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Prognostic Role of Minimal Residual Disease before and after Haematopoietic Stem Cell Transplantation in pediatric ALL patients and evaluation of droplet digital PCR applicability in pre-HSCT MRD monitoring

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ABSTRACT

Acute Lymphoblastic Leukemia (ALL) represents the most frequent cancer in childhood. Currently, more than 80% of children with ALL can be cured through intensive and risk-adapted chemotherapy protocols, but unfortunately, the remaining 20% ultimately relapse. Allogeneic hematopoietic stem cell transplantation (HSCT) is considered beneficial for approximately 10% of patients who are at high risk (HR) at frontline therapy according to the AIEOP-BFM protocol criteria, and for the majority of patients after ALL relapse. However, also after HSCT, relapse remains the leading cause of treatment failure in pediatric ALL.

The strongest prognostic factor in childhood ALL is the monitoring of Minimal Residual Disease (MRD). MRD is defined as the persistence, in bone marrow (BM), of leukemic cells not identifiable through cyto-morphological methods. MRD diagnostics has been implemented into major frontline treatment protocols for pediatric ALL, in which it is routinely used to stratify patients into different risk classes: standard risk (SR), medium risk (MR) or high risk (HR) of relapse. The aim of MRD-based stratification is to refine therapy based on risk-class, maximizing cure and minimizing toxicities.

Also for relapsed ALL patients and in patients undergoing HSCT, MRD assessment has been identified as one of the most relevant predictors of prognosis, useful to identify good and poor responders to the therapy. Nevertheless, the clinical significance of MRD in pediatric ALL patients given allogeneic HSCT has not yet been fully validated.

The most widely used approach to detect MRD is represented by real-time quantitative PCR (RQ-PCR), a very sensitive and specific molecular assay. RQ-PCR is based on the patient-specific junctional regions of Immunoglobulin (Ig) and T-cell Receptor (TCR) genes rearrangements, detected on BM aspirates collected at diagnosis (or relapse) of ALL patient.

In the first project of my PhD training (described in Chapter 1) we quantify MRD by RQ-PCR immediately before HSCT, in order to assess its clinical significance and impact on transplant outcome in a large cohort (119) of pediatric ALL patients in first, second or subsequent complete remission (respectively 1CR, 2CR or others CR). In addition, we consecutively analyzed MRD by RQ-PCR in 98/119 and 59/119 ALL patients,

respectively during the first (post-HSCT1) and third (post-HSCT3) trimester after HSCT. The aim of these analyses was to address the question of whether MRD evaluation could provide further information to predict the risk of post-transplant leukemia recurrence.

The overall 10-year event-free survival probability (EFSp) for patients with any level of positive MRD pre-HSCT was lower (39% for MRD < $1x10^{-3}$ and 18% for MRD ≥ $1x10^{-3}$) as compared with negative MRD patients (EFSp = 73%). When patients were analyzed according to the number of CR at HSCT, we observed that different levels of positivity had a different impact on EFSp: low-level MRD positivity had a negative impact only in patients transplanted in second or higher CR; while in first CR, only a high MRD positivity increased the risk of relapse. So pre-transplant MRD assessment confirmed to be a strong predictor of outcome and its effect was consistent throughout the different disease remissions.

We also evaluated the EFSp according to the MRD assessment at post-HSCT1 and post-HSCT3. MRD negativity at early post-transplant was associated with a good EFSp (63%), that was even better when negativity was confirmed also at 3th trimester post-HSCT (pEFS = 84%).

Also the variations of MRD levels over time were important. In particular the change between 1st and 3th trimester allowed to identify 2 categories of patients, with a dramatically different outcome: a group of patients with very poor prognosis (patients with an MRD increasing from post-HSCT1 to post-HSCT3) with an EFSp of only 8%, and a group of patients with very good prognosis (patients with unchanged negative MRD or decreasing to negative MRD and those with unchanged low-positive MRD) with an EFSp ≥ 80%.

Overall, these results confirm that MRD assessment is important both before and after transplant, for early identification of patients with the highest risk of ALL recurrence and with a strong indication to a prompt immunological intervention and to adoption of new drugs.

The second project (described in Chapter 2) was a preliminary study. We focused on a third generation PCR, the droplet digital PCR (ddPCR), that allows for an absolute quantification, with accurate concentration of target DNA. Instead, RQ-PCR allows for a relative quantification, since is based on the comparison with a calibration standard curve

made with the diagnostic DNA of patient, for MRD level quantification in follow-up sample. Thus, availability of diagnostic sample can limit RQ-PCR assay.

A broad spectrum of molecular markers has been yet interrogated using ddPCR for diagnostic purposes in various malignancies. Recently, the absolute method was evaluated for MRD quantification in lymphoproliferative disorders of adult, such as lymphomas and ALL; these reports showed a good correlation between quantitative PCR and ddPCR. However, there are still no studies in pediatric ALLs.

In the light of this, we performed ddPCR analyses on BM samples of 65 pediatric ALL transplanted patients with the same primers and probes used for RQ-PCR and in the same reaction conditions. Comparing head-to-head the MRD results obtained with the two molecular approaches, we aimed to investigate the applicability of ddPCR for MRD assessment also in this context. First, we evaluated if positive but not-quantifiable (PNQ) MRD performed by RQ-PCR can be quantified by ddPCR; then we also evaluated the prognostic impact of pre-HSCT MRD levels assessed by ddPCR.

