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TESIS DOCTORAL

Measurable residual disease for early relapse detection in childhood acute leukemia

Enfermedad medible residual para la detección precoz de la recaída en la leucemia aguda en la infancia

D. Eduardo Ramos Elbal

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UNIVERSIDAD DE MURCIA

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In Figure 19, on page 67, the number of patients in whom residual disease measured by multiparameter flow cytometry remains negative and who do not relapse is 15, instead of 14 (typo) and therefore the p value is 0.0012. This does not change the meaning of the conclusion.

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A todos los que me han acompañado en este viaje.

A mi familia, a mis amigos y a mi pareja.

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Resumen

Introducción

La leucemia aguda es la enfermedad maligna más frecuente en la infancia. Tanto la leucemia linfoblástica aguda (LLA) como la leucemia mieloide aguda (LMA) se caracterizan por una expansión clonal descontrolada de células inmaduras que invaden distintos tejidos y órganos, como la médula ósea o el sistema nervioso central. Aunque el pronóstico de estas enfermedades ha mejorado considerablemente gracias a diversos factores como la mejora en la eficacia de los regímenes de poliquimioterapia, el tratamiento de soporte, el manejo del trasplante de progenitores hematopoyéticos y la estratificación de los pacientes en diferentes grupos de riesgo de recaída e intensificación acorde a este, la recaída sigue siendo uno de los principales obstáculos para la curación de estos pacientes.

La enfermedad medible residual (EMR), definida como el número de células malignas remanentes tras el tratamiento por debajo del umbral de la observación citomorfológica, puede ser medida por diversas técnicas como la citometría de flujo multiparamétrica (CFM), la reacción en cadena de la polimerasa (PCR) en sus distintas modalidades, la hibridación in situ fluorescente (FISH) o la secuenciación de nueva generación (NGS), cada una con sus fortalezas y debilidades. Asimismo, estas técnicas han sido de vital importancia para la clasificación de los pacientes en distintos grupos de riesgo, tanto durante el tratamiento como previamente al trasplante de células progenitoras hematopoyéticas. Sin embargo, el papel que estos métodos diagnósticos puedan desempeñar en la monitorización de la enfermedad tras el tratamiento es aún un campo en desarrollo.

En esta tesis se describe nuestra experiencia en la monitorización de la enfermedad medible residual en leucemia aguda en la infancia mediante las técnicas disponibles en nuestro centro tanto en leucemia mieloide aguda como en leucemia linfoblástica aguda, su relación con la recaída y el resultado de diferentes intervenciones precoces realizadas en la práctica clínica diaria. Se analiza también el impacto de la genética de las células tumorales y el trasplante de progenitores hematopoyéticos en la cinética de recaída en una cohorte de pacientes con leucemia mieloide aguda (tanto adultos como niños) y su

posible implicación en la frecuencia de monitorización conveniente para detectar la recaída de manera precoz.

Material y métodos

Se revisaron los datos clínicos y de laboratorio de los pacientes pediátricos diagnosticados de LMA entre 2012 y 2022 y de los pacientes pediátricos diagnosticados de LLA entre junio de 2013 y febrero de 2022 en nuestro centro. En primer lugar, se realizó un estudio descriptivo de las principales variables clínicas y demográficas de estos pacientes. En segundo lugar, se empleó el método de Kaplan-Meier en nuestros pacientes para el análisis de supervivencia global y libre de evento y el método de Fine and Grey para el cálculo de las funciones de incidencia acumulada de recaída y mortalidad relacionada con el tratamiento.

A continuación, se analizaron las determinaciones de EMR durante todo el tratamiento y seguimiento de los pacientes con LMA y LLA diagnosticados y tratados en nuestro centro. Estas determinaciones se obtuvieron mediante CFM, FISH (en células totales en las leucemias mieloides y en las leucemias de células T, y en linfocitos B purificados en las leucemias de células B) y PCR (mediante reversotranscripción y PCR, o RT-PCR, para la determinación de reordenamientos recurrentes en la leucemia aguda, y mediante PCR cuantitativa al analizar la expresión de *WT1* en las leucemias mieloides agudas que lo sobreexpresaban). Se evaluó la asociación de la reaparición de la enfermedad mínima residual detectada mediante cada una de estas tres técnicas con la aparición de una recaída mediante la prueba exacta de Fisher. Complementariamente, se analizó descriptivamente el tiempo desde la primera muestra positiva hasta la recaída según las distintas técnicas y el efecto que tuvieron los distintos tratamientos instaurados antes de la recaída en la evolución de la enfermedad.

Por otra parte, se analizó el impacto de las diferentes alteraciones genéticas de las células leucémicas y el trasplante de progenitores hematopoyéticos en su cinética de recaída en una cohorte de pacientes de LMA, tanto pediátricos como adultos, diagnosticados y tratados en hospitales de los países nórdicos (Dinamarca, Suecia, Noruega y Finlandia). En estos pacientes, la EMR se determinaba sistemáticamente en sangre periférica aproximadamente cada 1-2 meses mediante PCR cuantitativa de alteraciones genéticas recurrentes de las células tumorales, desde el fin del tratamiento

hasta dos años después de este o la recaída, lo que antes ocurriese. Se incluyeron aquellos pacientes que presentaban al menos dos determinaciones positivas antes de la recaída sin recibir ningún tipo de tratamiento que pudiese modificar la velocidad de esta. Medimos la velocidad de recaída como incrementos en la EMR en escala logarítmica por cada treinta días.

Para el estudio de la relación entre las principales alteraciones genéticas de la leucemia mieloide aguda (leucemias con factor de unión nuclear, con reordenamientos del gen *KMT2A* o con mutaciones del gen *NPM1*) y su velocidad de recaída, se utilizó la prueba de la t de Student o el test de la suma de rangos de Wilcoxon según el cumplimiento de las condiciones de normalidad y homocedasticidad. Para el estudio del impacto de distintas alteraciones genéticas secundarias (mutaciones en *KIT*, *KRAS*, *NRAS*, *FLT3*-ITD y *FLT3*-D835) se realizó un análisis de regresión robusta lineal múltiple en el que la variable dependiente era la velocidad de recaída y las variables independientes la presencia o ausencia de dichas alteraciones genéticas. Para evaluar el papel del trasplante en la cinética de recaída, añadimos a este análisis el trasplante de progenitores hematopoyéticos como variable independiente. Y por el último, para el análisis de la relación exponencial negativa observada entre la velocidad de recaída y el tiempo tras el trasplante, se realizó un análisis de regresión robusta lineal simple con la velocidad de recaída en escala logarítmica como variable dependiente y el tiempo desde la fecha de infusión a la recaída como variable independiente.

Resultados

En los 20 pacientes con LMA diagnosticados y tratados en nuestro centro, la supervivencia global a tres años fue del 72,7% (IC95% 52,8%-100%) y la supervivencia libre de evento a tres años del 67,7% (IC95% 49,4%-93%). La tasa de recaída fue del 25% (5 de los 20 pacientes). Además, observamos una asociación positiva entre la reaparición de la enfermedad por CFM, la reaparición de reordenamientos del gen *KMT2A* o la t(9;9) y el incremento igual o superior a diez veces en la expresión del gen *WT1* en dos muestras consecutivas de médula ósea durante el seguimiento con la aparición de una recaída posterior (p-valores de 0,0016, 0,03 y 0,02, respectivamente). Sin embargo, no encontramos asociación alguna entre la reaparición de la t(8;21) asociada a las leucemias con factor de unión nuclear y la recaída.

La mediana de tiempo entre la reaparición de la EMR mediante cualquiera de estos tres métodos y la fecha de recaída fue directamente proporcional a la sensibilidad de la técnica empleada, siendo de 26 días (rango de 0-326 días) para la CFM, 111 días (rango de 90-575 días) para la RT-PCR y de 140 días (rango de 47-197) para la expresión de *WT1* medida mediante PCR cuantitativa.

En todos los casos menos en uno fuimos capaces de anticiparnos a la recaída mediante alguna de las tres técnicas empleadas. Sin embargo, las intervenciones instauradas (retirada de la inmunosupresión tras el trasplante, infusiones de linfocitos de donante, quimioterapia de rescate…) solo pudieron prolongar la supervivencia de los pacientes, sin evitar finalmente la recaída.

En los 80 pacientes con LLA diagnosticados y tratados en nuestro centro, la supervivencia global a los cinco años fue del 94% (IC95% 88,3%-100%), la supervivencia libre de evento a los cinco años fue del 84,1% (IC95% 75%-94,2%), la incidencia acumulada de recaída a los cinco años fue del 12,1% (IC95% 3,08-21,12%) y la mortalidad relacionada con el tratamiento a los cinco años fue del 3,8% (IC95% 0%-8,11%). Además, hallamos una asociación positiva entre la reaparición de la EMR por CFM y la recaída (p valor < 0,00001), así como entre la reaparición de reordenamientos específicos de la célula tumoral detectados mediante RT-PCR y FISH y la recaída ($p < 0,035$ y $p < 0,00001$, respectivamente).

El tiempo entre la reaparición de la enfermedad medible residual y la recaída también guardó relación con la sensibilidad de la técnica en aquellos casos en los que nos anticipamos a la recaída (12 días para la citometría de flujo y 40 días para la hibridación in situ fluorescentes en linfocitos B purificados en un caso y 21 días para la reacción en cadena de polimerasa con transcriptasa inversa en otro).

En cinco casos la continuación del tratamiento estándar o la instauración de tratamientos adicionales evitaron la recaída y en dos casos fuimos capaces de anticiparnos a esta pero no se pudo evitar.

En cuanto a la relación entre la genética de la célula leucémica y la cinética de recaída en la LMA, encontramos una mayor velocidad de recaída en las leucemias con reordenamientos del gen *KMT2A* respecto a las leucemias con factor de unión nuclear o *NPM1* mutado ($p=0,001$ y $p=0,05$, respectivamente). Al analizar el papel de las co-

mutaciones, el mejor modelo de regresión encontrado incluía como predictores todas las co-mutaciones estudiadas (mutaciones en *KIT*, *KRAS* o *NRAS, FLT3*-ITD y *FLT3*-D835) aunque ninguna de ellas alcanzó la significación estadística. En las leucemias agudas mieloides con factor de unión nuclear el mejor modelo incluía tanto las mutaciones en *KIT* como las mutaciones en *KRAS* y *NRAS*, pero sólo estas últimas estuvieron cerca de la significación estadística (p = 0,11). En las leucemias agudas mieloides con *NPM1* mutado, el mejor modelo encontrado presentaba a *FLT3*-D835 como único predictor y este resultaba ser estadísticamente significativo (p=0,04).

Al añadir el trasplante de progenitores hematopoyéticos al análisis anterior, se encontró que el trasplante previo era un predictor independiente de una mayor velocidad de recaída respecto a los pacientes no trasplantados (coeficiente de 0,88 y p = 0,042 en todos los pacientes y coeficiente de 0,97 y p = 0,071 en los pacientes con *NPM1* mutado) y, además, se observó una relación exponencial negativa entre la velocidad de recaída y el tiempo transcurrido desde la fecha de infusión, siendo más alta en los primeros días y meses tras esta y disminuyendo exponencialmente a medida que el tiempo transcurría desde ella (p = 0,041).

Conclusiones

En la LMA infantil, la CFM, la FISH y la PCR son métodos complementarios que pueden anticipar la recaída entre semanas y varios meses. Sin embargo, en nuestra serie, las terapias preventivas no pudieron prevenir la progresión de la enfermedad. Por lo tanto, se necesitan métodos de seguimiento de EMR más sensibles que anticipen aún más las recaídas y terapias preventivas más efectivas.

En la LLA pediátrica, la CFM, la FISH y la PCR son métodos complementarios para el seguimiento de la EMR. Aunque nuestros datos muestran claramente que la detección positiva de EMR se asocia con la recaída, la continuación del tratamiento estándar, la intensificación u otras intervenciones tempranas pudieron detener la recaída en pacientes con diferentes riesgos y antecedentes genéticos. Se necesitan métodos más sensibles y específicos para mejorar este enfoque. Sin embargo, es necesario evaluar en ensayos clínicos adecuadamente controlados si el tratamiento temprano de la EMR puede mejorar la supervivencia general en pacientes con LLA infantil.

La monitorización de la EMR es un enfoque útil para anticipar la recaída tanto en pacientes con LMA como con LLA. El tiempo desde la detección de EMR hasta la recaída es directamente proporcional a la sensibilidad de la técnica. Los métodos moleculares son los más sensibles y específicos, aunque su aplicabilidad está limitada por la presencia de alteraciones genéticas diana. Sin embargo, en la LMA con factor de unión nuclear la reaparición de la alteración genética no siempre implica una recaída.

La monitorización molecular de la EMR en sangre periférica durante el seguimiento es un enfoque factible para una detección temprana de la enfermedad y permitir un tratamiento preventivo. Los esquemas de seguimiento de EMR deben personalizarse de acuerdo con las características genéticas de la enfermedad de los pacientes (mutaciones definitorias, co-mutaciones) y el estado del paciente. Para que sea útil, el seguimiento de la EMR debería ser más frecuente en los primeros meses después del trasplante y más espaciado después.

Index

I. Introduction

1.1 Childhood acute leukemia

Acute leukemia is a clonal disease characterized by the uncontrolled proliferation and accumulation of immature white blood cells in the bone marrow, leading to a hematopoietic failure. Malignant cells can also emigrate to other tissues such as testes or central nervous system (CNS), resulting in an extramedullary disease.

There are two main types of acute leukemia: acute lymphoblastic leukemia (ALL) and acute myeloid leukemia (AML). The main difference between them is that ALL affects the production of lymphocytes and AML affects the production of myeloid cells (i.e granulocytes, monocytes, red blood cells or platelets). ALL is the most common type in children and adolescents, whereas AML is more common in adults.

1.1.1 Acute lymphoblastic leukemia: classification, treatment and prognosis.

Acute lymphoblastic leukemia is the most common cancer in children, accounting for about the 25% of malignancies in childhood and the 80% of acute leukemias in children (1). Its classification has evolved all over the time. Initially, the French-American-British (FAB) cooperative group proposed a classification based on individual cell features and the heterogeneity of these features in the leukemic-cell population (2,3) (Table 1).

Cytological features	*L1*	*L2*	*L3*
Cell size	Small cells predominate	Large, heterogeneous in size	Large and homogeneous
Nuclear chromatin	Homogeneous in any one case	Variable—heterogeneous in any one case	Finely stippled and homogeneous
Nuclear shape	Regular, occasional clefting or indentation	Irregular; clefting and indentation common	Regular—oval to round
Nucleoli	Not visible, or small and inconspicuous	One or more present, often large	Prominent; one or more vesicular
Amount of cytoplasm	Scanty	Variable; often moderately abundant	Moderately abundant
Basophilia of cytoplasm	Slight or moderate, rarely intense	Variable; deep in some	Very deep
Cytoplasmic vacuolation	Variable	Variable	Often prominent

Table 1. FAB classification for acute lymphoblastic leukemia. *Extracted from: Bennett JM, Catovsky D, Daniel M -T, Flandrin G, Galton DAG, Gralnick HR, et al. Proposals for the Classification of the Acute Leukaemias French-American-British (FAB) Co-operative Group. Br J Haematol. 1976;33(4):451–8.*

However, this classification offered little insight into the pathogenesis of ALL and less utility in its management. For this reason, the World Health Organization (WHO) developed a

classification for B-ALL based on genetic features that give us a much more precise view of its physiopathology and expected prognosis (4,5) (Table 2).

B-cell lymphoblastic leukaemias/lymphomas
B-lymphoblastic leukaemia/lymphoma, NOS
B-lymphoblastic leukaemia/lymphoma with high hyperdiploidy
B-lymphoblastic leukaemia/lymphoma with hypodiploidy
B-lymphoblastic leukaemia/lymphoma with iAMP21
B-lymphoblastic leukaemia/lymphoma with *BCR*::*ABL1* fusion
B-lymphoblastic leukaemia/lymphoma with *BCR*::*ABL1*-like features
B-lymphoblastic leukaemia/lymphoma with *KMT2A* rearrangement
B-lymphoblastic leukaemia/lymphoma with *ETV6*::*RUNX1* fusion
B-lymphoblastic leukaemia/lymphoma with *ETV6*::*RUNX1*-like features
B-lymphoblastic leukaemia/lymphoma with *TCF3*::*PBX1* fusion
B-lymphoblastic leukaemia/lymphoma with *IGH*::*IL3 fusion*
B-lymphoblastic leukaemia/lymphoma with *TCF3*::*HLF* fusion
B-lymphoblastic leukaemia/lymphoma with other defined genetic abnormalities
T-lymphoblastic leukaemia/lymphoma
T-lymphoblastic leukaemia / lymphoma, NOS
Early T-precursor lymphoblastic leukaemia / lymphoma

Table 2. WHO classification for ALL. *Extracted from: Alaggio R, Amador C, Anagnostopoulos I, Attygalle AD, Araujo IB de O, Berti E, et al. The 5th edition of the World Health Organization Classification of Haematolymphoid Tumours: Lymphoid Neoplasms. Leukemia. 2022;36(7):1720–48.*

For instance, high hyperdiploidy and *ETV6::RUNX1* fusion are associated with good response to treatment and, consequently, with good prognosis; while hypodiploidy, intrachromosomal amplification of chromosome 21 (iAMP21), *BCR::ABL1* fusion or *KMT2A* rearrangements are associated with the opposite (6–8). For T-ALL, sequencing studies are less advanced than in B-ALL, and the full range of genetic alterations remain to be defined. However, early T-cell precursor ALL, an immature stem or progenitor cell leukemia with a distinct gene-expression profile but, lacking specific genetic lesions and associated with a poor outcome, has been recently incorporated as a definitive entity (6–8).

Nowadays, treatment of childhood ALL generally consists in 4 to 6 weeks induction therapy whose goal is to achieve the complete remission of the patient, an 8-week consolidation phased based on repeated courses of methotrexate, a reinduction phase, similar to the initial one but shorter, and a low-intensity anti-metabolite maintenance therapy until 2 years from

diagnosis (9). Thanks to the collaborative efforts, its prognosis has dramatically improved in the last decades; from less than 10% in the 1960's to more than 90% at the present time (9–11).

1.1.2 Acute myeloid leukemia: classification, treatment and prognosis.

Acute myeloid leukemia accounts for the 5% of children cancer and about the 20% of childhood acute leukemias (1). Traditionally, AML has been classified by the FAB cooperative group classification, according to the direction of differentiation and the degree of maturation (2,12–14) (Table 3).

M0	Minimally differentiated acute myeloid leukemia
M1	Myeloblastic leukemia without maturation
M2	Myeloblastic leukemia with maturation
M3	Hypergranular promyelocytic leukemia
M4	Myelomonocytic leukemia
M5	Monocytic leukemia
M6	Erythroleukemia
M7	Acute leukemia of megakaryocytic lineage

Table 3. FAB classification of acute myeloid leukemia.

In the last decades, the WHO has updated and modified the FAB diagnostic criteria, adding a new classification based on recurrent genetic abnormalities (4,15) (Table 4).

Acute myeloid leukaemia with defining genetic abnormalities
Acute promyelocytic leukaemia with *PML::RARA* fusion
Acute myeloid leukaemia with *RUNX1::RUNX1T1* fusion
Acute myeloid leukaemia with *CBFB::MYH11* fusion
Acute myeloid leukaemia with *DEK::NUP214* fusion
Acute myeloid leukaemia with *RBM15::MRTFA* fusion
Acute myeloid leukaemia with *BCR::ABL1* fusion
Acute myeloid leukaemia with *KMT2A* rearrangement
Acute myeloid leukaemia with *MECOM* rearrangement
Acute myeloid leukaemia with *NUP98* rearrangement
Acute myeloid leukaemia with *NPM1* mutation
Acute myeloid leukaemia with *CEBPA* mutation
Acute myeloid leukaemia, myelodysplasia-related
Acute myeloid leukaemia with other defined genetic alterations

Table 4. Acute myeloid leukemia with defining genetic abnormalities. *Extracted from: Khoury JD, Solary E, Abla O, Akkari Y, Alaggio R, Apperley JF, et al. The 5th edition of the World Health Organization Classification of Haematolymphoid Tumours: Myeloid and Histiocytic/Dendritic Neoplasms. Leukemia. 2022;36(7):1703–19.*

Among these genetic abnormalities there are some of special relevance. Core binding factor (CBF) leukemias, which includes AML with *RUNX1::RUNX1T1* and *CBFB::MYH11* fusions, accounts for about 20% of AML in children and have a favorable outcome (16,17). Other relevant entities are *KMT2A* rearranged leukemias which account for approximately the 16% of children AML, are specially clustered in infants (16), and are associated with poor prognosis (17); or *NPM1* mutated leukemias which are much less frequent than in adults and confers a favorable prognosis in the absence of *FLT3* internal tandem duplications (ITD) (18,19).

Currently, the treatment of childhood AML include three to five courses of intensive therapy followed by allogenic hematopoietic stem cell transplantation for high-risk patients. Historically, chemotherapy cycles have been divided into one to two remission-induction cycles, whose objective is to reduce the tumor burden to less than 5% without precluding the hematological recovery, and one to three cytarabine-based consolidation cycles, whose objective is to consolidate this remission. Induction therapy usually includes three drugs: an anthracycline, cytarabine and etoposide (20,21). Unfortunately, despite improvements in survival in the last decades, overall survival remains around 60-70% (20,21).

These survival rates for both ALL and AML have been achieved thanks to the improvement in the efficacy of multiagent chemotherapy regimen, transplantation management, supportive care and the stratification of the patients into different groups of treatment based on their relapse risk. The relapse risk is estimated by several prognostic factors such as the clinical features of the patient, the biological features of the tumor, or the early response to treatment (22,23). The latter is an important and independent prognostic factor in both ALL, in children (24–26) and adults (27), and AML, in children (28–31) and adults (32,33). Early response to treatment is currently mostly measured in terms of measurable residual disease (MRD), which is crucial in the management of these children.

1.2 Measurable residual disease

1.2.1 Definition(s)

Measurable residual disease (MRD), previously termed as minimal residual disease, is defined as the number of leukemic cells persisting after chemotherapy below the sensitivity of routine morphology (33). It can be measured by diverse methods based on specific immunophenotypic or molecular aberrancies of the malignant cells. In addition, other

operational definitions regarding MRD behavior have been stablished for an adequate management of the disease.

In AML, initial recommendations on diagnosis and response assessment were made by the National Cancer Institute of the United States in 1990 (34). These recommendations were improved by a subsequent report of the International Working Group of AML in 2003 (35). In these reports, definitions of AML diagnosis, complete remission, refractory disease and relapse were established. The concepts of molecular complete remission and molecular relapse also began to be introduced; for example, molecular relapse was defined as the reappearance of a molecular or cytogenetic abnormality after achieving a molecular complete remission.

These definitions were later refined through the publication of a consensus document elaborated by the European Leukemia Net MRD Working Party (36). In this document, molecular relapse was defined as an increase of ≥ 1 $\log_{10}$ between 2 positive samples in a patient who previously tested negative in technically adequate samples. Also, molecular progression was defined as an increase of ≥ 1 $\log_{10}$ between any 2 positive samples in a patient with molecular persistence at low copy numbers (defined in turn as a relative increase < 1 $\log_{10}$ between any 2 positive samples collected after the end of treatment in a patient in morphological complete remission). An update over these recommendations was published in 2021 and, although specific recommendations for each MRD technology were made, definitions remained almost the same (37)

In ALL, until approximately the year 2000, cytology has been the main method for establishing the diagnosis of ALL and for evaluating response to treatment (38). The diagnosis of ALL has been classically defined by the presence of 25% or more lymphoblasts in the bone marrow and the same after achieving complete remission for relapse (39). Since the year 2000, new techniques with a greater sensitivity and specificity have emerged, so the majority of the collaborative groups have incorporated these techniques to the definition of relapse. In 2021, The Ponte-di-Legno Consortium revised all the definitions of remission, treatment failure and relapse of these groups and issued a consensus recommendation about them. The main novelty was that relapse could be diagnosed with less of 25% blasts if one or two additional MRD tests were positive: 1 test for 5-25% blasts and 2 tests for 1-5% blasts (38).

1.2.2 MRD methods

Measurable residual disease can be measured by diverse methods, including multiparameter flow cytometry, polymerase chain reaction-based methods such as amplification of antigen cell receptor rearrangements or fusion transcripts, and next-generation sequencing. All these methods are acceptable as long as quality standards have been stablished and each one has its own advantages and disadvantages (40–43) (Table 5).

1.2.2.1 Multiparameter flow cytometry

Multiparameter flow cytometry (MFC) is a widely expanded method to detect MRD, overall, in the United States and Asian countries (40). This method is based on the detection of cellular surface antigens by detecting the fluorescence emitted by a fluorochrome bound to antibodies against the aforementioned antigens when excited by a laser of one specific color (Figure 1).

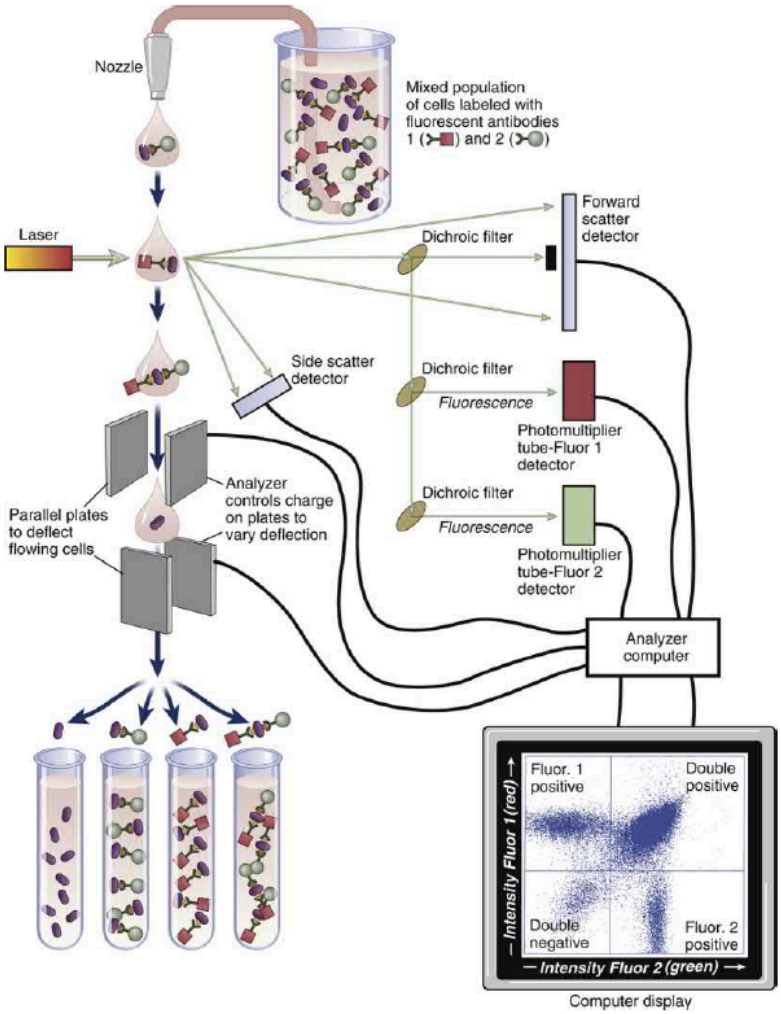

Figure 1. Multiparameter flow cytometry. The laser beam has a certain wavelength. The anterograde and lateral scattering of the light are analyzed in a two-dimensional dot plot, as well as the fluorescent lights that depends on the fluorochromes linked to the antibodies to identify specific cell immunophenotypes. *Extracted from: Abbas AK. Celullar and molecular immunology. Philadelphia, Pennsylvania: Elsevier, 2022.*

Its sensitivity ranges from 10^{-3}-10^{-4} in 3-4 color systems and closer to 10^{-4} in 6-8 color systems. It has the advantage of being a less laborious technique than the polymerase chain reaction of B-cell immunoglobulin or T-cell receptor gene rearrangements, but the disadvantage of having variable sensitivity due to similarities between regenerative cells and malignant cells in some cases, and of being a less standardized technique due to the big number of immunostaining protocols, antibody panels and gating strategies that can be used (40) (Table 5).

There are mainly two strategies to identify the malignant cells by MFC. The first consist in looking for a leukemia associated immunophenotype (LAIP) characterized by anomalies in the immunophenotype as cross-lineage expression, overexpression, lack of expression or asynchronous expression of surface antigens. The main problem of this approach is that clonal evolution of the leukemic cells can give us false positive or negative results during the follow-up. The second one consists in identifying abnormal leukemic cells based on established immunophenotypes different from normal cells irrespective to the original LAIP; this strategy is called the "different from normal" strategy and it has been overall applied in the transplant setting in which patients come from another hospital and the information about the original LAIP is limited (43).

As we have mentioned before, MFC is evolving from the primitive 3-4 color systems to the modern systems of more than 8 colors, improving sensitivities (40). Also, great efforts are being made in automating the analysis process through automated analysis algorithms such as SPADE or VisNE, but for now, the analysis has to be done manually, using biaxial plots, performing a gating strategy for identifying selected cell populations (43).

1.2.2.2 Polymerase chain reaction-based methods.

Polymerase chain reaction (PCR) is a cyclic process in which a specific fragment of a deoxyribonucleic acid (DNA) strand, delimited by two oligonucleotides called primers, can be amplified through the action of the DNA polymerase. It consists in consecutives cycles of denaturalization, amplification and renaturalization after which millions of copies of the original strand can be obtained (Figure 2). The starting point can be a DNA strand or a ribonucleic acid (RNA) strand, in which case it must be first retrotranscribed into complementary DNA (cDNA); this latter technique is called then reverse transcriptase PCR (RT-PCR).

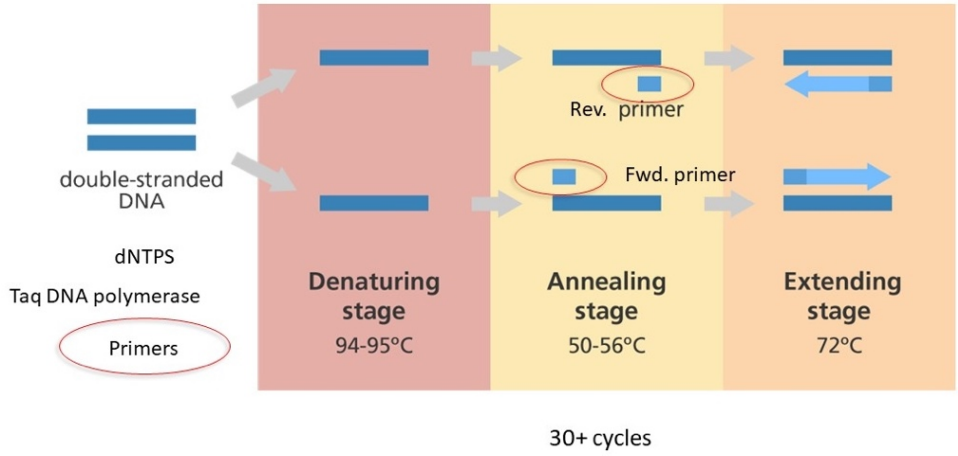

Figure 2. PCR usually uses 30-40 cycles of denaturing the DNA at high temperature (94-95º), lowering the temperature to allow the primers to bind (50-56º) and raising the temperature again (72º) to active the polymerase. Repeated cycles result in an exponential growth of the number of copies of the target DNA. *Extracted from: National Library of Medicine. The Polymerase Chain Reaction. Accessed on 15th December 2023. Available at: https://www.nlm.nih.gov/ncbi/workshops/2022-10_Primer-BLAST/PCR.html*

The final product of these techniques is independent of the initial amount of DNA or cDNA in the sample and depends mainly on the quantity of limiting reagents as the primers or the deoxynucleotides triphosphates (dNTPS). This problem has been solved with the introduction of fluorescent probes that emit fluorescence during the course of the reaction so the quantity of the amplified strand can be estimated in each cycle. This technique is called real-time or quantitative PCR (qPCR) (Figure 3) (44) and can be also performed from a DNA strand or a ARN strand, the latter with the intervention of a retrotranscriptase (RT-qPCR).

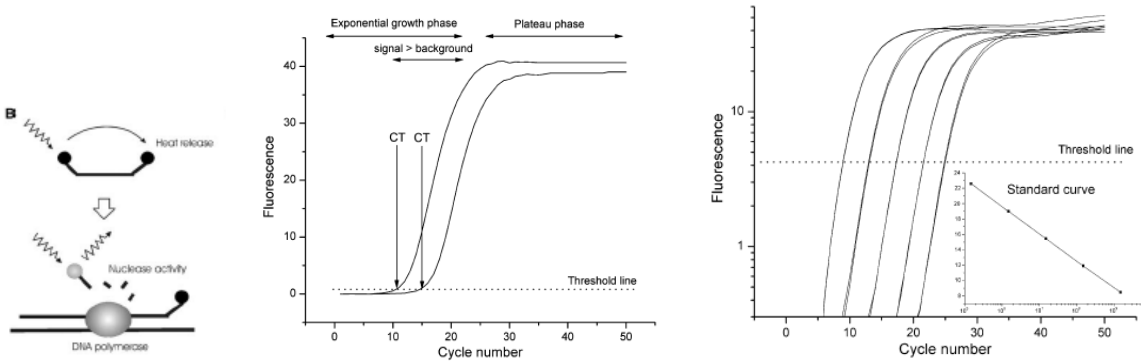

Figure 3. Taqman probe emits fluorescence as long as the polymerase synthetizes the targeted fragment. A number of cycles, proportional to the quantity of DNA, is required to reach the fluorescence threshold line. A standard curve is constructed based on samples with known DNA concentration that allow us to determine the DNA concentration in our sample. *Extracted from: Kubista M, Andrade JM, Bengtsson M, Forootan A, Jonák J, Lind K, et al. The real-time polymerase chain reaction. Mol Aspects Med. 2006;27(2–3):95–125.*

In ALL, qPCR of clonal antigen-receptor gene rearrangements based on the sequences of the junctional regions of the V, D and J fragments (Figure 4) is an approach that achieve sensitivities of 10^{-4}-10^{-5} using allele-specific oligonucleotides (ASO) as primers. Among its advantages is that it is a well-standardized technique, subject to periodic quality controls and virtually applicable to all acute lymphoblastic leukemias, but, on the other hand, it is a very expensive method that requires a lot of time for the preparation of allele-specific oligonucleotides, in addition to a great deal of experience and knowledge of it (40) (Table 5).

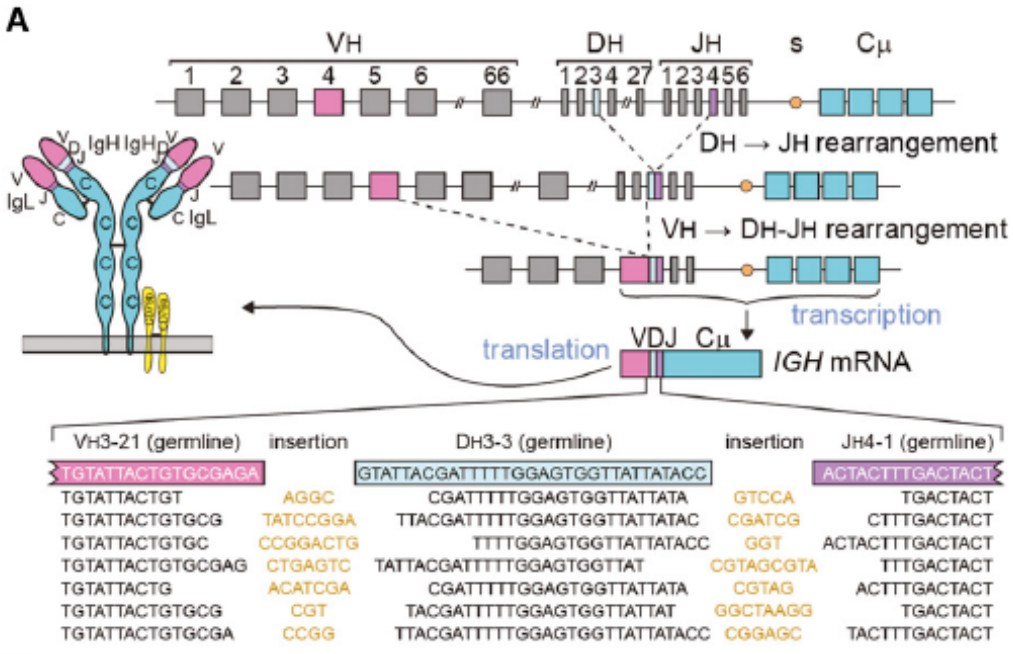

Figure 4. Schematic diagram of an IGH gene rearrangement, resulting in a V-D-J exon with highly diverse junctional regions, which differ in each individual B-cell, even if by coincidence the same V, D and J genes are used. *Extracted from: Van Dongen JJM, Van Der Velden VHJ, Brüggemann M, Orfao A. Minimal residual disease diagnostics in acute lymphoblastic leukemia: Need for sensitive, fast, and standardized technologies. Blood. 2015;125(26):3996–4009.*

It is important to note that the evaluation of the MRD by this technique requires the use of two or more rearrangements. This is due to the fact that, at diagnosis, multiple rearrangements occur in a non-negligible proportion of patients and these subclones may have a different response to treatment, causing a relapse due to another clone different from the initial one (40).

Polymerase chain reaction can also be used to assess MRD by the amplification of other recurrent gene rearrangements such as *RUNX1::RUNX1T1*, *CBFB::MYH11*, *MLL::MLLT3* in AML and *BCR::ABL1* in ALL, or other mutated genes such as *NPM1* in AML (32). The main advantage of this technique is a high sensitivity (up to 10^{-4}-10^{-6}) and a relatively simplicity of

execution since commercial kits have been available for decades. A landmark in the standardization of this technology for clinical application was the Europe Against Cancer Program (EAC), which designed a common primer and TaqMan probe for each fusion gene, established a common protocol for all steps and identified *ABL* as the most reliable control gene. They also recommended to run the assays in triplicate, for establishing criteria for the quality control of the measures (45,46).

However, its applicability is limited to 25-40% of B-ALL, 10-15% of T-ALL, 32-62% of adult AML and about 58% of childhood AML (40,43). Another disadvantage is that the standardization and quality control are not as well established as in ASO-PCR (40) (Table 5).

To overcome the limited applicability of the PCR of gene rearrangements or mutated genes in AML, the use of overexpressed genes in AML, such as *WT1*, has been proposed in cases in which a specific mutation or rearrangement is not available (36). However, as a certain level of expression is present in healthy progenitors, this method is not leukemia specific, so this technique is also limited to patients with a level of expression at diagnosis above that of the healthy progenitors. In 2002, Cilloni et al. determined the level of *WT1* expression in 79 AML and 48 ALL patients and compared it with the level of expression of normal controls, regenerative bone marrow cells and CD34-positive purified cells. The level of *WT1* expression was significantly higher than in the other situations, allowing *WT1* expression to be used as a leukemia marker (47,48). Later, two studies established a threshold to differ normal from pathological expression in both adults (49) and pediatric patients (50), what could be useful for MRD assessment.

