J, Anaissie EJ: Amphotericin B lipid complex for invasive fungal infections: analysis of safety and efficacy of in 556 cases. *Clin Infect Dis* 1998, 26: 1383-1396.
37) Maertens J, Raad I, Petrikkos G, Boogaerts M, Selleslag D, Petersen FB, Sable CA, Kartsonis NA, Ngai A, Taylor A, Patterson TF, Denning DW, Walsh TJ; Caspofungin Salvage Aspergillosis Study Group: Efficacy and safety of caspofungin for treatment of invasive aspergillosis in patients refractory or intolerant of conventional antifungal therapy. *Clin Infect Dis* 2004, 39: 1563-1571.
38) Kartsonis NA, Saah AJ, Lipka J, Taylor AF, Sable CA: Salvage therapy with caspofungin for invasive aspergillosis: results from the caspofungin compassionate use study. *J Infect* 2005, 50: 196-205.
39) Walsh TJ, Lutsar I, Driscoll T, Dupont B, Roden M, Ghahramani P, Hodges M, Groll AH, Perfect JR: Voriconazole in the treatment of aspergillosis, scedosporiosis and other invasive fungal infections in children. *Ped Infect Dis J* 2002, 21: 240-248.
40) Maertens J, Glasmacher A, Herbrecht R, Thiebaut A, Cordonnier C, Segal BH, Killar J, Taylor A, Kartsonis N, Patterson TF: Multicenter, noncomparative study of caspofungin in combination with other antifungals as salvage therapy in adults with invasive aspergillosis. *Cancer* 2006, 107:2888-2897
41) Abbassi S, Shenep JL, Hughes WT, Flynn PM: Aspergillosis in children with cancer: a 34-year experience. *Clin Infect Dis* 1999, 29: 1210-1219.
Cordonnier C, Ribaud P, Herbrecht R, Milpied N, Valteau-Couanet D, Morgan C, Wade A; Societe Francaise de Greffe de Moelle et de Therapie Cellulaire: Prognostic factors for death due to invasive aspergillosis after hematopoietic stem cell transplantation: a 1-year retrospective study of consecutive patients at French Transplantation Centers. *Clin Infect Dis* 2006, 42: 955-963.

2.1.d. Member of the *Writing Committee* of the Phase I-II study of Clofarabine, in combination therapy, for the treatment of Relapsed Acute Lymphoblastic Leukemia.

Background:

New treatment combinations are required for selective group of patients for which the treatment is at the moment unsatisfactory and outcome is almost uniformly poor.

Clofarabine is a promising new agent treatment of childhood leukemia, as evidenced by single agent activity in previous phase I and II studies, and it has been incorporated into several therapeutic trial protocols for treatment of leukemias, including refractory acute leukemia.

Clofarabine is a second generation purine nucleoside analogue, thought to work via three mechanisms: inhibition of ribonucleotide reductase; incorporation into DNA; and induction of apoptosis. Given these mechanisms of action, clofarabine would be predicted to act synergistically with other chemotherapeutic agents such as other purine nucleoside analogues and DNA damaging or cross linking agents such as anthracyclines and platinum-based compounds.

Kantarjian et al. conducted the first phase I study of clofarabine in adult patients with refractory-relapse solid and hematologic malignancies to determine the MTD and dose-limiting toxicities (DLTs) of clofarabine in adults (Kantarjian, 2003). Following the completion of the phase I study in adult leukemia, several phase II single-agent clofarabine and clofarabine combination studies were conducted (Jeha S, Blood 2004; Jeha S, J Clin Oncol 2006; Kearns P, Blood 2006; Faderl S, Blood 2005; Karp JE, Blood 2007; Powell BL, Blood 2006; Faderl S, Blood 2006; Agura E, Blood 2006).

A phase I study was initiated at M. D. Anderson in 2000 for pediatric patients with advanced leukemias (Jeha 2004). This phase I study followed the phase I study in adult acute leukemia, allowing treatment in a minimum number of children at dose levels identified to be safe in adults, in order to reach more active dose levels earlier. Six dose levels (11.25 mg/m^2 IV daily for 5 days escalating to 70 mg/m^2 IV daily for 5 days) were studied in 25 patients (8 with AML and 17 with ALL). Most patients were heavily pretreated, and over a third had undergone prior hematopoietic stem cell transplantation (SCT). Reversible Grade 3 and 4 DLTs of transaminitis and skin rash were observed in two patients at 70 mg/m^2 IV daily for 5 days. The MTD was identified as 52 mg/m^2 IV

daily for 5 days. Among the 13 patients treated at 52 mg/m^2 for 5 days, there were two cases of Grade 3 transaminitis and three cases of Grade 2 hyperbilirubinemia, all of which resolved within 14 days. Responses were observed in eight patients: five achieved a CR and three had a partial response (PR) for an overall response rate of 32%.