A good level of concordance was found in results of both analyses (Pearson $r = 0.98$, $P < 0.0001$) and ddPCR was also able to quantify a various number of sample not-quantifiable by conventional RQ-PCR. Our results suggest that ddPCR has sensitivity, accuracy and reproducibility at least comparable with RQ-PCR.

Statistical analyses have shown no significant differences in prognostic impact on outcome, if patients were stratified according to MRD levels detected by RQ-PCR and ddPCR, since EFSp of PNQ patients was very similar to that of MRD NEG by ddPCR (71% vs 68%, respectively). Despite this, the digital method was able to measure a positive and quantifiable value for 12 ALL patients who relapsed after HSCT, while RQ-PCR technique failed to identify relapse in advance.

These preliminary data confirm that ddPCR may be an accurate and applicable tool for MRD evaluation also in the context of pediatric ALL clinical trials, but highlight the importance of extending the analysis on other retrospectively collected cases, to better define the role of ddPCR for prospective MRD evaluation in pediatric ALLs.

SOMMARIO

La Leucemia Linfoblastica Acuta (LLA) rappresenta la patologia tumorale più frequente in età pediatrica. Oltre l'80% dei bambini affetti da LLA viene trattato con successo grazie agli attuali protocolli di chemioterapia intensiva e basata sul rischio di ricaduta, ma sfortunatamente, il restante 20% ricade. Il trapianto di cellule staminali ematopoietiche (TCSE) ha un ruolo fondamentale nella guarigione di circa il 10% dei pazienti definiti ad alto rischio di ricaduta LLA in prima linea e per gran parte dei pazienti recidivati. Sfortunatamente, anche dopo il TCSE, la ricaduta si conferma come principale causa di fallimento terapeutico nelle LLA pediatriche.

Il principale indicatore prognostico nelle LLA infantili è rappresentato dalla Malattia Residua Minima (MRM). La MRM è definita come la persistenza, all'interno del midollo osseo, di cellule leucemiche a livelli non identificabili attraverso esame citomorfologico. La valutazione della MRM è ormai parte integrante dei principali schemi terapeutici di prima linea, in cui viene usata per stratificare i pazienti in diverse classi di rischio di ricaduta (standard, intermedio o alto), con l'obiettivo di adattare la terapia al rischio individuale di ciascun paziente, ottimizzando le cure e riducendo al minimo la tossicità.

Il monitoraggio della MRM è stato identificato come uno dei maggiori fattori predittivi di prognosi, anche per i pazienti ricaduti e per quelli trapiantati, in cui risulta ulteriormente vantaggioso per valutare la risposta alla terapia dei pazienti LLA. Ciononostante, il significato clinico della MRM nei pazienti sottoposti al TCSE non è ancora stato pienamente validato.

L'approccio standard utilizzato per monitorare la MRM è attualmente rappresentato dalla real-time quantitative PCR (RQ-PCR), un saggio molecolare altamente sensibile e specifico, basato sulle regioni giunzionali dei riarrangiamenti dei geni delle immunoglobuline e del recettore dei linfociti T, identificati sugli aspirati midollari della diagnosi (o ricaduta) del paziente LLA.

Nel primo progetto (descritto nel capitolo 1) del mio percorso di dottorato, abbiamo quantificato la MRM mediante PCR quantitativa immediatamente prima del TCSE, per valutare il suo significato clinico e l'impatto sull'outcome in una vasta coorte di pazienti pediatrici affetti da LLA (119), in prima remissione completa (1RC), seconda (2RC) o

altre. Abbiamo poi analizzato MRM mediante RQ-PCR in 98/119 e 59/119 pazienti, rispettivamente durante il primo (post-TCSE1) e il terzo (post-TCSE3) trimestre dopo il trapianto. L'obiettivo di queste analisi è stato quello di capire se la valutazione MRM potesse fornire ulteriori informazioni, utili a identificare preventivamente i pazienti con maggior rischio di ricaduta leucemica dopo il trapianto.

Dalle analisi di sopravvivenza in relazione ai livelli di MRM pre-TCSE nei pazienti LLA, qualsiasi livello di positività correla con un outcome sfavorevole (pEFS = 39% per MRM positiva $< 1x10^{-3}$ e pEFS = 18% per MRM positiva $\geq 1x10^{-3}$), rispetto ai pazienti con MRM negativa (pEFS = 73%, P<0.0001). Inoltre, analizzando i pazienti in base al tipo di remissione al TCSE, livelli diversi di positività MRM correlano con un diverso impatto sulla pEFS: bassi livelli di positività MRM indicano infatti, una prognosi sfavorevole solo in pazienti trapiantati in seconda o altre RC, mentre in prima RC solo una positività alta si associa ad un aumentato rischio di ricaduta. Pertanto la MRM pre-TCSE si conferma come importante fattore predittivo di outcome e il suo effetto varia col variare del tipo di remissione al trapianto.

È stata valutata, inoltre, la pEFS dei pazienti in base ai livelli di MRM post-TCSE1 e post-TCSE3; MRM negativa post-TCSE correla significativamente con un outcome favorevole, sia al 1° trimestre (pEFS = 63%), che ancor più se riscontrata al 3° trimestre (pEFS = 84%).

Anche la valutazione prospettica del cambiamento di MRM è risultata significativa. In particolare, valutando la variazione di MRM dal 1° al 3° trimestre post-TCSE, i pazienti con MRM crescente hanno una prognosi sfavorevole (pEFS = 8%), mentre tutti gli altri gruppi correlano con una buona prognosi (pEFS $\geq$ 80%).

Questi risultati confermano l'importanza del monitoraggio della MRM sia nel periodo precedente che successivo al TCSE, nell'identificare preventivamente pazienti ad alto rischio di ricaduta, possibili beneficiari di interventi immunologici preventivi.