Two new PCR-based approaches are currently under investigation. High throughput sequencing of the antigen-receptor gene rearrangements is a novel technique with the aim of perform deep sequencing of all the V, D and J rearrangements through a multiplex PCR. This method allows to identify different subclones that are present in the diagnostic sample and can evolve in its proportion during treatment (40). Also, digital PCR is another promising technique. It consists in several PCR performed simultaneously in a nanofluidic chip which allows to calculate precisely the percentage of mutant allele copies with high sensitivities around 10^{-6} (43).

1.2.2.3 Next generation sequencing

Next generation sequencing (NGS) is a massive and parallel sequencing method that has emerged in the last decades. In it, prepared samples with adaptors bound to a surface in which

different clusters of sequences are generated by beads or bridge amplification. Then, simultaneously sequencing by synthesis of all these clusters is performed and extracted data is analyzed, comparing it with reference sequences (Figure 5) (51).

NGS can be used to sequence either antigen receptor genes or whatever other gene. Its sensitivity is as high as 10^{-6} and a specific primer for each patient is not needed which, together with a shorter sequencing time, simple operation, high throughput, stability and standardization overcomes the disadvantages of MFC and PCR-based methos in certain degree. It also can be used in virtually all acute leukemias due to the wide number of genes that can be screened simultaneously. On the other hand, the cost of NGS is relatively high, which is a disadvantage for the clinical implementation of this technique (41).

As NGS detect a large number of genetic alterations, it allows not only characterizing the biological profile of the leukemia at the diagnosis but also the clonal evolution of the disease at different points in the follow-up.

Figure 5. Next generation sequencing by bridge amplification. DNA is fragmentated and adaptors are bound to form the DNA library. This library is amplificated and amplified clusters are spatially distributed. Sequencing occurs in consecutive cycles in which fluorescent nucleotides are bound as the complementary strand is synthetized. Bioinformatic analysis of these short reads is necessary to ensemble the full sequence. *Modified with BioRender from: Ajmal Aseem. Next-generation sequencing and its application in genomics. Accessed on 16th December 2023. Available at: https://www.biotechreality.com /2023/06/next-generation-sequencing-ngs-and-its-application-in-genomics.html*

1.2.2.4 Other methods: FISH

Fluorescent in Situ Hybridization (FISH) is a technique in which structural chromosomal aberrations are identified through the hybridization of fluorescent probes; it can be done at interphase or at metaphase (Figure 6). Although it has been primarily used at diagnosis, some groups have explored its utility to measure MRD along the follow up in both ALL and AML (52,53). However, its limited applicability (determined by the presence of structural abnormalities) and sensitivity (dependent of the number of analyzed cells, ranging between 10^{-2}-10^{-4}) have precluded this technique to be an extended MRD monitoring method.

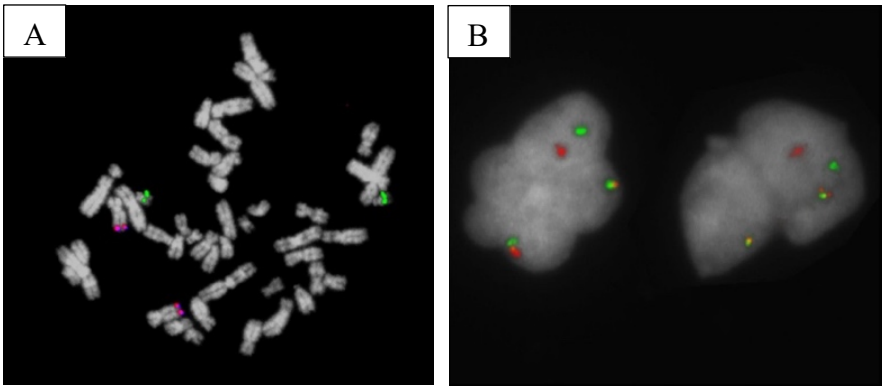

Figure 6. FISH. A. Metaphase FISH showing a normal metaphase. B. Interphase FISH showing two interphase cells with two fusion signals and separated red and green signals, indicating a BCR/ABL1 translocation. *Extracted from: Metasysistems-probes.com Accessed on 16th December 2024. Available at: https://metasystems-probes.com/en/probes/xl/d-5082-100-tc/ and https://metasystems-probes.com/en/probes/xl/d-5052-100-og/*

In the following table we resume the main characteristics, advantages and disadvantages of the main MRD methods (MFC, qPCR and NGS).

	Multiparameter flow cytometry	**qPCR of antigen-receptor genes junctional regions**	**qPCR of fusion transcripts or other aberrancies**	**NGS of antigen-receptor genes or other genes**
Estimated sensitivity	3-4 colors: 10^{-3}-10^{-4} 6-8 colors: 10^{-4}	10^{-4}-10^{-5}	10^{-4}-10^{-6}	10^{-6}
Applicability	>90% of ALL 80-90% of AML[33]	>95% of ALL Not applicable in AML	B-ALL: 25-40% T-ALL:10-15% AML: 60%[43]	>95% of ALL and AML
Advantages	Rapid Analysis at cell population or single cell level Information about the whole sample cellularity	High sensitivity Virtually applicable in all patients Well standardized, regular international quality assessment (QA) rounds	High sensitivity Simple and rapid Stable target gene	High sensitivity Rapid Not rely on specific primers

Disadvantages	Variable sensitivity, observer dependent, because similarities between regenerating and malignant cells Limited standardization	Expensive Time-consuming Requires extensive experience and knowledge	Limited standardization Limited QA rounds Limited applicability	Limited experience in the field Lack of standardization Higher cost

Table 5. Advantages and disadvantages of commonly used MRD monitoring methods. *Adapted from: Van Dongen JJM, Van Der Velden VHJ, Brüggemann M, Orfao A. Minimal residual disease diagnostics in acute lymphoblastic leukemia: Need for sensitive, fast, and standardized technologies. Blood. 2015;125(26):3996–4009 and Qin X, Zhang MY, Liu WJ. Application of minimal residual disease monitoring in pediatric patients with acute lymphoblastic leukemia. Eur Rev Med Pharmacol Sci. 2018;22(20):6885–95.*

1.2.3 Clinical applications of MRD

Measurable residual disease has diverse clinical applications, all of them based on its capacity to measure the cancer load with a higher sensitivity than traditional methods. It can be used to assess response to treatment as described before, but it can also be used as a prognostic factor prior to transplantation and to monitor the disease in the post-consolidation setting (after consolidation or after transplantation) in order to predict relapse and guide preemptive treatment.

1.2.3.1 MRD for the early response evaluation and risk stratification

Several studies have manifested the usefulness of MRD for early response evaluation and risk stratification. In childhood ALL, van Dongen et al. in 1998 demonstrated that MRD measured by PCR amplification of antigen-receptor gene rearrangements at the end of the induction and before consolidation was an independent prognostic factor in terms of relapse free survival (RFS) (24). Not much latter, in 2000, Coustan-Smith et al. discovered that MRD measured by MFC at the end of induction and other three timepoints along the treatment (weeks 14, 32 and 56) was also significantly associated with a higher likelihood of relapse in childhood ALL (25). Furthermore, in 2008, Borowitz et al. revealed that MRD measured by MFC at the end of induction was the most powerful prognostic factor when compared with others such as National Cancer Institute (NCI) risk group (which includes age and white blood cell count), day 8 MRD and other cytogenetic features such as 4 and 10 trisomies or presence of *ETV6::RUNX1* fusion (Figure 7) (26).

In adult ALL, Ribera et al. in 2014 reported the results of the largest prospective trial to that date including high-risk patients in which therapeutic decisions were made according to MFC-MRD. In that report, they described how MRD clearance after induction and after early

consolidation was the only variable remaining in multivariable analysis for disease-free survival (DFS) and overall survival (OS) (27).

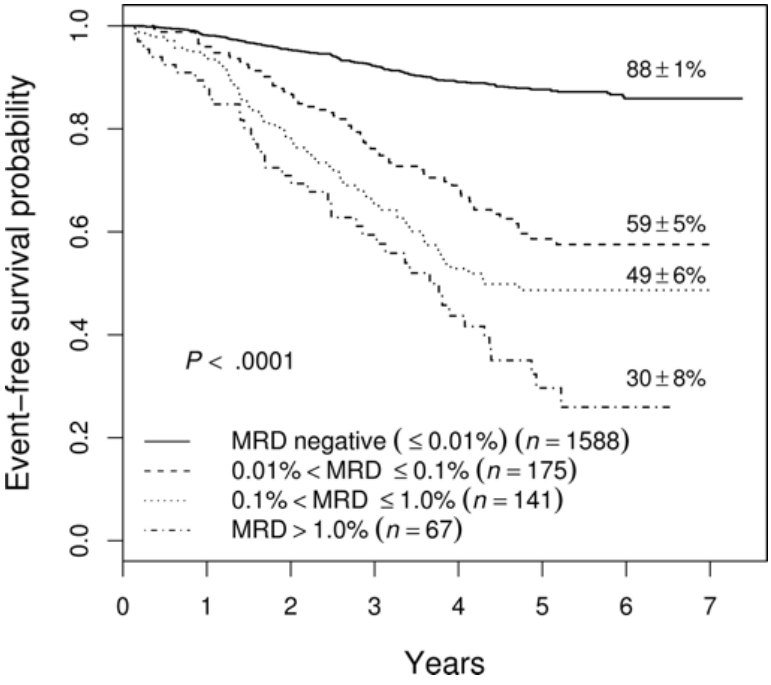

Figure 7. MFC-MRD at the end of induction is a strong prognostic factor in childhood ALL. *Extracted from: Borowitz MJ, Devidas M, Hunger SP, Bowman WP, Carroll AJ, Carroll WL, et al. Clinical significance of minimal residual disease in childhood acute lymphoblastic leukemia and its relationship to other prognostic factors: A Children's Oncology Group study. Blood. 2008;111(12):5477–85.*

Regarding childhood AML, Langebrake et al. in 2006 explored the utility of MRD measured by MFC. They described that bone marrow evaluation with this technique was mostly informative at the end of first induction and immediately before second induction, although it didn't retain its statistical significance in multivariable analysis (28). Nonetheless, in 2010, Rubnitz et al. published the results of the AML02 multicenter trial for, mostly, childhood AML. In this report, they conclude that, in its series, MRD measured by MFC at the end of first induction was the unique independent prognostic factor for both event-free survival (EFS) and OS (29). Similar results were later reported by the Children Oncology Group (30). Additionally, in 2015, Tierens et al. reported the results of the NOPHO-AML 2004 study. In this study, residual disease measured by flow-cytometry was an important prognostic factor both at the end of the first induction and before the consolidation (i.e. at the end of the two induction cycles), however, only the last retained its statistically significance in multivariable analysis (Table 6) (31).

Concerning adult AML, Krönke et al. in 2011 showed that MRD after double induction measured by RQ-PCR of *NPM1* mutations was an independent prognostic factor for OS and cumulative incidence of relapse (CIR) (32). Later, in 2013, Terwjin et al. communicated that

MRD measured by MFC after each treatment cycle (first induction, second induction and consolidation) was also an independent prognostic factor (33).

	Event-free survival		Overall survival	
	HR (95% CI)	*P* value	HR (95% CI)	*P* value
RD D15 ≥0·1%	0·78 (0·26–2·33)	0·651	0·77 (0·15–3·84)	0·748
RD BC ≥0·1%	5·00 (1·87–13·3)	0·001	6·97 (1·99–24·5)	0·002
Age	0·97 (0·91–1·04)	0·455	0·94 (0·85–1·03)	0·159
Sex	0·53 (0·20–1·38)	0·192	0·88 (0·27–2·84)	0·826
WBC	1·00 (0·99–1·01)	0·782	1·00 (0·99–1·01)	0·676
FLT3 ITD	1·41 (0·29–6·78)	0·666	2·86 (0·53–15·5)	0·223
CBF AML	0·77 (0·29–2·06)	0·603	0·66 (0·17–2·56)	0·543
BM morphology D15	1·65 (0·65–4·20)	0·297	1·29 (0·34–4·93)	0·711
BM morphology BC	3·09 (0·74–12·9)	0·121	0·65 (0·11–3·69)	0·624

Table 6. Residual disease (RD) before consolidation (BC) is an independent prognostic factor in childhood AML. *Extracted from Tierens A, Bjørklund E, Siitonen S, Marquart HV, Wulff-Juergensen G, Pelliniemi TT, et al. Residual disease detected by flow cytometry is an independent predictor of survival in childhood acute myeloid leukaemia; results of the NOPHO-AML 2004 study. Br J Haematol. 2016;174(4):600–9.*

1.2.3.2 MRD after first line therapy

Not all patients that have a good response to treatment are free of suffering a relapse in the future. That is the reason why continued monitoring of MRD after treatment have also been studied as a way to individualize therapy.

In 1998, Van Dongen et al. demonstrated that MRD measured by PCR amplification of antigen-receptor gene rearrangements in childhood ALL not only had important prognostic significance at the end of induction and before consolidation, but also correlated very well with relapse rates after first-line treatment, i.e. six of the eight MRD positive patients after completion of therapy subsequently relapsed (24).

However, most of the advances in the study of the ability of MRD monitoring after first-line treatment to predict relapse have occurred in the field of AML. For example, in 2003, Sievers et al. showed that flow cytometric evidence of AML at any time-point after induction treatment was an independent predictor of poor outcome (54).

With respect to the molecular level in AML, in 2006, Gorello et al. demonstrated that the expression level of *NPM1* mutations were directly correlated with the burden of disease and the clinical course in 13 AML patients (Figure 8) (55). In 2009, Schnittger et al. established that relapse after first-line therapy could be predicted through late assessment of MRD in *NPM1* mutated adult AML patients. In their study they found that a 100-fold increase of

NPM1 expression after one year since diagnosis was associated with a higher risk of relapse and a poorer EFS (56). Later, in 2011, Krönke et al. showed that MRD monitoring of *NPM1* mutations after conventional chemotherapy in 136 adult AML patients was able to anticipate relapse in an 84% of relapsed patients (36/43) when *NPM1* levels were above 200 copies *NPM1* mutated/10^4 *ABL* copies with a median time to relapse of 2.6 months (32). Finally, Shayegi et al. in 2012 found that a level of *NPM1* mutated expression relative to *ABL* expression above 1% after conventional chemotherapy was also associated with a poorer prognosis in terms of DFS and OS (57).

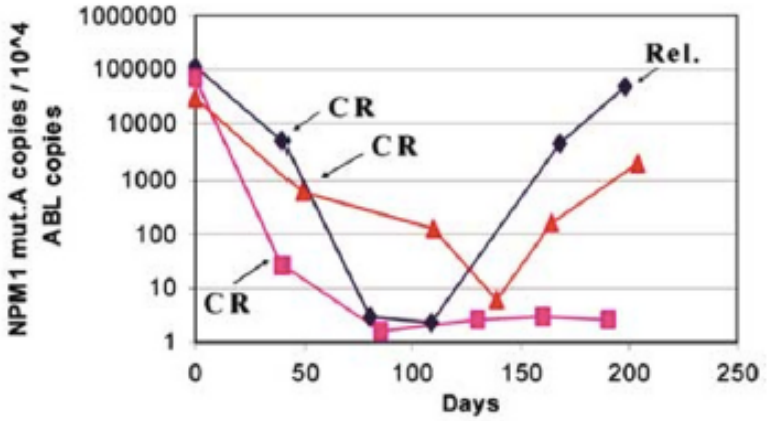

Figure 8. MRD measured by *NPM1* mutated expression is parallel to clinical course. CR: complete remission. Rel: relapse. Patient represented with triangles relapsed at day +234 albeit no material for molecular analysis was available. *Extracted from Gorello P, Cazzaniga G, Alberti F, Dell'Oro MG, Gottardi E, Specchia G, et al. Quantitative assessment of minimal residual disease in acute myeloid leukemia carrying nucleophosmin (NPM1) gene mutations. Leukemia. 2006;20(6):1103–*8.

In addition, other MRD molecular targets have demonstrated to be useful in predicting relapse in AML. In 2018, Matsuo et al. retrospectively analyzed the samples obtained from the pediatric patients enrolled in the Japanese Pediatric Leukemia/Lymphoma Study Group AML-05 study. In this report, they discovered that MRD levels of fusion gene transcripts of *RUNX1::RUNX1T1* and *MLL::AF9* were highly predictive of relapse; in the case of *RUNX1::RUNX1T1* when it exceeded from a threshold of 3000 copies and in the case of *MLL::AF9* with its simple detection (58). As well, in 2002, Cilloni et al. found that *WT1* levels during follow-up followed a similar pattern to that of other fusion gene transcripts in 10 AML patients and that *WT1* gene expression was highly consistent with clinical outcome of 5 AML patients who do not had a fusion gene transcript for monitoring (i.e. constantly in the normal range in three patients who remain in first complete remission, arising above the normal range four months before relapse in a patient who suffered a hematological relapse

and never reaching the normal range in the other patient who also suffered a hematological relapse) (47,48). In 2005, Weisser et al. corroborated this correlation between clinical course, fusion gene transcripts and *WT1* expression in a larger cohort of 116 adult AML patients (59) and, in 2019, similar results were reported by Juul-Dam et al. in a cohort of 30 children with AML (50).

1.2.3.3 MRD before transplantation

Over the years, MRD before transplantation has been positioned as one of the main prognostic factors for the outcome of transplantation, both in ALL and AML.

In ALL, three major retrospective studies reported initially an association between MRD before transplant and outcome (60–62). The first of them, analyzed the results of 64 children undergoing HSCT in the United Kingdom and found statistically significant differences between patients transplanted with positive and negative MRD in terms of EFS. A semi-quantitative approach of the PCR of the antigen-receptor rearrangements was used to measure MRD. As expected, those with pretransplant positive MRD had worse EFS (60). The second one reproduced the methodology of this study in 41 patients transplanted in Germany to clarify if different treatment protocols could influence these results, though similar results were found (61). The third study tested qPCR of antigen-receptor for this proposal in seventeen children transplanted by the Dutch Childhood Leukemia Study Group (DCLSG). They found also statistically significant differences among pretransplant MRD positive and negative patients, although MRD negative patients had a worse RFS than in the previous studies, probably due to the worse sensitivity of the technique (62). Krejci et al. pooled all this data and added the data of the pre-MRD BMT study group (involving centers from Czech Republic, Denmark, Germany, the Netherlands and the UK) to conclude a statistically significant association between pretransplant MRD classified into three levels (negative, low-level positive and high level positive) and 5-year EFS in the largest cohort published to that moment (Figure 9) (63).

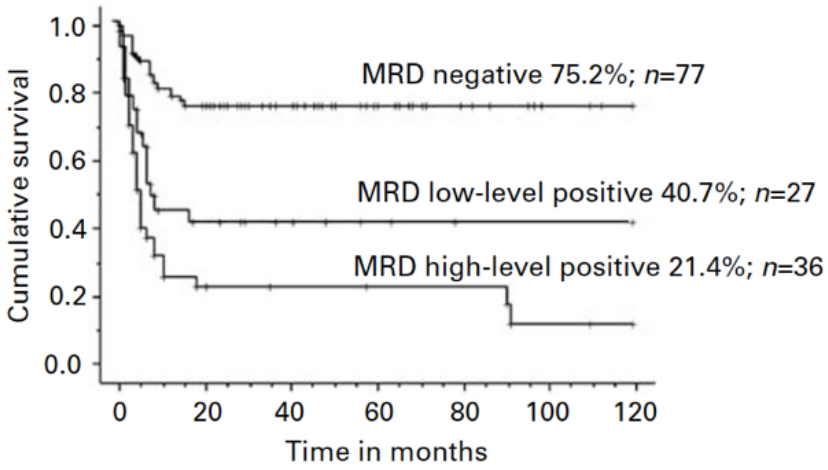

Figure 9. Pretransplant MRD is an important prognostic factor in ALL. MRD high level positive: $\geq 10^{-3}$. MRD low-level positive: $< 10^{-3}$. *Extracted from: Krejci O, van der Velden VHJ, Bader P, Kreyenberg H, Goulden N, Hancock J, et al. Level of minimal residual disease prior to haematopoietic stem cell transplantation predicts prognosis in paediatric patients with acute lymphoblastic leukaemia: A report of the Pre-BMT MRD Study Group. Bone Marrow Transplant. 2003;32(8):849–51.*

Later, other studies have corroborated these findings prospectively. In 2004, Gandemer et al. published the results of a French minimal residual disease-guided protocol in childhood ALL. They showed that a value of MRD 30 days before transplant measured by qPCR of the B cell receptor (BCR) or the T cell receptor gene rearrangements (TCR) $\geq 10^{-3}$ was associated with a reduction in OS and an increase in the CIR (64). In addition, between 1999 and 2005, Bader et al., conducted a prospective study in patients enrolled on the ALL-REZ 96 and 2002 protocols in which they showed that MRD over a threshold of 10^{-4} measured by qPCR of the BCR and TCR genes rearrangements was directly associated with a higher CIR and a lower probability of event-free survival (pEFS) (65). They also demonstrated that MRD before transplantation was an independent prognostic factor irrespective of sex, age at relapse, remission status, time point of relapse, immunophenotype, site of relapse, stem cell donor, T-cell depletion, time to transplantation and graft-versus-host disease (GVHD) (65). Regarding MFC, in 2016, Umeda et al. published a study in which they established that MRD status measured by MFC before transplantation also have a predictive value in DFS, OS and CIR, but no in non-relapse mortality (NRM) (66).

In AML, evidence that MRD before transplantation is a strong prognostic factor for the outcome is also available. In 2007, Buckley et al. published a meta-analysis of 19 studies including 1431 patients assessing prognostic significance of MRD before transplantation in childhood and adult AML, mainly assessed by MFC and *WT1* gene expression, in which they

showed that pre-transplant MRD was correlated with worse leukemia-free survival (LFS) and OS and a higher CIR, but not treatment related mortality (TRM) (67). In 2009, Jacobsohn et al. published a study in which they demonstrated that *WT1* gene expression levels in peripheral blood two weeks before transplantation were correlated with a lower EFS due to an increased rate of relapse (68). In 2011, Walter et al. conducted a retrospective study in 99 AML patients of all ages undergoing first myeloablative HSTC in which they proved that MRD measured by MFC before transplantation was also related with OS, DFS, CIR and NRM (69). And in 2015, Goswami et al. designed a multigene array improving the predictive value of *WT1* expression before transplantation. The multigene array, including *WT1*, *PRAME*, *CCNA1*, *PRTN3* and *MSLN* genes, identified the 100% of the patients who relapsed within 100 days after transplantation in contrast with the 57% using the *WT1* gene alone (70).

Finally, between 2002 and 2016, the I-BFM study group conducted a multicenter study in which they established three risk groups of relapse based upon MRD before transplantation measured by qPCR of AML recurrent genetic markers such as *RUNX1::RUNX1T1*, *CBFB::MYH11*, *KMT2A::MLLT3* or *FLT3*-ITD. The three established levels ($<2.1x10^{-4}$, $2.1x10^{-4}$ - $1x10^{-2}$, $>1x10^{-2}$) were associated with a 5-year EFS of 80.2%, 61.6% and 39.2% and a 5-year OS of 83.7%, 68.6% and 39.3%, respectively, illustrating how higher pre-transplant MRD was related with worse results (Figure 10). This study also demonstrated that MRD measured with this technique was an independent prognostic factor, together with disease status at transplantation (71).

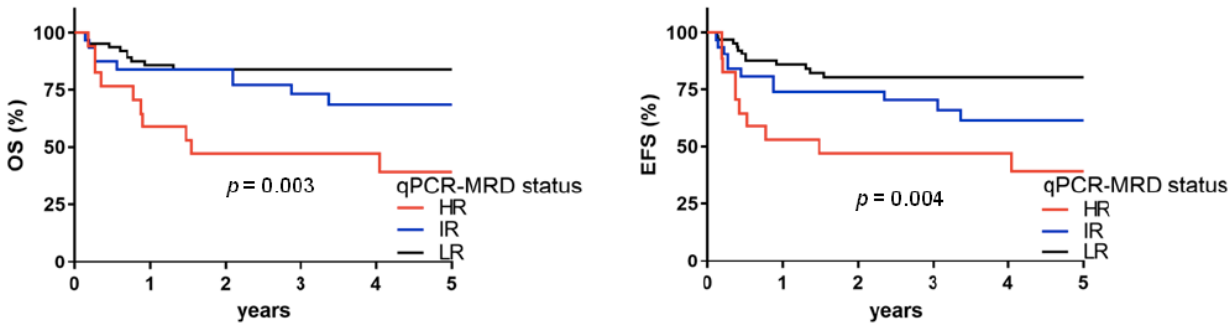

Figure 10. Pretransplant MRD measured through qPCR of recurrent genetic abnormalities is an important prognostic factor in AML. Low risk (LR): $<2.1x10^{-4}$, intermediate risk (IR): $2.1x10^{-4}$-$1x10^{-2}$, and high risk (HR): $>1x10^{-2}$. *Extracted from: Benetton M, Merli P, Walter C, Hansen M, Da Ros A, Polato K, et al. Molecular Measurable Residual Disease Assessment before Hematopoietic Stem Cell Transplantation in Pediatric Acute Myeloid Leukemia Patients: A Retrospective Study by the I-BFM Study Group. Biomedicines. 2022;10(7):1–15.*

There have also been studies addressing this issue for both ALL and AML. For example, in 2012, Leung et al. showed in a mixed cohort of ALL and AML patients that, although pretransplant MFC-MRD is an independent prognostic factor, even a fraction of patients with the highest pretransplant tumoral burden can be cured, not precluding the use of HSCT in very high-risk leukemia patients (72).

All these findings suggest that patients with a higher tumor burden before transplantation could benefit from both, treatment intensifications before transplantation and relapse prevention strategies after transplantation, as they have a higher risk of post-transplant relapse.

1.2.3.4 MRD after transplantation

Prognostic impact of MRD measures after transplantation have been another field of intense discussion and discovering. As early as in 1998, Knechtli et al., established a relationship between having persistence or rising levels of MRD, measured by PCR of antigen-receptor gene rearrangements, at any time after transplantation and relapse in children with ALL (73). Later, in 2015, Bader et al. demonstrated that MRD $\geq 10^{-4}$ measured by qPCR of BCR and TCR genes rearrangements after transplantation at different time points (+30, +60, +90, +180 and +365 days) was also associated with less EFS and higher CIR in childhood ALL (74).

Concerning to NGS, the same year, Pulsipher et al. showed that MRD measured through IgH-V(D)J NGS was superior to MFC to discriminate prognostic groups based upon negative or positive MRD both before and after transplantation (75). Additionally, in 2017, Kotrova et al. proved that NGS of antigen receptor rearrangements (both in B and T ALL) was more specific than qPCR of BCR or TCR genes rearrangements in the posttransplant setting, due to the presence of false positives in the latest technique when it was positive at low levels. They explained these false positives as an unspecific primer binding in regenerative cells during hematologic recovery (76).

Nonetheless, not only MRD monitoring after transplantation has been developed in ALL. MRD monitoring also has demonstrated its utility in the post-transplantation setting in AML. In 2009, Pozzi et al. showed that levels of *WT1* gene expression above 100 copies/10^4 ABL copies was the strongest predictor of relapse in the post-transplant setting in a cohort of 122 AML patients (77). Months later, on the subject of mutated genes, Shayegi et al. established a threshold of 10% associated with a high risk of relapse when monitoring MRD through RT-qPCR of *NPM1* mutations in transplanted patients (57).

There have been also studies that have simultaneously addressed the significance of MRD both before and after the HSCT. For instance, in 2015, Rossi et al. verified the relationship between a higher MRD both, before and after transplantation, and a minor DFS in 30 transplanted adult AML patients. Also, they compared the prognostic performance of MFC and *WT1* gene expression; they built ROC curves for each technique at every studied time-point (before transplantation and 1 and 3 months after transplantation) and calculated the diagnostics values, finding that MFC was more reliable for prognosis estimation before transplantation and *WT1* gene expression after transplantation (78).

Later, in 2018, Lovisa et al. corroborated the prognostic value of pre-transplant and post-transplant MRD measured by qPCR of antigen receptor rearrangements in childhood ALL. They found statistically significant differences in 10-year EFS according to three different MRD groups before transplantation (MRD negative, MRD positive $< 10^{-3}$ and MRD positive $\geq 10^{-3}$) and statistically significant differences in EFS according to MRD in the first trimester post-transplant, in the third trimester post-transplant and all the possible variations of the aforementioned three groups between pretransplant MRD and first trimester MRD, and first trimester MRD and third trimester MRD (Figure 11, in the next page) (79).

In addition, there have been studies assessing the predictive values of other techniques in both, ALL and AML. For instance, in 2009, Börnhauser et al. demonstrated that a decrease of donor chimerism in transplanted patients in CD34+ sorted peripheral blood cells, was almost an unavoidable sign of relapse within a median time of 61 days (80).

In general, with different cutoffs and at different time points, the higher MRD, the higher the risk of relapse.

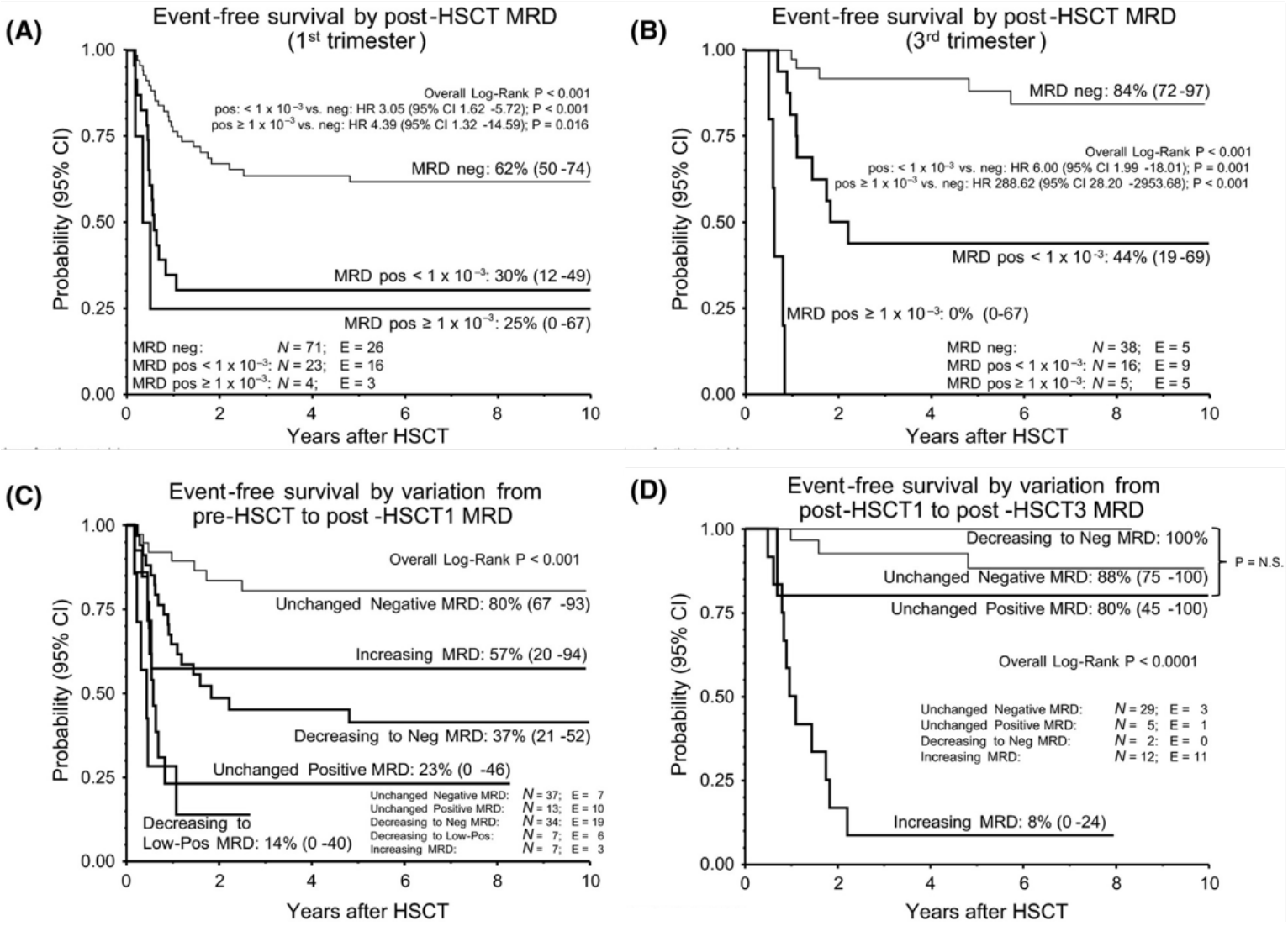

Figure 11. EFS according to post-transplantation MRD in childhood ALL. A. EFS according to post-transplant MRD level in the 1st trimester. B. EFS according to post-transplant MRD in the third trimester. C. EFS according to the variation from pre-transplant MRD to 1st trimester MRD. D. EFS according to evolution from 1st trimester MRD to 3rd trimester MRD. *Extracted from: Lovisa F, Zecca M, Rossi B, Campeggio M, Magrin E, Giarin E, et al. Pre-and post-transplant minimal residual disease predicts relapse occurrence in children with acute lymphoblastic leukaemia. Br J Haematol. 2018;180(5):680–93.*

1.2.4 MRD study to guide preemptive therapy

As long as not only MRD at fixed timepoints (i.e. at the end of induction or pre-transplantation), but also MRD evolution (i.e. after first-line therapy or after transplantation) predicts outcome, the possibility of administering preemptive treatment in those with an unfavorable evolution to improve outcome has arisen.

1.2.4.1 A case of success: acute promyelocytic leukemia (APL)

Acute promyelocytic leukemia is a paradigm of success of preemptive treatment after front-line therapy. After proving that the reappearance of the associated genetic alteration (*PML-RARa*) to this subtype of leukemia was directly correlated with the risk of relapse (81,82), physicians decided to intervene before hematological relapse was reached. In 1999, Lo Coco et al. treated with ATRA (all-trans retinoic acid) and adjuvant chemotherapy 14 APL patients in molecular relapse after first complete remission and found that survival from molecular relapse was superior to that present in a previous study in which patients were treated at frank relapse (83). Later, in 2007, Esteve et al. corroborated this finding in a larger cohort of 52 patients (16 in molecular relapse and 36 in hematological relapse); treating acute promyelocytic leukemia in molecular relapse, rather than in hematological relapse, offered advantages in OS and in CIR when a molecular complete response was achieved (84).

These two major studies led to the inclusion of the preemptive treatment in the recommendations formulated in 2009 by an expert panel (85). Afterwards, research has continued to improve the management of these patients. For example, later the same year these recommendations were published, Grimwade et al. showed in a cohort of 406 APL patients that, although persistent or recurrent MRD positivity was the strongest and the most independent prognostic factor for clinical relapse and RFS, preemptive treatment with arsenic trioxide (ATO) and gentuzumab ozogamincin (GO) avoided the relapse in a high proportion of patients (10/14) when administered (86).

1.2.4.2 MRD study to guide preemptive therapy after conventional chemotherapy

MRD guided preemptive therapy after conventional chemotherapy is less developed than in the post-transplantation setting. In part, because efforts have been focused on patients at highest risk of relapse, who are usually those who undergo transplantation.

In 2018, Platzbecker et al. demonstrated in a cohort of 172 AML and 26 MDS adult patients, in complete remission, that treatment with azacytidine could prevent, or at least delay, relapse (55% of treated patients in this cohort received conventional chemotherapy as previous treatment). Patients were monitored prospectively through qPCR of *NPM1* mutations, leukemia-specific fusion genes such as *RUNX1::RUNX1T1* or *CBFB::MYH11* and analysis of donor-chimerism at intervals between 1 and 3 months (depending on the source of material, marker and level of expression) for 24 months. Treatment was administered with six cycles of azacytidine when an increase above 1% in NPM1 mutations or fusion genes or a drop of 80% or less in donor chimerism was detected. RFS at 6 months was significantly superior to that reported in previous observational studies (58% vs 30%) and a median time to relapse much longer (422 vs 61 days) (87). In childhood AML, an international collaborative phase II clinical trial to address this question is currently in progress (Eudra CT Number 2017-003422-32).

1.2.4.3 MRD study to guide preemptive therapy after HSCT.

The post-transplant setting offers a unique opportunity to preemptive therapy. Transplanted patients are usually high-risk of relapse and well-monitored patients in whom MRD allows to institute different treatments before frank relapse. Also, a main feature of HSCT, graft-versus-leukemia effect, can be used as a basis of a therapeutic approach. Immune modulation through early immunosuppression withdrawal and donor lymphocyte infusions (DLI) have been essayed as an application of this effect.

Early immunosuppression withdrawal has been proposed in adult ALL as a mechanism to avoid post-transplant relapse in nonmyeloablative HSCT (88,89). Treatment of post-transplant hematological relapse through immunosuppression withdrawal in adult AML and ALL patients have also been reported, although graft-versus-host disease blurred the results (90). On the contrary, Boatsman et al. reported a case of success in an infant ALL (91) and Juul-Dam et al. communicated a case of preemptive treatment in an 8-year-old AML patient monitored through *WT1* expression in peripheral blood with partial success (the patient initially responded to cyclosporin withdrawal but relapsed a few months later) (92). These studies show limited efficacy of this therapeutic approach on its own.

The results are different when combined with DLI, for which we have a little more evidence. For instance, in 2007, Dominietto et al. showed that DLI after immunosuppression withdrawal could improve OS and decrease CIR in a mixed cohort of 80 AML and ALL patients (44 MRD-, 19 MRD+ without DLI and 17 MRD+ with DLI) when administered

following an MRD-guided strategy in the post-transplant setting. MRD was measured through clone-specific PCR in ALL patients and *WT1* gene overexpression in AML patients (93). Patriarca et al. also recently reported an improvement in OS due to this approach when established prophylactically or preemptively in the post-transplant setting of acute leukemia in a retrospective study of the experience of 34 Italian centers. They compared the outcome of 24 AML and ALL (and mixed phenotype) patients receiving prophylactic or preemptive DLI with the outcome of 172 patients receiving this type of treatment in hematological relapse (94).

In a cohort of just AML patients, in 2012, Pozzi et al. also demonstrated that MRD-guided DLI could improve OS in the posttransplant setting. They monitored MRD through *WT1* expression in 122 AML patients; 84 were MRD negative, 21 were MRD positive but not received DLI and 16 were MRD positive and received DLI. Despite the fact that the absolute relapse rate was almost equal for the two groups, it occurred later in the DLI group, suggesting a partially effective graft-versus-leukemia effect (77).

Finally, Pochon et al. in 2015 proposed a MRD guided protocol for ALL in France and, although they corroborated that MRD positivity after transplantation was associated to smaller OS and higher CIR, unfortunately, they were not able to show any advantage on survival of patients receiving preventive/preemptive immune intervention (early cyclosporine withdrawal and DLI) based on pre- and post-transplant MRD, respectively (95).