A phase II study of 61 pediatric patients with refractory or relapsed ALL treated with clofarabine 52 mg/m^2 IV daily for 5 days was initiated in 2002 (Jeha 2006). Cycles were repeated every 2 - 6 weeks for up to 12 cycles. Approximately one third of the patients had received a prior allogeneic SCT; 35 of the 62 patients (57%) were refractory to their last course of chemotherapy. Responses occurred in 18 patients (7 CR, 5 CRp (complete response with incomplete platelet recovery), and 6 PR) for an overall response rate of 30%. The most common Grades 3 - 4 adverse events were febrile neutropenia, anorexia, hypotension, and nausea.

Based on these results, clofarabine was approved by the FDA in 2004 for the treatment of pediatric patients with relapsed or refractory ALL after at least two prior regimens.

Furthermore, in adults, clofarabine has shown significant efficacy in hematologic malignancies including acute myeloid leukemia (AML) and myelodysplastic syndrome (MDS) alone and in combinations (Powell BL, Blood 2006; Faderl S, Blood 2006; Agura E, Blood 2006).

Objectives

We are going to design a phase I and II study of clofarabine used in combination with idarubicine and cytarabine, to determine the maximum tolerated dose (MTD) and dose limiting toxicities (DLT). Moreover, the study would evaluate the efficacy of the treatment in term of overall remission rate, response duration and survival.

Patients, Materials and Methods

A selected group of paediatric patients (aged between 1 and 18 years) affected by relapsed or refractory Acute Lymphoblastic Leukemia (ALL) should be enrolled, after protocol's approval by the institutional ethical committees, and patients' informed consent.

Summary of the patient eligibility

- ALL patients with >25% blasts in the bone marrow
- Patients affected by second bone marrow relapse, isolated or combined, that previously received second line treatment protocols
- Patients with by refractory ALL

- Patients relapsed after hematopoietic stem cell transplantation (HSCT) (at least 6 months later)

Treatment Plan

A modified 3+3 design should be followed to determine the safe dose of clofarabine when used in combination. The starting dose level of clofarabine is 20 mg/m^2/daily intravenously for 5 consecutive days. Doses for idarubicine and cytarabine are maintained.

Clofarabine would be escalated of 5 mg/m^2/daily for each dose level, until the MTD is defined.

Once the paediatric MTD is reached, the subsequent study will be directed to define the efficacy of the treatment with this dose .

References:

-Agura E, Berryman B, Brougher L: Phase II Study of Clofarabine and Cytosine Arabinoside in Adult Patients with Relapsed AML and in Elderly Patients with Untreated AML Who Are al High Risk of Anthracycline Toxicity. Blood 2006; 108 (11) [Abstract 1951]

-Faderl S, Gandhi V, O'Brien S, et al: Results of phase 1-2 study of clofarabine in combination with cytarabine (ara-C) in relapsed and refractory acute leukemias. Blood 2005; 105(3): 940-947.

-Faderl S, Ferrajoli A, Ravandi-Kashani F, et al: Clofarabina Plus Anthracycline Combinations in Acute Myeloid Leukemia (AML)Salvage. Blood 2006; 108 (11) [Abstract 159]

-Jeha, S., Gandhi, V., Chan, K., et al. Clofarabine, a novel nucleoside analog, is active in pediatric patients with advanced leukemia. Blood 2004; 103 , pp. 784-789.

-Jeha, S., Gaynon, P., Razzouk, B., et al. Phase II study of clofarabine in pediatric patients with refractory or relapsed acute lymphoblastic leukemia. J Clin Oncol 2006 24 , pp. 1917-1923.

-Kantarjian, H. M., Gandhi, V., Kozuch, P., et al. Phase I clinical and pharmacology study of clofarabine in patients with solid and hematologic cancers. J Clin Oncol 2003; 21, pp. 1167-1173.

-Karp JE, Ricklis RM, Balakrishnan K , et al. A phase 1 clinical-laboratory study of clofarabine followed by cyclophosphamide for adults with refractory acute leukemias, Blood 2007; 110 (6)

-Kearns P, Michel G, Nelken B, et al: BIOV-111 A European Phase II Trial of Clofarabine (Evoltra ®) in Refractory and Relapsed Chilhood AcuteLlymphoblastic Leukemia . Blood 2006; 108 (11) [Abstract 1859]

-Powell BL, Lovato J, Kimbrough C et al: Limited Phase I Trial of Sequential High Dose Cytarabine and Clofarabine (HiDAC →CLOF) in Relapsed or Refractory Acute Myeloid Leukemia. Blood 2006; 108 (11) [Abstract 4565]

PUBLICATIONS

* Zangrando A, Luchini A, Buldini B, Rondelli R, Pession A, Bicciato S, te KronnieG, Basso G. Immunopenotype signature as a tool to define prognostic subroups in childhood acute myeloid leukemia. *Leukemia* 2006 May, 20 (5): 888-91

* Pozzi S, Lisini D, Podesta M, Bernardo ME, Sessarego N, Piaggio G, Cometa A, Giorgiani G, Mina T, Buldini B, Maccario R, Frassoni F, Locatelli F. Donor multipotent mesencymal stromal cells may engraft in pediatric patients given either cord blood or bone marrow transplantation. *Exp Hematol* 2006 Jul: 34 (7):934-42