Il secondo progetto trattato (descritto nel capitolo 2) è stato uno studio preliminare, focalizzato su una PCR di terza generazione, la Droplet Digital PCR (ddPCR). Essa consente una quantifica di tipo assoluto, con un'accurata concentrazione del DNA target. La RQ-PCR fornisce, invece, una quantifica di tipo relativo, basata su una curva standard di calibrazione fatta con il DNA della diagnosi del paziente, per la quantificazione dei

livelli di MRM di ciascun follow-up. Per cui, la PCR quantitativa può essere limitata dalla disponibilità di materiale diagnostico.

Un ampio spettro di marcatori molecolari è già stato indagato mediante ddPCR per scopi diagnostici in varie patologie tumorali. Studi recenti hanno valutato l'applicabilità della ddPCR nell'ambito delle malattie linfoproliferative dell'adulto, come i linfomi e le LLA, mostrando una buona correlazione dei risultati fra le due metodiche in entrambi gli ambiti. Tuttavia, non sono ancora disponibili lavori che valutino questa correlazione nel campo delle leucemie pediatriche.

Alla luce di questo, abbiamo eseguito analisi ddPCR sugli aspirati midollari di 65 pazienti pediatrici sottoposti a TCSE, utilizzando stessi primer e stesse sonde fluorescenti usati negli esperimenti RQ-PCR, nelle medesime condizioni di reazione. Mettendo a confronto i livelli di MRM emersi coi due approcci molecolari, si è investigata l'applicabilità della metodica assoluta per il monitoraggio della MRM anche in questo contesto. Inizialmente, sono stati valutati campioni risultati, mediante RQ-PCR, positivi ma non quantificabili (PNQ), per verificare se invece si potessero quantificare mediante ddPCR. Successivamente, è stato valutato anche l'impatto prognostico dei livelli MRM pre-TCSE ottenuti tramite ddPCR.

Un buon livello di concordanza è emerso dai risultati ottenuti con entrambe le metodiche (Pearson $r = 0.98$, $P < 0.0001$); la ddPCR ha permesso, inoltre, di quantificare numerosi campioni risultati non quantificabili tramite RQ-PCR. I risultati suggeriscono che il metodo assoluto possieda sensibilità, accuratezza e riproducibilità almeno paragonabili alla PCR quantitativa convenzionale.

I pazienti LLA analizzati sono stati stratificati sulla base dei livelli di MRD ottenuti con le due tecniche molecolari, ma nelle analisi di sopravvivenza non sono emerse differenze significative sulla prognosi. Infatti le pEFS dei pazienti con MRM negativa e positiva quantificabile per i due metodi risultano molto simili (rispettivamente 71% e 68%).

Ciononostante, dal presente studio è emerso che col metodo digital sia stato possibile misurare un valore di MRM positivo e quantificabile per almeno 12 pazienti LLA che, in seguito al trapianto, hanno presentato una recidiva; viceversa, la RQ-PCR non era stata in grado di identificare anticipatamente la ricaduta di questi pazienti.

Questi dati preliminari mostrano che la ddPCR possa essere un valido strumento per il monitoraggio della MRM e applicabile anche nel contesto dei trials clinici per pazienti LLA pediatrici. Tuttavia una prosecuzione dello studio ddPCR, con estensione della casistica analizzata, potrebbe essere utile a definire con precisione la significatività delle misurazioni con questa recente metodica.

CHAPTER 1

Role of MRD in pediatric patients with ALL and relapse ALL before and after HSCT

1.1 Acute Lymphoblastic Leukemia

Acute Lymphoblastic Leukemia (ALL) is a clonal malignant disease of bone marrow (BM), that originates from early lymphoid precursors, and is characterized by a neoplastic proliferation of blast cells. It results in suppression of normal haematopoiesis and infiltration of various extramedullary sites. ALL is the most frequent cancer in childhood, accounting for 25% of all malignancies occurring before age 15 years and 19% among those younger than age 20 years.[1]

More than 80% of childhood ALLs is of B-cell lineage (B-cell Precursor ALL or pB-ALL), whereas T-ALL accounts for 10-15% of the cases.[2,3]

ALL is frequently associated with recurrent genetic alterations, including whole chromosomal gains and losses, and translocations resulting in the expression of chimeric fusion genes (i.e. ETV6-RUNX1, TCF3-PBX1, BCR-ABL1 and rearrangements of the gene KMT2A) or in dysregulation of genes by juxtaposition to antigen receptor gene loci.[4] Lesions as hypodiploidy and some KMT2A rearrangements confer a high risk of treatment failure and relapse.

In recent years genome-wide approaches have been widely used to identify the full spectrum of structural genetic lesions present in ALL. The majority of ALL cases harbor a relatively low number of alterations, mostly focal and affecting genes involved in lymphoid development and leukemogenesis.[5]

Progress in the management of childhood ALL has been made over the past 20 years, mainly through refinement of risk relapse stratification and risk-adapted chemotherapy. The international collaborative treatment protocol for children and adolescents with ALL is currently represented by the AIEOP-BFM ALL 2009 protocol (Associazione Italiana di Emato-Oncologia Pediatrica e Berlin-Frankfurt-Münster Acute Lymphoblastic Leukemia 2009 – EudraCT Number: 2007-004270-43), in which patients with newly diagnosed leukemia are stratified in risk-relapse groups. Stratification is based on following information regarding biological features and therapy response assessment by morphology and Minimal Residual Disease (MRD) detection:

- Immunophenotype (T-ALL or pB-ALL);

- Prednisone response at day 8 (good or poor responder);
- Flow-cytometry (FCM) MRD on day 15;
- Morphological remission status (defined as less than 5% blasts by morphological examination) on day 33 (time-point 1, TP1);
- PCR-MRD on day 33 and 78 (time-point 2, TP2);
- Ploidy;
- Presence or absence of MLL/AF4 (now called KMT2A and AFF1, respectively) or t(4;11) translocation.