However, not only preemptive therapy in the post-transplant setting is based on immune modulation. Several assays have been conducting to prove the efficacy of tyrosine kinase inhibitors (TKI) as preventive or preemptive treatment in the post-transplant setting in Philadelphia-positive (Phi+) ALL (96). For instance, Chen et al. investigated the role of imatinib in the post-transplant setting in a cohort of 82 Phi+ ALL patients. They found an improvement in OS and DFS in treated patients, preventively or preemptively, with the first generation TKI (97). Other studies have been conducted to evaluate the efficacy of other second-class inhibitors such as nilotinib or dasatinib in the posttransplant setting. Kang et al. published a case of a 23-year-old Phi+ ALL patient in who nilotinib achieved a molecular remission after a second allogeneic transplantation (98) and Caocci et al. reported a series of 10 patients in whom treatment with dasatinib negativized MRD after transplantation in 2 of 4 positive patients (99). As it can be seen, this is a very limited evidence and based on case series, and the usefulness of these approaches remains to be demonstrated in larger cohorts.

Ph-like ALL (but Phi-), a novel subtype of B-cell ALL with poor outcome, in which the gene expression profile is similar to that of Phi+ patients, carries many kinase-activating lesions that can also be targeted with this type of therapies (100).

In AML, the principal drug essayed in the post-transplant setting has been azacytidine. In 2012, Platzbecker et al. conducted a prospective study in which they treated AML patients with azacytidine when donor chimerism in sorted CD34+ peripheral blood cells was less than 80% (101). They didn't achieve to reduce the relapse rate, but they achieved to prolong the time to relapse from the 61 days in their previous study (80) to approximately 6 months, which could be a benefit from the patient to recover itself from early toxicities of the firs transplantation and undergo a second HSCT.

Another advantage of this type of therapies (either after conventional chemotherapy or after transplantation) is to provide us more time for donor search, which is sometimes a critical factor when making decisions about these patients.

1.2.5 Other aspects to consider in the monitoring of MRD

1.2.5.1 Bone marrow vs peripheral blood sampling

As far as more sensitive MRD detection methods were available, peripheral blood instead bone marrow sampling has been proposed in order to avoid this invasive procedure for patients.

In 1997, Brisco et al. showed in 35 paired samples of B-ALL that levels of MRD measured through PCR of the antigen receptor by the use of limiting dilutions (102) were approximately ten-fold lower in peripheral blood than in bone marrow (103). Later, in 2001, the same group communicated that, in 8 patients with B-ALL studied with the same method, hematological relapse was preceded in all cases by MRD detection in peripheral blood and that, in 7 out of those 8 patients, MRD increased exponentially during 8-10 weeks prior to relapse (104).

When studying both B and T-ALL, in 2002, van der Velden et al. analyzed paired samples of 62 precursor B-ALL (pB-ALL) and 22 T-ALL and found that, although MRD measured with qPCR of antigen-receptors levels were comparable between peripheral blood and bone marrow in T-ALL, this difference was wider in pBALL (105). The same year, Coustan Smith et al. conducted a similar study in 173 children with B-ALL and 53 children with T-ALL measuring MRD with flow cytometry obtaining similar results (106). These studies

suggested that MRD levels in bone marrow and peripheral blood are comparable in T-ALL because its thymic origin, whereas, in B-ALL, bone marrow levels are consistently higher than peripheral blood levels due to its bone marrow origin.

In AML, as early as 1993, Adriaansen et al. described how the course of MRD detected by double immunofluorescence staining for terminal deoxynucleotidyl transferase and some myeloid markers (CD13 and CD33) was parallel in both bone marrow and peripheral blood and related to the course of the disease (107). Later, in 2013, Shayegi et al. found that MRD evaluation through *NPM1* mutation analysis using qPCR, was almost ten folds lower in peripheral blood than in bone marrow when comparing 138 paired samples (57). Afterwards, in 2020, Juul-Dam et al, studied the utility of qPCR of recurrent fusion transcripts (*RUNX1::RUNX1T1*, *CBFB::MYH11*, *KMT2A::MLLT3* and *KMT2A::ELL*) monitoring during follow up and found that the persistence or the reappearance after primary treatment of these rearrangements in peripheral blood predicted relapse in all patients (Figure 12) (108).

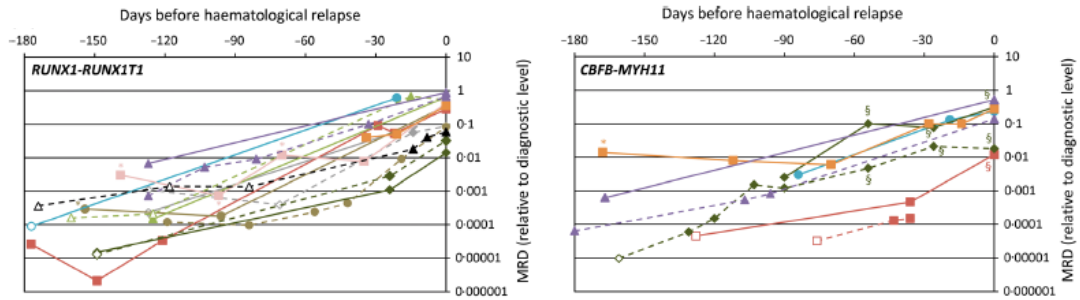

Figure 12. MRD monitoring of *RUNX1::RUNXT1* and *CBFB-MYH11* fusion transcript in peripheral blood (dashed lines) and bone marrow (solid lines) six months before hematological relapse. *Extracted from: Juul-Dam KL, Ommen HB, Nyvold CG, Walter C, Vålerhaugen H, Kairisto V, et al. Measurable residual disease assessment by qPCR in peripheral blood is an informative tool for disease surveillance in childhood acute myeloid leukaemia. Br J Haematol. 2020;190(2):198–208.*

However, authors found MRD positivity at low levels in bone marrow in CBF leukemias in continuous complete remission, proposing a threshold of $5x10^{-4}$ to establish the diagnosis of molecular relapse in bone marrow in this genetic subgroup. These findings were consistent with those described by Miyamoto et al. in 1996, in which multipotent progenitors of patients treated with chemotherapy continued to express *RUNX1::RUNX1T1* during long-term complete remission (109).

Regarding *WT1* expression, as aforementioned, the course of *WT1* expression has proved to be parallel to that of the disease and highly predictive of relapse in case of persistence or reappearance after first line therapy, also in peripheral blood (47,48,50,110). In addition, as

expression in peripheral blood is ten folds lower than in bone marrow, detection of *WT1* expression in peripheral blood cells is quite specific of disease (49,50).

All these findings support that MRD levels in peripheral blood are parallel to those detected in bone marrow and related to the clinical course of the disease, offering us an opportunity to monitor it without increase patient suffering. For this approach to be possible, sensible methods that can overcome the differences between peripheral blood and bone marrow MRD levels should be available.

1.2.5.2 Relapse kinetics and monitoring frequency

The study of relapse kinetics is crucial to plan and adequate monitoring frequency in order to detect MRD before relapse as earlier as possible. Slower relapses will require a wider frequency of monitoring while faster relapses will require a narrower monitoring frequency.

This topic has been extensively studied by Ommen et al. In 2008, they published a mathematical model based on the quantification of *WT1* expression in 89 AML patients, which allowed them to calculate the relapse detection fraction and the median time to relapse for different sampling intervals (Figure 13). They found that, although every four-month bone marrow sampling was required to obtain relapse detection fractions above 93% and median times to relapse above 74 days, an acceptable relapse detection fraction of 81% and a median time to relapse of 44 days could be obtained by bimonthly peripheral blood sampling (111).

$$\text{a) } RDR = \frac{\int_{t_a}^{0} F(t)dt}{0-t_a} \qquad \text{b) } \int_{t_m}^{0} F_1(t)dt = \int_{t_0}^{t_m} F_1(t)dt$$

Figure 13. a) Relapse detection rate for a given timespan, t_a to 0, is the integral of the relapse detection function (*F(t)*) in this timespan divided by it. Relapse detection function is inferred from the data of the study. In b) median time to relapse (t_m) can be solved from the equation. For more mathematical details see *Ommen HB, Nyvold CG, Brændstrup K, Andersen BL, Ommen IB, Hasle H, et al. Relapse prediction in acute myeloid leukaemia patients in complete remission using WT1 as a molecular marker: Development of a mathematical model to predict time from molecular to clinical relapse and define optimal sampling intervals. Br J Haematol. 2008;141(6):782–91.*

Afterwards, this group applied the same methodology to specific recurrent genetic abnormalities. In 2010, they studied the behavior of MRD analyzing *NPM1* mutations, *PML::RARA*, *RUNX1::RUNX1T1* and *CBFB::MYH11*. Based on 74 AML patients, they reported different median doubling times (the time in which the tumor burden doubles) for each subgroup and different relapse detection rates and median times to relapse depending on the genetic subgroup, the source of sample and the sampling interval. For example, they

inferred that bone marrow or peripheral blood monitoring every six months was sufficient in *CBFB::MYH11,* whereas bone marrow monitoring every four months was required to obtain similar relapse detection rates (around 90%) in *RUNX1::RUNX1T1* AML (112). In 2013, they did the same with MLL rearrangements finding a very high speed of relapse (doubling times of 12 days for *MLL::MLLT3* and 13 days for *MLL::MLLT4*) and, consequently, a need for a very frequent monitoring (every month in peripheral blood or bone marrow for a relapse detection rate of 85% and a median time to relapse of 25 days) (113).

1.2.5.3 Oligoclonality and clonal evolution

Oligoclonality and clonal evolution are two main issues when assessing MRD in acute leukemia. Oligoclonality refers to the presence of more than one clone at diagnosis and clonal evolution refers to the presence of changes in the genotype of these over the time. Models of clonal evolution have been proposed (114). Both situations together can cause the immunophenotype or the genetic alterations of the predominant clone in the relapse to be different from those of the initial diagnosis (Figure 14). This is a problem for MRD monitoring all along follow up, because these situations can be the cause of false negatives.

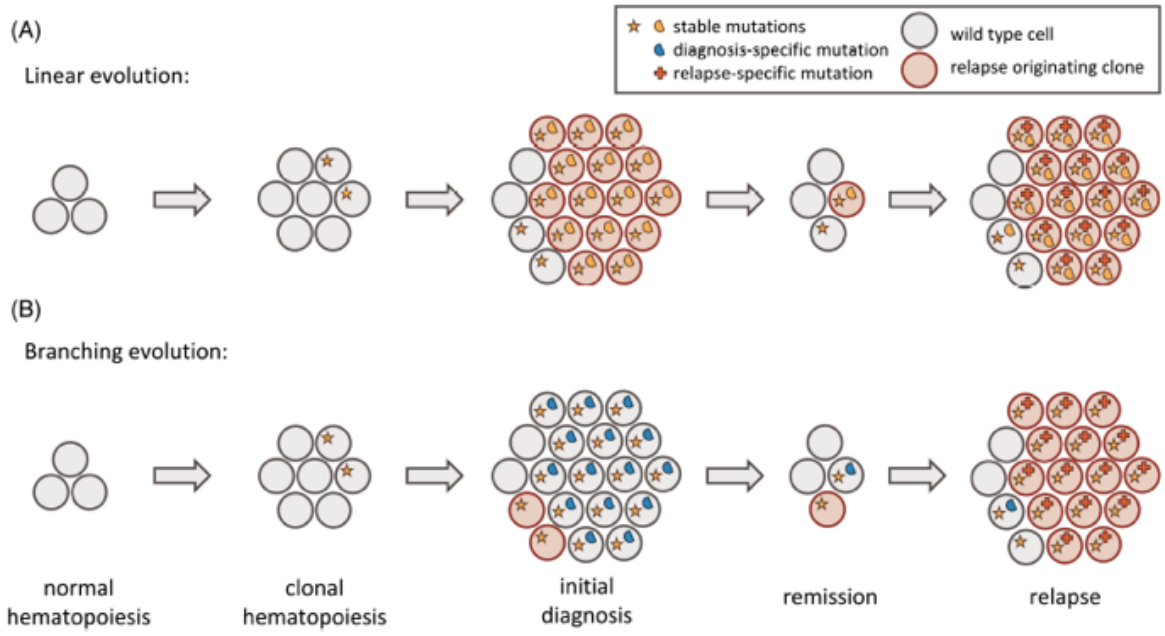

Figure 14. Models of clonal evolution over time. *Extracted from: Vosberg S, Greif PA. Clonal evolution of acute myeloid leukemia from diagnosis to relapse. Genes Chromosomes Cancer. 2019;58(12):839–49*

Regarding ALL, in 1994, Beishuizen et al. found differences in Ig and/or TcR gene rearrangements between diagnosis and relapse in 67% of pB-ALL and 50% of T-ALL in a cohort of 30 pB-ALL and 10 T-ALL. Despite this high frequency of immunogenotypic

changes, they observed the stability of at least one rearrangement in the three studied genes (IgH, TcR-γ and TcT-δ) in around 90% of leukemias. Consequently, they proposed to use at least two junctional regions of different genes to monitor MRD in ALL (115). Afterwards, in 1998, von Dongen corroborated the stability of at least one rearrangement in 34 of 36 patients (94%) when using two or three PCR targets, depending on their availability (24).

In AML, stability of *CBFB::MYH11* (116), *RUNX1::RUNX1T1* (116–118), *MLL* rearrangements (113) and *NPM1* mutations (19,56,119) has been described. In contrast, several changes between diagnosis and relapse have been found in *KIT* and *FLT3* status (116–118,120). These findings suggest that stable genetic alterations may play an essential role in primary leukemogenesis related to impairment in differentiation, while co-mutations are secondary hits that confer and advantage in proliferation (119). For that reason, only recognized stable genetic alterations should be used as MRD markers, although investigation about oligoclonality and clonal evolution based on wide genetic profiling is encouraged.

In summary, MRD monitoring after consolidation (either after conventional chemotherapy or HSCT) provides the opportunity to intervene when the tumor burden is low. Interventions cover a wide range (from immune-based therapies to target therapies passing through donor search). Moreover, MRD studies not only provide useful information regarding the tumor burden, which is directly correlated with the risk of relapse, but also important information about the biology of the re-emerging clone, which can be very useful to decide the appropriate targeted therapy.

In this thesis we have reviewed the current state of MRD monitoring in our hospital and explored new ways of undertaking this methodology through an international stay in a pioneering center in this topic.

II. Justification

Justification

Despite the improvements in survival in both lymphoblastic and myeloblastic childhood acute leukemia, relapse continues to be one of the main causes of treatment failure and death. The appearance of new diagnostic methods with increased sensitivity, together with the fact that tumor burden is an important prognostic factor, makes it advisable to update the concept of relapse and test new approaches to these situations.

Each one of the available MRD methods has its own strengths and weakness, and a deeper understanding of these differences is necessary to choose wisely the appropriate method according to our needs. In addition, as aforementioned, the utility of the different MRD methods not only reach the diagnostic level, but also the treatment level, providing different information about the biology of the tumor cell; information that can be used to bring forward tailored strategies against each individual cancer.

Furthermore, peripheral blood monitoring of MRD is a promising approach to avoid invasive procedures. Improved sensitivities of the new MRD techniques have allowed to obtain similar efficacy of detection in peripheral blood than in bone marrow with the commonly used methods in clinical practice. However, to delineate relapse kinetics according to different factors is important to design adequate personalized follow-up schemes.

This thesis was born with the intention of making a diagnosis of the current situation in this regard in our center and exploring new approaches to it, as peripheral blood MRD monitoring, with the goal of improving our clinical practice and contributing to the development of this important field in pediatric oncology.

For this purpose, we have divided this research into three parts, to make its reading more understandable and to be able to cover the entire range of aspects that this subject requires.

III. Objectives

Objectives

1. To review MRD measurements by the three methods available in our series of childhood AML and ALL treated in our center.

2. To evaluate if those measurements are predictable of an impending relapse, trying to establish which are the advantages or disadvantages for each method.

3. To analyze the impact of preemptive treatments when guided by the MRD monitoring

4. To evaluate the feasibility of MRD monitoring in peripheral blood as an incruent method for AML patients.

5. To analyze the role of not defining genetic alterations (co-mutations) and hematopoietic stem cell transplantation in AML relapse kinetics by monitoring defining genetic alterations in peripheral blood.

IV. Materials and methods

4.1 MRD to anticipate relapse and guide preemptive therapy in childhood AML

4.1.1 Patients and samples

For this purpose, we reviewed the clinical and laboratory data of pediatric patients with AML treated in the Clinical University Hospital Virgen de la Arrixaca between 2012 and 2022. Diagnostic criteria for the type and subtype of AML were based on the WHO classification of tumors of hematopoietic and lymphoid tissues (121,122). A patient initially diagnosed as AML and later reclassified as mixed phenotype acute leukemia was also included. Children with t(15;17) PML-RARα AML acute promyelocytic leukemia or Down Syndrome related AML were excluded since these AML subtypes are biologically different and receive specific treatment schedules. The end of data collection was on July 1st, 2022.

Cytomorphology, immunophenotype, cytogenetics (karyotype and FISH) and molecular results of BM aspirates were recorded at diagnosis, at the end of induction (day +21 or day +22) and other timepoints during therapy established by the corresponding treatment protocol. Transplanted patients were evaluated at days +30, +60, +90, +180 and +360 after transplantation. Other extra evaluations after therapy completion were performed according to clinician's criteria based on the presence of unexplained anemia, thrombopenia or neutropenia during follow-up. A total of 297 samples from 20 patients were evaluated.

Treatment was administered according to the SHOP-LMA-2007 protocol (https://www.recerca.com/shop/entrar/prot_pdf/LMA2007_PROTOCOLO.pdf) or the more recent NOPHO-DBH AML 2012 protocol (EudraCT number: 2012-002934-35). Patients were classified as low, high or very high risk in the SHOP-LMA-2007 protocol and as standard or high risk in the NOPHO-DBH-AML-2012 protocol according to the presence of particular cytogenetic abnormalities at diagnosis and treatment response (see table 7 for details). Patients diagnosed between 2016 and 2017 received an infusion of NK cells as consolidation instead of autologous BM transplantation or at the end of the protocolized treatment, as part of a phase II clinical trial (NCT02763475) (123,124). The patient diagnosed as the mixed phenotype acute leukemia received the first induction from the NOPHO-DBH AML 2012 protocol and consolidation, reinduction and maintenance from the current acute lymphoblastic leukemia protocol in our country, the SEHOP-PETHEMA 2013 protocol. In case of suspected regrowth of the disease after HSCT, early cyclosporine withdrawal, DLIs and/or boost of donor cells were applied as preemptive therapy.

Protocol	Risk	Details
SHOP-LMA-2007	Low	Patients with t(8;21) or inv(16), absence of -5 and -7, good response in peripheral blood at day +7 and in BM at day +21 and CR after 1st induction cycle.
	High	All patients not included as low risk or very high risk.
	Very High	Patients with -5 and -7.
NOPHO-DBH-AML	Standard	All patients not included as high risk.
	High	Patients that achieve CR after two induction courses and either of the following: poor response after course 1(>15%), intermediate response after course 2 (0.1-4.9%) or *FLT3*-ITD without NMP1 mutation.

Table 7. Criteria for risk classification according to treatment protocol in AML.
CR: Complete remission.

4.1.2 Immunophenotype and MRD studies

Immunophenotyping and MRD studies were performed in BM aspirates obtained at diagnosis and during follow-up by 8-color FACSCanto-II (from 2012 to June 2020) or 12-color FACSLyric (from June 2020 to 2022) flow cytometers (Becton Dickinson, BD, San Jose, CA). Photomultiplier (PMT) voltages were adjusted daily using CS&T beads (BD). Fluorescence compensations were adjusted using FC beads (BD) every two months and finely adjusted on a daily base using negative events as reference for each fluorochrome, as previously described (125). For cell surface staining, 100µL of BM samples diluted with PBS-1%BSA or concentrated using bulk lysing with ammonium chloride (BD) to contain 3 million total white cells were labeled during 10 minutes at room temperature in the dark with the appropriate amount of antibodies to detect the following molecules: CD3, CD4, CD7, CD11b, CD13, CD14, CD16, CD19, CD33, CD34, CD36, CD38, CD41, CD45, CD56, CD58, CD61, CD64, CD66, CD71, CD81, CD117, CD123, CD203c, CD235a (Glycophorin-A), CD300e (IREM2), CD371 and HLA-DR. Antibodies were purchased from BD, Beckman Coulter (Brea, California) or Dako (Glostrup, Denmark). Finally, samples were lysed with 3 ml FACSLysing (BD), washed with 3 ml of FACSFlow (BD) and 1 to 2 million cells acquired for each tube. For intracellular staining of myeloperoxidase (MPO), terminal deoxynucleotidyl transferase (Tdt) or CD3, IntraStaing kit (Dako, Denmark) was used following the manufacturer's instructions. The same antibody-fluorochrome combinations at diagnosis and follow-up were used. Details on combination and source of monoclonal antibodies can be seen in table 8 and 9.

Diva™ software (BD) was used for sample analysis. LAIPs were defined as clusters of cells displaying patterns of antigenic expression separated from normal myeloid maturation stages

(126). As previously described (127), aberrant immunophenotypes were divided into four main subgroups: 1) cross-lineage antigen expression, 2) asynchronous antigen expression, 3) antigen dim/strong expression and 4) antigen expression missing. Abnormal forward-scattered and side-scattered patterns were also included in the LAIP. MRD was defined in presence of a distinct cluster of at least 20 cells showing a compatible LAIP, to reach theorical maximum sensitivities ranging from 1 to 2 x 10^{-5}.

Canto-II (8 colors)	**Tube-1**	**Tube-2**	**Tube-3**	**Tube-4**	**Tube-5**
FITC	CD7[a]	CD66[c]	cyMPO[c]	cyTdt[a]	CD36[b]
PE	CD56[a]	CD13[a]	HLA-DR[a]	CD41/42/61[a]	CD123[a]
PE-Cy5 or PerCP	CD38[a]	CD38[a]	CD38[a]	CD19[a]	CD11b[b]
PE-Cy7	CD34[a]	CD34[a]	CD34[a]	CD34[a]	CD34[a]
APC or AF647	CD33[b]	CD33[b]	CD33[b]	GlicoA[a]	CD64[a]
APC-H7	CD45[a]	CD45[a]	CD45[a]	CD45[a]	CD45[a]
BV421 or V500	CD14[a]/CD19[a]	CD117[a]	CD14[a]	cyCD3[a]	CD4[a]
BV510	CD16[a]/CD3[a]	CD16[a]	CD16[a]	CD3[a]	CD3[a]

Lyric (12 colors)	**Tube-1**	**Tube-2**	**Tube-3**
FITC	CD4[a]/19[a]/66[c]/71[a]	CD36[b]	cyMPO[c]
PE	CD13/CD235a[a]	CD117[c]	CD41/CD61[a]
PE-Cy5 or PerCp	CD11b[a]	CD11b[b]	HLA-DR[a]
PE-Cy7	CD34[a]	CD34[a]	CD34[a]
APC or AF647	CD117[a]	CD64[a]	CD14[a]
APC-R700	CD38[a]	CD38[a]	CD38[a]
APC-H7	CD45[a]	CD45[a]	CD45[a]
BV421 or V450	CD16[a]	CD7[a]	CD58[a]
BV510	CD3[a]/CD300e[a]	CD123[a]	CD123[a]
BV605	CD33[a]	CD371[a]	CD4[a]
BV711	CD56[a]	CD56[a]	TIM-3[a]
BV786	HLA-DR[a]	HLA-DR[a]	CD203c[a]

Monoclonal antibodies were purchased from: [a] Becton Dickinson (San Jose, CA); [b] Beckman Coulter (Brea, California); or [c] Dako (Glostrup, Denmark).

Table 8. Combinations of monoclonal antibodies used in 8 or 12 color studies.

Monoclonal antibody	Manufacturer, reference	Clone
CD7 FITC	Becton Dickinson, 347483	124-1D1
CD66 FITC	Dako, F7112	Kat4c
cyMPO FITC	Dako, F0714	MPO-7
cyTdt FITC	Becton Dickinson, 332789	E17-1519
CD36 FITC	Beckman Coulter, B77224	FA6.152
CD56 PE	Becton Dickinson, 345810	MY31
CD13 PE	Becton Dickinson, 347406	L138
HLA-DR PE	Becton Dickinson, 347401	L243
CD41 PE	Becton Dickinson, 555467	HIP8
CD42 PE	Becton Dickinson, 555473	HIP27
CD61 PE	Immunostep, 61PE	VIPL2
CD123 PE	Becton Dickinson, 561050	9F5
CD38 PE-Cy5	Becton Dickinson, 551400	HIT2
CD19 PerCp	Becton Dickinson, 332780	4G7
CD11b PE-Cy5	Becton Dickinson, 555389	ICRF44
CD34 PE-Cy7	Becton Dickinson, 348811	8G12
CD33 APC	Beckman Coulter, IM2471	D3HL60.251
Glico-A APC	Immunostep, CD235A-100T	HI264
CD64 APC	Becton Dickinson, 561189	10,1
CD45 APC-H7	Becton Dickinson, 641417	2D1
CD14 BV421	Becton Dickinson, 565283	M5E2
CD19 BV421	Becton Dickinson, 562440	HIB19
CD117 BV421	Becton Dickinson, 562434	YB5.B8
cyCD3 V500	Becton Dickinson, 561417	UCHT1
CD4 BV421	Becton Dickinson, 562425	RPA-T4
CD16 BV510	Becton Dickinson, 563830	3G8
CD3 BV510	Becton Dickinson, 563109	UCHT1
CD4 FITC	Becton Dickinson, 345768	SK3
CD19 FITC	Becton Dickinson, 345776	4G7
CD71 FITC	Becton Dickinson, 333151	L01.1
CD235a PE	Becton Dickinson, 555570	GA-R2 (HIR2)
CD117 PE	Dako, R7145	104D2
HLA-DR PerCp	Becton Dickinson, 339216	L243
CD117 APC	Becton Dickinson, 333233	104D2
CD14 APC	Becton Dickinson, 345787	MΦP9
CD38 APC-R700	Becton Dickinson, 564979	HIT2
CD16 BV421	Becton Dickinson, 562874	3G8
CD7 BV421	Becton Dickinson, 562635	M-T701
CD58 BV421	Becton Dickinson, 566239	1C3
CD300e BV510	Becton Dickinson, 744993	UP-H1
CD123 BV510	Becton Dickinson, 563072	9F5
CD33 BV605	Becton Dickinson, 745229	P67.6
CD371 BV605	Becton Dickinson, 742931	50C1
CD4 BV605	Becton Dickinson, 562658	RPA-T4
CD56 BV711	Becton Dickinson, 563169	NCAM16.2
TIM-3 BV711	Becton Dickinson, 565566	7D3
HLA-DR BV785	Biolegend, 307642	L243
CD203c BV786	Becton Dickinson, 744244	NP4D6

Table 9. Details and source of monoclonal antibodies used in for immunophenotyping.

4.1.3 Fluorescent in situ hybridization (FISH)

Cytogenetic abnormalities were evaluated in interphase nucleus from total BM cells following standard procedures previously validated (128). The following FISH probes from Metasystems (Altlussheim, Germany) were used to evaluate 5q31 deletion (XL 5q31/5q33/5p15, cut-off 10%), 7q deletion (XL del(7)(q22q31), cut-off 9%), Cr-3 inversion (XL t(3;3) GATA2/MECOM DF, cut-off 10%), KMT2A rearrangements (XL KMT2A BA, cut-off 1%), t(8;21)(q22;q22) (XL AML1/ETO, cut-off 1%) and inv(16)(p13;q22) (XL CBFB/MYH11, cut-off 1%). For each probe 250 cells were analyzed with Metafer system (Metasystems). Up to 3000 cells were captured to increase sensitivity when needed for t(8;21) or KMT2A rearrangements.

4.1.4 Molecular studies

FLT3 internal tandem duplication mutations (*FLT3*-ITD) and mutation in the tyrosine kinase domain (*FLT3*-TKD) were evaluated in genomic DNA extracted from whole BM samples using QIAmp® DNA blood mini kit and QIAsymphony® (QIAgen, GmbH, Hilden, Germany). Previously described methods were used for detection of both FLT3-ITD (129) and FLT3-TKD (130) mutations with slight modification labelling forward primers at 5´ end with FAM fluorochrome to be resolved in ABI PRISM® 3500 Genetic Analyzer (Applied Biosystems, Foster City, CA).

NPM1 and CEBPA mutations and WT1 expression were evaluated in total RNA extracted from BM samples using QIAmp® RNA blood mini kit and QICube® (QIAgen). Complementary DNA (cDNA) was synthesized starting with 1µg of RNA using M-MLV reverse transcriptase and random primers (Thermofisher Scientific, MA, USA), and RNase inhibitor (MerK, Darmstadt, Germany). Previously described methods were used for detection of both NPM1 (55) and CEBPA (131). WT1 expression was evaluated using WT1 Profile QuantTM Kit (Ipsogen, Marseille, France) according to the manufacturer's protocol so WT1 levels were calculated conforming to the standard curve method.

Common chromosomal translocations in childhood leukemia as del(1p32) (STIL-TAL1), t(1;11)(p32;q23) (KMT2A-EPS15), t(1;11)(q21;q23) (KMT2A-MLLT11), t(1;19)(q23;p13) (TCF3-PBX1), t(3;5)(q25;q34) (NPM1-MLF1), t(3;21)(q26;q22) (RUNX1-MECOM), t(4;11)(q21;q23) (KMT2A-AFF1), t(5;12)(q33;p13) (ETV6-PDGFRB), t(5;17)(q35;q21) (NPM1-RARA), t(6;9)(p23;q34) (DEK-NUP214), t(6;11)(q27;q23) (KMT2A-AFDN), t(8;21)(q22;q22) (RUNX1-RUNX1T1), t(9;9)(q34;q34) (SET-NUP214), t(9;11)(p22;q23)

(KMT2A-MLLT3), t(9;12)(q34;p13) (ETV6-ABL1), t(9,22)(q34;q11) (BCR-ABL1), t(10;11)(p12;q23) (KMT2A-MLLT10), t(11;17)(q23;q21) (KMT2A-MLLT6), t(11;17)(q23;q21) (ZBTB16-RARA), t(11;19)(q23;p13.1) (KMT2A-ELL), t(11;19)(q23;p13.3) (KMT2A-MLLT1), t(12;21)(p13;q22) (ETV6-RUNX1) t(12;22)(p13;q11) (ETV6-MN1), t(15;17)(q24;q21) (PML-RARA), inv(16)(p13;q22) (CBFB-MYH11), t(16;21)(p11;q22) (FUS-ERG), t(17;19)(q22;p13) (TCF3-HLF), t(X;11)(q13;q23) (KMT2A-FOXO4) were evaluated using multiplex nested RT-PCR HemaVision®-28N Chromosomal Translocations kits (DNA Technology, Aarhus, Denmark) which detects 80 splice variants. In this case, reverse transcription was performed with a mixture of translocation-specific primers using the HemaVision® reagent module.

4.1.5 Definitions and statistical analysis

Complete remission was defined as < 5% leukemic cells in bone marrow with clear evidence of regeneration in the bone marrow or in the peripheral blood. Bone marrow relapse was defined as the presence of >5% leukemic cells in the BM cytomorphology after complete remission was achieved. Extramedullary relapse was defined as the development of extramedullary disease once complete remission was achieved.

The Kaplan-Meier method was used for survival estimation. Overall survival (OS) was defined as the time from diagnosis to death, with living patients censored on the date of last follow-up. Event-free survival (EFS) was defined as the time from diagnosis to relapse, progression or death, with event-free patients censored on the date of last follow-up. Cumulative incidence functions were calculated and compared according to the Fine and Gray method and the Gray's test, respectively. Comparison between qualitative variables were performed by the two-tailed Fisher's exact test. Data were collected in Excel (Microsoft Corporation, Redmond, USA) and analyzed in R version 3.6.3 (R Foundation for Statistical Computing, open-source software).

4.2 MRD to anticipate relapse and guide preemptive therapy in childhood ALL

4.2.1 Patients and samples

Regarding ALL, clinical and laboratory data of pediatric patients with ALL diagnosed in the Clinical University Hospital Virgen de la Arrixaca (Murcia, Spain) between June 2013 and February 2022 were reviewed. Diagnostic criteria for the type and subtype of ALL were based on the WHO classification of tumors of hematopoietic and lymphoid tissues (121,122). The end of data collection was on April 1st, 2022.

Treatment was administered according to the SEHOP-PETHEMA 2013 protocol in patients between 1 and 19 years old and according to our Spanish National INTERFANT-06 based treatment guidelines in patients under 1 year old. Patients were classified as standard, intermediate or high risk in SEHOP-PETHEMA 2013 protocol according to their age, white blood cell (WBC) count at diagnosis, immunophenotype, extramedullary infiltration, cytogenetics and early response to treatment. Patients younger than 1 year were classified as low, medium or high risk according to the *KMT2A* status, age, WBC count and treatment response. Details on risk classification can be seen in table 10.

Cytomorphology, immunophenotype, cytogenetics (karyotype and FISH) and molecular results of BM aspirates were recorded at each evaluation. Patients treated with the SHEOP-PETHEMA 2013 protocol were evaluated at different timepoints according to their risk group. Standard and intermediate risk patients were evaluated at diagnosis and at days +15, +33 and +78. High risk patients were evaluated at diagnosis, at days +15, +33, +52 (if complete remission was not achieved at day 33), +78, before the second high-risk intensification block if MRD was ≥ 0.01% at day +78, and at hematological recovery after the third intensification block. Patients treated with the INTERFANT-06 based treatment guidelines were evaluated at the following different timepoints: at diagnosis, at days +15 and +33, before MARMA and OCTADAD consolidation blocks, before maintenance, at week 43 of maintenance chemotherapy and at the end of the treatment. Patients undergoing stem cell transplantation were evaluated at days +30, +60, +90, +180 and +360 after transplantation. Other extra evaluations were performed according to clinician's criteria based on the presence of unexplained anemia, thrombocytopenia or neutropenia during follow-up.

Protocol	Risk	Details
SEHOP-PETHEMA 2013	Standard	The patient must meet each and every one of the following criteria: • Age >1 year and < 10 years • Leukocytes count < 20x109/L at diagnosis • Non-T immunophenotype • Absence of infiltration of the CNS and/or testes • Cytogenetics (one of the two criteria is sufficient): • High hyper diploidy (51-67 chromosomes), DNA index 1.10-1.44 (always confirmed by other cytogenetic techniques) • t(12;21) *ETV6::RUNX1* • No (1;19) *TCF3::PBX1* • No *KMT2A* rearrangement • Presence of < 1000 blasts/mm3 on day +8 of induction in peripheral blood • Presence of < 5% blasts and < 0.1% MRD in bone marrow on day +15 of induction and at the end of induction I'A.
	Intermediate	All patients not included as low risk or high risk.
	High	The existence of any of the following criteria determines the inclusion of the patients in this group: t(4;11) *KMT2A::AFF1* Hypodiploidy <44 chromosomes or DNA index <0.81 (confirmation by other techniques is required) ≥ 1000 blasts on day +8 of induction in peripheral blood > 25% blasts and >10% MRD on day +15 of induction in bone marrow MRD ≥ 1% on day +33 of induction in bone marrow MRD ≥ 0.1% before consolidation in bone marrow. Patients with ALL Ph+ until the COG/EsPhALL international protocol is available
INTERFANT-06	Low	*KMT2A* not rearranged
	High	*KMT2A* rearranged AND Age at diagnosis < 6 months AND WBC ≥ 300 x 109/L and/or prednisone poor response
	Medium	All other cases so including those with: *KMT2A* status unknown OR *KMT2A* rearranged AND age > 6 months OR *KMT2A* rearranged AND age < 6 months AND WBC < 300 x 109/L AND prednisone good response

Table 10. Criteria for risk classification according to treatment protocol.
MRD: measurable residual disease WBC: white blood cells.

4.2.2 Immunophenotype and MRD studies

Immunophenotyping and MRD studies were performed in BM aspirates obtained at diagnosis and during follow-up using 8-color FACSCanto-II (from June 2013 to June 2020)

or 12-color FACSLyric (from June 2020 to April 2022) flow cytometers (Becton Dickinson, BD, San Jose, CA). Photomultiplier (PMT) voltages were adjusted daily using CS&T beads (BD). Fluorescence compensations were adjusted using FC beads (BD) every two months and finely adjusted on a daily base using negative events as reference for each fluorochrome, as previously described (125). For cell surface staining, 100 uL of BM samples diluted with PBS-1%BSA or concentrated using bulk lysing with ammonium chloride (BD) to contain 3 million total white cells were labeled during 10 minutes at room temperature in the dark with the appropriate amount of antibodies to detect the following molecules: CD3, CD4, CD7, CD8, CD10, CD11b, CD13, CD14, CD15, CD16, CD19, CD20, CD22, CD33, CD34, CD38, CD45, CD45RA, CD56, CD58, CD66, CD71, CD79a, CD79b, CD81, CD117, CD235a (Glycophorin-A), CD300e (IREM2), Tdt, IgM, MPO, HLA-DR and CRLF2 (see tables 11 and 12 for details). Antibodies were purchased from BD, Beckman Coulter (Brea, California) or Dako (Glostrup, Denmark). Finally, samples were lysed with 3 ml FACSLysing (BD), washed with 3 ml of FACSFlow (BD) and 1 to 2 million cells acquired for each tube. For intracellular staining of myeloperoxidase (MPO), terminal deoxynucleotidyl transferase (Tdt) or CD3, IntraStaing kit (Dako, Denmark) was used following the manufacturer's instructions.

The same antibody-fluorochrome combinations at diagnosis and follow-up were used. DIVA^{TM} Software (BD) was used for sample analysis. LAIPs were defined as clusters of cells displaying patterns of antigenic expression separated from normal lymphoid maturation stages. Aberrant immunophenotypes were divided into four main subgroups: (1) cross-lineage antigen expression, (2) asynchronous antigen expression, (3) antigen dim/strong expression and (4) antigen expression missing. Abnormal forward-scattered and side-scattered patterns were also included in the LAIP. MRD was defined in presence of a distinct cluster of at least 20 cells showing a compatible leukemia associated immunophenotype (LAIP), to reach theorical maximum sensitivities ranging from 1 to 2 x 10-5.