* Cesaro S, Giacchino M, Locatelli F, Spiller M, Buldini B, Castellini C, Caselli D, Giraldi E, Tucci F, Tridello G, Rossi MR, Castagnola E. Safety and efficacy of caspofungin-based combination therapy for treatment of proven or probable aspergillosis in pediatric hematological patients. *BMC Infect Dis* 2007 Apr 18;7:28

* Veltroni M, Sainati L, Zecca M, Fenu S, Tridello G, Di Cesare-Merlone A, Buldini B, Leszl A, Lo Nigro L, Longoni D, Bernini G, Basso G. Paediatric myelodysplastic syndromes: can immunophenotypic characterization of blast cells be a diagnostic and prognostic tool? (*submitted for publication*)

PARTICIPATION IN CONFERENCES AND COURSES

* L.Zanesco, B.Buldini. L'EMOCROMO…COSA CI DICE?. 2°Congresso Nazionale ONSP and 1st European Meeting on Pediatric Emergency Medicine and Neonatal Intensive Care Training. Sciacca, 27-29 maggio 2005

* MMR nella LAL pediatrica: come e quando intervenire? Presentazione di casi clinici e discussione interattiva. G. Basso, B.Buldini. Grandangolo in Ematologia (V edizione), promosso da Accademia nazionale di medicina, Roma, 15-16 febbraio 2007

* Relazione a 61° Congresso Nazionale della Società Italiana di Pediatria "Cosa fare in caso di…Cosa fare in caso di un bambino pallido", F.Locatelli, B.Buldini, D.Pagliara, Montecatini Terme (PT), 28 settembre-2 ottobre 2005 (pubblicato su *Quaderni di Pediatria* vol.4 N.1-2005)

* Abstract selezionato per comunicazione orale "Uno strano caso di dispnea: case report", S.Sartori, B.Buldini, P.Cogo, L.Da Dalt. 3°Congresso nazionale della Società Italiana di Medicina d'Urgenza ed Emergenza Pediatrica.SIMEUP. Montesilvano (PE), 18-20 novembre 2005. Vincitore del 1° Premio per il migliore lavoro presentato nell'ambito del Congresso.

* Relazione a 22° Convegno Interregionale di Studio, Società Italiana di Medicina di Laboratorio, Patologia Clinica dell'Età Pediatrica "Dalla morfologia ai microarray: come e perchè si sta modificando l'approccio diagnostico alle leucemie" G.Basso e B.Buldini, 24-25 maggio 2007 (La Rivista Italiana della Medicina di Laboratorio, Vol.3, N.1, pp 1-72, gennaio-marzo 2007)

COMMUNICATIONS TO NATIONAL AND INTERNATIONAL CONGRESS

* R.Maccario, A.Moretta, A.Cometa, M.E.Bernardo, M.Podesta', S.Pozzi, M.A.Avanzini, G.Piaggio, B.Buldini, P.Comoli, D.Montagna, F.Frassoni, F.Locatelli. EVALUATION OF INTERACTION PATTERNS BETWEEN HUMAN MESENCHYMAL STEM CELLS AND ANTIGEN-SPECIFIC LYMPHOCYTES DURING ACTIVATION IN VITRO. EBMT, Hamburg 19-22 marzo 2006 (pubblicato su *Bone Marrow Transplantation* March 2006 vol. 37 Supplement 1)

* P.Comoli, R.Maccario, M.Zecca, M.Labirio, S.Basso, A.Moretta, D.Montagna, D.Pagliara, B.Buldini, F.Baldanti, G.Gerna, F.Locatelli. PRE-EMPTIVE TREATMENT OF EBSTEIN-BARR VIRUS-RELATED POST-TRANSPLANT LYMPHOPROLIFERATIVE DISEASE IN PAEDIATRIC RECIPIENTS OF HAPLOIDENTICAL HAEMATOPOIETIC STEM CELL TRANSPLANTATION. EBMT, Hamburg 19-22 marzo 2006 (pubblicato su *Bone Marrow Transplantation* March 2006 vol. 37 Supplement 1)

* S.Cesaro, M.Spiller, M.Giacchino, B.Buldini, C.Castellini, D.Caselli, E.Giraldi, F.Tucci, G.Tridello, F.Locatelli, M.R.Rossi, E.Castagnola. SAFETY AND EFFICACY OF A CASPOFUNGIN-BASED COMBINATION THERAPY FOR THE TREATMENT OF INVASIVE MYCOSES IN PEDIATRIC HEMATOLOGICAL PATIENTS. 14 th International Symposium of Infections in the Immunocompromised Host, July 2-5, 2006, Crans Montana, Switzerland (*International Journal of Infectious Diseases* vol.10 Suppl.1 July 2006)

* Cesaro S, Giacchino M, Spiller M, Buldini B, Castellini C, Caselli D, Giraldi E, Tucci F, Tridello G, Rossi MR, Castagnola E. LA TERAPIA DI COMBINAZIONE NEL TRATTAMENTO DELLE INFEZIONI FUNGINE. XXXIII Congresso Nazionale Associazione Italiana Ematologia Oncologia Pediatrica, Abano Terme 22-24 ottobre 2006 (*Haematologica* vol.91, Suppl.4, October 2006)