On the bases of these parameters, patients enrolled in the AIEOP-BFM ALL 2009 protocol are so stratified:

- **High-risk (HR) patients**: prednisone poor-responder (PPR), FCM ≥ 10% on day 15, no complete remission on day 33, positivity for KMT2A/AFF1, hypodiploidy, high risk by PCR-MRD response;
- **T/non-HR patients**: T-ALL in absence of any HR criteria (see above);
- **pB/non-HR patients**: pB-ALL in absence of any HR criteria (see above);

  Standard-risk (SR) patients: PCR-MRD-SR or, if no PCR-MRD result available, FCM < 0.1% on day 15;

  Medium-risk (MR) patients: no SR or HR criteria (see above).

The fundamental contribute of MRD results to the final risk group assignment are reported in the paragraph 1.1.3.

1.1.1 ALL Recurrence

Current treatment strategies result in long-term remission for nearly 80% of children with ALL, but, unfortunately, the remaining 20% ultimately relapse.

Leukemia recurrence represents the outgrowth of a clonal cell population not completely eliminated by treatment. Retrospective studies suggested that many leukemia relapses may be the result of the selection of a relatively drug-resistant clone already present at initial diagnosis.[6] In only a minority of ALL cases (6%), the relapse clone represents the emergence of a genetically distinct and thus unrelated second leukemia.[7]

Anyway, relapse represents exactly the most common cause of treatment failure in pediatric ALL, resulting in an incidence of approximately 0.7 of 100.000 children per year in Europe.[8]

The BFM-ALL Relapse Study Group identified, as poor prognostic factors after first relapse, a short duration of first remission, an isolated bone marrow relapse and T-cell immunophenotype.[9] These statements are also confirmed by further studies.[4, 10, 11, 12]

According to the Berlin-Frankfurt- Munster relapse risk stratification[13], patients with a first leukemia relapse were stratified into 4 different groups of risk (S1, S2, S3, S4) and treated according to the AIEOP ALL REC 2003 protocol (EudraCT Number: 2012-000793-30). Re-induction treatment for patients who presented a second ALL relapse varied for single patient.

In the most recent protocols for treatment of relapsed ALL, with intensive combination of chemotherapy and allogeneic HSCT, 30%-50% of all children can be cured.[13, 14, 15, 16, 6, 17, 9] However, most children still die despite aggressive chemo-radiotherapy approaches. Thus, novel therapeutic strategies are needed, not only in salvage regimens, but also in frontline protocols, especially for those patients who are at high risk of relapse.

1.1.2 Minimal Residual Disease

Besides risk factors associated with the patient (eg, sex, age at diagnosis) and the disease (eg, white blood cell count at diagnosis, immunophenotype, structural and numeric chromosomal aberrations), measurement of *in vivo* treatment effectiveness has been shown to be of high significance in predicting patient outcome and risk of relapse in childhood ALL.[18, 19, 20, 21, 22, 23]

Around 95% of ALL patients achieve the Complete Remission (CR), which is defined as less than 5% leukemic blasts by morphological examination of bone marrow smears. However, such finding can correspond to a residual leukemic cell burden. Many patients, despite achieving the CR, subsequently relapse. The presence of leukemic cells not identifiable through cyto-morphological methods is defined Minimal Residual Disease (MRD).

In recent years, to assess more accurately the treatment response by monitoring MRD in pediatric patients with ALL, a lot of effort has been applied to develop novel sensitive techniques. Accurate measurement of low frequencies of leukemic cells, ≤ 1 blast cells in 10.000 normal cells (≤ 0.01% or ≤ 10-4), requires highly specific markers for discrimination between leukemic cells and normal leukocytes both in peripheral blood and BM. Markers of leukemia are aberrant immunophenotypes, specific genetic aberrations and/or clone-specific antigen receptor gene rearrangements, which are currently detectable by quantitative flow cytometry (FCM) or PCR-based molecular methodologies.[24]

Cytofluorimetric markers are represented by leukemia-associated phenotypes, which are not expressed by normal hematopoietic cells.[25] Moreover, blast cells carry genetic abnormalities resulting in the overexpression of aberrant mRNA transcripts which can be used for MRD detection. The fusion-transcripts most widely used are BCR-ABL1, KMT2A-AFF1, TCF3-PBX1 and ETV6-RUNX.[26] Such recurrent abnormalities suitable for MRD evaluation are present in approximately 40% of pediatric ALL patients.