Canto-II (8 colors)	Tube-1	Tube-2	Tube-3	Tube-4	Tube-5
FITC	CD15[a]	CD66[b]	CD19[c]	CD79a[a]	Tdt-Cyt[b]
PE	CD79b[b]	CD13[c]	IgM-Sup[b]	IgM-Cyt[b]	MPO[b]
PE-Cy5 or PerCP	CD38[c]	HLA-DR[c]	CD58[d]	CD10[c]	CD10[c]
PE-Cy7	CD19[c]	CD34[c]	CD10[c]	CD19[c]	CD19[c]
APC/AF647	CD14c/CD8[c]	CD33[d]	CD22[c]	CD34[c]	CD34[c]
APC-H7	CD45[c]	CD45[c]	CD45[c]	CD45[c]	CD45[c]
BV421/VB/PB	CD4[c]	CD19[c]	CD20[e]	CD20[e]	CD3-Cyt[c]
BV510	CD3[c]	CRLF2[c]	-	-	-
Lyric (12 colors)	**Tube-1**	**Tube-2**	**Tube-3**		
FITC	CD4[c]/19[c]/66b/71[c]	CD66[b]	Tdt-Cyt[b]		
PE	CD13c/CD235a[c]	CD22[c]	MPO-Cyt[b]		
PE-Cy5 or PerCp	CD11b[c]	CD10[c]	CD10[c]		
PE-Cy7	CD34[c]	CD34[c]	CD34[c]		
APC/AF647	CD117[c]	CD19[c]	CD19[c]		
APC-R700	CD38[c]	CD38[c]	CD38[c]		
APC-H7	CD45[c]	CD45[c]	CD45[c]		
BV421 or V450	CD16[c]	CD58[c]	CD7[c]		
BV510	CD3c/CD300e[c]	CD45RA[c]	CD45RA[c]		
BV605	CD33[c]	CD20[c]	CD20[c]		
BV711	CD56[c]	CD81[c]	CD3-Cyt[c]		
BV786	HLA-DR[f]	HLA-DR[f]	IgM-Cyt[c]		

Monoclonal antibodies were purchased from: [a]Cytognos (Salamanca, Spain); [b]Dako (Glostrup, Denmark); [c]Beckton Dickinson (San Jose, CA); [d]Beckman Coulter (Brea, California); [e]Miltenyi (Bergisch Gladbach, Germany); [f] BioLegend (San Diego, CA).

Table 11. Combinations of monoclonal antibodies used in 8 or 12 color studies.

Monoclonal antibody	Manufacturer, reference	Clone
CD15 FITC	Cytognos, CYT15F4	MCS-1
CD66 FITC	Dako, F7112	Kat4c
CD19 FITC	Beckton Dickinson, 345776	1D3
CD79a FITC	Cytognos, CYT79aF4	HM57
Tdt-Cyt FITC	Dako, F713950-2	HT-6
CD79b PE	Dako, R7272	SN8
CD13 PE	Becton Dickinson, 347406	L138
IgM-Sup, Cyt PE	Dako, R5111	Rabbit anti-Human
MPO, -Cyt PE	Dako, R720901	MPO-7
CD38 PE-Cy5	Becton Dickinson, 551400	HIT2
HLA-DR PerCp	Becton Dickinson, 339216	G46-6
CD58 PE-Cy5	Beckman Coulter, IM3702	AICD58
CD10 PerCp	Becton Dickinson, 563508	HI10a
CD19 PE-Cy7	Becton Dickinson, 341113	SJ25C1
CD34 PE-Cy7	Becton Dickinson, 348811	8G12
CD10 PE-Cy7	Becton Dickinson, 341112	HI10a
CD14 APC	Becton Dickinson, 345787	MφP9
CD8 APC	Becton Dickinson, 345775	SK1
CD33 APC	Beckman Coulter, IM2471	D3HL60.251
CD22 APC	Becton Dickinson, 333145	S-HCL-1
CD34 APC	Becton Dickinson, 345804	8G12
CD45 APC-H7	Becton Dickinson, 641417	2D1
CD4 BV421	Becton Dickinson, 562425	RPA-T4
CD19 BV421	Becton Dickinson, 562440	HIB19
CD20 VioBlue	Miltenyi, 130-113-378	LT20
CD3-Cyt Pacific Blue	Becton Dickinson, 558117	UCHT1
CD3 BV510	Becton Dickinson, 563109	UCHT1
CRLF2 BV510	Becton Dickinson, 563340	1F11
CD4 FITC	Becton Dickinson, 345768	SK3
CD71 FITC	Becton Dickinson, 333151	L01.1
CD235a PE	Becton Dickinson, 555570	GA-R2
CD22 PE	Becton Dickinson, 337899	S-HCL-1
CD11b PE-Cy5	Becton Dickinson, 555389	ICRF44
CD117 APC	Becton Dickinson, 333233	104D2
CD19 APC	Becton Dickinson, 345791	SJ25C1
CD38 APC-R700	Becton Dickinson, 564979	HIT2
CD16 BV421	Becton Dickinson, 562874	3G8
CD58 BV421	Becton Dickinson, 566239	1C3
CD7 BV421	Becton Dickinson, 562635	M-T701
CD300e BV510	Becton Dickinson, 744993	UP-H1
CD45RA BV510	Becton Dickinson, 563031	HI100
CD33 BV605	Becton Dickinson, 745229	P67.6
CD20 BV605	Becton Dickinson, 740333	L27
CD56 BV711	Becton Dickinson, 563169	NCAM16.2
CD81 BV711	Becton Dickinson, 740789	JS-81
CD3-Cyt BV711	Becton Dickinson, 563725	UCHT-1
HLA-DR BV786	BioLegend, 307642	L243
IgM-Cyt BV786	Becton Dickinson, 740998	G20-127

Table 12. Details and source of monoclonal antibodies used in for immunophenotyping.

4.2.3 Fluorescent in-situ hybridization (FISH)

Cytogenetic abnormalities were evaluated in interphase nucleus from purified B or T lymphocytes using RosetteStepTM Human B Cell Enrichment Cocktail or RosetteStepTM Human T Cell Enrichment Cocktail (Stemcell Technologies, Grenoble, France), respectively, following standard procedures previously validated (128). The following probes from Metasystems (Altlussheim, Germany) were used for the diagnosis of B ALL: XCE 4/10/17 (cut-off 2% for trisomies and 10% for monosomies), XL t(12;21)ETV6/RUNX1 DF (cut-off 1%), XL E2A BA (cut-off 10%), BCR/ABL1/ASS1 (cut-off 1%), XL KMT2A BA (cut-off 10%) and XL CDKN2A Deletion Probe (cut-off 9%). In the case the former assays were negative, since June 2019, we tried to identify genetic alterations linked to Ph-like B-ALL through the following probes: XL 5q32 PDGFRB BA (cut-off 10%), XL JAK2 BA (cut-off 10%) and XL ABL2 BA (cut-off 10%) from Metasystems and CRLF2 Breakapart probe (cut-off 10%) from Cytocell. For the diagnosis of T-ALL we used the following probes from Metaysystems: XL BCR/ABL1/ASS1 (cut-off 1%), XL KMT2A BA (cut-off 10%), XL CDKN2A Deletion Probe (cut-off 9%), XL 6q21/6q23 Deletion Probe (cut-off 10%) and XL TLX3 BA (cut-off 10%). For each probe 250 cells were analyzed with Metafer system (Metasystems). Up to 3000 purified B or T cells were captured to increase sensitivity when needed.

4.2.4 Molecular studies

Common chromosomal translocations in childhood leukemia as del(1p32) (STIL-TAL1), t(1;11)(p32;q23) (KMT2A-EPS15), t(1;11)(q21;q23) (KMT2A-MLLT11), t(1;19)(q23;p13) (TCF3-PBX1), t(3;5)(q25;q34) (NPM1-MLF1), t(3;21)(q26;q22) (RUNX1-MECOM), t(4;11)(q21;q23) (KMT2A-AFF1), t(5;12)(q33;p13) (ETV6-PDGFRB), t(5;17)(q35;q21) (NPM1-RARA), t(6;9)(p23;q34) (DEK-NUP214), t(6;11)(q27;q23) (KMT2A-AFDN), t(8;21)(q22;q22) (RUNX1-RUNX1T1), t(9;9)(q34;q34) (SET-NUP214), t(9;11)(p22;q23) (KMT2A-MLLT3), t(9;12)(q34;p13) (ETV6-ABL1), t(9,22)(q34;q11) (BCR-ABL1), t(10;11)(p12;q23) (KMT2A-MLLT10), t(11;17)(q23;q21) (KMT2A-MLLT6), t(11;17)(q23;q21) (ZBTB16-RARA), t(11;19)(q23;p13.1) (KMT2A-ELL), t(11;19)(q23;p13.3) (KMT2A-MLLT1), t(12;21)(p13;q22) (ETV6-RUNX1) t(12;22)(p13;q11) (ETV6-MN1), t(15;17)(q24;q21) (PML-RARA), inv(16)(p13;q22) (CBFB-MYH11), t(16;21)(p11;q22) (FUS-ERG), t(17;19)(q22;p13) (TCF3-HLF), t(X;11)(q13;q23) (KMT2A-FOXO4) were evaluated using multiplex nested RT-PCR HemaVision®-28N Chromosomal Translocations kits (DNA Technology, Aarhus,

Denmark) which detects 80 splice variants. In this case, reverse transcription was performed with a mixture of translocation-specific primers using the HemaVision® reagent module.

4.2.5 Definitions and statistical analysis

Complete remission was defined as < 5% leukemic cells in bone marrow with clear evidence of regeneration in bone marrow or in peripheral blood. Bone marrow relapse was defined as the presence of ≥ 25% leukemic cells in the BM cytomorphology after complete remission was achieved. Extramedullary relapse was defined as the development of extramedullary disease once complete remission was achieved. Extramedullary disease was defined according to standard criteria.

The Kaplan-Meier method was used for survival estimation. Overall survival (OS) was defined as the time from diagnosis to death, with living patients censored on the date of last follow-up. Event-free survival (EFS) was defined as the time from diagnosis to relapse, progression or death, with event-free patients censored on the date of last follow up. Cumulative incidence functions were calculated and compared according to the Fine and Gray method and the Gray's test, respectively. Comparisons between qualitative variables were performed by the two-tailed Fisher's exact test. MRD negative patients were defined as those with no reappearance while MRD positive are those with reappearance after previous negative results. Detectable FISH below the established threshold of sensitivity were considered as negative results. Data were collected in Excel (Microsoft Corporation, Redmond, USA) and analyzed in R version 3.6.3 (R Foundation for Statistical Computing, open-source software).

4.3 Impact of co-mutations and HSCT in AML relapse kinetics

4.3.1 Patients and samples

This part of the thesis includes childhood and adult AML patients who achieved a complete response and subsequently experienced relapse preceded by available real-time quantitative polymerase chain reaction (qPCR) MRD measurements in peripheral blood (PB) and or bone marrow (BM) of fusion transcripts (*RUNX1::RUNX1T1*, *CBFB::MYH11* and *MLL::AF9*) or *NPM1*mut. For the adult cohort, we reviewed the laboratory data of all patients under 75 years treated at the Department of Haematology of Aarhus University Hospital from the 1st of January 2012 to 1st of November 2022. For the children's cohort, we used the data from children treated in the Nordic Countries (Denmark, Sweden, Norway and Finland) previously reported (108) and additional patients enrolled in the NOPHO-DBH AML 2012 Protocol in the Nordic countries and The Netherlands from the end of data collection of the aforementioned study (December 2015) until 1st of November 2022.

Peripheral blood samples were collected for qPCR MRD analyses every 1-2 months from end of therapy until 2 years from diagnosis or until relapse. Patients with at least two positive MRD samples in PB before relapse without receiving any type of treatment were included. In cases where informative PB qPCR MRD values were not available, MRD measurements from BM were used if available.

4.3.2 Polymerase chain reaction

qPCR reactions were performed according to the standardized protocol established by the Europe Against Cancer (EAC) program (45). Details on preparatory procedures and the qPCR reactions were conducted as previously described (108,112). MRD quantification was performed using the relative quantification (ΔΔCt) method, where the MRD level at diagnosis was set to one and all subsequent measurements were expressed relative to this.

4.3.3 KIT, KRAS, NRAS and FLT3 status determination

KIT, *KRAS*, *NRAS* and *FLT3* genes status was determined by next generation sequencing (NGS) or, in the case of mutations of *KIT* and *FLT3* in some patients, by PCR.

The PCR-based *KIT* and *FLT3* mutation analysis were performed by PCR amplification of genomic DNA and subsequent fragment analysis as described previously (130,132,133). *FLT3* mutation analysis by this latter method included (internal tandem duplication) ITD and tyrosine kinase domain (TKD) mutations (D835 and I836).

For the adults, all but five (n=25) have complete molecular characterization via the Sophia Genetics Myeloid Panel. Among the other five patients, *FLT3* gene was studied by PCR in two patients. For the children, Danish children (n= 8) were analyzed using either the PCR method or the Sophia Genetics Panel. All other (Sweden, Norway, Finland and The Netherlands, n= 16) were analyzed with whole exome sequencing (WES) according to the following reference (134).

4.3.4 Relapse kinetics and statistical analyses

Hematological relapse was defined according to standard criteria (135). Definitions on molecular MRD assessment were used as established by the European Leukemia Net in its latest recommendations (37). The exponential increase of MRD was calculated based on samples leading up to either hematological or molecular relapse before institution of any therapy (including tapering of immunosuppression or donor lymphocyte infusion). Based on positive measurements, we estimated the slope of the regression line of the logarithmic transformation of the MRD and assessed the regrowth rate by $\log_{10}$ increments normalized to 30 days intervals (Figure 15).

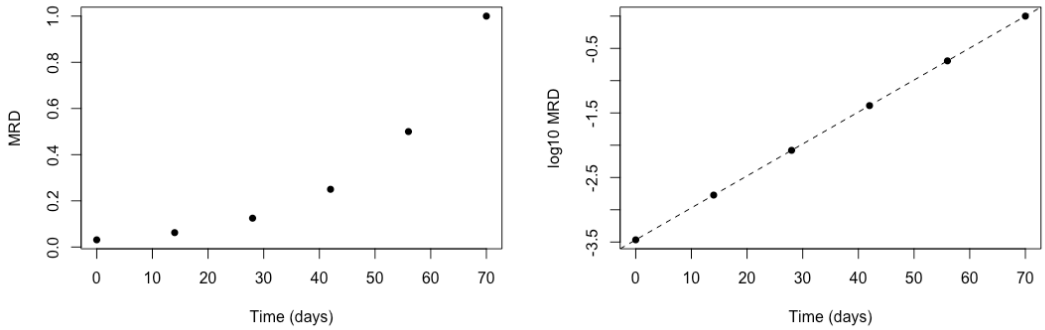

Figure 15. The slope of the MRD log-transformed regression line was used to assess the regrowth rate by $\log_{10}$ MRD increments, normalized to 30 days intervals.

To compare the $\log_{10}$ increments per 30 days between the main genetic subgroups, *i.e.* core binding factor leukemias (CBF), *KMT2A* rearranged (*KMT2A*-r) and *NPM1* mutated (*NPM1*mut), we used the student's t-test when the prerequisites of normality and homoscedasticity were fulfilled and the Wilcoxon rank-sum test when not. Subsequent relapses in the same patient were excluded to avoid violation of the principle of independence between groups. For the association of the speed of relapse with the studied genes status and HSTC, we used the $\log_{10}$ increment per 30 days as a dependent variable and the *KIT*, *RAS* genes (*KRAS* and *NRAS* combined into a variable) and *FLT3* status (*FLT3*-ITD and *FLT3*-D835 considered separately), and HSCT as independent variables in a

multivariable linear regression analysis. To minimize the impact of outliers, we used the *lmrob* robust linear regression method included in the robust base package (136,137). All the analysis were performed with R software version 4.2.2.

V. Results

5.1 MRD to anticipate relapse and guide preemptive therapy in childhood AML

5.1.1 Characteristics and outcomes

We included 20 AML patients with a mean age at diagnosis of 6.58 years (range 0.3-16.58 years) and a male:female ratio of 1.5. Biological, clinical, therapeutic and evolutionary characteristics according to the FAB classification can be seen in table 13.

	Myeloblastic (M0-2)[1]	**Promyelo and myelomonocytic (M3-4)**[1]	**Monocytic (M5a/b)**[1]	**Megakarioblastic (M7)**[1]
Number of cases	9	3	4	4
Mean age (years)	7.2	13.2	5.7	1.7
Gender (male, n (%))	4 (44)	3 (100)	3 (75)	2 (50)
Admission in our hospital				
At diagnosis, n (%)	8 (89)	2 (66)	4 (100)	4 (100)
After diagnosis, n (%)	1 (11)	1 (33)	0 (0)	0 (0)
PB-WBC at diagnosis				
<100 x 10^3/µl	6	2	3	4
>100 x 10^3/µl	2	-	1	-
Cytogenetics				
t(8;21)(q22;q22)	4	-	1	-
inv(16)(p13q22)	1	-	-	-
KTM2A-r	1	1	3	-
t(9;9)(q34;q34)	1	-	-	-
Risk stratification				
Standard, n (%)	7 (78)	1 (33)	2 (50)	-
High, n (%)	2 (22)	2 (66)	2 (50)	2 (50)
Very high, n (%)	-	-	-	2 (50)
Treatments				
LMA-SHOP-2007	2	2	2	4
NOPHO-DBH-AML-2012	7	1	2	-
HSCT, n (%)	3 (33)	1 (33)	0 (0)	4 (100)
Clinical outcome				
Mean follow-up (days)	1090	1635	1501	1765
Relapse, n (%)	2 (22)	1 (33)	0 (0)	2 (50)
Death, n (%)	2 (22)	1 (33)	0 (0)	2 (50)

Table 13. Biological, clinical, therapeutic, and evolutionary features of the included AML patients.
[1] M1, n=2; M2, n=7; M3 without t(15;17), n=1; M4, n=2; M5a, n=2; M5b, n=2; M7, n=4.

The diagnosis of AML was established before referral to our hospital in two patients. Twelve out of 20 patients (60%) had a specific chromosomal rearrangement, distributed as follows: five patients had a t(8;21) *RUNX1::RUNX1T1* (25%), five patients had *KMT2A* rearrangement (25%), one patient had an inv(16) *CBFB::MYH11* (5%) and one patient the t(9;9) *SET::CAN* (5%). Among patients with a *KMT2A* rearrangements, we found two t(9;11)(p21;q23) *KMT2A::MLLT3*, one t(11;17)(q23;q12) *KMT2A::MLLT6*, one

t(6;11)(q27;q23) *KMT2A::AFDN* and one with a rare *KMT2A* rearrangement detected by FISH whose partner was identified in the Diagnostic Center for Acute Leukemia in Frankfurt as ins(11;X)(q23;q28q12) *KMT2A::FLNA.*

Ten patients were treated according to the SHOP-LMA-2007 protocol (8 high risk and 2 very high risk) and ten patients were treated according to the NOPHO-DBH AML 2012 protocol (9 standard-risk and 1 high-risk patient). Three out of ten patients treated with the SHOP-LMA-2007 protocol and one of ten patients treated with the NOPHO-DBH AML 2012 protocol received infusions of NK cells as part of a phase II clinical trial (NCT02763475). In total, 8 children out of 20 (40%) received allogeneic HSCT, 5 in first complete remission and 3 in second complete remission.

Pre-emptive treatment was used in four transplanted patients guided by the MRD results. In two patients, early cyclosporine withdrawal was used 113 days and 47 days before relapse respectively, and DLI were used 100 days and 34 days before relapse respectively. In a patient with a t(6;11), DLI were used 75 days before relapse. In a patient with a t(9;9), DLI were used 16 days before relapse. Mean follow-up was 1389 days (range 85-3446).

With the aforementioned treatment protocols, our patients with AML showed an estimated 3-year OS of 72.7% (IC95% 52.8-100) and an estimated 3-year EFS of 67.7% (IC95% 49.4-93) (Figure 16). The global relapse rate was of 25% (5 out of 20 cases). According to their genetic alteration, the relapse rate was as follows: one out 5 (20%) patients with t(8;21), one out of 5 (20%) patients with a *KMT2A* rearrangement, and the only patient (100%) with the t(9;9) relapsed. The patient with inv(16) did not relapse (0%). Among patients with a *KMT2A* rearrangement, the patient with the t(6;11) *KMT2A::AFDN* relapsed. Besides, 2 patients without specific chromosomal alterations relapsed.

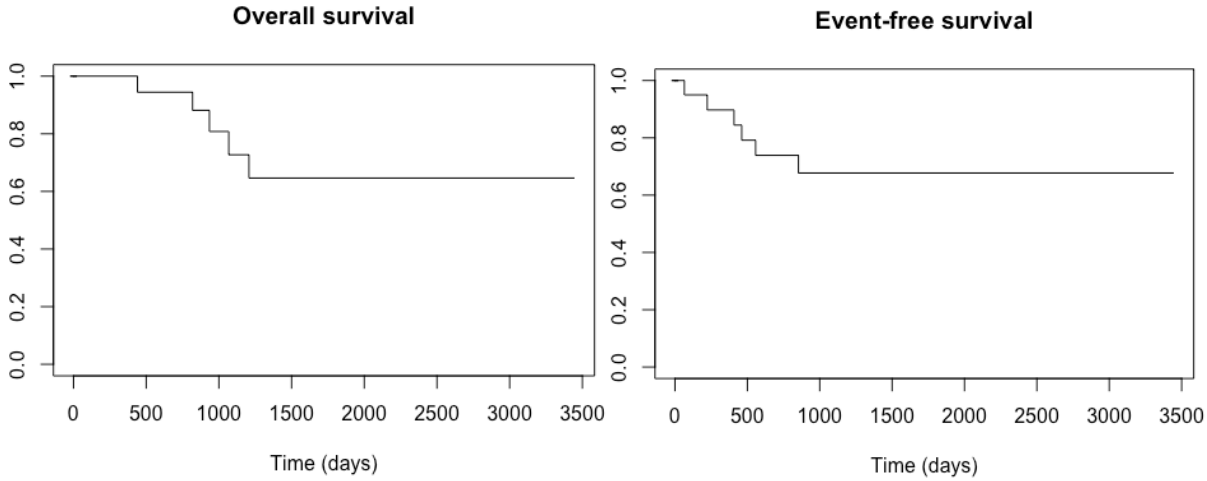

Figure 16. OS and EFS of the included AML patients.

5.1.2 Evolutionary patterns of MRD in AML patients

We detected two patterns of MRD presentation during the follow-up. In Figure 17 we illustrate these patterns with two representative cases (patients 10 and 13, respectively). In the first pattern, patients enter in complete remission and stay until the end of the follow up (Figure 17A). In the second pattern, relapse is preceded by the reappearance of the specific molecular and cytogenetic markers and the detection of MRD in the immunophenotype analysis, followed by a progressive normalization of these parameters after treatment intensification (Figure 17B).

As observed in Figure 17B, some patients experienced more than one relapse. In total, 10 relapses were observed in 5 patients: 9 BM relapses and 1 extramedullary relapse

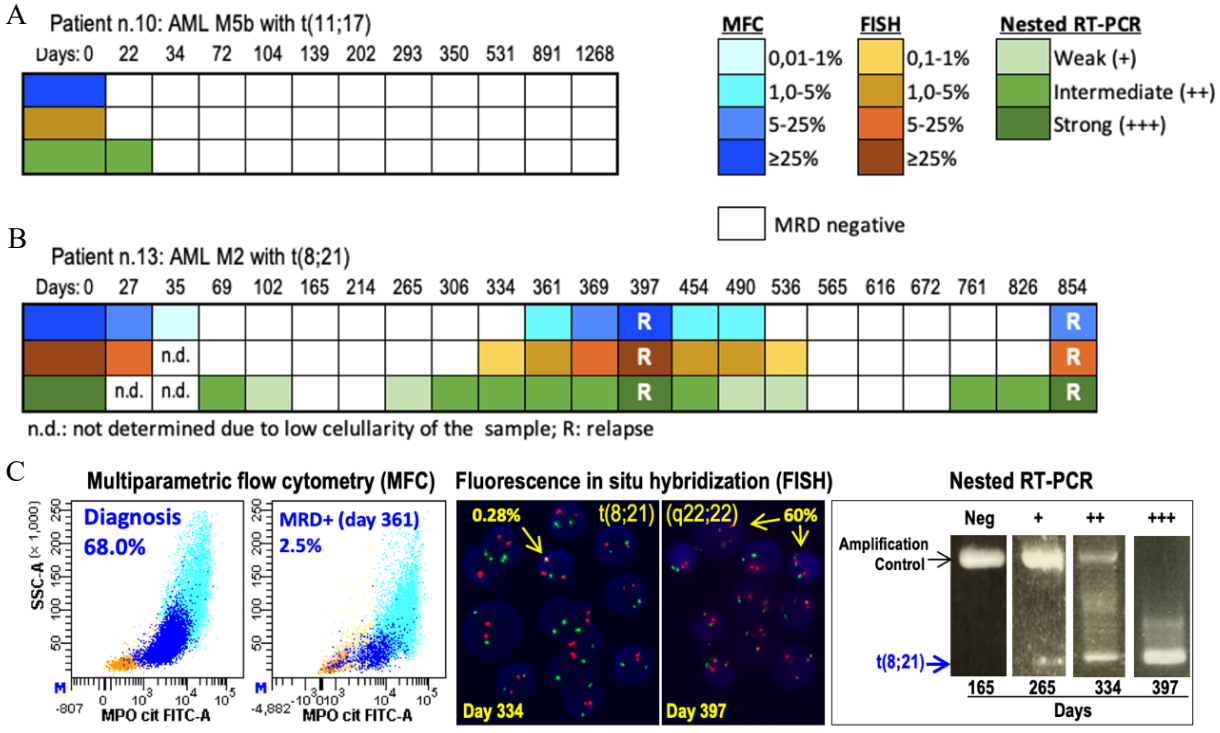

Figure 17. Evolutionary patterns of MRD and relapse in AML patients. A) The patient enters in complete remission and stays in remission until the end of follow up. B) Relapse is preceded by the reappearance and progressive increment of the measurable residual disease detected by nested reverse transcriptase polymerase chain reaction (RT-PCR), fluorescent in situ hybridization (FISH) and/or multiparametric flow cytometry (MFC). Progressive normalization of those markers is observed during the resolution of the relapse. C) Shows representative results for MFC, FISH and RT-PCR in bone marrow samples with different levels of leukemic infiltration.

See Figure 18 in the next page for a summary of the main events of all patients.

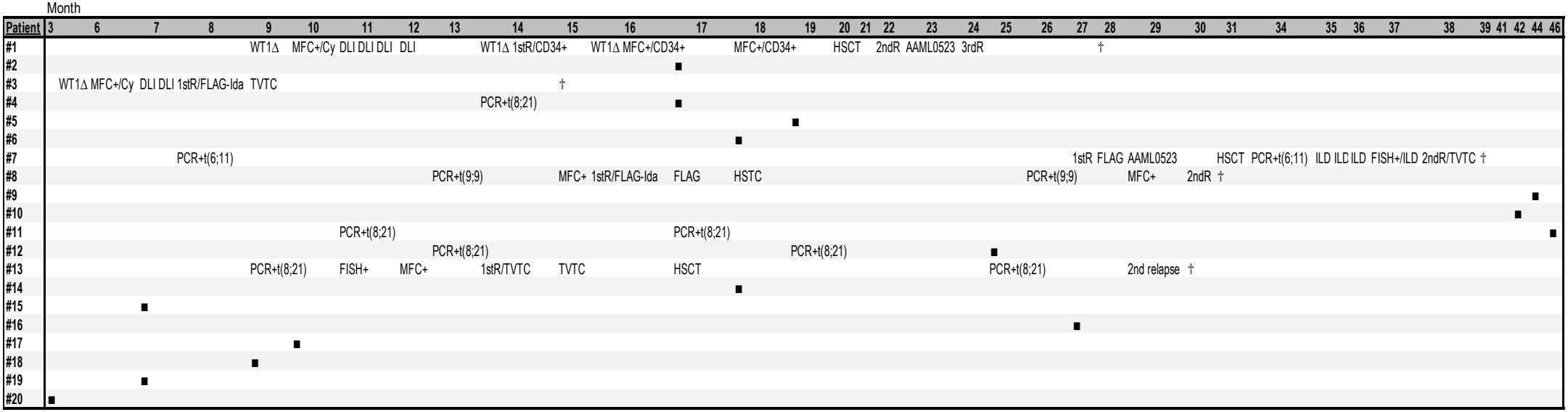

Figure 18. Time course of main events in the 20 included AML patients. R: relapse; MFC+: MRD reappearance in MFC after first line treatment; FISH+: MRD reappearance in FISH after first line treatment; PCR+: MRD reappearance in PCR after firs line treatment; WT1Δ: 1 log increase in two consecutives samples; DLI: donor lymphocyte infusions; Cy: early cyclosporine withdrawal; CD34+: boost of CD34+ cells; AAML0523: clofarabine and cytarabine; FLAG: fludarabine, cytarabine and G-CSF with or without idarubicin (Ida); TVTC: topotecan, vinorelbine, thiotepa and clofarabine; HSTC: hematopoietic stem cell transplantation after firs line treatment; ■ last bone marrow evaluation; and † death.

5.1.2.1 Multiparameter flow cytometry

Five of the nine BM relapses and the extramedullary relapse were preceded by the reappearance of MRD by multiparametric flow cytometry (p=0.0016) (Figure 19A). Though, the latter was probably a combined relapse but could not be confirmed due to the clinical status of the patient. The cumulative incidence of relapse according to MFC-MRD evolution is showed in Figure 19B. The median time from MRD detection by flow cytometry to relapse was 26 days (range 0-326). Of note, fifteen out of the twenty patients (75%) had an identifiable leukemia associated immunophenotype LAIP (see table 14). One of two patients in whom MFC failed to anticipate MRD detection had no specific LAIP.

Patient id.	LAIP	Personalized LAIP	Relapse
#1	Yes	CD45- CD34- DR- CD56++	Yes
#2	Yes	CD45dim CD34- DR- CD41/42/61++	No
#3	Yes	CD45dim CD34- DR- CD41/42/61+++	Yes
#4	No	-	No
#5	Yes	CD45dim CD34- DR- MPO++	No
#6	Yes	CD45dim CD34- CD41/42/61+	No
#7	No	-	Yes
#8	Yes	CD45dim CD7+	Yes
#9	Yes	CD34- CD 117+ DR+++ NG2+	No
#10	Yes	CD34- DR++ CD64+++ NG2+	No
#11	No	-	No
#12	No	-	No
#13	Yes	CD45dim CD34+ CD33- MPO+	Yes
#14	Yes	CD45dim CD34+ CD13+++ CD19+ MPO+	No
#15	Yes	CD45dim CD34+ CD33- CD56+ MPO+	No
#16	Yes	CD45 dim CD34+ CD13- CD19++ MPO++	No
#17	Yes	CD45dim CD34- CD4+ CD56+ MPO-	No
#18	Yes	CD45dim CD34- DR- CD38- MPO+++	No
#19	No	-	No
#20	Yes	CD45dim CD34- CD33- CD38-/+dim MPO+	No

Table 14. Leukemia associated immunophenotype (LAIPs).

5.1.2.2 RT-PCR and FISH

In patients showing a specific rearrangement that could be monitored by RT-PCR or FISH, all the relapses were preceded by the reappearance of the alteration. Also, all the reappearances of the genetic alteration after first-line treatment were followed by a

relapse (p=0.03), except in the case of core binding factor leukemias (t(8;21) *RUNX1::RUNXT1* and inv(16) *CBFB::MYH11*) where only 1 patient with a t(8;21) relapsed (Figure 19A). The cumulative incidence of relapse according to specific rearrangements is showed in Figure 19B. The median time from the reappearance of the specific translocation detected by RT-PCR to relapse was 111 days (range 90-575).

5.1.2.3 WT1 expression

In the seven patients monitored trough *WT1*/*ABL* expression ratio on BM cells, all the relapses were preceded by a molecular increase of *WT1*/*ABL*, but not all the molecular increases of *WT1* were followed by a relapse. However, one log increment of *WT1* expression in two consecutive evaluations was always associated with subsequent frank hematological relapse (p = 0.02) (Figure 19A). The cumulative incidence of relapse regarding the increment of *WT1* expression is showed in Figure 19B. The median time from a 1 log increment of *WT1* expression in two consecutive evaluations to relapse was 140 days (range 47-197). See Figure 20 to see evolution in *WT1*/*ABL* expression in all patients.

A

	Flow cytometry		KTM2A or t(9;9)		t(8;21)		1 log WT1 increment	
	MRD⁻	**MRD⁺**	**MRD⁻**	**MRD⁺**	**MRD⁻**	**MRD⁺**	**No**	**Yes**
No Relapse	14	0	4	0	1	4	5	0
Relapse	4	6	0	4	0	1	0	3
	p = 0.0016		p = 0.03		p = 1.00000		p = 0.02	

B

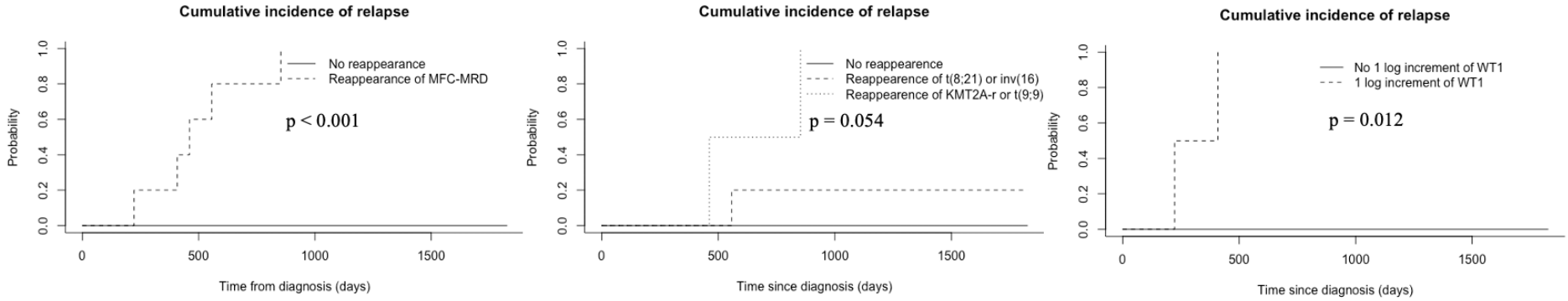

Figure 19. Association between MRD detection and posterior AML relapse. A. Crosstab showing the relationship between the reappearance of MRD detected by multiparametric flow cytometry, RT-PCR and FISH for KTM2A or t(9;9) and t(8;21) or inv(16), as well as by the one log increment of WT1 expression in two consecutive samples and the AML relapse. B. Cumulative incidence of relapse according to the reappearance of these rearrangements (left) or the one log increment of WT1 expression (right).

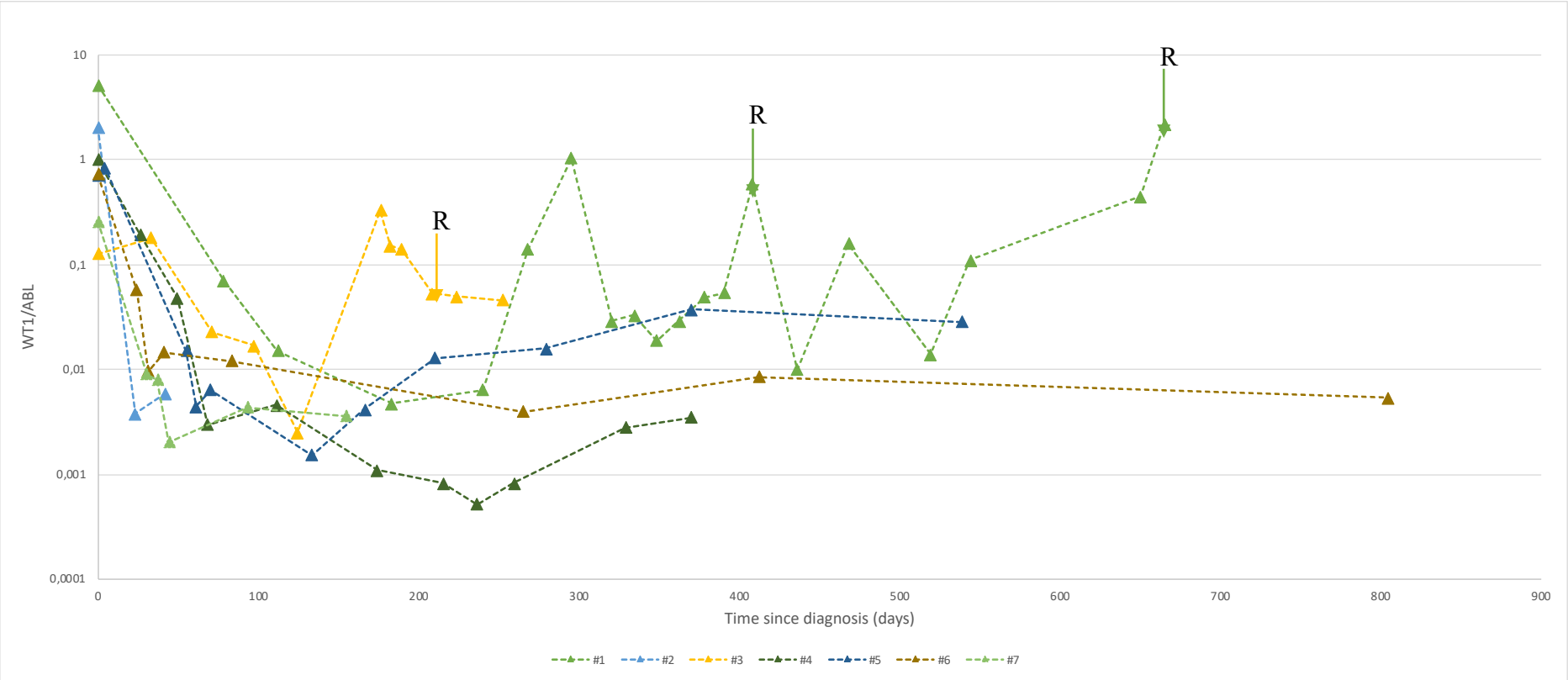

Figure 20. WT1/ABL expression in the seven patients monitored trough this diagnostic method.
R: relapse.

5.1.2.4 Comparison between the three MRD methods

Comparing the results of the three methods to evaluate MRD (MFC, RT-PCR and FISH) in BM aspirates of patients with a specific rearrangement, we identified seven samples with negative MFC and positive FISH and only two with positive MFC and negative FISH. Besides, we identified forty-nine samples with negative MFC and positive RT-PCR and just one with positive MFC and negative RT-PCR. Also, we identified forty-four samples with positive RT-PCR and negative FISH and no samples with positive FISH and negative RT-PCR (Table 15).

Patient id.	**Rearrangement**	**N. of evaluations[1]**	**MFC+**	**LAIP**	**FISH+**	**RT-PCR+**	**Relapse**
#4	t(8;21)	11	1	No	2	8	No
#7	t(6;11)	15	1	No	2	11	Yes
#8	t(9;9)	1	1	Yes	-[2]	1	Yes
#9	t(9;11)	1	1	Yes	1	1	No
#10	t(11;17)	13	1	Yes	1	2	No
#11	t(8;21)	19	1	No	3	11	No
#12	t(8;21)	18	1	No	2	10	No
#13	t(8;21)	20	7	Yes	8	14	Yes
#15	t(8;21)	8	1	Yes	2	6	No
#17	t(9;11)	8	4	Yes	2	3	No
#18	ins(11;X)	1	1	Yes	1	-[2]	No
#20	inv(16)	1	1	Yes	1	1	No

Table 15. Comparative analysis of MRD results detected by MFC, FISH and RT-PCR in patients with specific chromosomal rearrangement.

[1]Only evaluations during and after therapy in which the three diagnostic methods (MFC, FISH and RT-PCR) were carried out were considered in this analysis. This excludes evaluations in which the MFC analysis undoubtedly detected MRD+; in these cases, FISH or RT-PCR was not performed.

[2]In patients 8 and 18, t(9;9) and ins(11;X) could not be detected by FISH and RT-PCR respectively, because they were not included among the studied translocations by these methods as described in the methods section.

5.2 MRD to anticipate relapse and guide preemptive therapy in childhood ALL

5.2.1 Characteristics and outcome of patients

We included 80 patients with a mean age at diagnosis of 80 months (range 3 – 189 months). The cohort consisted of 47 male and 33 female patients. A total of 544 bone marrow samples were evaluated. Clinical and biological characteristics of patients and outcome are shown in table 16.

	B-ALL	T-ALL
Number of cases, n (%)[a]	67 (83.75%)	13 (16.25%)
Mean age at diagnosis (months)	81	75
Gender (male, n (%))	37 (55.2%)	9 (69.2%)
WBC at diagnosis		
<10 x 10^3/uL	41 (61.2%)	2 (15.4%)
10-50 x 10^3/uL	15 (22.4%)	4 (30.8%)
>50 x 10^3/uL	11 (16.4%)	7 (53.8%)
Treatment protocol		
SEHOP-PETHEMA 2013	65 (97%)	13 (100%)
INTERFANT-06	2 (3%)	0 (0%)
Risk at diagnosis[b]		
Standard, n (%)	21 (31.3%)	0 (0%)
Intermediate, n (%)	41 (61.2%)	13 (100%)
High, n (%)	5 (7.5%)	0 (0%)
Clinical outcome		
Mean follow-up (months)	46	60
Relapse, n (%)	5 (7.5%)	2 (15.4%)
Death, n (%)	4 (6%)	1 (7.7%)

Table 16. Clinical and biological characteristics of patients and outcome.
[a] All cases were included in the study at first diagnosis
[b] Low and medium risk infants are included in the table as standard and intermediate risk, respectively
Abbreviations: ALL, acute lymphoblastic leukemia, B, B-cell precursor; T, T-cell immunophenotype; WBC, white blood cell count

The prevalence of genetic alterations in the sixty-seven patients with B-ALL was as follows: fourteen patients (21%) had the t(12;21)(p13;q22) *ETV6::RUNX1*, two patients (3%) the t(1;19)(q23;p13) *TCF3::PBX1*, two patients (3%) the t(9;22)(q34;q11) *BCR::ABL*, two patients (3%) the t(4;11)(q21;23) *KMT2A::AFF1*, four patients (6%) the iAMP21 and twelve patients (18%) the 16p deletion. In the thirty patients in whom Ph-like related alterations were investigated we found one *JAK2* rearrangement (3%) and one *CRLF2* rearrangement (3%). Regarding the thirteen patients with T-ALL we found two patients (15%) with interstitial 1p32 deletion, two patients (15%) with *TLX3* rearrangement, one patient (8%) with t(12;21) *ETV6::RUNX1*, one patient (8%) with a

KMT2A rearrangement and nine patients (69%) with 16p deletion; four patients from this group had more than one alteration. Numeric alterations of the chromosomes (monosomies, trisomies or tetrasomies) were also found by FISH in 35 (52%) of the B-ALL and 1 (8%) of the T-ALL patients.

With the aforementioned treatment protocols, our patients with ALL showed an estimated 5-year OS of 94% (IC95% 88.3-100) and an estimated 5-year EFS of 84.1% (IC95% 75-94.2). The 5-year CIR was 12.1% (SE±4.6%) and the 5-year TRM was 3.8% (SE±2.2%). These results are presented in Figure 21.

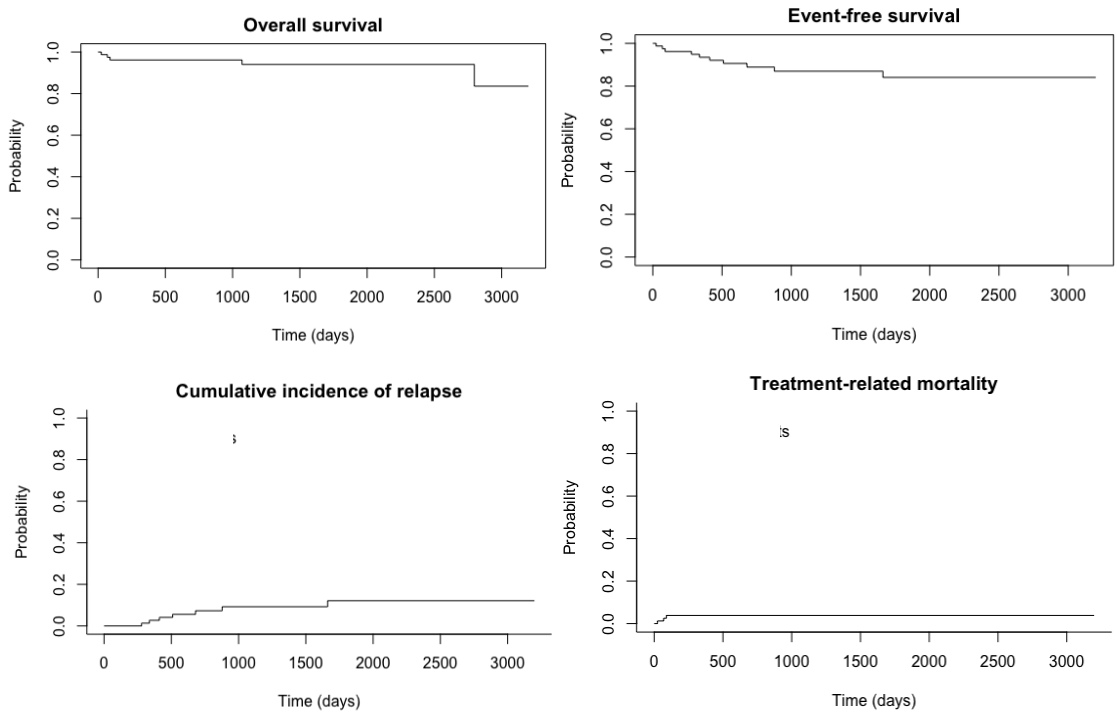

Figure 21. OS, EFS, CIR and TRM of ALL patients.

5.2.2 Evolutionary patterns of MRD in ALL patients

We observed two patterns of MRD presentation during follow-up. In figure 22 we illustrate these patterns with two representative cases (patients 52 and 61, respectively). In the first pattern (91.25% in our series), patients achieve complete remission and remain in complete remission until the end of follow-up (Figure 22A). In the second pattern, the reappearance of the specific molecular marker, cytogenetic alteration or characteristic immunophenotype is followed or coincident in time with overt relapse, except in some cases in which the continuation of the standard treatment or the introduction of additional interventions avoid this. Also, MRD normalized progressively after intensification of treatment in responders. A representative patient of this pattern is shown in figure 22B, a

B-ALL with a t(4;11), in which relapse was transiently aborted twice with Blinatumomab and hematopoietic stem cell transplantation (HSCT), although finally the patient relapsed. After relapse, cytoreductive and CAR-T therapies led to a new complete remission (MRD evaluations not shown because were performed at the CAR-T therapy center), but four months later the patient relapsed again with a myeloid immunophenotype. She achieved a new MRD-negative complete remission with a combination of fludarabine, cytarabine and idarubicin (FLAG-Ida) with Gentuzumab Ozogamycin and underwent a second HSCT as consolidation.

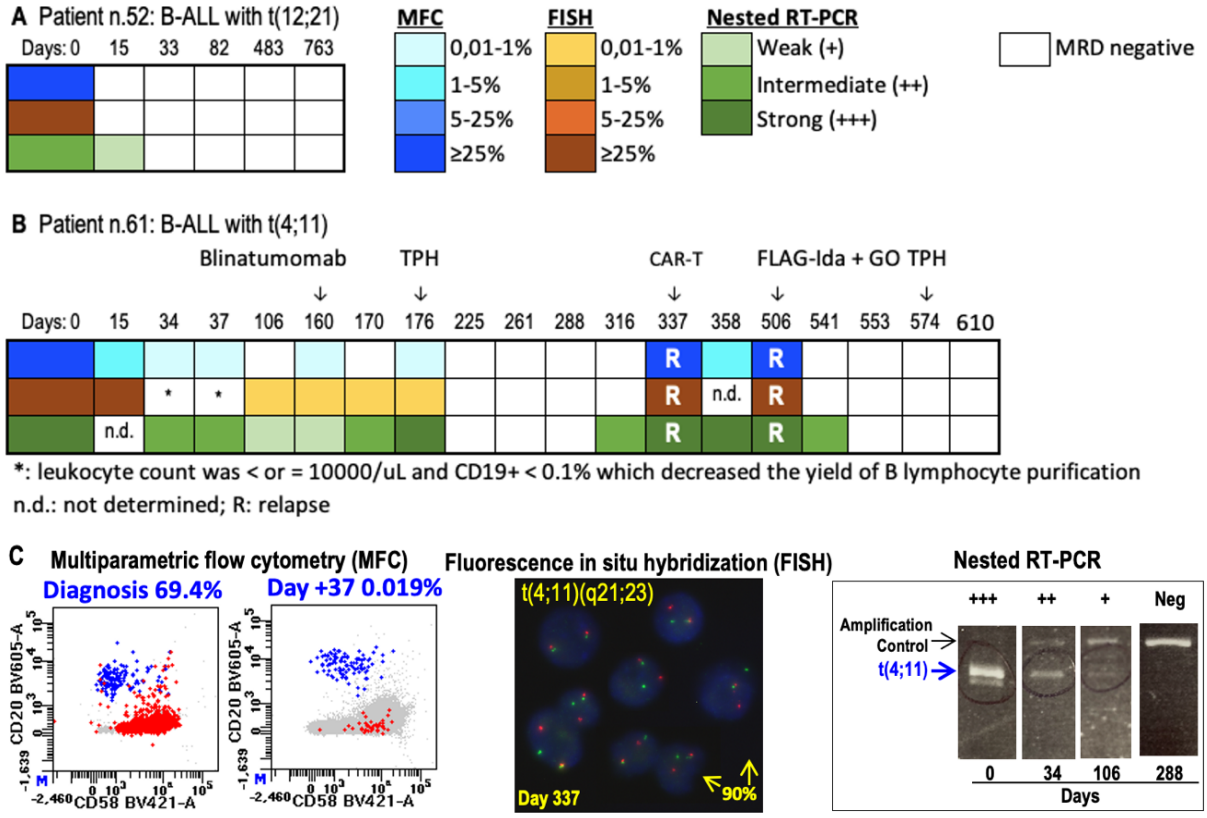

Figure 22. Evolutionary patterns of MRD and relapse in ALL patients. A) The patient achieves complete remission and stays until the end of follow up. B) Relapse is preceded by the reappearance and progressive increment of the minimal residual disease detected by multiparametric flow cytometry (MFC), fluorescent in situ hybridization (FISH) in purified B lymphocytes (or in total nucleated cells after myeloid shift) and/or nested reverse transcriptase polymerase chain reaction (RT-PCR). Progressive normalization of those markers is observed during the resolution of the relapse. C) Representative results for MFC, FISH and RT-PCR in bone marrow samples with different level of leukemic infiltration.

A summary of the main events of all patients is shown in Figure 23.

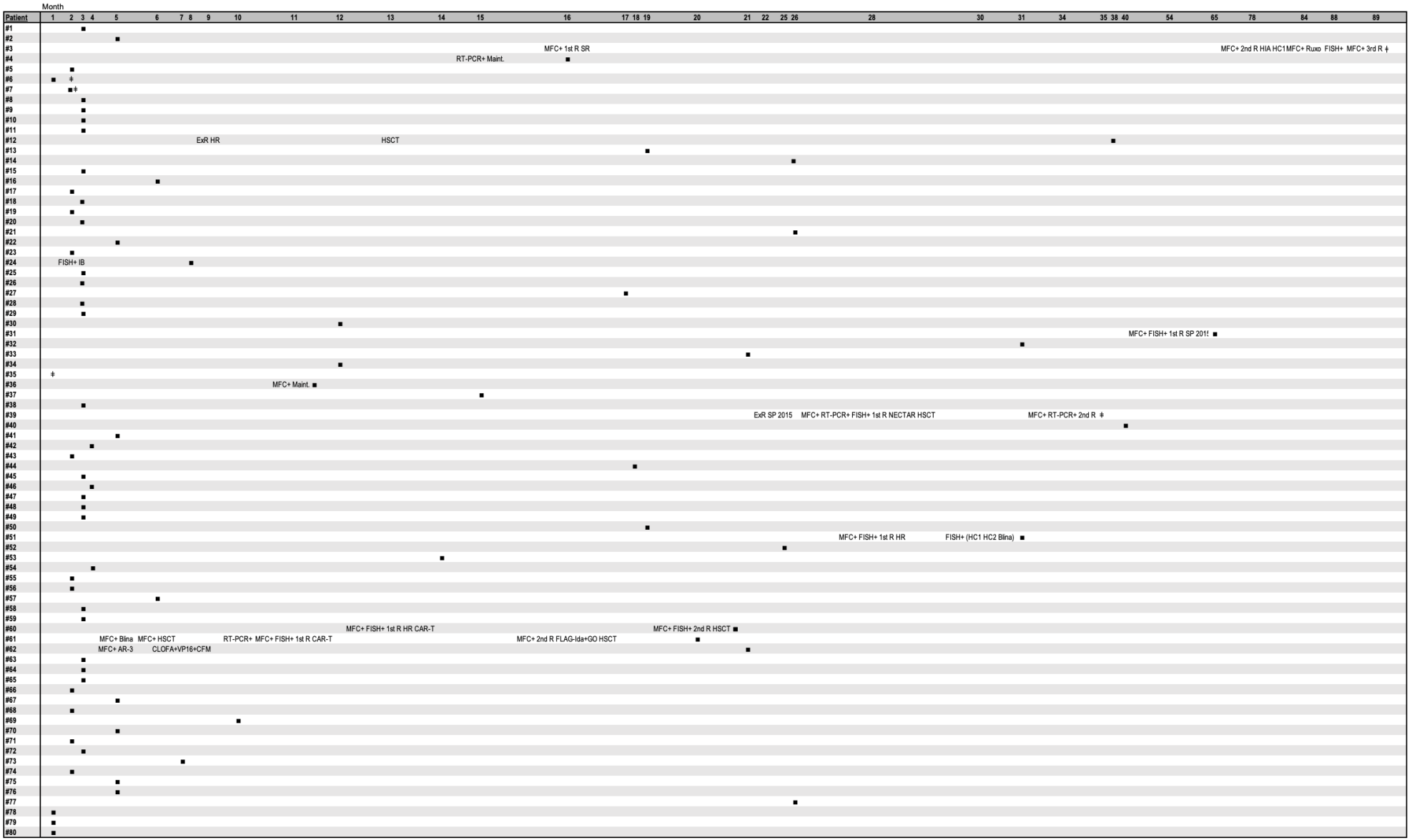

Figure 23. Time course of main events in the 80 included ALL patients. R: relapse; ExR: extramedullary relapse; MFC+: MRD reappearance in MFC; FISH+: MRD reappearance in FISH; RT-PCR+: MRD reappearance in RT-PCR; IB: Induction IB; AR-3: High-risk reinduction 3; Maint: maintenance; SR: IntReALL 2010 SR; HR: IntReALL 2010 HR; HIA: High-risk induction A; HC1: High-risk consolidation 1; HC2: High-risk consolidation 2; SP 2015: SEHOP-PETHEMA 2015; Ruxo: Ruxolitinb; NECTAR: nelarabine, etoposide and cyclophosphamide; HSCT: hematopoietic stem cell transplantation; Blina: Blinatumomab; CAR-T: chimeric antigen receptor T-cell therapy; FLAG: fludarabine, cytarabine and G-CSF with or without idarubicin (Ida); GO: Gemtuzumab Ozogamizin; CLOFA+VP16+CFM: clofarabine, etoposide and cyclophosphamide; ■ last bone marrow evaluation; and ǂ death.

5.2.2.1 Multiparameter flow cytometry

As observed in Figure 22B, some patients experienced more than one relapse. We identified a total of 12 relapses in 7 patients. As expected, all six patients with bone marrow involvement at relapse had a concordant positive MFC result, while one isolated extramedullary relapse was negative ($p<0.00001$) (Figure 24A). Moreover, we were able to anticipate relapse 12 days in one case.

It is noteworthy that in three cases the MRD reappearance by MFC became negative again with the continuation of the standard treatment or after further intervention with additional treatments: 1) maintenance therapy in a patient with intermediate risk without any genetic marker 2) third intensification block and salvage therapy with clofarabine, etoposide and cyclophosphamide in a patient with T-ALL and *TLX3* rearrangement; and 3) mercaptopurine plus ruxolitinib in a patient with a *JAK2* rearrangement, although this last patient relapsed after the interruption of the treatment.

5.2.2.2 Reverse transcription polymerase chain reaction

Twenty-two out of 80 (27.5%) patients showed a monitorable specific alteration by RT-PCR. The reappearance of *TAL1* deletion and *KMT2A::AFF11* rearrangement were associated with relapse in two patients, which was anticipated 21 days by RT-PCR in the former. Also, the reappearance of *TCF3::PBX1* rearrangement returned to negative during maintenance treatment without further intervention in another patient. No patient with persistently negative RT-PCR relapsed except for the aforementioned extramedullary relapse ($p=0.013$) (Figure 24A).

5.2.2.3 Fluorescent in situ hibridization

Sixty-five out of 80 (81.25%) patients showed monitorable alterations detected by this method. The reappearance of MRD detected by FISH was present in all six patients experiencing a bone marrow relapse while, with the exception of one isolated extramedullary relapse, no patient with persistently negative results relapsed ($p<0.00001$) (Figure 24A). The patient with the *JAK2* rearrangement showed a positive FISH result 40 days before overt relapse. The continuation of standard first-line treatment (early consolidation) was able to normalize MRD reappearance in one patient with a chromosome 4 tetrasomy.

The cumulative incidence of relapse according to the reappearance of measurable residual disease through the three different methods is showed in Figure 24B.

A

	Flow cytometry		RT-PCR		FISH	
	MRD⁻	MRD⁺	MRD⁻	MRD⁺	MRD⁻	MRD⁺
No Relapse	71	2	18	1	57	1
Relapse	1[a]	6	1[a]	2	1[a]	6
	p < 0.00001		p = 0.038		p < 0.00001	

B

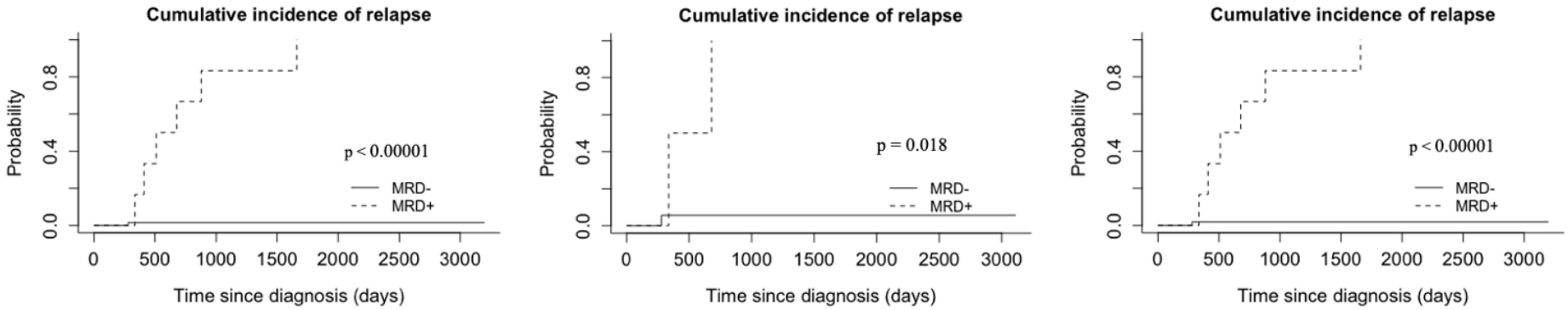

Figure 24. Association between measurable residual disease (MRD) detection and ALL relapse. A. Crosstab showing the relationship between ALL relapse and the reappearance of MRD detected by multiparametric flow cytometry (MFC), RT-PCR and FISH. [a] Extramedullary relapse. B. Cumulative incidence of relapse according to the reappearance of MRD detected by MFC (left), RT-PCR (mid) and FISH (right). MRD-: No MRD reappearance. MRD+: MRD reappearance.

Two patients showed detectable growing levels of p16 deletion below the established threshold of the FISH probe (9%) before relapse. If we take these two later patients into account, FISH on purified B or T cells was able to anticipate relapse with a median time of 39.5 days. Twelve additional patients had MRD reappearance below the sensitivity threshold. Continuation of standard first-line treatment was able to control disease in eight of these patients: one patient with iAMP21+, one patient with a t(12;21), three patients with p16 deletion and three patients with numeric alterations of chromosomes 4, 10, 14, 16, 17 and 21. In addition, second line treatments were able to control disease in two patients: one with a numeric alteration of chromosome 21 who received two cycles of high-risk consolidation therapy plus blinatumomab, and another with a *KMT2A* rearrangement who received blinatumomab plus HSCT, although this last patient relapsed afterwards. Furthermore, immunosuppression withdrawal after HSCT was able to negativize emerging disease in another patient with p16 deletion without posterior relapse. Finally, in the patient with the *TLX3* rearrangement, the continuation of the first line treatment, salvage therapy and immunosuppression withdrawal after HSCT, negativized MRD reappearance below the sensitivity threshold in three different occasions. A summary of interventions and outcome for FISH reappearances is shown in Table 17.

FISH alteration	MRD reappearance	Early intervention	MRD outcome	Relapse
Tetrasomy 4	Yes	No*	Negative	No
JAK2-r	Yes	Ruxolitinib	Negative	Yes
p16 deletion	Below the sensitivity threshold	No	Positive	Yes
p16 deletion	Below the sensitivity threshold	No	Positive	Yes
iAMP21	Below the sensitivity threshold	No*	Negative	No
t(12;21)	Below the sensitivity threshold	No*	Negative	No
p16 deletion	Below the sensitivity threshold	No*	Negative	No
p16 deletion	Below the sensitivity threshold	No*	Negative	No
p16 deletion	Below the sensitivity threshold	No*	Negative	No
Aneuploidy	Below the sensitivity threshold	No*	Negative	No
Aneuploidy	Below the sensitivity threshold	No*	Negative	No
Aneuploidy	Below the sensitivity threshold	No*	Negative	No
Chromosome 21 numeric alteration	Below the sensitivity threshold	Yes (intensified chemotherapy & Blinatumomab)	Negative	No
KMT2A-r	Below the sensitivity threshold	Yes (Blinatumomab)	Negative	Yes
p16 deletion	Below the sensitivity threshold	Yes (immunosuppression withdrawal)	Negative	No
TLX3-r	Below the sensitivity threshold	Yes (intensified chemotherapy, immunosuppression withdrawal)	Negative	No

Table 17. Interventions and outcome for patients with MRD reappearance detected by FISH
* Continuation of standard first-line treatment

5.2.2.4 Comparison between the three MRD methods

A high level of concordance was observed among samples with available data from the three MRD evaluation methods (Table 18). Due to its higher sensitivity RT-PCR showed positive results in 10 samples with negative results in MFC and FISH analysis. One sample was a t(1;19), three samples were a t(9;22), one sample was t(12;21) and five samples were a t(4;11).

Patient id.	Rearrangement	N. of samples	LAIP	MFC+	FISH+	RT-PCR+
#4	t(1;19)	6	Yes	1	1	2
#9	t(12;21)	3	Yes	1	1	1
#12	t(12;21)	14	Yes	1	1	1
#20	t(12;21)	3	Yes	1	1	1
#27	t(12;21)	4	Yes	1	1	1
#33	t(9;22)	8	Yes	1	1	4
#34	t(12;21)	2	Yes	1	1	1
#42	t(12;21)	4	No[b]	1	1	1
#47	t(12;21)	3	No[b]	1	1	1
#52	t(12;21)	5	Yes	1	1	2
#56	t(12;21)	2	Yes	1	1	1
#57	t(12;21)	4	Yes	1	1	1
#58	t(12;21)	2	Yes	1	1	1
#61	t(4;11)	17	Yes	7	3	11
#64	t(12;21)	3	Yes	1	1	1
#68	t(12;21)	2	Yes	1	1	1
#72	t(12;21)	2	Yes	1	1	1
#74	t(12;21)	3	Yes	1	1	1
#79	t(4:11)	3	Yes	1	1	2

Table 18. Comparative analysis MRD results detected by MFC, FISH and RT-PCR.[a]

[a] This analysis includes samples in which the three methods were performed and offered a valid result in patients with an identifiable rearrangement by FISH and RT-PCR (n=90).
[b] Patients with no LAIP were assessed in the basis of aberrant immunophenotypes as described in the patients and methods section.

Two to two comparisons of the results obtained with the three MRD methods offered the following results: 1) when comparing samples analyzed with MFC and FISH, 12 out of 330 samples (3.6%) were MFC positive and FISH negative, whereas only 2 out of 330 samples (0.6%) were MFC negative and FISH positive; 2) when comparing samples with a valid result for MFC and RT-PCR, 12 out 130 samples (10%) were MFC negative and RT-PCR positive, whereas no samples with MFC positive and RT-PCR negative were found (0%); and 3) when comparing samples with a valid result for the same genetic alteration in FISH and RT-PCR, 10 out 90 samples (11%) were FISH negative and RT-PCR positive, whereas no samples were found with FISH positive and RT-PCR negative (0%). Genetic alterations identified in these discrepancies are shown in table 19.

Discrepancy / Patient id.	Alteration
MFC-negative & FISH-positive	
#11	p16 deletion
#24	Tetrasomy 4
MFC-positive & FISH-negative	
#3	JAK2 rearrangement
#12	t(12;21) ETV6-RUNX1
#36	Trisomy 10, 17 and n-somy 14
#41	p16 deletion
#51	Tri- or tetrasomy 21 and trisomy 4, 10, 17
#61	KTM2A rearrangement
#62	TLX3 rearrangement
#68	t(12;21) ETV6-RUNX1 and RUNX1 duplication
MFC-negative & RT-PCR-positive	
#4	TCF3/PBX1
#33	BCR/ABL
#34	t(12;21) ETV6-RUNX1
#52	t(12;21) ETV6-RUNX1
#61	KTM2A rearrangement
#79	KTM2A rearrangement
MFC-positive & RT-PCR-negative	
No patients	
FISH-negative & RT-PCR-positive	
#4	TCF3/PBX1
#33	BCR/ABL
#52	t(12;21) ETV6-RUNX1
#61	KTM2A rearrangement
#79	KTM2A rearrangement
FISH-positive & RT-PCR-negative	
No patients	

Table 19. Genetic alterations in patients with discrepancies in the MRD detection between two techniques.

5.3 Impact of co-mutations and HSCT in AML relapse kinetics

5.3.1 Characteristics and outcomes

We found 63 patients who suffered a hematological relapse and 20 patients who experienced a molecular relapse but further deterioration to hematological relapse was prevented through pre-emptive therapy (n=83) of which 38 had a CBF AML, 11 had a *KMT2A*-rearranged AML and 34 had a *NPM1*mut AML. Of these, 54 patients meet the inclusion criteria detailed in the materials and methods section. Main characteristics of these 54 patients are shown in table 20. As can be seen in this table, 24 patients were children, and 30 patients were adults. Most of *NPM1*mut were adults (95%) and none of the children were transplanted in first complete remission. Also, 35 patients had full molecular analysis performed covering all the studied genes, but 19 patients missed at least the status of one gene.

	All patients N=54		***RUNX1::RUNX1T1* N= 16**		***CBFB::MYH11* N= 12**		***MLL::AF9* N= 6**		***NPM1mut* N = 20**	
	n	%	n	%	n	%	n	%	n	%
Sex										
Male	31	57	12	75%	7	58%	3	50%	9	45%
Female	23	43	4	25%	5	42%	3	50%	11	55%
Age										
Median age in years			10.5		14.5		3.5		56	
<18 years	24	44	12	75%	7	58%	4	67%	1	5%
>18 years	30	56	4	25%	5	42%	2	33%	19	95%
SCT before relapse	8	15	0	0%	0	0%	0	0%	8	40%
Time to relapse										
early	26	48	6	37.5%	4	33%	3	50%	13	65%
late	28	52	10	62.5%	8	67%	3	50%	7	35%
Co-mutation status (mutated/tested)										
KIT	7/40	18	6/14	43%	1/6	16%	0/2	0%	0/18	0%
KRAS/NRAS	7/36	19	1/11	9%	1/4	25%	1/3	33%	4/18	22%
FLT3-ITD	8/47	17	1/14	7%	0/7	0%	0/6	0%	7/20	35%
FLT3-D835	3/46	7	1/14	7%	1/7	14%	0/6	0%	1/19	5%
Relapse kinetics										
Log increment/30 days (range)	1.06(0.11-3.85)		1.04(0.2-1.50)		0.76(0.11-2.29)		1.65 (1.11-3.71)		1.14 (0.35-3.85)	

Table 20. Demographic, clinical and laboratory characteristics of included patients.

A more detailed description of the genetics and relapse kinetics of each individual patient can be found in table 21.

Patient id.	AML subgroup (MRD marker)	Age group	KIT, KRAS, NRAS and FLT3 status	Log_{10} increment /30 days
#1	*RUNX1::RUNX1T1*	Children	KIT mutated, unspecified	1.49
#2	*RUNX1::RUNX1T1*	Children	KIT mutated Exon 8	0.87
#3	*RUNX1::RUNX1T1*	Children	KIT mutated Exon 17	0.40
#4	*RUNX1::RUNX1T1*	Children	KIT mutated Exon 17 ††	0.87
#5	*RUNX1::RUNX1T1*	Children	NRAS mutated Exon 3	1.08
#6	*RUNX1::RUNX1T1*	Children	FLT3-ITD †††	0.59
#7	*RUNX1::RUNX1T1*	Children	Wild type †††	0.21
#8	*RUNX1::RUNX1T1*	Children	Wild type	0.67
#9	*RUNX1::RUNX1T1*	Children	Wild type	1.06
#10	*RUNX1::RUNX1T1*	Children	Wild type	1.04
#11	*RUNX1::RUNX1T1*	Children	††††	1.04
#12	*RUNX1::RUNX1T1*	Children	††††	1.32
#13	*RUNX1::RUNX1T1*	Adults	KIT mutated Exon 17	1.39
#14	*RUNX1::RUNX1T1*	Adults	KIT mutated Exon 17	1.37
#15	*RUNX1::RUNX1T1*	Adults	FLT3-ITD	0.20
#16	*RUNX1::RUNX1T1*	Adults	FLT3-D835	1.23
#17	*CBFB::MYH11*	Children	KIT mutated Exon 8 ††	2.1
#18	*CBFB::MYH11*	Children	Wild type ††	0.69
#19	*CBFB::MYH11*	Children	Wild type †††	0.34
#20	*CBFB::MYH11*	Children	Wild type	1.07
#21	*CBFB::MYH11*	Children	Wild type	2.29
#22	*CBFB::MYH11*	Children	††††	0.27
#23	*CBFB::MYH11*	Children	††††	0.69
#24	*CBFB::MYH11*	Adults	KRAS & NRAS mutated Exons 2&3	1.27
#25	*CBFB::MYH11*	Adults	Wild type	0.67
#26	*CBFB::MYH11*	Adults	††††	2.16
#27	*CBFB::MYH11*	Adults	††††	0.11
#28	*CBFB::MYH11*	Adults	††††	0.83
#29	*KMT2A::MLLT3*	Children	Wild type †	1.58
#30	*KMT2A::MLLT3*	Children	Wild type †††	3.71
#31	*KMT2A:MLLT3*	Children	Wild type †††	1.68
#32	*KMT2A::MLLT3*	Children	Wild type †††	1.11
#33	*KMT2A::MLLT3*	Adults	KRAS mutated Exon 3	1.63
#34	*KMT2A::MLLT3*	Adults	Wild type	3.57
#35	*NPM1 mutated*	Children	NRAS mutated Exon 1	0.93
#36	*NPM1 mutated*	Adults	KRAS mutated Exon 2	0.53
#37	*NPM1 mutated*	Adults	KRAS mutated, Exon 2	1.58
#38	*NPM1 mutated*	Adults	NRAS mutated Exon 2, FLT3-D835	1.71
#39	*NPM1 mutated*	Adults	FLT3-ITD	1.45
#40	*NPM1 mutated*	Adults	FLT3-ITD *	1.57
#41	*NPM1 mutated*	Adults	FLT3-ITD	3.47
#42	*NPM1 mutated*	Adults	FLT3-ITD	0.75
#43	*NPM1 mutated*	Adults	FLT3-ITD	0.35
#44	*NPM1 mutated*	Adults	FLT3-ITD	2.48
#45	*NPM1 mutated*	Adults	FLT3-ITD	0.55
#46	*NPM1 mutated*	Adults	Wild type †††	3.85
#47	*NPM1 mutated*	Adults	Wild type	1.01
#48	*NPM1 mutated*	Adults	Wild type	0.35

#49	*NPM1 mutated*	Adults	Wild type	2.84
#50	*NPM1 mutated*	Adults	Wild type	0.73
#51	*NPM1 mutated*	Adults	Wild type	1.26
#52	*NPM1 mutated*	Adults	Wild type	2.27
#53	*NPM1 mutated*	Adults	Wild type	1.01
#54	*NPM1 mutated*	Adults	Wild type	0.55
† KIT status not available †† KRAS and NRAS status not available ††† KIT, KRAS and NRAS status not available ††††FLT3, KIT, KRAS and NRAS status not available * Only FLT3-ITD studied.				

Table 21. Details of genetics and relapse kinetics of the included patients.

5.3.2 Impact of co-mutations in AML relapse kinetics

Median MRD increments were higher in *KMT2A*-r (log10 1.65/30 days) compared to CBF (log10 0.96/30 days, P = 0.001) and *NPM1*mut (log10 1.14/30 days, P = 0.05) patients. There were no significant differences between CBF and *NPM1*mut patients in relapse kinetics (P = 0.186) (Figure 25).

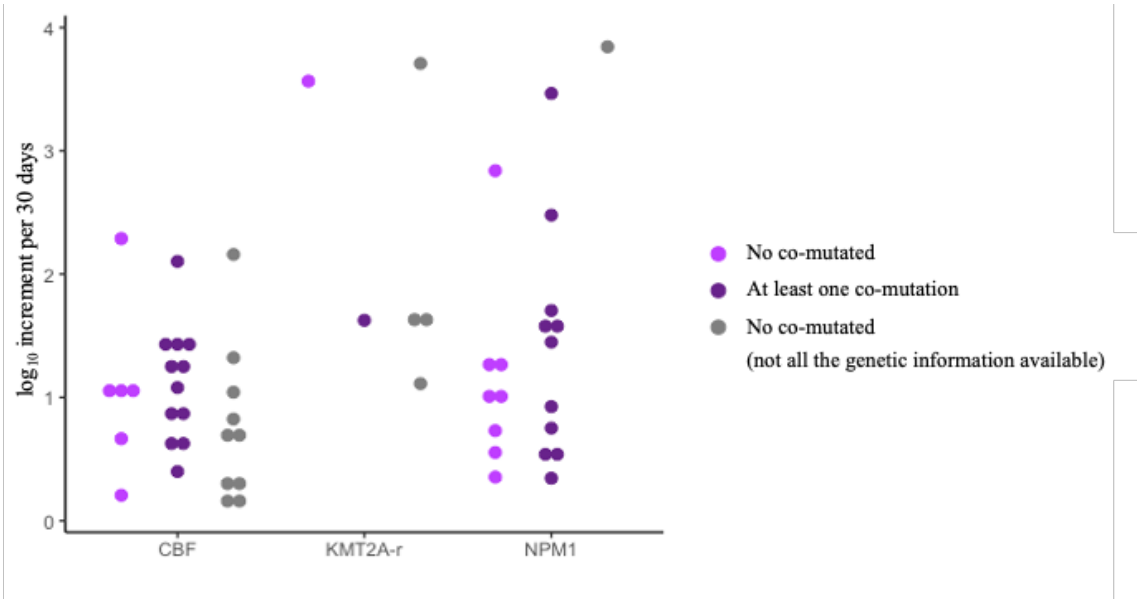

Figure 25. Dot plot of log10 increments per 30 days in the main AML genetic subgroups according to the co-mutation status.

When we analyzed the impact of the studied co-mutations in all included patients, irrespective of genetic subgroup, the best regression model found (i.e. the model that minimized the robust residual standard error and maximized the multiple R squared) included all these co-mutations with the following coefficients: 0.19 for *KIT*, 0.3 for *RAS* mutations, 0.01 for *FLT3*-ITD and 0.17 for *FLT3*-D835, starting from an intercept of 0.94 (P = < 0.001), although none of these coefficients were statistically significant (P = 0.478, 0.163, 0.979 and 0.449 respectively).

Next, we stratified for the main genetic AML subgroups (i.e CBF, *KMT2A*-r and *NPM1*mut). In CBF patients, the best model found included *KIT*, and *RAS* genes as predictors (coefficients of 0.23 and 0.26, respectively, starting from an intercept of 0.91 (P = < 0.001) with the coefficient of *RAS* genes being close to statistical significance (P = 0.11). In *NPM1*mut patients, the best model found included *FLT3*-D835 as the only predictor (coefficient of 0.66, P = 0.04) starting from an intercept of 1.05 (P = 0.002). In the *KMT2A*-r patients, there was no reliable model due to the scarce information available.

5.3.3 Impact of HSCT in AML relapse kinetics

Moreover, when we add the transplant to the explanatory variables, we found that transplantation before relapse was an independent predictor of a higher speed of relapse in all patients (coefficient 0.88, P = 0.042) and it almost reached statistical significance in *NPM1*mut patients (coefficient 0.97, P = 0.071, Figure 26).

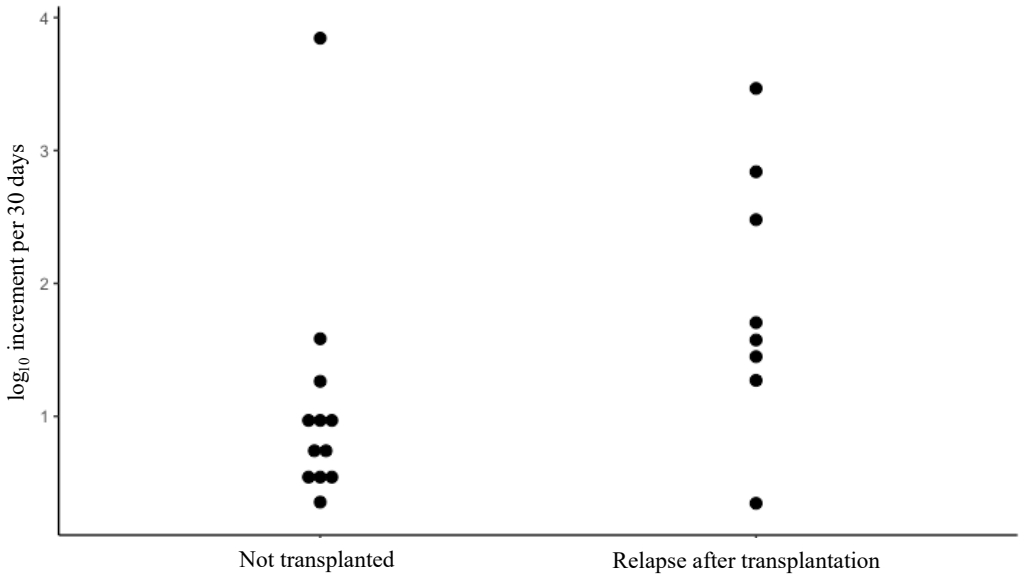

Figure 26. Dot plot of log_{10} increments per 30 days in the NPM1mut patients in relation to HSCT.