PCR targets for MRD studies in ALL are represented by the junctional regions deriving by Immunoglobulin (IG) and T-cell receptor (TCR) gene rearrangements; junctional regions are specific to the leukemic clone.[27] The most common approach includes a PCR-based screening of diagnostic samples with primers matching the V and J regions of the various antigen-receptor genes to determine if rearrangements are present. The identified rearrangements are tested for clonality by heteroduplex analysis,[28] and the sequence obtained for each rearranged gene contains a specific junctional region. The highly diverse size and composition of the junctional regions result in higher specificities,[29] particularly because of allele-specific oligonucleotides (ASO-primers) were designed complementary to the individual junctional region sequences. Quantitative MRD data can

be obtained by using real-time quantitative PCR (RQ-PCR), a very sensitive and specific molecular assay, which combines ASO-primers with fluorescent labeled probes, obtaining a reading system for improved quantification of MRD levels.[30, 31, 32, 33] Mixtures of diagnostic leukemic and normal DNAs are tested in parallel to assess the sensitivity of the assay: high sensitivities of 10^{-4} to 10^{-5} could be reached.[30]

The first large-scale PCR-based MRD studies were performed in childhood ALL, using IGH (VH-JH), TRG and TRD gene rearrangements as PCR targets, mainly because of the limited number of primers needed to detect these rearrangements.[19, 34] After several European collaborations (BIOMED-1, International Berlin-Frankfurt-Münster Study Group (I-BFM-SG), and BIOMED-2 Concerted Actions), additional PCR targets could be introduced, such as IGK, TRB, incomplete IGH (DH-JH) and unusual TRD (Vd2-Ja) rearrangements.[33, 35, 36, 37, 38, 39]

Droplet Digital PCR is a third generation PCR, recently applied in a wide variety of cancers, including adult ALL, because of its high sensitivity and specificity.[40, 41, 42, 43, 44] ddPCR will be widely discuss in Chapter 2.

Among the different approaches available for MRD assessment, the detection of Ig/TCR rearrangements by RQ-PCR is the most widely used, as it is feasible in 90-95% of childhood ALL cases, monitoring patients with at least two sensitive MRD-PCR targets.[45, 33]

MRD diagnostics has proven to be the strongest prognostic factor in childhood ALL and it has been implemented into major frontline treatment protocols for pediatric ALL.[46, 19] The large-scale AIEOP-BFM-ALL 2000 studies have shown that MRD-based treatment strategies further improve outcome in the involved patients, both in BCP-ALL and T-ALL patients (Figure 4).[19, 47, 48]

As mentioned in paragraph 1.1, in the current international therapeutic protocol AIEOP-BFM ALL 2009 for pediatric ALL treatment, the MRD assessment is routinely used to stratify patients into different risk classes (standard, medium or high-risk of relapse)[47], with the aim to refine therapy based on risk of relapse maximizing cure and minimizing toxicities. Protocol criteria for assigning the risk-group are defined according to MRD levels on day 33 (TP1) and on day 78 (TP2) from the beginning of the treatment. Based on MRD analyses performed by RQ-PCR, 3 PCR-MRD risk classes are identified:

- **Standard-risk** (MRD-SR): negative-MRD at both time points, with at least one, two if possible, markers with sensitivity at least 10^{-4};
- **Intermediate or Medium-risk** (MRD-MR): positive-MRD at TP1 and/or at TP2, and MRD levels < 10^{-3} at TP2;
- **Slow early responder** (MRD-SER): positive MRD ≥ 10^{-3} at TP1 and any level of MRD positivity < 10^{-3} at TP2;
- **High-risk** (MRD-HR): positive MRD ≥10^{-3} at TP2. (EudraCT Number: 2007-004270-43)

In the case of not-availability of at least two sensitive MRD markers (sensitivity at least 10^{-4}), MRD risk group stratification can also be based on only one sensitive marker.
To assign the final risk-group to the patient (SR, MR o HR), high-risk clinical/biological parameters qualify HR patients, regardless of MRD levels.
Furthermore, MRD assessment is a strong prognostic factor also for relapsed ALL patients and in patients undergoing HSCT, useful to identify good and poor responders to therapy and to correlate MRD levels with outcome.[46, 49, 50, 51]

1.1.3 Minimal Residual Disease in HSCT

Although current front-line chemotherapy is able to cure a large proportion (80 – 90%) of children affected by ALL,[52, 48] allogeneic hematopoietic stem cell transplantation still plays a pivotal role as the curative therapy of choice for 10% of the patients who are at high risk at first-line treatment protocols and for the majority of patients after relapse.[53, 54, 55, 56]

According to the AIEOP-BFM ALL 2009 protocol, allogeneic HSCT should be offered to ALL patients presenting at least one of the following criteria:

- No CR at TP1;
- HR PCR-MRD;

- t(4;11) translocation + MR, SER or HR PCR-MRD;
- Hypodiploidy + MR, SER or HR PCR-MRD;
- T-ALL, PPR + HR PCR-MRD or no MRD results.

In ALL relapses, allogeneic HSCT is indicated to those patients with a high risk of subsequent relapse, namely those with early or very early relapse or with T-cell precursor leukemia, or to patients with standard risk disease but with a persistently positive MRD during treatment.

Since 1998, MRD diagnostics has been identified as a relevant prognostic factor also in the transplantation setting.[49] Several groups reported the unfavorable prognostic significance of high MRD levels before allogeneic HSCT in children with high-risk ALL.[57, 49, 50, 58] Studies exploring the significance of post-transplant MRD showed that detectable MRD at any time after HSCT represents a substantial risk of post-HSCT relapse.[59, 60] Consequently, MRD measurements are now guiding the extent of pre-transplant chemotherapy administration or post-transplant pre-emptive immunomodulation, in order to prevent a new ALL relapse.[61, 62]

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1.2 Pre and post-transplant minimal residual disease predicts relapse occurrence in children with acute lymphoblastic leukemia