To deepen into this observation, we investigated if there was any relationship between the time from transplant to the first MRD detection and the speed of relapse, and we found a negative exponential relationship between days from transplant to the start of the relapse and it speed (estimate of -0.01 for the time in days with an intercept of 0.87, p-value = 0.041, see Figure 27). This means that the speed of relapse declines exponentially as time since transplant increases.

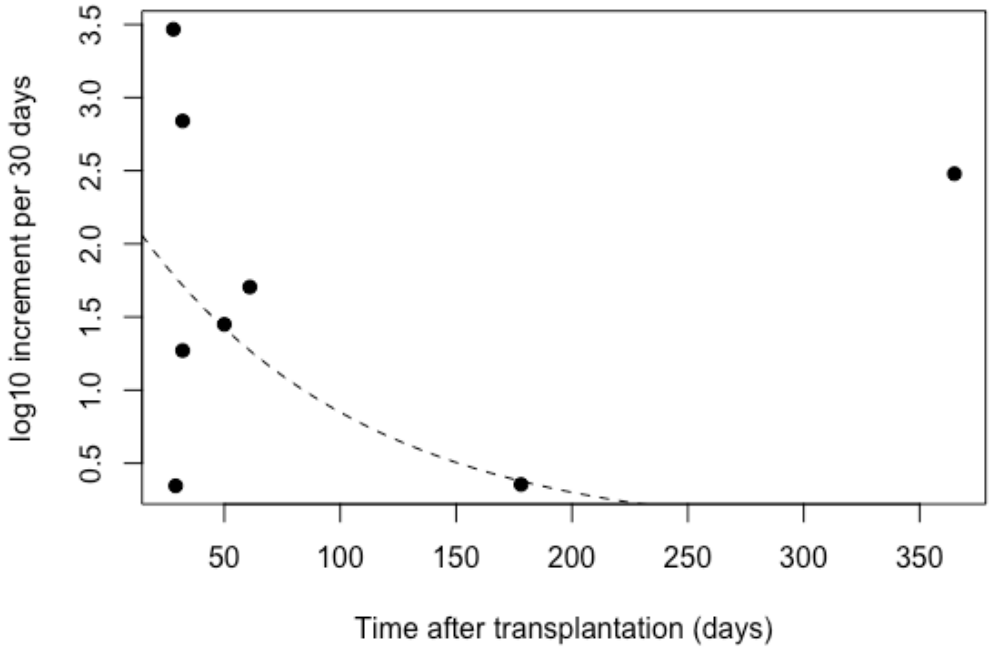

Figure 27. Negative exponential relationship between the $\log_{10}$ increment per 30 days and time from transplant to the start of the relapse.

VI. Discussion

6.1 MRD to anticipate relapse and guide preemptive therapy in childhood AML

Monitoring MRD during the follow-up of childhood AML to predict relapse is challenging. While MFC is an almost universal method for the evaluation of MRD because most AMLs have a specific LAIP, its lower sensitivity compared to molecular methods delay the detection of the reappearance of the disease. In our cohort, MFC was able to detect MRD positivity 26 days (median time) before relapse, compared to 111 days of molecular methods. Unfortunately, only 40% of AMLs express a specific translocation (36) that can be detected with sensitive molecular methods; therefore, we should use two different strategies, one for patients that present specific translocations and other for those that do not.

Regarding AMLs with a specific translocation, our results obtained by nested RT-PCR show that the reappearance of t(8;21)(q22;q22) *RUNX1::RUNX1T1*, did not systematically imply a relapse. These findings are consistent with the study of Miyamoto et al., in which some patients in remission positivized the t(8;21) without experiencing relapse (109). This is supported by the fact that t(8;21), in the absence of other secondary events, is insufficient for leukemogenesis (138). However, when the t(8;21) exceeds a certain threshold measured by qPCR, it is indicative of relapse (58,108), probably reflecting an uncontrolled proliferation of tumor cells that have suffered additional events. Similar results have been described for the inv(16) or *CBFB::MYH11* (58,108).

In contrast, our results show that the reappearance of t(9;9)(q34;q34) *SET::NUP214* and *KMT2A* rearrangements, if untreated, conducts irremediably to the relapse. These results are also in agreement with those of Matsuo et al. and Juul-Dam et al. in which the reappearance of *KMT2A* rearrangements was followed or accompanied by a relapse in all cases (58,108). Therefore, even though the technique used in this study do not allow MRD quantification of these genes, our results suggest that the reappearance of these rearrangements in BM seems to inevitably lead to relapse in contrast to core binding factor leukemias.

Differences between core binding factor leukemias and other leukemias draw attention to the fact that each translocation has a different significance regarding the biology of the tumor cell and should be interpreted in consequence.

For patients with AML lacking a specific molecular biomarker an alternative approach might be the measurement of *WT1* expression, which is a specific biomarker of immature myeloid cells (139). *WT1* overexpression is present in up to 90% of AMLs (140). Many studies have demonstrated its usefulness as a marker to anticipate hematological relapse; however, each study uses a different approach. For instance, in the study of Weisser et al, 16 of 44 relapses were preceded by a one log increment of the transcript in two consecutive bone marrow samples (59), whereas in the study published by Mashima et al., the cumulative incidence of relapse was significantly higher in patients with two consecutive values greater than 100 copies per μg of RNA in peripheral blood (110). In our study, all the relapses monitored with this marker were preceded by a one log increase and none of the patients in complete remission showed a one log increase between two consecutive samples.

However, we could not see how the MRD reappearance was immediately followed by a relapse in all cases because of the use of pre-emptive treatment (cyclosporine-A withdrawal, DLIs or boost of donor cells). Pre-emptive treatments in our series could probably have extended the median time from MRD reappearance to relapse. Unfortunately, pre-emptive treatments in our study only temporarily halted the progression of the disease, and patients finally relapsed and died. This is indicative either that new and more effective therapies should be investigated, or that these treatments should be provided earlier to be effective. Accordingly, more sensitive MRD monitoring methods that further anticipate relapse such as NGS and digital droplet PCR should be introduced to provide more time for second line strategies, like stem cell transplantation, where time for donor search is crucial.

Although theoretically sensitivity of MFC is higher than that of FISH, in our study seven samples with negative MFC were detected positive for the specific chromosomal translocation by FISH. This is indicative that in some AML patients without well-defined LAIP, an increase in the number of cells to be analyzed by FISH may offer an alternative method for MRD evaluation that could help to anticipate relapse, most particularly if molecular methods are not available.

6.2 MRD to anticipate relapse and guide preemptive therapy in childhood ALL

MRD assessment is a useful tool to assess early treatment response and adjust therapy during first line treatment, although its relevance later on the follow-up in real-life ALL patients has been far less investigated (41). After first line treatment, pediatric patients with ALL are usually monitored with periodical peripheral blood cell counts (141). This approach has the disadvantage that unspecific alterations as neutropenia or thrombocytopenia are frequently found in association to other conditions (infections, toxicity…) instead of relapse. Although MRD analysis can be attempted on peripheral blood (142), generally, bone marrows aspirates are performed to clarify if we are facing a relapse or another transient condition. However, the interpretation of these results is not always straightforward, as no consensus has been established on the clinical interventions to be taken when we find re-emerging MRD.

In this study in pediatric ALL we found that positive detection of MRD by any of the three methods tested (MFC, FISH or RT-PCR) at any time during follow-up was strongly correlated with a subsequent relapse. Furthermore, both the continuation of the standard therapy and the implementation of additional interventions were successful in preventing overt relapse in some patients. However, due to the highly heterogenous nature of this retrospective study, definitive recommendations regarding preemptive interventions cannot be made. A prospective study is needed to determine to what extent more intensive treatment is necessary to prevent disease progression.

Although MRD assessment by MFC is a useful and widely implemented approach, its sensitivity is inferior compared with new molecular methods. In our series, MFC was able to anticipate relapse in one case just by 12 days, the shortest time compared to the other two techniques. RT-PCR, however, has demonstrated to be the most sensitive and specific method to monitor MRD. In fact, in our study we did not find any false positive and we were able to anticipate relapse in one case by 21 days. Nonetheless, it was only one case, so probably the lack of monitorable specific alterations have precluded early detection of MRD in other patients. This strongly supports that a universal and sensitive molecular method to assess MRD status in every patient with B or T ALL should be available in all centers where MRD evaluation is performed. In this regard, next-generation sequencing (NGS) to detect immunoglobulin and T-cell receptor

rearrangements has proven to be the most sensitive and specific method (143), although is not as well implemented as qPCR of these rearrangements which is the gold standard in many collaborative groups (144). This last technique is time and cost consuming, which has hindered a wide implementation in our country, making it a challenge.

Alternatively, our results clearly show that MRD assessment trough FISH in purified B or T cells is a complementary method that can also be useful to anticipate relapse. In this study, we were able to anticipate relapse in one case by 40 days and in up to three cases with a median time of 39.5 days when considering the growing reappearances of the specific genetic alteration below the established sensitivity threshold of the FISH probe. Although theoretically sensitivity of MFC is higher than that of FISH, in our study two patients with negative MFC were detected positive for the specific chromosomal translocation by FISH on purified B or T cells. This is indicative that in some ALL patients without well-defined LAIP, an increase in the number of cells to be analyzed by FISH may offer an alternative method for MRD evaluation that could help to anticipate relapse, particularly when molecular methods are not available.

It is important to highlight that contrary to children with AML in whom preemptive treatments only temporarily halted disease progression (145), probably due to the higher chemoresistance of AML cells (146), several ALL patients turned MRD negative again with the continuation of standard treatment, such in cases with t(1;19), iAMP21, t(12;21), p16 deletion or numeric alterations of chromosomes 4, 10, 14, 16, 17 and 21. Also, in four patients with emerging MRD by FISH and/or RT-PCR (one translocation t(4;11), one *JAK2* rearrangement, one chromosome 21 aneuploidy and one 16p deletion), early interventions such blinatumomab plus HSCT, targeted therapy with ruxolitinib in combination with mercaptopurine, chemotherapy intensification and blinatumomab, and immunosuppression withdrawal after HSCT respectively, were able to yield transient (two former patients) and sustained (two later patients) disease control. Finally, in the T-ALL patient with a *TLX3* rearrangement, both, the continuation of the first line treatment and early interventions adapted to MRD along the follow up were able to control disease progression in up to three times. However, early treatment of MRD continues to be debated and whether it can offer improved global survival in childhood ALL patients should be established in properly controlled clinical trials.

6.3 Impact of co-mutations and HSCT in AML relapse kinetics

In this part of the thesis, we investigated the role of co-mutations and HSCT in the relapse kinetics of AML in a mixed cohort of children and adults using regression analysis. Concerning the impact of co-mutations, we were able to demonstrate an almost statistically significant effect of *RAS* mutations and a statistically significant effect of *FLT3*-D835 in relapse kinetics in CBF and *NPM1*mut AML patients, respectively. However, we were not able to demonstrate a statistically significant effect of *KIT* mutations and *FLT3*-ITD, which reveals some limitations of our research such as co-mutations instability between diagnosis and relapse or incomplete genetic information for some patients.

Regarding the effect of transplantation on relapse kinetics, we have observed a higher speed of relapse in transplanted patients. Factors of potential impact are the inherent high proportion of high-risk disease entities included in this group and the state of immunosuppression in the first days and months after transplantation. In line with this, the speed of relapse exponentially decays according to time from HSCT, probably reflecting an immune settlement of the allogeneic immune system and the appearance of the graft versus leukemia effect. Nonetheless, these findings should be taken with caution, since all post-transplant relapses occurred in *NPM1*mut patients, and the negative exponential relationship found is based on only 8 patients and only 2 of them relapsed beyond day 75. Larger studies with post-transplant relapses in all genetics subgroups and longer times from transplant to relapse would be desirable.

Knowledge about factors affecting the speed of relapse is important in order to establish and adjust disease monitoring schedule in these patients and intervene early with pre-emptive therapies which seems feasible in both adults and children (87,147,148). Our findings suggest that *RAS* genes and *FLT3*-D835 may have an impact on relapse speed in CBF and *NPM1*mut respectively, although whether the monitoring schedules should be modified for this reason remains to be elucidated. Additionally, this study provides evidence that supports that, in the posttransplant setting, MRD should be monitored more closely in the months just after the transplant and then expand the time interval in the following months.

VII. Conclusions

Conclusions

1. MFC, FISH and PCR are complementary methods that can anticipate relapse of childhood AML by weeks to several months. However, in our series, pre-emptive therapies were not able to prevent disease progression. Therefore, more sensitive MRD monitoring methods that further anticipate relapse and more effective pre-emptive therapies are needed.

2. MFC, FISH and RT-PCR are complementary methods for MRD monitoring in pediatric ALL. Although, our data clearly show that MDR positive detection is associated with relapse, continuation of standard treatment, intensification or other early interventions were able to halt relapse in patients with different risks and genetic background. More sensitive and specific methods are warranted to enhance this approach. However, whether early treatment of MRD can improve overall survival in patients with childhood ALL needs to be evaluated in adequately controlled clinical trials.

3. MRD monitoring is a useful approach to anticipate relapse in both AML and ALL patients. Time from MRD detection to relapse is directly proportional to the sensitivity of the technique. Molecular methods are the most sensitive and specific although its applicability is limited by the presence of target genetic alterations. However, in core biding factor AML the reappearance of the genetic alteration does not always imply a relapse.

4. Molecular MRD monitoring in peripheral blood during follow up is a feasible approach for an early detection of the disease and to enable preemptive treatment. MRD monitoring schemes should be personalized according to the genetic features of patients' disease (defining mutations, co-mutations) and the status of the patient. To be useful, MRD monitoring should be more frequent in the first months after transplantation and more spaced afterwards.

VIII. References

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IX. Annex I: Publications

First publication

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RESEARCH ARTICLE

Measurable residual disease study through three different methods can anticipate relapse and guide pre-emptive therapy in childhood acute myeloid leukemia

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Abstract

Purpose Although outcomes of children with acute myeloid leukemia (AML) have improved over the last decades, around one-third of patients relapse. Measurable (or minimal) residual disease (MRD) monitoring may guide therapy adjustments or pre-emptive treatments before overt hematological relapse.

Methods In this study, we review 297 bone marrow samples from 20 real-life pediatric AML patients using three MRD monitoring methods: multiparametric flow cytometry (MFC), fluorescent in situ hybridization (FISH) and polymerase chain reaction (PCR).

Results Patients showed a 3-year overall survival of 73% and a 3-year event-free survival of 68%. Global relapse rate was of 25%. All relapses were preceded by the reappearance of MRD detection by: (1) MFC ($p=0.001$), (2) PCR and/or FISH in patients with an identifiable chromosomal translocation ($p=0.03$) and/or (3) one log increase of Wilms tumor gene 1 (WT1) expression in two consecutive samples ($p=0.02$). The median times from MRD detection to relapse were 26, 111, and 140 days for MFC, specific PCR and FISH, and a one log increment of WT1, respectively.

Conclusions MFC, FISH and PCR are complementary methods that can anticipate relapse of childhood AML by weeks to several months. However, in our series, pre-emptive therapies were not able to prevent disease progression. Therefore, more sensitive MRD monitoring methods that further anticipate relapse and more effective pre-emptive therapies are needed.

Keywords Childhood acute myeloid leukemia · Measurable residual disease · Multiparameter flow cytometry · Fluorescent in situ hybridization · Polymerase chain reaction · Relapse

Introduction

Acute myeloid leukemia (AML) accounts for approximately 20% of pediatric acute leukemias in children but causes a disproportionately high mortality. Although outcomes for children with AML have improved over the last decades, overall survival remains near 70% [1]. Monitoring measurable residual disease (MRD, previously termed minimal residual disease), defined as the leukemic cells persisting during or after chemotherapy below the sensitivity of bone marrow (BM) cytomorphology, is essential in the management of these patients. MRD positivity after induction treatment predicts an adverse clinical outcome in AML [2–4] and the presence of MRD before transplantation is associated with a higher risk of relapse and, therefore, with a lower leukemia-free survival and overall survival [5–7]. In addition,

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detection of leukemic cells in patients who are in morphologic remission is predictive of a more rapid relapse [8].

Several disease monitoring methods with increasing sensitivity are available: cytomorphology and immunochemistry assisted light microscopy, cytogenetics, fluorescent in situ hybridization (FISH), multiparameter flow cytometry (MFC) and diverse methods based on polymerase chain reaction (PCR) [9]. MFC is able to identify the leukemia-associated immunophenotype (LAIP) that distinguish leukemic cells from healthy hematopoietic progenitors. Finding a LAIP to all AMLs requires an extensive panel of antibodies [10], but 80–90% of patients can be followed using a reasonably sized panel [11]. Leukemic cells are detectable by MFC with a sensitivity ranging from 10^{-2} to 10^{-4} [10]. By PCR, any nucleic acid sequence can be amplified to generate a large number of identical copies that can readily be analyzed. However, while conventional PCR cannot estimate the initial amount of the targeted sequence, real-time PCR can offer a quantitative measurement [12]. These methods can achieve sensitivities of 10^{-4} to 10^{-6} [13]. Chromosomal alterations can also be analyzed by FISH. Despite its lower sensitivity (up to 10^{-3}), some researchers defend its usefulness in MRD monitoring [14].

Among the genetic alterations detected by PCR or FISH, we find the t(8;21)(q22;q22) and the inv(16)(p13;q22), the rearrangements of *KMT2A* (formerly known as *MLL)* and the t(9;9)(q34;q34). The t(8;21) or *RUNX1::RUNX1T1* and the inv(16) or *CBFB::MYH11* (also known as core binding factor AML) are together the most common cytogenetic abnormalities in AML. They occur in approximately 25% of cases and are associated with a good prognosis [15, 16]. Rearrangements of *KMT2A* are present in approximately 20% of pediatric AML patients and may involve more than 100 different translocation partners, although the most common are the t(9;11)(p22;q23), t(10;11)(p13;q14), t(11;19)(q23;p13.1) and t(6;11)(q27;q23) [15–17]; t(6;11)(q27;q23), t(10;11)(p12;q23) and t(10;11)(p11.2;q23) have the highest rates of relapse, whereas patients with t(1;11)(q21;q23) have an excellent outcome [18]. The t(9;9)(q34;q34) or *SET-CAN/NUP214*, is a rare genetic event in AML, and it is associated with poor prognosis [19], nonetheless, this alteration is insufficient for leukemogenesis, and secondary genetic mutations are needed for disease development [20].

Unfortunately, not all AMLs have molecular alterations suitable for specific MRD monitoring. For this purpose, the *Wilms tumor gene 1* (WT1) is a near pan-leukemic marker overexpressed in most AMLs [21]. *WT1* expression has been shown to predict disease progression in AML patients treated with conventional chemotherapy [22] or HSCT [23]. Furthermore, quantification of *WT1* expression shows comparable sensitivity predicting AML relapse to MFC [24].

MRD study is useful at specific time points to guide therapy adjustments, although is also useful to guide pre-emptive treatments before overt hematological relapse [9]. Donor lymphocyte infusions (DLI) have been used as pre-emptive/prophylactic treatment in AML with better results than when used in overt relapse [25]. Hypomethylating agents such as azacytidine or decitabine, rapid withdrawal of immunosuppression after transplantation and the so-called "microtransplantation" are alternative approaches to prevent or treat relapse [25–28].

This study is aimed at evaluating the usefulness of standard MRD monitoring methods, such as MFC, FISH and RT-PCR, for the early prediction of relapse in real-life pediatric patients with AML.

Materials and methods

Patients and samples

In this retrospective, observational and analytical study, clinical and laboratory data of pediatric patients with AML treated in the Clinical University Hospital Virgen de la Arrixaca between 2012 and 2022 were reviewed. Diagnostic criteria for the type and subtype of AML were based on the WHO classification of tumors of hematopoietic and lymphoid tissues [29, 30]. A patient initially diagnosed as AML and later reclassified as mixed phenotype acute leukemia was also included. Children with t(15;17)/PML-RARα acute promyelocytic leukemia or Down Syndrome-related AML were excluded since these AML subtypes are biologically different and receive specific treatment schedules. The end of data collection was on July 1st, 2022. The institutional review board (IRB-00005712) approved the study. Written informed consent was obtained from all patients (and/or their parents) in accordance with the Declaration of Helsinki.

Cytomorphology, immunophenotype, cytogenetics (karyotype and FISH) and molecular results of BM aspirates were recorded at diagnosis, at the end of induction (day + 21 or + 22) and at other timepoints during therapy established by the corresponding treatment protocol. Transplanted patients were evaluated at days + 30, + 60, + 90, + 180 and + 360 after transplantation. Other extra evaluations after therapy completion were performed according to clinician's criteria based on the presence of unexplained anemia, thrombocytopenia or neutropenia during follow-up. A total of 297 samples from 20 patients were evaluated. See supplementary file 1 for treatment details.

Immunophenotype and MRD studies

Immunophenotyping and MRD studies were performed in BM aspirates obtained at diagnosis and during follow-up by using 8-color FACSCanto-II (from 2012 to June 2020) or

12-color FACSLyric (from June 2020 to 2022) flow cytometers (Becton Dickinson, BD, San Jose, CA). See supplementary file 1 for details.

Fluorescent in situ hybridization (FISH)

Cytogenetic abnormalities were evaluated in interphase nucleus from total BM cells following standard procedures previously validated [31]. The following FISH probes from Metasystems (Altlussheim, Germany) were used to evaluate 5q31 deletion (XL 5q31/5q33/5p15, cut-off 10%), 7q deletion (XL del(7)(q22q31), cut-off 9%), Cr-3 inversion (XL t(3;3) GATA2/MECOM DF, cut-off 10%), *KMT2A* rearrangements (XL KMT2A BA, cut-off 1%), t(8;21)(q22;q22) (XL AML1/ETO, cut-off 1%) and inv(16)(p13;q22) (XL CBFB/MYH11, cut-off 1%). For each probe 250 cells were analyzed with Metafer system (Metasystems). Up to 3000 cells were captured to increase sensitivity when needed for t(8;21) or *KMT2A* rearrangements.

Molecular studies

FMS-like tyrosine kinase internal tandem duplication mutations (FLT3-ITD) and mutation in the tyrosine kinase domain (FLT3-TKD) were evaluated in genomic DNA extracted from BM samples using QIAmp® DNA blood mini kit and QIAsymphony® (QIAgen, GmbH, Hilden, Germany). NPM1 and CEBPA mutations, WT1 expression and common chromosomal translocations in childhood leukemia were evaluated in total RNA extracted from BM samples using QIAmp® RNA blood mini kit and QICube® (QIAgen). See supplementary file 1 for details.

Definitions and statistical analysis

Complete remission was defined as < 5% leukemic cells in bone marrow with clear evidence of regeneration in the bone marrow or in the peripheral blood. Bone marrow relapse was defined as the presence of ≥ 5% leukemic cells in the BM cytomorphology after complete remission was achieved. Extramedullary relapse was defined as the development of extramedullary disease once complete remission was achieved.

The Kaplan–Meier method was used for survival estimation. Overall survival (OS) was defined as the time from diagnosis to death, with living patients censored on the date of last follow-up. Event-free survival (EFS) was defined as the time from diagnosis to relapse, progression or death, with event-free patients censored on the date of last follow-up. Cumulative incidence functions were calculated and compared according to the Fine and Gray method and the Gray's test, respectively. Comparisons between qualitative variables were performed by the two-tailed Fisher's exact test. Data were collected in Excel (Microsoft Corporation, Redmond, USA) and analyzed in R version 3.6.3 (R Foundation for Statistical Computing, open-source software).

Results

Characteristics and outcome of patients according to the type of AML

This study included 20 AML patients with a mean age at diagnosis of 6.58 years (range 0.3–16.58 years) and a male:female ratio of 1.5. Biological, clinical, therapeutic and evolutionary characteristics according to the FAB stages are shown in Table 1. The diagnosis of AML was established before referral to our hospital in two patients. Twelve out of 20 patients (60%) had a specific chromosomal rearrangement, distributed as follows: five patients had the t(8;21) *RUNX1::RUNX1T1* (25%), five patients a *KMT2A*-rearrangement (25%), one patient had an inv(16) *CBFB::MYH11* (5%) and one patient the t(9;9) *SET::CAN/NUP214* (5%). Among patients with a *KMT2A*-rearrangement, we found two t(9;11)(p21;q23) *KMT2A::MLLT3*, one t(11;17)(q23;q12) *KMT2A::MLLT6*, one t(6:11)(q27;q23) *KMT2A::AFDN* and one with a rare *KMT2A*-rearrangement detected by FISH whose partner was identified in the Diagnostic Center for Acute Leukemia in Frankfurt as ins(11;X)(q23;q28q12) *KMT2A::FLNA*.

Ten patients were treated according to the SHOP-LMA-2007 protocol (8 high risk and 2 very high risk patients) and ten patients were treated according to the NOPHO-DBH AML 2012 protocol (9 standard-risk and 1 high-risk patient). Three out of the ten patients treated with the SHOP-LMA-2007 protocol and one out of the ten patients treated with the NOPHO-DBH AML 2012 protocol received infusions of NK cells as part of a phase II clinical trial (NCT02763475). In total, 8 children out of 20 (40.0%) received allogeneic HSCT, 5 in first complete remission and 3 in second complete remission. Pre-emptive treatment was used in four transplanted patients guided by the MRD results. In two patients early cyclosporine withdrawal was used 113 days and 47 days before relapse respectively, and DLI were used 100 days and 34 days before relapse, respectively. In a patient with a t(6;11), DLI were used 75 days before relapse. In a patient with a t(9;9), DLI were used 16 days before relapse. Mean follow-up was 1389 days (range 85–3446).

With the aforementioned treatment protocols, our patients with AML showed an estimated 3-year OS of 72.7% (IC 95% 52.8–100) and an estimated 3-year EFS of 67.7% (IC 95% 49.4–93). The global relapse rate was of 25% (5 out of 20 cases). According to their genetic alteration, the relapse rate was as follows: one out 5 (20%) patients with t(8;21),

Table 1 Biological, clinical, therapeutic, and evolutionary features of patients

	Myeloblastic (M0–2)[1]	Promyelo- and myelo-monocytic (M3–4)[1]	Monocytic (M5a/b)[1]	Mega-karyoblastic (M7)[1]
Number of cases	9	3	4	4
Mean age (years)	7.2	13.2	5.7	1.7
Gender (male, *n* (%))	4 (44)	3 (100)	3 (75)	2 (50)
Admission in our hospital				
At diagnosis, *n* (%)	8 (89)	2 (66)	4 (100)	4 (100)
After diagnosis, *n* (%)	1 (11)	1 (33)	0 (0)	0 (0)
PB-WBC at diagnosis				
$< 100 \times 10^3$/µl	6	2	3	4
$> 100 \times 10^3$/µl	2	–	1	–
Cytogenetics				
t(8;21)(q22;q22)	4	–	1	–
inv(16)(p13q22)	1	–	–	–
KTM2A rearrangement	1	1	3	–
t(9;9)(q34;q34)	1	–	–	–
Risk stratification[2]				
Standard, *n* (%)	7 (78)	1 (33)	2 (50)	–
High, *n* (%)	2 (22)	2 (66)	2 (50)	2 (50)
Very high, *n* (%)	–	–	–	2 (50)
Treatments				
LMA-SHOP-2007	2	2	2	4
NOPHO-DBH-AML-2012	7	1	2	–
HSCT, *n* (%)	3 (33)	1 (33)	0 (0)	4 (100)
Clinical outcome				
Mean follow-up (days)	1090	1635	1501	1765
Relapse, *n* (%)	2 (22)	1 (33)	0 (0)	2 (50)
Death, *n* (%)	2 (22)	1 (33)	0 (0)	2 (50)

[1]M1, $n=2$; M2, $n=7$; M3 without t(15;17), $n=1$; M4, $n=2$; M5a, $n=2$; M5b, $n=2$; M7, $n=4$

[2]The risk classification depends on the protocol (see supplementary table 1)

one out of 5 (20%) patients with a KMT2A-rearrangement, and the only patient (100%) with the t(9;9) relapsed. The patient with inv(16) did not relapse (0%). Among patients with a KMT2A-rearrangement, the patient with the t(6;11) KMT2A/AFDN relapsed. Besides, 2 patients without specific chromosomal alterations relapsed.

Evolutionary patterns of MRD in AML patients

We detected two patterns of MRD presentation during the follow-up. In Fig. 1, we illustrate these patterns with two representative cases (patients 10 and 13, respectively). In the first pattern, patients enter in complete remission and stay until the end of the follow-up (Fig. 1a). In the second pattern, relapse is preceded by the reappearance of the specific molecular and cytogenetic markers and the detection of MRD in the immunophenotype analysis, followed by a progressive normalization of these parameters after treatment intensification (Fig. 1b). See Table S1 for a summary of the main events of all patients.

As observed in Fig. 1b, some patients experienced more than one relapse. In total, 10 relapses were observed in 5 patients: 9 BM relapses and 1 extramedullary relapse.

Multiparameter flow cytometry

Five of the nine BM relapses and the extramedullary relapse were preceded by the detection of MRD by multiparametric flow cytometry ($p=0.001$) (Fig. 2a). Though, the latter was probably a combined relapse but could not be confirmed due to the clinical status of the patient. The median time from MRD detection by flow cytometry to relapse was 26 days (range 0–326). Of note, fifteen out of the twenty patients (75%) had an identifiable leukemia associated immunophenotype LAIP (see Table 2). One of the two patients in whom MFC failed to anticipate MRD detection had no specific LAIP.

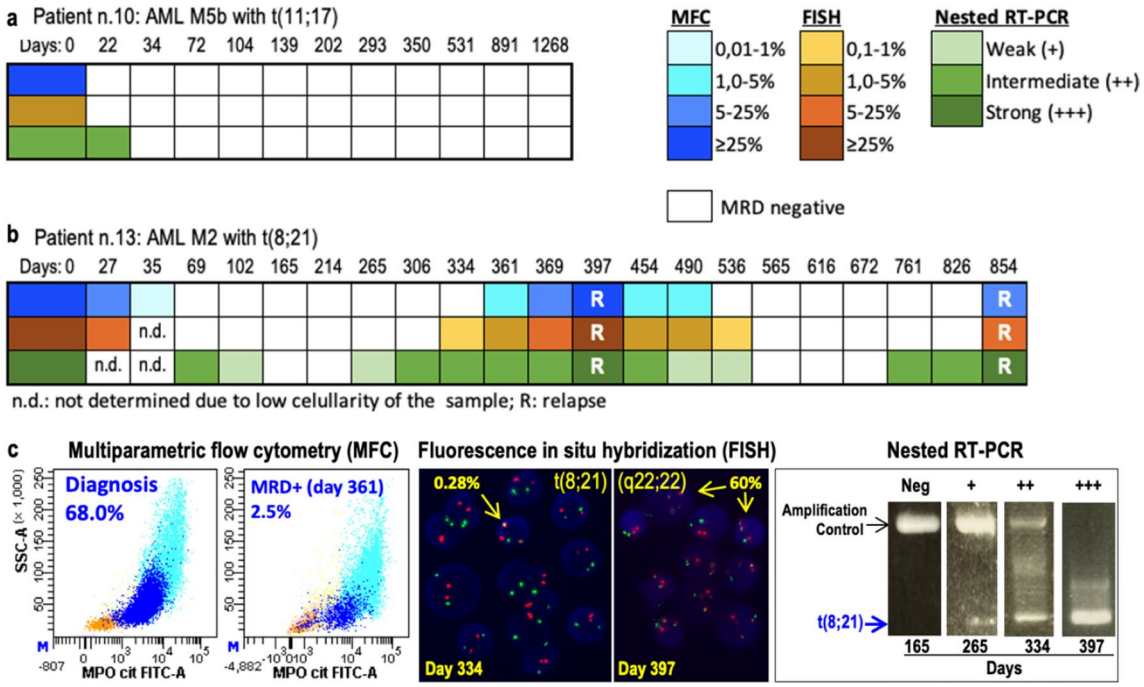

Fig. 1 Evolutionary patterns of MRD and relapse in AML patients. **a** The patient enters in complete remission and stays in remission until the end of follow-up. **b** Relapse is preceded by the reappearance and progressive increment of the measurable residual disease detected by nested reverse transcriptase polymerase chain reaction (RT-PCR), fluorescent in situ hybridization (FISH) and/or multiparametric flow cytometry (MFC). Progressive normalization of those markers is observed during the resolution of the relapse. **c** Shows representative results for MFC, FISH and RT-PCR in bone marrow samples with different levels of leukemic infiltration

a

	Flow cytometry		KTM2A or t(9;9)		t(8;21) or inv(16)		1 log WT1 increment	
	MRD⁻	MRD⁺	MRD⁻	MRD⁺	MRD⁻	MRD⁺	No	Yes
No Relapse	15	0	4	0	1	4	5	0
Relapse	4	6	0	4	0	1	0	3
	p = 0.001		p = 0.03		p = 1.000		p = 0.02	

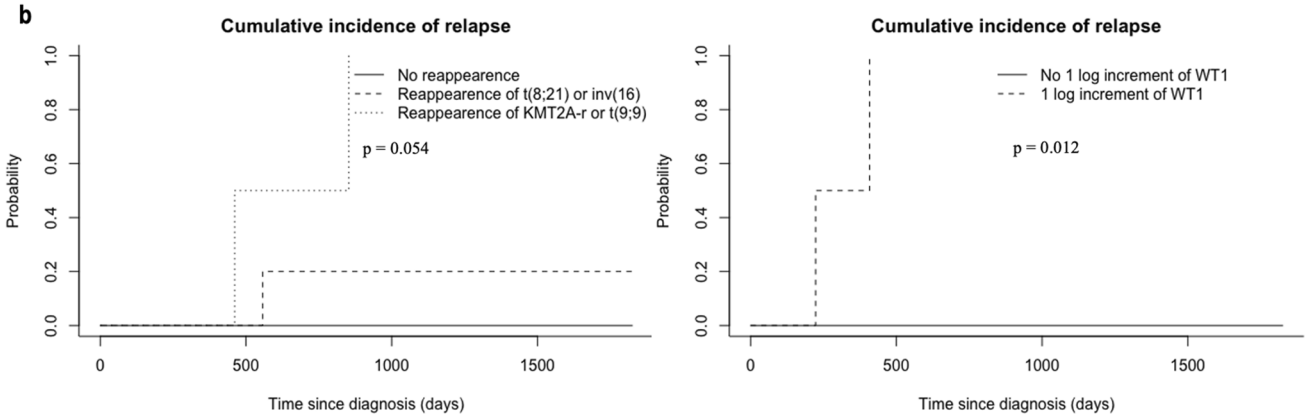

Fig. 2 Association between MRD detection and posterior AML relapse. **a** Crosstab showing the relationship between the reappearance of MRD detected by multiparametric flow cytometry, RT-PCR and FISH for KTM2A or t(9;9) and t(8;21) or inv(16), as well as by the one log increment of WT1 expression in two consecutive samples and the AML relapse. **b** Cumulative incidence of relapse according to the reappearance of these rearrangements (left) or the one log increment of WT1 expression (right)

Table 2 Leukemia-associated immunophenotype (LAIPs)

Patient id	LAIP	Personalized LAIP	Relapse
#1	Yes	CD45−CD34−DR−CD56 + +	Yes
#2	Yes	CD45dim CD34−DR−CD41/42/61 + +	No
#3	Yes	CD45dim CD34−DR−CD41/42/61 + + +	Yes
#4	No	–	No
#5	Yes	CD45dim CD34−DR−MPO + +	No
#6	Yes	CD45dim CD34−CD41/42/61 +	No
#7	No	–	Yes
#8	Yes	CD45dim CD7 +	Yes
#9	Yes	CD34−CD 117 + DR + + + NG2 +	No
#10	Yes	CD34−DR + + CD64 + + + NG2 +	No
#11	No	–	No
#12	No	–	No
#13	Yes	CD45dim CD34 + CD33−MPO +	Yes
#14	Yes	CD45dim CD34 + CD13 + + + CD19 + MPO +	No
#15	Yes	CD45dim CD34 + CD33−CD56 + MPO +	No
#16	Yes	CD45 dim CD34 + CD13−CD19 + + MPO + +	No
#17	Yes	CD45dim CD34−CD4 + CD56 + MPO-	No
#18	Yes	CD45dim CD34−DR−CD38−MPO + + +	No
#19	No	–	No
#20	Yes	CD45dim CD34−CD33−CD38−/ + dim MPO +	No

RT-PCR and FISH

In patients showing a specific rearrangement that could be monitored by RT-PCR or FISH, all the relapses were preceded by the reappearance of the alteration. Also, all the reappearances of the genetic alteration after first-line treatment were followed by a relapse ($p = 0.03$), except in the case of core binding factor leukemias (t(8;21) RUNX1:RUNXT1 and inv(16) CBFB/MYH11) where only 1 patient with a t(8;21) relapsed (Fig. 2a). The cumulative incidence of relapse according to specific rearrangements is shown in Fig. 2b. The median time from the reappearance of the specific translocation detected by RT-PCR to relapse was 111 days (range 90–575).

WT1 expression

In the seven patients monitored trough WT1/ABL expression ratio on BM cells, all the relapses were preceded by a molecular increase of WT1/ABL, but not all the molecular increases of WT1 were followed by a relapse. However, one log increment of WT1 expression in two consecutive evaluations was always associated with subsequent frank hematological relapse ($p = 0.02$) (Fig. 2a). The cumulative incidence of relapse regarding the increment of WT1 expression is shown in Fig. 2b. The median time from a 1 log increment of WT1 expression in two consecutive evaluations to relapse was 140 days (range 47–197). See Figure S1 to see evolution in WT1/ABL expression in all patients.

Comparing the results of the three methods to evaluate MRD (MFC, RT-PCR and FISH) in BM aspirates of patients with a specific rearrangement, we identified seven samples with negative MFC and positive FISH and only two with positive MFC and negative FISH. Besides, we identified forty-nine samples with negative MFC and positive RT-PCR and just one with positive MFC and negative RT-PCR. Also, we identified forty-three samples with positive RT-PCR and negative FISH and no samples with positive FISH and negative RT-PCR (Table 3).

Discussion

Monitoring MRD during the follow-up of childhood AML to predict relapse is challenging. While MFC is an almost universal method for the evaluation of MRD because most AMLs have a specific LAIP, its lower sensitivity compared to molecular methods delay the detection of the reappearance of the disease. In this study of real-life clinical practice, MFC was able to detect MRD positivity 26 days (median time) before relapse, compared to 111 days of molecular methods. Unfortunately, only 40% of AMLs express a specific translocation [32] that can be detected with sensitive molecular methods; therefore, we should use two different strategies, one for patients that present specific translocations and other for those that do not.