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Summary

Relapse remains the leading cause of treatment failure in children with acute lymphoblastic leukaemia (ALL) undergoing allogeneic haematopoietic stem cell transplantation (HSCT). We retrospectively investigated the prognostic role of minimal residual disease (MRD) before and after HSCT in 119 children transplanted in complete remission (CR). MRD was measured by polymerase chain reaction in bone marrow samples collected pre-HSCT and during the first and third trimesters after HSCT (post-HSCT1 and post-HSCT3). The overall event-free survival (EFS) was 50%. The cumulative incidence of relapse and non-relapse mortality was 41% and 9%. Any degree of detectable pre-HSCT MRD was associated with poor outcome: EFS was 39% and 18% in patients with MRD positivity $<1x10^{-3}$ or $\geq 1x10^{-3}$, respectively, *versus* 73% in MRD-negative patients ($P<0.001$). This effect was maintained in different disease remissions, but low-level MRD had a very strong negative impact only in patients transplanted in second or further CR. Also, MRD after HSCT enabled patients to be stratified, with increasing MRD between post-HSCT1 and post-HSCT3 clearly defining cohorts with a different outcome. MRD is an important prognostic factor both before and after transplantation. Given that MRD persistence after HSCT is associated with dismal outcome, these patients could benefit from early discontinuation of immunosuppression, or pre-emptive immuno-therapy.

Introduction

Currently, conventional front-line chemotherapy cures a large proportion of children affected by acute lymphoblastic leukaemia (ALL).[1,2] Furthermore, second-line treatment followed by allogeneic haematopoietic stem cell transplantation (HSCT) can be effective in rescuing 30-50% of relapsed patients.[3,4] Nevertheless, relapse remains the most frequent cause of treatment failure for children affected by ALL, even after allogeneic HSCT.[5,6]

During the last 2 decades, minimal residual disease (MRD) quantification has progressively acquired a pivotal role in the assessment the early treatment response and defining risk stratification of children with newly diagnosed ALL,[1,7–11] as well as of relapsed patients receiving chemotherapy according to second-line protocols.[12,13] Pre-transplant MRD status has also been shown to predict the risk of relapse and final outcome of children affected by ALL and given allogeneic HSCT.[7,8,14–16]

In light of these considerations, MRD has been recently proposed as a tool to guide the extent of pre-transplant chemotherapy administration or post-transplant pre-emptive immunomodulation or immunotherapy, in order to prevent a new disease relapse.[17,18,19,20]

The aim of this study was to quantify MRD by real time quantitative polymerase chain reaction (RQ-PCR) immediately before allogeneic HSCT, in order to assess its clinical significance and impact on the risk of relapse and transplant outcome in a cohort of paediatric ALL patients transplanted in first, second or subsequent complete remission (CR). Furthermore, we analysed MRD in the same patients during the first and third trimester after transplantation, to address the question of whether MRD evaluation could provide further information to predict the risk of post-transplant leukaemia relapse.

Patients and methods

Patients

This study included 119 consecutive patients aged between 1 and 18 years, affected by ALL in first, second, or subsequent morphological CR (CR1, CR2 or other CR) given

allogeneic HSCT in one of the Italian Association for Paediatric Haematology/Oncology *(Associazione Italiana di Ematologia e Oncologia Pediatrica, AIEOP)* transplant centres in Padua, Pavia and Turin. Inclusion criteria were: morphological CR at time of HSCT, defined as less than 5% blasts by morphological examination, allogeneic HSCT from a matched family donor (MFD), an unrelated donor (UD) or a partially matched (haploidentical) family donor (PMFD) and the availability of bone marrow (BM) aspirates for MRD assessment within 30 days before HSCT. In 98 of the 119 patients MRD was also assessed within the first 3 months after HSCT (post-HSCT1), in 59 between the 7th and the 9th month after HSCT (post-HSCT3), and at both these time points in 48 patients. All parents or guardians signed the appropriate informed consent, approved by the local ethics committee or Institutional Review Board. Details on clinical characteristics of patients enrolled in the study are reported in Table I.

Treatment protocols

All patients had been enrolled in one of the following first-line treatment protocols: AIEOP ALL 2000,[21] AIEOP ALL R2006, AIEOP-Berlin-Frankfurt-Munster (BFM) ALL 2009 or EsPhALL (Safety and Efficacy of Imatinib Added to Chemotherapy in Treatment of Ph+ Acute Lymphoblastic Leukaemia in Children).[22] Eligibility criteria for transplantation in CR1 have been reported elsewhere.[23] Patients with first leukaemia relapse were stratified according to the BFM relapse risk stratification,[24,4] and treated according to the AIEOP ALL REC 2003 protocol. Re-induction treatment for patients who presented a second relapse before HSCT varied between centres. Transplants were performed between January 2001 and June 2014. In all donor-recipient pairs, histocompatibility was determined by high-resolution molecular typing of HLA-A, B, C, DRB1 and DQB1 loci. Forty-five patients (38%) received HSCT from a MFD, 59 (49%) from an UD and 15 (13%), lacking a compatible donor, were transplanted from a PMFD. Forty-three patients (36%) were transplanted in CR1, 65 (55%) in CR2 and 11 (9%) in other CR. Conditioning regimen included total body irradiation (TBI) in 113 cases (95%) and chemotherapy alone in the remaining 6 cases (5%). Details on the transplant procedure and graft-versus-host disease (GVHD) prophylaxis are reported in Table I. In

absence of GVHD, ciclosporin A tapering was started within 3 months after HSCT and the drug discontinued within 6 months after HSCT.

Because of the retrospective nature of the study, clinicians were not informed of the results of MRD before or after HSCT and no decision concerning immunosuppressive treatment tapering and discontinuation was based on MRD results. No patients received additional post-transplant consolidation treatment, including donor lymphocyte infusion (DLI) and tyrosine-kinase inhibitors.