Regarding AMLs with a specific translocation, our results obtained by nested RT-PCR show that the reappearance of

Table 3 Comparative analysis of MRD results detected by MFC, FISH and RT-PCR in patients with specific chromosomal rearrangement

Patient id	Rearrangement	N. of evaluations[1]	MFC +	LAIP	FISH +	RT-PCR +	Relapse
#4	t(8;21)	11	1	No	2	8	No
#7	t(6;11)	15	1	No	2	11	Yes
#8	t(9;9)	1	1	Yes	–[2]	1	Yes
#9	t(9;11)	1	1	Yes	1	1	No
#10	t(11;17)	13	1	Yes	1	2	No
#11	t(8;21)	19	1	No	3	11	No
#12	t(8;21)	18	1	No	2	10	No
#13	t(8;21)	20	7	Yes	8	14	Yes
#15	t(8;21)	8	1	Yes	2	6	No
#17	t(9;11)	8	4	Yes	2	3	No
#18	ins(11;X)	1	1	Yes	1	–[2]	No
#20	inv(16)	1	1	Yes	1	1	No

[1]Only evaluations during and after therapy in which the three diagnostic methods (MFC, FISH and RT-PCR) were carried out were considered in this analysis. This excludes evaluations in which the MFC analysis undoubtedly detected MRD + ; in these cases, FISH or RT-PCR was not performed.

[2]In patients 8 and 18, t(9;9) and ins(11;X) could not be detected by FISH and RT-PCR, respectively, because they were not included among the studied translocations by these methods as described in the methods section.

t(8;21)(q22;q22) (RUNX1-RUNX1T1), did not systematically imply a relapse. These findings are consistent with the study of Miyamoto et al. in which some patients in remission positivized the t(8;21) without experiencing relapse [33]. This is supported by the fact that t(8;21), in the absence of other secondary events, is insufficient for leukemogenesis [34]. However, when the t(8;21) exceeds a certain threshold measured by qPCR, it is indicative of relapse [35, 36], probably reflecting an uncontrolled proliferation of tumor cells that have suffered additional events. Similar results have been described for the inv(16) or *CBFB/MYH11* genetic alteration [35, 36].

In contrast, our results show that the reappearance of t(9;9)(q34;q34) (SET-NUP214) and MLL rearrangements, if untreated, conducts irremediably to the relapse. These results are also in agreement with those of Matsuo et al. and Juul-Dam et al. in which the reappearance of MLL rearrangements was followed or accompanied by a relapse in all cases [35, 36]. Therefore, even though the technique used in this study do not allow MRD quantification of these genes, our results suggest that the reappearance of these rearrangements in BM seems to inevitably lead to relapse in contrast to core binding factor leukemias.

Differences between core binding factor leukemias and other leukemias draw attention to the fact that each translocation has a different significance regarding the biology of the tumor cell and should be interpreted in consequence.

For patients with AML lacking specific molecular biomarker an alternative approach might be the measurement of WT1 expression, which is a specific biomarker of immature myeloid cells [37]. WT1 overexpression is present in up to 90% of AMLs [21]. Many studies have demonstrated its usefulness as a marker to anticipate hematological relapse; however, each study uses a different approach. For instance, in the study of Weisser et al. 16 of 44 relapses were preceded by a one log increment of the transcript in two consecutive bone marrow samples [38], whereas in the study published by Mashima et al. the cumulative incidence of relapse was significantly higher in patients with two consecutive values greater than 100 copies per µg of RNA in peripheral blood [39]. In our study, all the relapses monitored with this marker were preceded by a one log increase and none of the patients in complete remission showed a one log increase between two consecutive samples.

However, we could not see how the MRD reappearance was immediately followed by a relapse in all cases because of the use of pre-emptive treatment (cyclosporine-A withdrawal, DLIs or boost of donor cells). Pre-emptive treatments in our series could probably have extended the median time from MRD reappearance to relapse. Unfortunately, pre-emptive treatments in our study only temporarily halted the progression of the disease, and patients finally relapsed and died. This is indicative either that new and more effective therapies should be investigated, or that these treatments should be provided earlier to be effective. Accordingly, more sensitive MRD monitoring methods that further anticipate relapse such as NGS and digital droplet PCR should be introduced to provide more time for second line strategies, like stem cell transplantation, where time for donor search is crucial.

Although theoretically sensitivity of MFC is higher than that of FISH, in our study seven samples with negative MFC

were detected positive for the specific chromosomal translocation by FISH. This is indicative that in some AML patients without well-defined LAIP, an increase in the number of cells to be analyzed by FISH may offer an alternative method for MRD evaluation that could help to anticipate relapse, most particularly if molecular methods are not available.

Conclusions

This study of real-life clinical practice clearly shows that MFC, FISH and RT-PCR, although with different sensitivity, are complementary methods to analyze MRD monitoring and anticipate childhood AML relapse. Although molecular methods can anticipate relapse by weeks or months, pre-emptive therapies in our series were not able to prevent disease progression. Therefore, more sensitive MRD monitoring methods that further anticipate relapse should be applied to provide more time for new and more effective pre-emptive or second-line strategies.

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Data availability The data that support the findings of this study are available from the corresponding author, upon reasonable request.

Declarations

Conflict of interest The authors report no conflict of interest.

Ethical approval The study was performed in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent Written informed consent was obtained from all patients (and/or their parents) in accordance with the Declaration of Helsinki.

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SUPPLEMENTARY MATERIALS FOR THE FIRST PUBLICATION

Treatment details

Treatment was administered according to the SHOP-LMA-2007 protocol (https://www.recerca.com/shop/entrar/prot_pdf/LMA2007_PROTOCOLO.pdf) or the more recent NOPHO-DBH AML 2012 protocol (EudraCT number: 2012-002934-35). Patients were classified as low, high or very high risk in the SHOP-LMA-2007 protocol and as standard or high risk in the NOPHO-DBH-AML-2012 protocol according to the presence of particular cytogenetic abnormalities at diagnosis and treatment response (see table 1 for details). Patients diagnosed between 2016 and 2017 received an infusion of NK cells as consolidation instead of autologous BM transplantation or at the end of the protocolized treatment, as part of a phase II clinical trial (NCT02763475) [1-4]. The patient diagnosed as the mixed phenotype acute leukemia received the first induction from the NOPHO-DBH AML 2012 protocol and consolidation, reinduction and maintenance from the current acute lymphoblastic leukemia protocol in our country, the SEHOP-PETHEMA 2013 protocol. In case of suspected regrowth of the disease after HSCT, early cyclosporine withdrawal, DLIs and/or boost of donor cells were applied as preemptive therapy.

Immunophenotype and MRD studies

Immunophenotyping and MRD studies were performed in bone marrow (BM) aspirates obtained at diagnosis and during follow-up by using 8-color FACSCanto-II (from 2012 to June 2020) or 12-color FACSLyric (from June 2020 to 2022) flow cytometers (Becton Dickinson, BD, San Jose, CA). Photomultiplier (PMT) voltages were adjusted daily using CS&T beads (BD). Fluorescence compensations were adjusted using FC beads (BD) every two months and finely adjusted on a daily base using negative events as reference for each fluorochrome, as previously described [5]. For cell surface staining, 100µL of BM samples diluted with PBS-1%BSA or concentrated using bulk lysing with ammonium chloride (BD) to contain 3 million total white cells were labeled during 10 minutes at room temperature in the dark with the appropriate amount of antibodies to detect the following molecules: CD3, CD4, CD7, CD11b, CD13, CD14, CD16, CD19, CD33, CD34, CD36, CD38, CD41, CD45, CD56, CD58, CD61, CD64, CD66, CD71, CD81, CD117, CD123, CD203c, CD235a (Glycophorin-A), CD300e (IREM2), CD371 and HLA-DR (see tables 1 and 2 in this supplementary file for

details). Antibodies were purchased from BD, Beckman Coulter (Brea, California) or Dako (Glostrup, Denmark). Finally, samples were lysed with 3 ml FACSLysing (BD), washed with 3 ml of FACSFlow (BD) and 1 to 2 million cells acquired for each tube. For intracellular staining of myeloperoxidase (MPO), terminal deoxynucleotidyl transferase (Tdt) or CD3, IntraStaing kit (Dako, Denmark) was used following the manufacturer's instructions. The same antibody-fluorochrome combinations at diagnosis and follow-up were used.

DiVA™ Software (BD) was used for sample analysis. LAIPs were defined as clusters of cells displaying patterns of antigenic expression separated from normal myeloid maturation stages [6]. As previously described [7], aberrant immunophenotypes were divided into four main subgroups: 1) cross-lineage antigen expression, 2) asynchronous antigen expression, 3) antigen dim/strong expression and 4) antigen expression missing. Abnormal forward-scattered and side-scattered patterns were also included in the LAIP. MRD was defined in presence of a distinct cluster of at least 20 cells showing a compatible LAIP, to reach theorical maximum sensitivities ranging from 1 to 2 x 10^{-5}.

Molecular studies

FLT3 internal tandem duplication mutations (FLT3-ITD) and mutation in the tyrosine kinase domain (FLT3-TKD) were evaluated in genomic DNA extracted from whole BM samples using QIAmp® DNA blood mini kit and QIAsymphony® (QIAgen, GmbH, Hilden, Germany). Previously described methods were used for detection of both FLT3-ITD [8] and FLT3-TKD [9] mutations with slight modification labelling forward primers at 5′ end with FAM fluorochrome to be resolved in ABI PRISM® 3500 Genetic Analyzer (Applied Biosystems, Foster City, CA).

NPM1 and CEBPA mutations and WT1 expression were evaluated in total RNA extracted from BM samples using QIAmp® RNA blood mini kit and QICube® (QIAgen). Complementary DNA (cDNA) was synthesized starting with 1µg of RNA using M-MLV reverse transcriptase and random primers (Thermofisher Scientific, MA, USA), and RNase inhibitor (MerK, Darmstadt, Germany). Previously described methods were used for detection of both NPM1 [10] and CEBPA [11]. WT1 expression was evaluated using WT1 Profile Quant™ Kit (Ipsogen, Marseille, France) according to the manufacturer's protocol so WT1 levels were calculated conforming to the standard curve method.

Common chromosomal translocations in childhood leukemia as del(1p32) (STIL-TAL1), t(1;11)(p32;q23) (KMT2A-EPS15), t(1;11)(q21;q23) (KMT2A-MLLT11), t(1;19)(q23;p13) (TCF3-PBX1), t(3;5)(q25;q34) (NPM1-MLF1), t(3;21)(q26;q22) (RUNX1-MECOM), t(4;11)(q21;q23) (KMT2A-AFF1), t(5;12)(q33;p13) (ETV6-PDGFRB), t(5;17)(q35;q21) (NPM1-RARA), t(6;9)(p23;q34) (DEK-NUP214), t(6;11)(q27;q23) (KMT2A-AFDN), t(8;21)(q22;q22) (RUNX1-RUNX1T1), t(9;9)(q34;q34) (SET-NUP214), t(9;11)(p22;q23) (KMT2A-MLLT3), t(9;12)(q34;p13) (ETV6-ABL1), t(9,22)(q34;q11) (BCR-ABL1), t(10;11)(p12;q23) (KMT2A-MLLT10), t(11;17)(q23;q21) (KMT2A-MLLT6), t(11;17)(q23;q21) (ZBTB16-RARA), t(11;19)(q23;p13.1) (KMT2A-ELL), t(11;19)(q23;p13.3) (KMT2A-MLLT1), t(12;21)(p13;q22) (ETV6-RUNX1) t(12;22)(p13;q11) (ETV6-MN1), t(15;17)(q24;q21) (PML-RARA), inv(16)(p13;q22) (CBFB-MYH11), t(16;21)(p11;q22) (FUS-ERG), t(17;19)(q22;p13) (TCF3-HLF), t(X;11)(q13;q23) (KMT2A-FOXO4) were evaluated using multiplex nested RT-PCR HemaVision®-28N Chromosomal Translocations kits (DNA Technology, Aarhus, Denmark) which detects 80 splice variants. In this case, reverse transcription was performed with a mixture of translocation-specific primers using the HemaVision® reagent module.

Table 1. Criteria for risk classification according to treatment protocol.

Protocol	Risk	Details
SHOP-LMA-2007	Low	Patients with t(8;21) or inv(16), absence of -5 and -7, good response in PB at day +7 and in BM at day +21 and CR after 1st induction cycle.
	High	All patients not included as low risk or very high risk.
	Very High	Patients with -5 and -7.
NOPHO-DBH-AML	Standard	All patients not included as high risk.
	High	Patients that achieve CR after two induction courses and either of the following: poor response after course 1(>15%), intermediate response after course 2 (0.1-4.9%) or FLT3-ITD without NMP1 mutation.

CR: Complete remission.

Table 2. Combinations of monoclonal antibodies used in 8 or 12 color studies.

Canto-II (8 colors)	**Tube-1**	**Tube-2**	**Tube-3**	**Tube-4**	**Tube-5**
FITC	CD7[a]	CD66[c]	cyMPO[c]	cyTdt[a]	CD36[b]
PE	CD56[a]	CD13[a]	HLA-DR[a]	CD41/42/61[a]	CD123[a]
PE-Cy5 or PerCP	CD38[a]	CD38[a]	CD38[a]	CD19[a]	CD11b[b]
PE-Cy7	CD34[a]	CD34[a]	CD34[a]	CD34[a]	CD34[a]
APC or AF647	CD33[b]	CD33[b]	CD33[b]	GlicoA[a]	CD64[a]
APC-H7	CD45[a]	CD45[a]	CD45[a]	CD45[a]	CD45[a]
BV421 or V500	CD14[a]/CD19[a]	CD117[a]	CD14[a]	cyCD3[a]	CD4[a]
BV510	CD16[a]/CD3[a]	CD16[a]	CD16[a]	CD3[a]	CD3[a]

Lyric (12 colors)	**Tube-1**	**Tube-2**	**Tube-3**
FITC	CD4[a]/19[a]/66[c]/71[a]	CD36[b]	cyMPO[c]
PE	CD13/CD235a[a]	CD117[c]	CD41/CD61[a]
PE-Cy5 or PerCp	CD11b[a]	CD11b[b]	HLA-DR[a]
PE-Cy7	CD34[a]	CD34[a]	CD34[a]
APC or AF647	CD117[a]	CD64[a]	CD14[a]
APC-R700	CD38[a]	CD38[a]	CD38[a]
APC-H7	CD45[a]	CD45[a]	CD45[a]
BV421 or V450	CD16[a]	CD7[a]	CD58[a]
BV510	CD3[a]/CD300e[a]	CD123[a]	CD123[a]
BV605	CD33[a]	CD371[a]	CD4[a]
BV711	CD56[a]	CD56[a]	TIM-3[a]
BV786	HLA-DR[a]	HLA-DR[a]	CD203c[a]

Monoclonal antibodies were purchased from: [a] Becton Dickinson (San Jose, CA); [b] Beckman Coulter (Brea, California); or [c] Dako (Glostrup, Denmark).

Table 3. Details and source of monoclonal antibodies used in for immunophenotyping.

Monoclonal antibody	Manufacturer, reference	Clone
CD7 FITC	Becton Dickinson, 347483	124-1D1
CD66 FITC	Dako, F7112	Kat4c
cyMPO FITC	Dako, F0714	MPO-7
cyTdt FITC	Becton Dickinson, 332789	E17-1519
CD36 FITC	Beckman Coulter, B77224	FA6.152
CD56 PE	Becton Dickinson, 345810	MY31
CD13 PE	Becton Dickinson, 347406	L138
HLA-DR PE	Becton Dickinson, 347401	L243
CD41 PE	Becton Dickinson, 555467	HIP8
CD42 PE	Becton Dickinson, 555473	HIP27
CD61 PE	Immunostep, 61PE	VIPL2
CD123 PE	Becton Dickinson, 561050	9F5
CD38 PE-Cy5	Becton Dickinson, 551400	HIT2
CD19 PerCp	Becton Dickinson, 332780	4G7
CD11b PE-Cy5	Becton Dickinson, 555389	ICRF44
CD34 PE-Cy7	Becton Dickinson, 348811	8G12
CD33 APC	Beckman Coulter, IM2471	D3HL60.251
Glico-A APC	Immunostep, CD235A-100T	HI264
CD64 APC	Becton Dickinson, 561189	10,1
CD45 APC-H7	Becton Dickinson, 641417	2D1
CD14 BV421	Becton Dickinson, 565283	M5E2
CD19 BV421	Becton Dickinson, 562440	HIB19
CD117 BV421	Becton Dickinson, 562434	YB5.B8
cyCD3 V500	Becton Dickinson, 561417	UCHT1
CD4 BV421	Becton Dickinson, 562425	RPA-T4
CD16 BV510	Becton Dickinson, 563830	3G8
CD3 BV510	Becton Dickinson, 563109	UCHT1
CD4 FITC	Becton Dickinson, 345768	SK3
CD19 FITC	Becton Dickinson, 345776	4G7
CD71 FITC	Becton Dickinson, 333151	L01.1
CD235a PE	Becton Dickinson, 555570	GA-R2 (HIR2)
CD117 PE	Dako, R7145	104D2
HLA-DR PerCp	Becton Dickinson, 339216	L243
CD117 APC	Becton Dickinson, 333233	104D2
CD14 APC	Becton Dickinson, 345787	МΦР9
CD38 APC-R700	Becton Dickinson, 564979	HIT2
CD16 BV421	Becton Dickinson, 562874	3G8
CD7 BV421	Becton Dickinson, 562635	M-T701
CD58 BV421	Becton Dickinson, 566239	1C3
CD300e BV510	Becton Dickinson, 744993	UP-H1
CD123 BV510	Becton Dickinson, 563072	9F5
CD33 BV605	Becton Dickinson, 745229	P67.6
CD371 BV605	Becton Dickinson, 742931	50C1
CD4 BV605	Becton Dickinson, 562658	RPA-T4
CD56 BV711	Becton Dickinson, 563169	NCAM16.2
TIM-3 BV711	Becton Dickinson, 565566	7D3
HLA-DR BV785	Biolegend, 307642	L243

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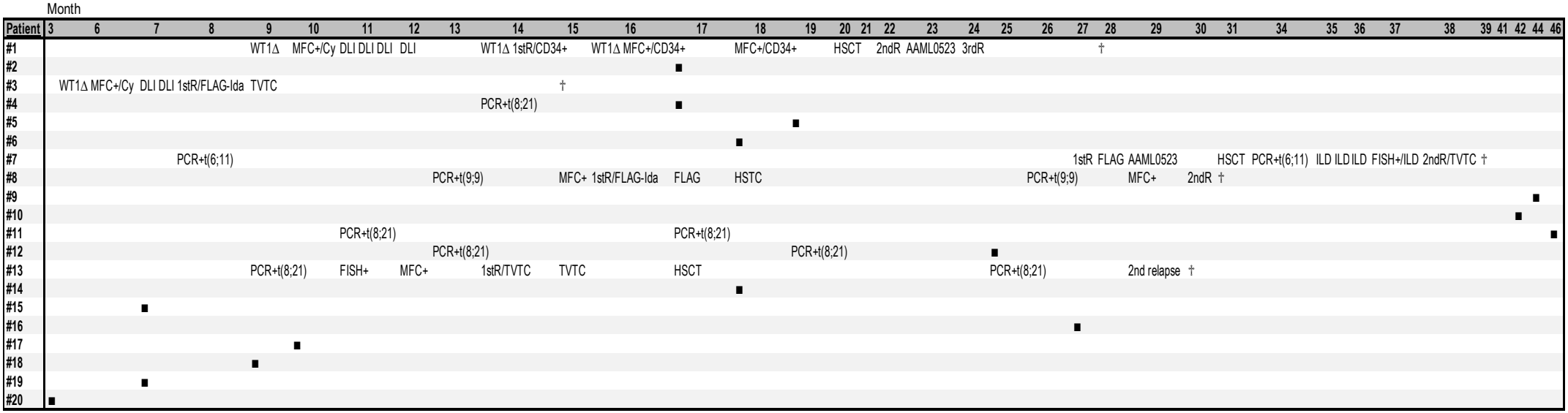
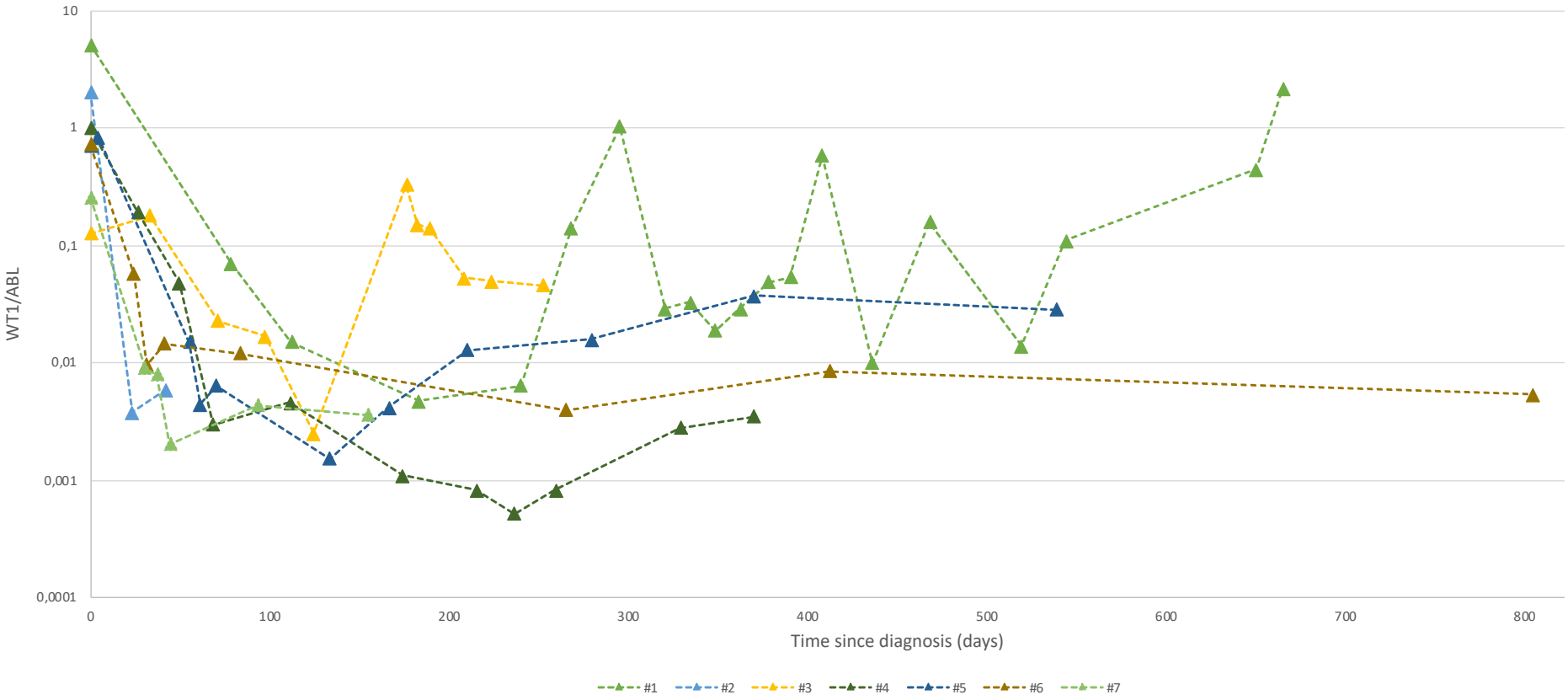
Table S1. Time course of main events in the 20 included patients.



R: relapse; MFC+: MRD reappearance in MFC after first line treatment; FISH+: MRD reappearance in FISH after first line treatment; PCR+: MRD reappearance in PCR after firs line treatment; WT1Δ: 1 log increase in two consecutives samples; DLI: donor lymphocyte infusions; Cy: early cyclosporine withdrawal; CD34+: boost of CD34+ cells; AAML0523: clofarabine and cytarabine; FLAG: fludarabine, cytarabine and G-CSF with or without idarubicin (Ida); TVTC: topotecan, vinorelbine, thiotepa and clofarabine; HSTC: hematopoietic stem cell transplantation after firs line treatment; ■ last bone marrow evaluation; and ✝ death.

Figure S1. WT1/ABL expression in the seven patients monitored trough this diagnostic method.



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RESEARCH ARTICLE

Measurable residual disease study through three different methods can anticipate relapse and guide early interventions in childhood acute lymphoblastic leukemia

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Abstract

Introduction Acute lymphoblastic leukemia (ALL) is the most common cancer among children. Measurable residual disease (MRD, previously named minimal residual disease) study can guide therapy adjustments or preemptive interventions that might avoid hematological relapse.

Methods Clinical decision making and patient outcome were evaluated in 80 real-life childhood ALL patients, according to the results observed in 544 bone marrow samples analyzed with three MRD methods: multiparametric flow cytometry (MFC), fluorescent in-situ hybridization (FISH) on B or T-purified lymphocytes and patient-specific nested reverse transcription polymerase chain reaction (RT-PCR).

Results Estimated 5 year overall survival and event-free survival were 94% and 84.1%, respectively. A total of 12 relapses in 7 patients were associated with positive MRD detection with at least one of the three methods: MFC ($p < 0.00001$), FISH ($p < 0.00001$) and RT-PCR ($p = 0.013$). MRD assessment allowed the anticipation of relapse and adapted early interventions with different approaches including chemotherapy intensification, blinatumomab, HSCT and targeted therapy to halt relapse in five patients, although two of them relapsed afterwards.

Conclusion MFC, FISH and RT-PCR are complementary methods for MRD monitoring in pediatric ALL. Although, our data clearly show that MDR positive detection is associated with relapse, continuation of standard treatment, intensification or other early interventions were able to halt relapse in patients with different risks and genetic background. More sensitive and specific methods are warranted to enhance this approach. However, whether early treatment of MRD can improve overall survival in patients with childhood ALL needs to be evaluated in adequately controlled clinical trials.

Keywords Childhood acute lymphoblastic leukemia · Measurable residual disease · Multiparameter flow cytometry · Fluorescent in-situ hybridization · Polymerase chain reaction · Relapse

Introduction

Acute lymphoblastic leukemia (ALL) is the most common cancer among children [1]. Five-year survival is around 90% in most developed countries with current treatment protocols [2]. However, relapse occurs in 15–20% of patients and cure rates are much lower after this [1]. Personalized therapy based on patients' risk stratification has mostly contributed to improve survival after first-line treatment [3]. Risk stratification is usually based on clinical and biological findings at diagnosis (age, white blood cell count -WBC-, immunophenotype, extramedullar involvement or genetic alterations) and on early response to treatment at different timepoints [3], through the monitoring of the measurable residual disease (MRD, previously named minimal residual disease). However, little research has been done on the utility of extending the MRD analysis further during the follow-up [4].

MRD is defined as the leukemic cells persisting below the sensitivity of bone marrow morphology after chemotherapy. MRD can be measured trough different methods as polymerase chain reaction (PCR), multiparametric flow cytometry (MFC) or fluorescent in-situ hybridization (FISH).

Extended author information available on the last page of the article

Clone-specific PCR of the immunoglobulin/T-cell receptor gene rearrangements is a well-established method to monitor MRD in ALL with an estimated sensitivity of 10^{-4}–10^{-5} [5]. Also, PCR based methods can be used to monitor recurrent translocations with sensitivities ranging from 10^{-4} to 10^{-6} [5]. MFC has a sensibility up to 10^{-4}, inferior to molecular methods, but it is also a widely employed method as a faster technique [5]. FISH is not a commonly used method due to its low sensitivity. However, sensitivity can be improved by performing analysis on purified B or T lymphocytes according to the leukemia immunophenotype and increasing the number of analyzed cells using automated FISH signal analysis.

Any specific genetic alteration has the potential to be used as a marker to monitor MRD trough molecular methods. Main recurrent translocations in B-cell precursor ALL (B-ALL) are t(12;21)(p13;q23) (*ETV6/RUNX1)*, t(1;19(q23;p12) (*TCF3/PBX1),* t(9;22)(q34;q11.2) (*BCR/ABL1*) and *KMT2A* gene rearrangements and their prevalence, role in leukemogenesis and prognostic significance are widely discussed in the literature [6–8].

Intrachromosomal amplification of *RUNX1* (iAMP21) is defined as the presence of three or more copies of the *RUNX1* gene within a chromosome 21 [6] and it is usually diagnosed when five or more copies of the *RUNX1* gene are detected by FISH [9]. The prevalence of iAMP21 is about 2% and it is associated with a high risk of relapse, which can be counteracted with high-risk treatment protocols [9, 10].

Ph-like ALL is defined by a gene-expression profile similar to *BCR-ABL* leukemias, but in the absence of this rearrangement [11]. Prognosis of Ph-like ALL is as bad as *BCR-ABL* leukemias [11]. This entity is not defined by a single rearrangement or genetic alteration, but it associates a wide range of alterations such as rearrangements of *JAK2, EPOR* and *CRLF2* and other point mutations [7] that can be used as therapeutic targets [8, 12]. Most of these alterations can be detected by FISH [12].

In contrast, genetic alterations in T-cell ALL (T-ALL) have been less studied and are mostly different to those of B-ALL. Recurrent genetic alterations in T-ALL are, for instance, interstitial 1p32 deletion with the consequent *TAL1* dysregulation and the t(5;14) (q35;q32) translocation which conduces to a *TLX3* dysregulation [7].

MRD study is useful at specific time points to guide therapy adjustments but can be also useful to guide preemptive treatments before overt hematological relapse. Although this approach is not as developed in acute lymphoblastic leukemia as in acute myeloblastic leukemia, some previous experiences of preemptive treatment have already been carried out, especially with tyrosine kinase inhibitors [13].

This study is aimed at evaluating the usefulness of three MRD monitoring methods (MFC, FISH and PCR) for the early prediction of relapse in a subset of real-life pediatric ALL patients and its importance in introducing early personalized relapse preemptive treatments.

Patients and methods

Patients and samples

In this retrospective, observational and analytical study, clinical and laboratory data of pediatric patients with ALL diagnosed in the Clinical University Hospital Virgen de la Arrixaca (Murcia, Spain) between June 2013 and February 2022 were reviewed. Diagnostic criteria for the type and subtype of ALL were based on the WHO classification of tumors of hematopoietic and lymphoid tissues [14, 15]. The end of data collection was on April 1st, 2022. The institutional review board (IRB-00005712) approved the study. Written informed consent was obtained from all patients (and/or their parents) in accordance with the Declaration of Helsinki.

Treatment was administered according to the SEHOP-PETHEMA 2013 protocol in patients between 1 and 19 years old and according to our Spanish National INTERFANT-06 based treatment guidelines in patients under 1 year old. Patients were classified as standard, intermediate or high risk in the SEHOP-PETHEMA 2013 protocol according to their age, WBC at diagnosis, immunophenotype, extramedullary infiltration, cytogenetics and early response to treatment. Patients younger than 1 year were classified as low, medium and high risk according to the *KMT2A* status, age, WBC count and treatment response. See supplementary table S1 for details.

Cytomorphology, immunophenotype, cytogenetics (karyotype and FISH) and molecular results of bone marrow (BM) aspirates were recorded at each evaluation. Patients treated with the SEHOP-PETHEMA 2013 protocol were evaluated at different timepoints according to their risk group. Standard and intermediate risk patients were evaluated at diagnosis and at days + 15, + 33 and + 78. High risk patients were evaluated at diagnosis, at days + 15, + 33, + 52 (if complete remission was not achieved at day 33), + 78, before the second high-risk intensification block if MRD was ≥ 0.01% at day + 78, and at hematological recovery after the third intensification block. Patients treated with the INTERFANT-06 based treatment guidelines were evaluated at seven different timepoints: at diagnosis, at days + 15 and + 33, before MARMA and OCTADAD consolidation blocks, before maintenance, at week 43 of maintenance chemotherapy and at the end of the treatment. Patients undergoing stem cell transplantation were evaluated at days + 30, + 60, + 90, + 180 and + 360 after transplantation. Other extra evaluations were performed according to clinician's criteria based on

the presence of unexplained anemia, thrombocytopenia or neutropenia during follow-up.

Immunophenotype and MRD studies

Immunophenotyping and MRD studies were performed in BM aspirates obtained at diagnosis and during follow-up using 8-color FACSCanto-II (from June 2013 to June 2020) or 12-color FACSLyric (from June 2020 to April 2022) flow cytometers (Becton Dickinson, BD, San Jose, CA). Photomultiplier (PMT) voltages were adjusted daily using CS&T beads (BD). Fluorescence compensations were adjusted using FC beads (BD) every two months and finely adjusted on a daily base using negative events as reference for each fluorochrome, as previously described [16]. For cell surface staining, 100 µL of BM samples diluted with PBS-1%BSA or concentrated using bulk lysing with ammonium chloride (BD) to contain 3 million total white cells were labeled during 10 min at room temperature in the dark with the appropriate amount of antibodies to detect the following molecules: CD3, CD4, CD7, CD8, CD10, CD11b, CD13, CD14, CD15, CD16, CD19, CD20, CD22, CD33, CD34, CD38, CD45, CD45RA, CD56, CD58, CD66, CD71, CD79a, CD 79b, CD81, CD117, CD235a (Glycophorin-A), CD300e (IREM2), Tdt, IgM, MPO, HLA-DR and CRLF2 (see supplementary tables S2 and S3 for details). Antibodies were purchased from BD, Beckman Coulter (Brea, California) or Dako (Glostrup, Denmark). Finally, samples were lysed with 3 ml FACSLysing (BD), washed with 3 ml of FACSFlow (BD) and 1 to 2 million cells acquired for each tube. For intracellular staining of myeloperoxidase (MPO), terminal deoxynucleotidyl transferase (Tdt) or CD3, IntraStaing kit (Dako, Denmark) was used following the manufacturer's instructions. The same antibody-fluorochrome combinations at diagnosis and follow-up were used. DiVA™ Software (BD) was used for sample analysis. LAIPs were defined as clusters of cells displaying patterns of antigenic expression separated from normal lymphoid maturation stages. Aberrant immunophenotypes were divided into four main subgroups: (1) cross-lineage antigen expression, (2) asynchronous antigen expression, (3) antigen dim/strong expression and (4) antigen expression missing. Abnormal forward-scattered and side-scattered patterns were also included in the LAIP. MRD was defined in presence of a distinct cluster of at least 20 cells showing a compatible leukemia associated immunophenotype (LAIP), to reach theorical maximum sensitivities ranging from 1 to 2×10^{-5}.

Fluorescent in-situ hybridization (FISH)

Cytogenetic abnormalities were evaluated in interphase nucleus from purified B or T lymphocytes using RosetteSep™ Human B Cell Enrichment Cocktail or RosetteSep™ Human T Cell Enrichment Cocktail (Stemcell Technologies, Grenoble, France), respectively, following standard procedures previously validated [17]. The following FISH probes from Metasystems (Altlussheim, Germany) were used for the diagnosis of B ALL: XCE 4/10/17 (cut-off 2% for trisomies and 10% for monosomies), XL t(12;21) ETV6/RUNX1 DF (cut-off 1%), XL E2A BA (cut-off 10%), BCR/ABL1/ASS1 (cut-off 1%), XL KMT2A BA (cut-off 10%) and XL CDKN2A Deletion Probe (cut-off 9%). In case the former assays were negative, since June 2019, we tried to identify genetic alterations linked to Ph-like B-ALL through the following probes: XL 5q32 PDGFRB BA (cut-off 10%), XL JAK2 BA (cut-off 10%) and XL ABL2 BA (cut-off 10%) from Metasystems and CRLF2 Breakapart probe (cut-off 10%) and EPOR Breakapart probe (cut-off 10%) from Cytocell. For the diagnosis of T-ALL we used the following probes from Metasystems: XL BCR/ABL1/ASS1 (cut-off 1%), XL KMT2A BA (cut-off 10%), XL CDKN2A Deletion Probe (cut-off 9%), XL 6q21/6q23 Deletion Probe (cut-off 10%) and XL TLX3 BA (cut-off 10%). For each probe 250 cells were analyzed with Metafer system (Metasystems). Up to 3000 purified B or T cells were captured to increase sensitivity when needed.

Molecular studies

Common chromosomal translocations in childhood leukemia as del(1p32) (*STIL-TAL1*), t(1;11)(p32;q23) (*KMT2A-EPS15*), t(1;11)(q21;q23) (*KMT2A-MLLT11*), t(1;19)(q23;p13) (*TCF3-PBX1*), t(3;5)(q25;q34) (*NPM1-MLF1*), t(3;21)(q26;q22) (*RUNX1-MECOM*), t(4;11)(q21;q23) (*KMT2A-AFF1*), t(5;12)(q33;p13) (*ETV6-PDGFRB*), t(5;17)(q35;q21) (*NPM1-RARA*), t(6;9)(p23;q34) (*DEK-NUP214*), t(6;11)(q27;q23) (*KMT2A-AFDN*), t(8;21)(q22;q22) (*RUNX1-RUNX1T1*), t(9;9)(q34;q34) (*SET-NUP214*), t(9;11)(p22;q23) (*KMT2A-MLLT3*), t(9;12)(q34;p13) (*ETV6-ABL1*), t(9,22)(q34;q11) (*BCR-ABL1*), t(10;11)(p12;q23) (*KMT2A-MLLT10*), t(11;17)(q23;q21) (*KMT2A-MLLT6*), t(11;17)(q23;q21) (*ZBTB16-RARA*), t(11;19)(q23;p13.1) (*KMT2A-ELL*), t(11;19)(q23;p13.3) (*KMT2A-MLLT1*), t(12;21)(p13;q22) (*ETV6-RUNX1*) t(12;22)(p13;q11) (*ETV6-MN1*), t(15;17)(q24;q21) (*PML-RARA*), inv(16)(p13;q22) (*CBFB-MYH11*), t(16;21)(p11;q22) (*FUS-ERG*), t(17;19)(q22;p13) (*TCF3-HLF*), t(X;11)(q13;q23) (*KMT2A-FOXO4*) were evaluated using multiplex nested RT-PCR HemaVision®-28N Chromosomal Translocations kits (DNA Technology, Aarhus, Denmark) which detects 80 splice variants. In this case, reverse transcription was performed with a mixture of translocation-specific primers using the HemaVision® reagent module.

Definitions and statistical analysis

Complete remission was defined as < 5% leukemic cells in bone marrow with clear evidence of regeneration in bone marrow or in peripheral blood. Bone marrow relapse was defined as the presence of ≥ 25% leukemic cells in the BM cytomorphology after complete remission was achieved. Extramedullary relapse was defined as the development of extramedullary disease once complete remission was achieved. Extramedullary disease was defined according to standard criteria.

The Kaplan–Meier method was used for survival estimation. Overall survival (OS) was defined as the time from diagnosis to death, with living patients censored on the date of last follow-up. Event-free survival (EFS) was defined as the time from diagnosis to relapse, progression or death, with event-free patients censored on the date of last follow-up. Cumulative incidence functions were calculated and compared according to the Fine and Gray method and the Gray's test, respectively. Comparisons between qualitative variables were performed by the two-tailed Fisher's exact test. MRD-negative patients were defined as those with no reappearance while MRD positive are those with reappearance after previous negative results. Detectable FISH below the established threshold of sensitivity were considered as negative results.

Data were collected in Excel (Microsoft Corporation, Redmond, USA) and analyzed in R version 3.6.3 (R Foundation for Statistical Computing, open-source software).

Results

Characteristics and outcome of patients

We included 80 patients with a mean age at diagnosis of 80 months (range 3–189 months). The cohort consisted of 47 male and 33 female patients. A total of 544 bone marrow samples were evaluated. Clinical and biological characteristics of patients and outcome are shown in Table 1.

The prevalence of genetic alterations in the sixty-seven patients with B-ALL was as follows: fourteen patients (21%) had the t(12;21)(p13;q22) *ETV6/RUNX1*, two patients (3%) the t(1;19)(q23;p13) *TCF3/PBX1*, two patients (3%) the t(9;22)(q34;q11) *BCR/ABL*, two patients (3%) the t(4;11)(q21;23) *KMT2A/AFF1*, four patients (6%) the iAMP21 and twelve patients (18%) the 16p deletion. In the thirty patients in whom Ph-like related alterations were investigated we found one *JAK2* rearrangement (3%) and one *CRLF2* rearrangement (3%). Regarding the thirteen patients with T-ALL we found two patients (15%) with interstitial 1p32 deletion, two patients (15%) with *TLX3* rearrangement, one patient (8%) with t(12;21) *ETV6/RUNX1*, one patient (8%) with a *KMT2A* rearrangement and nine patients (69%) with 16p deletion; four patients from this group had more than one alteration. Numeric alterations of the chromosomes (monosomies, trisomies or tetrasomies) were also found by FISH in 35 (52%) of the B-ALL and 1 (8%) of the T-ALL patients.

Table 1 Clinical and biological characteristics of patients and outcome

	B-ALL	T-ALL
Number of cases, *n* (%)[a]	67 (83.75%)	13 (16.25%)
Mean age at diagnosis (months)	81	75
Gender (male, *n* (%))	37 (55.2%)	9 (69.2%)
WBC at diagnosis		
$< 10 \times 10^3$/uL	41 (61.2%)	2 (15.4%)
$10–50 \times 10^3$/uL	15 (22.4%)	4 (30.8%)
$> 50 \times 10^3$/uL	11 (16.4%)	7 (53.8%)
Treatment protocol		
SEHOP-PETHEMA 2013	65 (97%)	13 (100%)
INTERFANT-06	2 (3%)	0 (0%)
Risk at diagnosis[b]		
Standard, *n* (%)	21 (31.3%)	0 (0%)
Intermediate, *n* (%)	41 (61.2%)	13 (100%)
High, *n* (%)	5 (7.5%)	0 (0%)
Clinical outcome		
Mean follow-up (months)	46	60
Relapse, *n* (%)	5 (7.5%)	2 (15.4%)
Death, *n* (%)	4 (6%)	1 (7.7%)

[a]All cases were included in the study at first diagnosis

[b]Low and medium risk infants are included in the table as standard and intermediate risk, respectively

ALL acute lymphoblastic leukemia, *B* B-cell precursor; *T* T-cell immunophenotype; *WBC* white blood cell count

With the aforementioned treatment protocols, our patients with ALL showed an estimated 5 year OS of 94% (IC95% 88.3–100) and an estimated 5 year EFS of 84.1% (IC95% 75–94.2). The 5 year cumulative incidence of relapse (CIR) was 12.1% (SE ± 4.6%) and the 5 year treatment-related mortality (TRM) was 3.8% (SE ± 2.2%). These results are presented in Fig. S1.

Evolutionary patterns of MRD in ALL patients

We observed two patterns of MRD presentation during follow-up. In Fig. 1 we illustrate these patterns with two representative cases (patients 52 and 61, respectively). In the first pattern (91.25% in our series), patients achieve complete remission and remain in complete remission until the end of follow-up (Fig. 1A). In the second pattern, the reappearance of the specific molecular marker, cytogenetic alteration or characteristic immunophenotype is followed or coincident in time with overt relapse, except

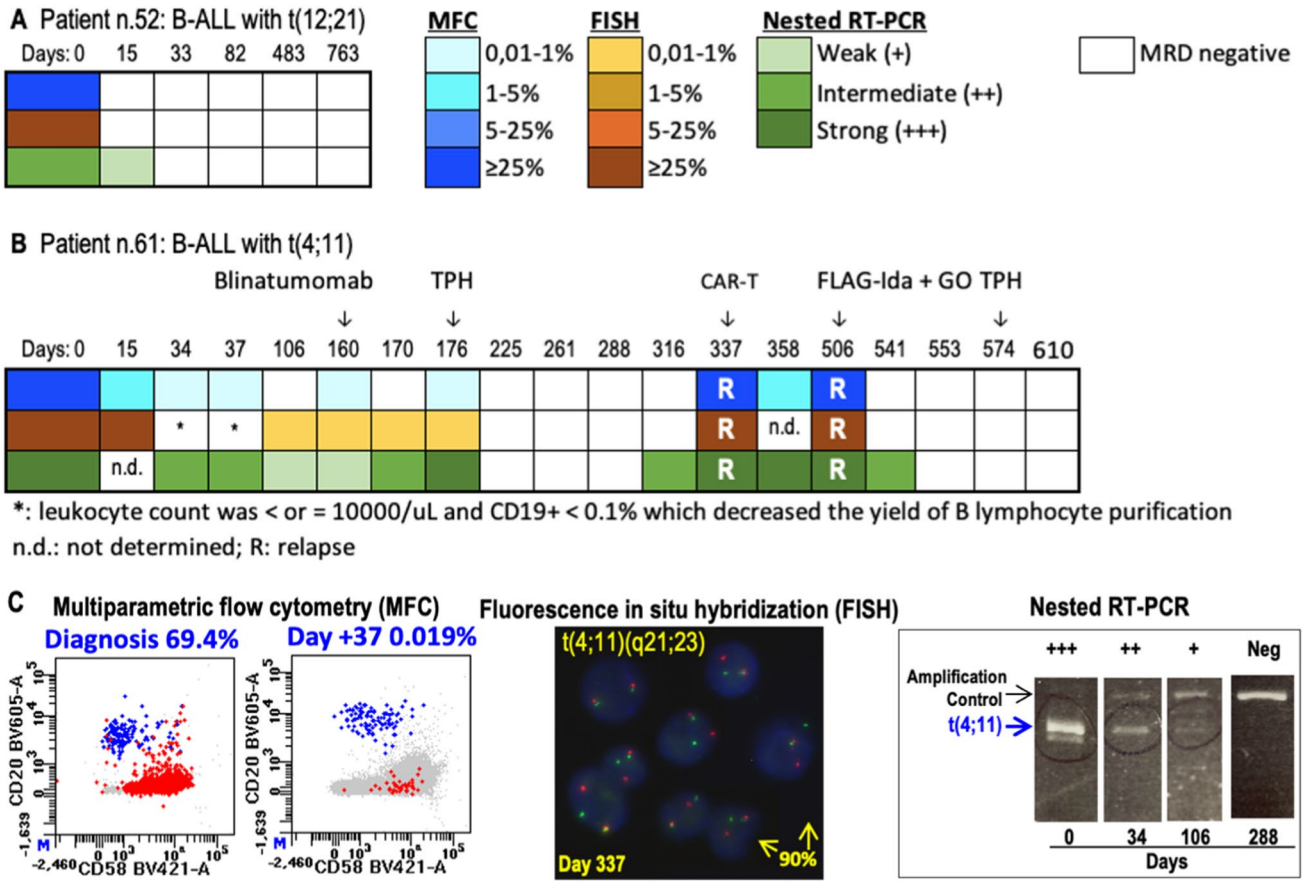

Fig. 1 Evolutionary patterns of MRD and relapse in ALL patients. **A** The patient achieves complete remission and stays until the end of follow-up. **B** Relapse is preceded by the reappearance and progressive increment of the minimal residual disease detected by multiparametric flow cytometry (MFC), fluorescent in-situ hybridization (FISH) in purified B lymphocytes (or in total nucleated cells after myeloid shift) and/or nested reverse transcriptase polymerase chain reaction (RT-PCR). Progressive normalization of those markers is observed during the resolution of the relapse. **C** Representative results for MFC, FISH and RT-PCR in bone marrow samples with different level of leukemic infiltration

in some cases in which the continuation of the standard treatment or the introduction of additional interventions avoided this. Also, MRD normalized progressively after intensification of treatment in responders. A representative patient of this pattern is shown in Fig. 1B, a B-ALL with a t(4;11), in which relapse was transiently aborted twice with Blinatumomab and hematopoietic stem cell transplantation (HSCT), although finally the patient relapsed. After relapse, cytoreductive and CAR-T therapies led to a new complete remission (MRD evaluations not shown because were performed at the CAR-T therapy center), but four months later the patient relapsed again with a myeloid immunophenotype. She achieved a new MRD-negative complete remission with a combination of fludarabine, cytarabine and idarubicin (FLAG-Ida) with Gentuzumab Ozogamycin and underwent a second HSCT as consolidation. A summary of the main events of all patients is shown in Fig. S1.

Detection and management

Multiparameter flow cytometry

As observed in Fig. 1B, some patients experienced more than one relapse. We identified a total of 12 relapses in 7 patients. As expected, all six patients with bone marrow involvement at relapse had a concordant positive MFC result, while one isolated extramedullary relapse was negative ($p < 0.00001$) (Fig. 2A). Moreover, we were able to anticipate relapse 12 days in one case.

It is noteworthy that in three cases the MRD reappearance by MFC became negative again with the continuation of the standard treatment or after further intervention with additional treatments: (1) maintenance therapy in a patient with intermediate risk without any genetic marker (2) third intensification block and salvage therapy with clofarabine, etoposide and cyclophosphamide in a patient with T-ALL and TLX3 rearrangement and (3) mercaptopurine

A

	Flow cytometry		RT-PCR		FISH	
	MRD-	MRD+	MRD-	MRD+	MRD-	MRD+
No Relapse	71	2	18	1	57	1
Relapse	1[a]	6	1[a]	2	1[a]	6
	p < 0.00001		p = 0.038		p < 0.00001	

B

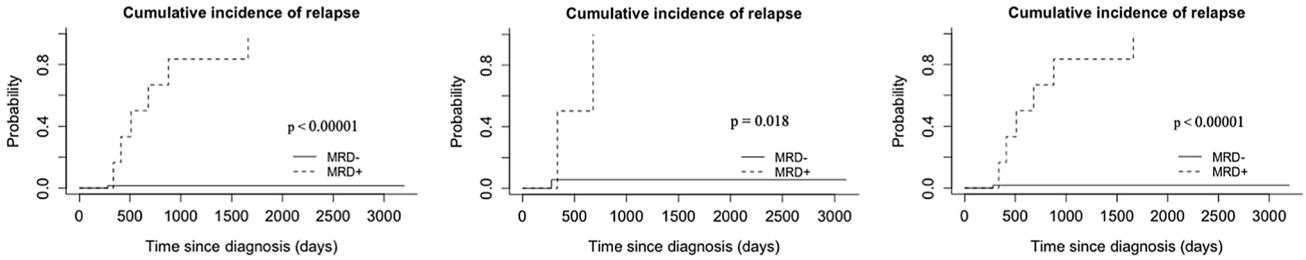

Fig. 2 Association between measurable residual disease (MRD) detection and ALL relapse. **A** Crosstab showing the relationship between ALL relapse and the reappearance of MRD detected by multiparametric flow cytometry (MFC), RT-PCR and FISH. [a]Extramedullary relapse. **B** Cumulative incidence of relapse according to the reappearance of MRD detected by MFC (left), RT-PCR (mid) and FISH (right). *MRD−* No MRD reappearance. *MRD+*: MRD reappearance

plus ruxolitinib in a patient with a JAK2 rearrangement, although this last patient relapsed after the interruption of the treatment.

Reverse transcription polymerase chain reaction

Twenty-two out of 80 (27.5%) patients showed a monitorable specific alteration by RT-PCR. The reappearance of *TAL1* deletion and KMT2A-AFF11 rearrangement were associated with relapse in two patients, which was anticipated 21 days by RT-PCR in the former. Also, the reappearance of TCF3-PBX1 rearrangement returned to negative during maintenance treatment without further intervention in another patient. No patient with persistently negative RT-PCR relapsed except for the aforementioned extramedullary relapse ($p = 0.013$) (Fig. 2A).

Fluorescent in-situ hybridization

Sixty-five out of 80 (81.25%) patients showed monitorable alterations detected by this method. The reappearance of MRD detected by FISH was present in all six patients experiencing a bone marrow relapse while, with the exception of one isolated extramedullary relapse, no patient with persistently negative results relapsed ($p < 0.00001$) (Fig. 2A). The patient with the *JAK2* rearrangement showed a positive FISH result 40 days before overt relapse. The continuation of standard first-line treatment (early consolidation) was able to normalize MRD reappearance in one patient with a chromosome 4 tetrasomy.

Two patients showed detectable growing levels of p16 deletion below the established threshold of the FISH probe (9%) before relapse. If we take these two later patients into account, FISH on purified B or T cells was able to anticipate relapse with a median time of 39.5 days. Twelve additional patients had MRD reappearance below the sensitivity threshold. Continuation of standard first-line treatment was able to control disease in eight of these patients: one patient with iAMP21+, one patient with a t(12;21), three patients with p16 deletion and three patients with numeric alterations of chromosomes 4, 10, 14, 16, 17 and 21. In addition, second line treatments were able to control disease in two patients: one with a numeric alteration of chromosome 21 who received two cycles of high-risk consolidation therapy plus blinatumomab, and another with a *KMT2A* rearrangement who received blinatumomab plus HSCT, although this last patient relapsed afterwards. Furthermore, immunosuppression withdrawal after HSCT was able to negativize emerging disease in another patient with p16 deletion without posterior relapse. Finally, in the patient with the *TLX3* rearrangement, the continuation of the first-line treatment, salvage therapy and immunosuppression withdrawal after HSCT, negativized MRD reappearance below the sensitivity threshold in three different occasions. A summary of interventions and outcome for FISH reappearances is shown in Supplementary Table 4.

The cumulative incidence of relapse according to the reappearance of measurable residual disease through the three different methods is showed in Fig. 2B.

A high level of concordance was observed among samples with available data from the three MRD evaluation methods (Table 2). Due to its higher sensitivity RT-PCR showed positive results in 10 samples with negative results in MFC and FISH analysis. One sample was a t(1;19), four samples were a t(12;21) and five samples were a t(4;11).

Table 2 Comparative analysis MRD results detected by MFC, FISH and RT-PCR

Patient id	Rearrangement	N. of samples	LAIP	MFC+	FISH+	RT-PCR+
#4	t(1;19)	6	Yes	1	1	2
#9	t(12;21)	3	Yes	1	1	1
#12	t(12;21)	14	Yes	1	1	1
#20	t(12;21)	3	Yes	1	1	1
#27	t(12;21)	4	Yes	1	1	1
#33	t(9;22)	8	Yes	1	1	4
#34	t(12;21)	2	Yes	1	1	1
#42	t(12;21)	4	No[a]	1	1	1
#47	t(12;21)	3	No[a]	1	1	1
#52	t(12;21)	5	Yes	1	1	2
#56	t(12;21)	2	Yes	1	1	1
#57	t(12;21)	4	Yes	1	1	1
#58	t(12;21)	2	Yes	1	1	1
#61	t(4;11)	17	Yes	7	3	11
#64	t(12;21)	3	Yes	1	1	1
#68	t(12;21)	2	Yes	1	1	1
#72	t(12;21)	2	Yes	1	1	1
#74	t(12;21)	3	Yes	1	1	1
#79	t(4:11)	3	Yes	1	1	2

This analysis includes samples in which the three methods were performed and offered a valid result in patients with an identifiable rearrangement by FISH and RT-PCR ($n=90$)

[a]Patients with no LAIP were assessed in the basis of aberrant immunophenotypes as described in the patients and methods section

Two to two comparisons of the results obtained with the three MRD methods offered the following results: (1) when comparing samples analyzed with MFC and FISH, 12 out of 330 samples (3.6%) were MFC positive and FISH negative, whereas only 2 out of 330 samples (0.6%) were MFC negative and FISH positive; (2) when comparing samples with a valid result for MFC and RT-PCR, 12 out 130 samples (10%) were MFC negative and RT-PCR positive, whereas no samples with MFC positive and RT-PCR negative were found (0%); and (3) when comparing samples with a valid result for the same genetic alteration in FISH and RT-PCR, 10 out 90 samples (11%) were FISH negative and RT-PCR positive, whereas no samples were found with FISH positive and RT-PCR negative (0%). Genetic alterations identified in these discrepancies are shown in Supplementary Table 5.

Discussion

MRD assessment is a useful tool to assess early treatment response and adjust therapy during first-line treatment, although its relevance later on the follow-up in real-life ALL patients has been far less investigated [18]. After first-line treatment, pediatric patients with ALL are usually monitored with periodical peripheral blood cell counts [19]. This approach has the disadvantage that unspecific alterations as neutropenia or thrombocytopenia are frequently found in association to other conditions (infections, toxicity…) instead of relapse. Although MRD analysis can be attempted on peripheral blood [20], generally, bone marrows aspirates are performed to clarify if we are facing a relapse or another transient condition. However, the interpretation of these results is not always straightforward, as no consensus has been established on the clinical interventions to be taken when we find re-emerging MRD. Moreover, as far as we know, in ALL there is no international consensus establishing the definition of molecular relapse as is the case of AML [21].

In this real-life study in pediatric ALL we found that positive detection of MRD by any of the three methods tested (MFC, FISH or RT-PCR) at any time during follow-up was strongly correlated with a subsequent relapse. Furthermore, both the continuation of the standard therapy and the implementation of additional interventions were successful in preventing overt relapse in some patients. However, due to the highly heterogenous nature of this retrospective study, definitive recommendations regarding preemptive interventions cannot be made. A prospective study is needed to determine to what extent more intensive treatment is necessary to prevent disease progression.

Although MRD assessment by MFC is a useful and widely implemented approach, its sensitivity is inferior

compared with new molecular methods. In our series, MFC was able to anticipate relapse in one case just by 12 days, the shortest time compared to the other two techniques. RT-PCR, however, has demonstrated to be the most sensitive and specific method to monitor MRD. In our study we did not find any false positive and we were able to anticipate relapse in one case by 21 days. Nonetheless, it was only one case, so probably the lack of monitorable specific alterations have precluded early detection of MRD in other patients. This strongly supports that a universal and sensitive molecular method to assess MRD status in every patient with B or T ALL should be available in all centers where MRD evaluation is performed. In this regard, next-generation sequencing (NGS) to detect immunoglobulin and T-cell receptor rearrangements has proven to be the most sensitive and specific method [22], although is not as well implemented as qPCR of these rearrangements which is the gold standard in many collaborative groups [23]. This last technique is time and cost consuming, which has hindered a wide implementation in our country, making it a challenge.

Alternatively, our results clearly show that MRD assessment trough FISH in purified B or T cells is a complementary method that can also be useful to anticipate relapse. In this study, we were able to anticipate relapse in one case by 40 days and in up to three cases with a median time of 39.5 days when considering the growing reappearances of the specific genetic alteration below the established sensitivity threshold of the FISH probe. Although theoretically sensitivity of MFC is higher than that of FISH, in our study two patients with negative MFC were detected positive for the specific chromosomal translocation by FISH on purified B or T cells. This is indicative that in some ALL patients without well-defined LAIP, an increase in the number of cells to be analyzed by FISH may offer an alternative method for MRD evaluation that could help to anticipate relapse, particularly when molecular methods are not available.

It is important to highlight that contrary to children with AML in whom preemptive treatments only temporarily halted disease progression [24], probably due to the higher chemoresistance of AML cells [25], several ALL patients turned MRD-negative again with the continuation of standard treatment, such in cases with t(1;19), iAMP21, t(12;21), p16 deletion or numeric alterations of chromosomes 4, 10, 14, 16, 17 and 21. Also, in four patients with emerging MRD by FISH and/or RT-PCR (one translocation t(4;11), one *JAK2* rearrangement, one chromosome 21 anueploidy and one 16p deletion), early interventions such blinatumomab plus HSCT, targeted therapy with ruxolitinib in combination with mercaptopurine, chemotherapy intensification and blinatumomab, and immunosuppression withdrawal after HSCT respectively, were able to yield transient (two former patients) and sustained (two later patients) disease control. Finally, in the T-ALL patient with a TLX3 rearrangement, both, the continuation of the first-line treatment and early interventions adapted to MRD along the follow-up were able to control disease progression in up to three times. However, early treatment of MRD continues to be debated and whether it can offer improved global survival in childhood ALL patients should be established in properly controlled clinical trials.

Conclusion

MFC, FISH and RT-PCR offer complementary data for MRD monitoring in pediatric ALL. Although, our data clearly show that MDR positive detection with any of the three methods is associated with relapse, continuation of standard treatment, intensification or other preemptive treatments were able to halt relapse in patients with different risks and genetic background. For this reason, controlled clinical trials should be carried out to determine if treating emerging residual disease detected with highly sensitive methods could offer advantages in patient survival.

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Data availability The data that supports the findings of this study are available from the corresponding author, upon reasonable request.

Declarations

Conflict of interest The authors report no conflict of interest.

Ethical approval The study was performed in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent Written informed consent was obtained from all patients (and/or their parents) in accordance with the Declaration of Helsinki.

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SUPPLEMENTARY MATERIALS FOR THE SECOND PUBLICATION

Table S1. Criteria for risk classification according to treatment protocol.

Protocol	Risk	Details
SEHOP-PETHEMA 2013	Standard	The patient must meet each and every one of the following criteria: • Age >1 year and < 10 years • Leukocytes count < 20x109/L at diagnosis • Non-T immunophenotype • Absence of infiltration of the CNS and/or testes • Cytogenetics (one of the two criteria is sufficient): • -High hyper diploidy (51-67 chromosomes), DNA index 1.10-1.44 (always confirmed by other cytogenetic techniques) • -t(12;21) ETV6-RUNX1 • No (1;19) TCF3-PBX1 • No KMT2A rearrangement • Presence of < 1000 blasts/mm3 on day +8 of induction in peripheral blood • Presence of < 5% blasts and < 0.1% MRD in bone marrow on day +15 of induction and at the end of induction I'A.
	Intermediate	All patients not included as low risk or high risk.
	High	The existence of any of the following criteria determines the inclusion of the patients in this group: t(4;11) KMT2A-AFF1 Hypodiploidy <44 chromosomes or DNA index <0.81 (confirmation by other techniques is required) ≥ 1000 blasts on day +8 of induction in peripheral blood > 25% blasts and >10% MRD on day +15 of induction in bone marrow MRD ≥ 1% on day +33 of induction in bone marrow MRD ≥ 0.1% before consolidation in bone marrow. Patients with ALL Ph+ until the COG/EsPhALL international protocol is available
INTERFANT-06	Low	KMT2A germline
	High	KMT2A rearranged AND Age at diagnosis < 6 months AND WBC ≥ 300 x 109/L and/or prednisone poor response
	Medium	All other cases so including those with: KMT2A status unknown OR KMT2A rearranged AND age > 6 months OR KMT2A rearranged AND age < 6 months AND WBC < 300 x 109/L AND prednisone good response

MRD: measurable residual disease WBC: white blood cells

Table S2. Combinations of monoclonal antibodies used in 8 or 12 color studies.

Canto-II (8 colors)	**Tube-1**	**Tube-2**	**Tube-3**	**Tube-4**	**Tube-5**
FITC	CD15[a]	CD66[b]	CD19[c]	CD79a[a]	Tdt-Cyt[b]
PE	CD79b[b]	CD13[c]	IgM-Sup[b]	IgM-Cyt[b]	MPO[b]
PE-Cy5 or PerCP	CD38[c]	HLA-DR[c]	CD58[d]	CD10[c]	CD10[c]
PE-Cy7	CD19[c]	CD34[c]	CD10[c]	CD19[c]	CD19[c]
APC/AF647	CD14c/CD8[c]	CD33[d]	CD22[c]	CD34[c]	CD34[c]
APC-H7	CD45[c]	CD45[c]	CD45[c]	CD45[c]	CD45[c]
BV421/VB/PB	CD4[c]	CD19[c]	CD20[e]	CD20[e]	CD3-Cyt[c]
BV510	CD3[c]	CRLF2[c]	-	-	-

Lyric (12 colors)	**Tube-1**	**Tube-2**	**Tube-3**
FITC	CD4[c]/19[c]/66b/71[c]	CD66[b]	Tdt-Cyt[b]
PE	CD13c/CD235a[c]	CD22[c]	MPO-Cyt[b]
PE-Cy5 or PerCp	CD11b[c]	CD10[c]	CD10[c]
PE-Cy7	CD34[c]	CD34[c]	CD34[c]
APC/AF647	CD117[c]	CD19[c]	CD19[c]
APC-R700	CD38[c]	CD38[c]	CD38[c]
APC-H7	CD45[c]	CD45[c]	CD45[c]
BV421 or V450	CD16[c]	CD58[c]	CD7[c]
BV510	CD3c/CD300e[c]	CD45RA[c]	CD45RA[c]
BV605	CD33[c]	CD20[c]	CD20[c]
BV711	CD56[c]	CD81[c]	CD3-Cyt[c]
BV786	HLA-DR[f]	HLA-DR[f]	IgM-Cyt[c]

Monoclonal antibodies were purchased from: [a]Cytognos (Salamanca, Spain); [b]Dako (Glostrup, Denmark); [c]Beckton Dickinson (San Jose, CA); [d]Beckman Coulter (Brea, California); [e]Miltenyi (Bergisch Gladbach, Germany); [f] BioLegend (San Diego, CA).

Table S3. Details and source of monoclonal antibodies used in for immunophenotyping.

Monoclonal antibody	Manufacturer, reference	Clone
CD15 FITC	Cytognos, CYT15F4	MCS-1
CD66 FITC	Dako, F7112	Kat4c
CD19 FITC	Beckton Dickinson, 345776	1D3
CD79a FITC	Cytognos, CYT79aF4	HM57
Tdt-Cyt FITC	Dako, F713950-2	HT-6
CD79b PE	Dako, R7272	SN8
CD13 PE	Becton Dickinson, 347406	L138
IgM-Sup, Cyt PE	Dako, R5111	Rabbit anti-Human
MPO, -Cyt PE	Dako, R720901	MPO-7
CD38 PE-Cy5	Becton Dickinson, 551400	HIT2
HLA-DR PerCp	Becton Dickinson, 339216	G46-6
CD58 PE-Cy5	Beckman Coulter, IM3702	AICD58
CD10 PerCp	Becton Dickinson, 563508	HI10a
CD19 PE-Cy7	Becton Dickinson, 341113	SJ25C1
CD34 PE-Cy7	Becton Dickinson, 348811	8G12
CD10 PE-Cy7	Becton Dickinson, 341112	HI10a
CD14 APC	Becton Dickinson, 345787	MφP9
CD8 APC	Becton Dickinson, 345775	SK1
CD33 APC	Beckman Coulter, IM2471	D3HL60.251
CD22 APC	Becton Dickinson, 333145	S-HCL-1
CD34 APC	Becton Dickinson, 345804	8G12
CD45 APC-H7	Becton Dickinson, 641417	2D1
CD4 BV421	Becton Dickinson, 562425	RPA-T4
CD19 BV421	Becton Dickinson, 562440	HIB19
CD20 VioBlue	Miltenyi, 130-113-378	LT20
CD3-Cyt Pacific Blue	Becton Dickinson, 558117	UCHT1
CD3 BV510	Becton Dickinson, 563109	UCHT1
CRLF2 BV510	Becton Dickinson, 563340	1F11
CD4 FITC	Becton Dickinson, 345768	SK3
CD71 FITC	Becton Dickinson, 333151	L01.1
CD235a PE	Becton Dickinson, 555570	GA-R2
CD22 PE	Becton Dickinson, 337899	S-HCL-1
CD11b PE-Cy5	Becton Dickinson, 555389	ICRF44
CD117 APC	Becton Dickinson, 333233	104D2
CD19 APC	Becton Dickinson, 345791	SJ25C1
CD38 APC-R700	Becton Dickinson, 564979	HIT2
CD16 BV421	Becton Dickinson, 562874	3G8
CD58 BV421	Becton Dickinson, 566239	1C3
CD7 BV421	Becton Dickinson, 562635	M-T701
CD300e BV510	Becton Dickinson, 744993	UP-H1
CD45RA BV510	Becton Dickinson, 563031	HI100
CD33 BV605	Becton Dickinson, 745229	P67.6
CD20 BV605	Becton Dickinson, 740333	L27
CD56 BV711	Becton Dickinson, 563169	NCAM16.2
CD81 BV711	Becton Dickinson, 740789	JS-81
CD3-Cyt BV711	Becton Dickinson, 563725	UCHT-1
HLA-DR BV786	BioLegend, 307642	L243
IgM-Cyt BV786	Becton Dickinson, 740998	G20-127

Table S4. Interventions and outcome for patients with MRD reappearance detected by FISH

FISH alteration	**MRD reappearance**	**Early intervention**	**MRD outcome**	**Relapse**
Tetrasomy 4	Yes	No*	Negative	No
JAK2-r	Yes	Ruxolitinib	Negative	Yes
p16 deletion	Below the sensitivity threshold	No	Positive	Yes
p16 deletion	Below the sensitivity threshold	No	Positive	Yes
iAMP21	Below the sensitivity threshold	No*	Negative	No
t(12;21)	Below the sensitivity threshold	No*	Negative	No
p16 deletion	Below the sensitivity threshold	No*	Negative	No
p16 deletion	Below the sensitivity threshold	No*	Negative	No
p16 deletion	Below the sensitivity threshold	No*	Negative	No
Aneuploidy	Below the sensitivity threshold	No*	Negative	No
Aneuploidy	Below the sensitivity threshold	No*	Negative	No
Aneuploidy	Below the sensitivity threshold	No*	Negative	No
Chromosome 21 numeric alteration	Below the sensitivity threshold	Yes (intensified chemotherapy + Blinatumomab)	Negative	No
MLL-r	Below the sensitivity threshold	Yes (Blinatumomab)	Negative	Yes
p16 deletion	Below the sensitivity threshold	Yes (immunosuppression withdrawal)	Negative	No
TLX3-r	Below the sensitivity threshold	Yes (intensified chemotherapy, immunosuppression withdrawal)	Negative	No

* Continuation of standard first-line treatment

Table S5. Genetic alterations in patients with discrepancies in the MRD detection between two techniques.

Patient # / Discrepancy	**Alteration**
MFC-negative & FISH-positive	
#11	p16 deletion
#24	Tetrasomy 4
MFC-positive & FISH-negative	
#3	JAK2 rearrangement
#12	t(12;21) ETV6-RUNX1
#36	Trisomy 10, 17 and n-somy 14
#41	p16 deletion
#51	Tri- or tetrasomy 21 and trisomy 4, 10, 17
#61	KTM2A rearrangement
#62	TLX3 rearrangement
#68	t(12;21) ETV6-RUNX1 and RUNX1 duplication
MFC-negative & RT-PCR-positive	
#4	TCF3/PBX1
#33	BCR/ABL
#34	t(12;21) ETV6-RUNX1
#52	t(12;21) ETV6-RUNX1
#61	KTM2A rearrangement
#79	KTM2A rearrangement
MFC-positive & RT-PCR-negative	
No patients	
FISH-negative & RT-PCR-positive	
#4	TCF3/PBX1
#33	BCR/ABL
#52	t(12;21) ETV6-RUNX1
#61	KTM2A rearrangement
#79	KTM2A rearrangement
FISH-positive & RT-PCR-negative	
No patients	

Figure S1. Overall survival, event-free survival, cumulative incidence of relapse and treatment related mortality of all patients.

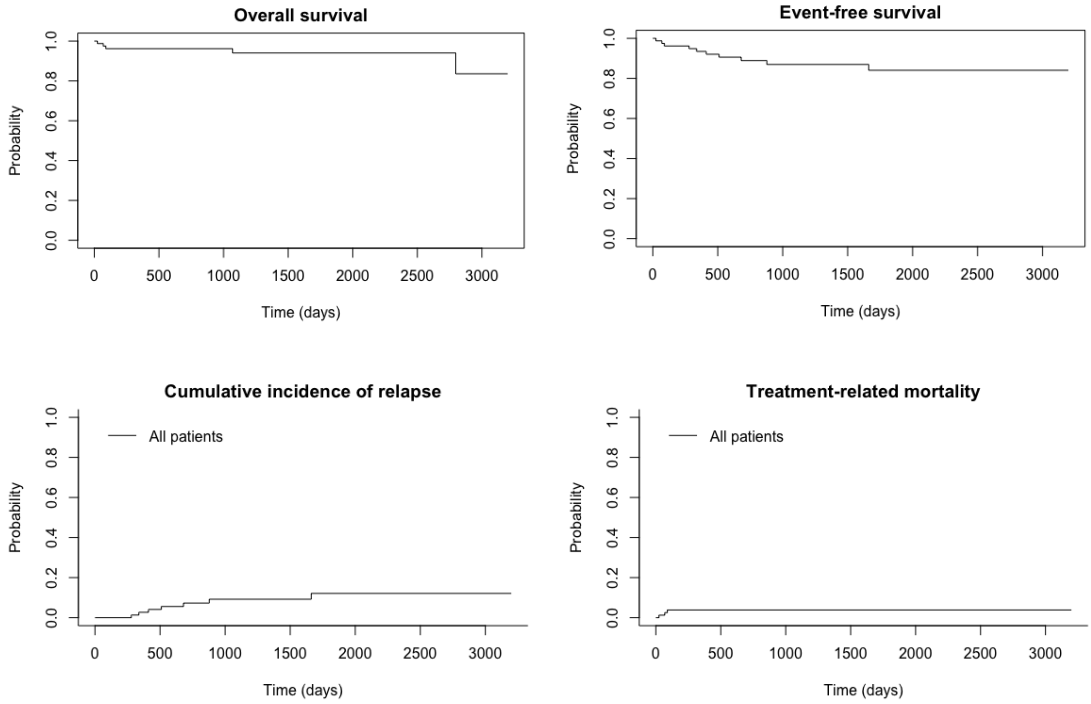
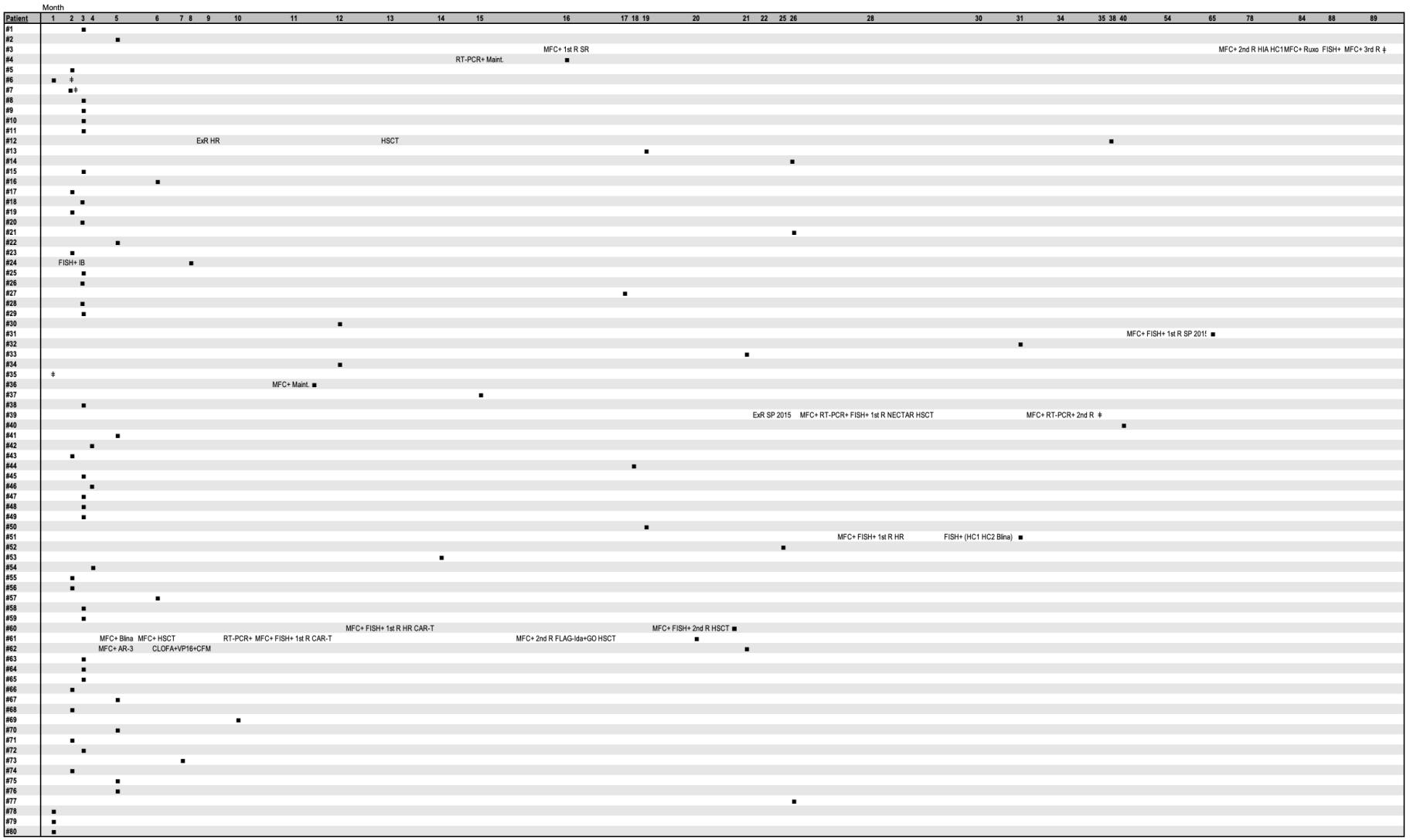


Figure S2. Time course of main events in the 80 included patients.



R: relapse; ExR: extramedullary relapse; MFC+: MRD reappearance in MFC; FISH+: MRD reappearance in FISH; RT-PCR+: MRD reappearance in RT-PCR; IB: Induction IB; AR-3: High-risk reinduction 3; Maint: maintenance; SR: IntReALL 2010 SR; HR: IntReALL 2010 HR; HIA: High-risk induction A; HC1: High-risk consolidation 1; HC2: High-risk consolidation 2; SP 2015: SEHOP-PETHEMA 2015; Ruxo: Ruxolitinb; NECTAR: nelarabine, etoposide and cyclophosphamide; HSCT: hematopoietic stem cell transplantation; Blina: Blinatumomab; CAR-T: chimeric antigen receptor T-cell therapy; FLAG: fludarabine, cytarabine and G-CSF with or without idarubicin (Ida); GO: Gemtuzumab Ozogamizin; CLOFA+VP16+CFM: clofarabine, etoposide and cyclophosphamide; ■ last bone marrow evaluation; and ǂ death.