MRD analysis

A total of 276 BM aspirates, collected before and after HSCT, and previously stored in the biological bank "*BioBanca Oncologica Pediatrica BBOP*" were retrospectively analysed for MRD.

DNA samples from BM mononuclear cells were obtained as previously reported.[25] Clonal immune gene rearrangements identified at diagnosis/relapse[26,27] were used for MRD assessment by RQ-PCR, and the results were interpreted according to the EuroMRD guidelines, as previously published. Briefly, a set of PCR reactions were performed on diagnosis/relapse DNA to identify IGH, IGK, TRG, TRD, and TRB rearrangements. Clonal gene rearrangements, confirmed by homo/heteroduplex analysis, were sequenced and patient-specific primers were designed complementary to the junctional regions of each target. Specific and sensitive RQ-PCR assays were developed and the 2 best performing targets were selected for MRD quantification. As for relapsed patients, we used at least one molecular marker confirmed at the time of relapse. MRD positivity was defined according to the one Ct below background rule.[28] Patients were categorized into 3 groups according to their MRD results: (i) MRD-high: patients with positive quantifiable MRD $\geq 1x10^{-3}$; (ii) MRD-low: patients with positive quantifiable or not-quantifiable MRD $< 1x10^{-3}$; (iii) MRD-negative: patients with a negative MRD result.[29]

Statistical analysis

The reference date used for analysis was 31 January 2016. Quantitative variables were reported as median value and range, while categorical variables were expressed as

absolute value and percentage. Demographic and clinical characteristics of patients were compared using the Chi-square test or Fisher's exact test for categorical variables, while the Mann-Whitney rank sum test or the Student's t-test were used for continuous variables as appropriate. Overall survival (OS) and EFS were calculated according to the Kaplan-Meier method,[30] while the risk of relapse (REL) and death in remission, defined as non-relapse mortality (NRM) were calculated as cumulative incidences in order to adjust the analysis for the 2 competing risks.[31] Comparisons between different OS and EFS probabilities were performed using the Log-Rank test,[32] while Gray's test was used to assess, in univariable analyses, differences between cumulative incidences.[33] Multivariable analysis was performed using the Cox proportional hazard regression model.[34] All results were expressed as 10-year probabilities or 10-year cumulative incidences (%) and 95% confidence interval (95% CI). $P < 0{\cdot}05$ were considered to be statistically significant. Statistical analysis was performed using NCSS [NCSS 10 Statistical Software (2015). NCSS, LLC. Kaysville, Utah, ncss.com/software/ncss.] and Stata MP/14 (StataCorp LP, College Station, TX, USA, www.stata.com).

Results

Overall outcome

The median observation time for surviving patients was 7.8 years (range, 1·2-13·4 years). All patients engrafted. Grade II-IV acute GVHD developed in 57 out of the 119 patients, with a cumulative incidence of 50% (95% CI, 40-58). Grade III-IV acute GVHD was observed in 13 patients (CI 11%; range 7-18). Chronic GVHD developed in 17 of the 111 patients surviving in remission for at least 100 days (CI 15%; 95% CI, 10-24), with 12 of them experiencing the extensive form of the disease (CI 11%; 95% CI, 6-18).

Overall, 67 of the 119 patients (56%) are alive, 61 of whom are disease-free after transplantation, resulting in an estimated 10-year OS and EFS probability of 54% (95% CI, 45-63) and 50% (95% CI, 41-59), respectively (Fig 1A). Forty-eight patients relapsed (REL 41%; 95% CI, 33-51) at a median of 7 months after HSCT (range, 1.8-58 months). Ten patients died in remission from transplantation-related causes, at a median of 7

months after transplantation (range, 1-68 months), resulting into a NRM of 9% (95% CI, 5-16) (Fig 1B).

Table II summarizes the results of the univariate analysis for EFS. Only 3 variables were found to be associated with a statistically different EFS: age <10 years at HSCT [EFS = 59% (95% CI, 49-70%) vs. 30% (95% CI, 15-46) age >10 years at HSCT; P = 0·01] (Fig 1C); disease phase at HSCT [EFS = 60% (95% CI, 45-75) for patients transplanted in CR1, 76% (95% CI, 62-90) for patients transplanted in CR2 and belonging to the S1-S2 BFM risk groups, 9% (95% CI, 0-21) for those transplanted in 2nd CR and belonging to the S3-S4 risk groups and 18% (95% CI, 0-41) for children transplanted in subsequent CR; P <0·0001] (Fig 1D); the use of TBI during the conditioning regimen [EFS = 52% (95% CI, 43-61) vs no TBI 17% (95% CI, 0-46); P = 0·04].

Results of MRD analysis

A total of 172 RQ-PCR targets were used for MRD assessment. Most of them were IGH rearrangements (60%), followed by TRD and TRG (16% and 10%, respectively). MRD could be evaluated by 2 markers in 53/119 patients, and in 30/53 cases the PCR results were concordant. In cases with discordant results, the highest MRD value was considered for patient categorization into the appropriate MRD group.

Pre-HSCT MRD was negative (MRD-neg) in 51/119 patients (43%), positive $<1x10^{-3}$ (MRD-low) in 46 (31%), and positive $\geq 1x10^{-3}$ (MRD-high) in 22 (18%). As shown in Table III, we observed a strong correlation between disease phase and pre-transplant MRD level. Negative MRD was observed more frequently in patients transplanted in CR1 or in those transplanted in CR2 and belonging to the S1-S2 risk groups, while MRD $\geq 1x10^{-3}$ was more frequent in patients transplanted in CR2 and belonging to the S3-S4 risk groups (P = 0·0009).

MRD was also assessed after HSCT in 109/119 patients (92%) either during the first trimester (post-HSCT1) or the third trimester (post-HSCT3). MRD at post-HSCT1 was analysed in 98 patients: 71 were negative, 23 were MRD-low (22/23 with not-quantifiable MRD levels), and 4 were MRD-high. BM aspirates at post-HSCT3 were available for 59 patients (32 patients relapsed or died in remission before post-HSCT3,

while the BM aspirate was not performed or not available in 28 cases). MRD was negative in 38 patients (64%), MRD-low in 16 (27%; not quantifiable levels in 12/16) and MRD-high in 5 (9%).

BM aspirate was consecutively analysed at the first 2 time points (before HSCT and at post-HSCT1) in 71 of the 119 patients, and at all the 3 time points in 48. Details on the evolution of MRD in these 71 patients presented in Fig 2.

Twenty-six of these patients were MRD-neg before HSCT, 20 of whom (77%) remained negative both at post-HSCT1 and post-HSCT3. Two (2%) patients were MRD-neg at post-HSCT1, but one became MRD-low at post-HSCT3 and subsequently relapsed, and one had an overt relapse between post-HSCT1 and post-HSCT3. Four additional patients (4%) became MRD-low already at post-HSCT1: 2 relapsed shortly after, while the other 2 remained MRD-low at post-HSCT3 and are alive in complete remission at the time of last follow-up (9 and 11 years after HSCT, respectively).

Thirty-one patients were MRD-low before HSCT; 19 of them (61%) became MRD-neg, 11 (36%) remained MRD-low and 1 (3%) presented a very early marrow relapse at post-HSCT1. Nine of the 19 patients who were MRD-neg at post-HSCT1 remained MRD-neg at post-HSCT3; 7 remained in remission at last follow-up. The MRD level of the other 10 patients increased at post-HSCT3 and only 1 of them is still in remission. Of the 11 patients who remained stable MRD-low at post-HSCT1, only 4 remained MRD low or became negative at post-HSCT3 (1 subsequently relapsed) while 7 ultimately relapsed.

Fourteen of these 71 children were MRD-high at the pre-transplant evaluation. Seven (50%) became negative at post-HSCT1, but only 1 remained negative at post-HSCT3 and is currently alive and in remission, while the MRD level of other 6 patients increased at post-HSCT3 and they ultimately relapsed. Of the other 7 children who were pre-transplant MRD-high, 5 improved to MRD-low and 2 remained MRD-high. Only one patient was still MRD-low at post-HSCT3 and is currently alive and in remission, while the remaining 6 patients ultimately relapsed.

Prognostic significance of pre-transplant MRD

Considering the whole study population, a negative MRD evaluation before transplantation was associated with better outcome: 38 out of 51 patients (75%) with

negative MRD at time of HSCT are still alive in complete remission. Persistence of any MRD level at pre-HSCT was associated with a lower probability to be alive and in remission: 19/46 patients (41%) with MRD-low are alive and disease free, while only 4/22 patients (18%) with MRD-high values are alive in complete remission. The 10-year EFS probability was 73% (95% CI, 61-86) for MRD-neg patients, 39% (95% CI, 25-54) for MRD-low patients and 18% (95% CI, 2-34) for MRD-high patients, P < 0·001 (Fig 3A). The difference in EFS was entirely due to a different relapse risk, the cumulative incidence of relapse being 20% (95% CI, 11-35) for MRD-neg patients, 50% (95% CI, 37-67) for MRD-low patients and 73% (95% CI, 56-94) for MRD-high patients, P < 0·001 (Fig 3B). No difference in NRM was observed among the 3 MRD groups (Table IV).

As shown in Table IV and Fig 3C, D, the predictive value of pre-HSCT MRD level was confirmed also when patients were analysed according to disease phase at HSCT (first, second or subsequent CR). However, the impact of pre-HSCT MRD level was different in patients transplanted in CR1 or CR2. In detail, considering patients transplanted in CR1, the EFS probability was similar for MRD-neg and MRD-low patients [74% (95% CI, 55-94) vs. 63% (95% CI, 41-85), respectively] while it was 0% for MRD-high patients (P < 0·0001). Conversely, for patients transplanted in CR2, EFS probability was significantly better for MRD-neg patients [78% (95% CI, 62-94), P = 0·001], while it was almost identical for MRD-low and MRD-high patients [24% (95% CI, 3-45) vs. 25% (95% CI, 4-46), respectively].

Grade II-IV acute GVHD demonstrated a protective effect against relapse, especially in patients with pre-transplant low-level MRD positivity, where the effect was statistically significant [relapse incidence = 67% (95% CI, 50-88) vs. 27% (95% CI, 14-54) in grade 0-I and grade II-IV patients respectively, P = 0·018] (Table V). Also, chronic GVHD seems to have a protective impact against relapse in pre-HSCT low-level MRD patients, even though the advantage associated with chronic GVHD occurrence was not statistically significant (Table V).

Prognostic significance of post-transplant MRD

The probability of EFS was evaluated according to the MRD level at post-HSCT1 and post-HSCT3. Considering the 98 patients evaluated at post-HSCT1, the 71 who had a negative MRD had a 10-year EFS probability of 63% (95% CI, 52-75), while EFS was 30% (95% CI, 12-49) and 25% (95% CI, 0-67) for the 23 and 4 patients with low positive MRD and high positive MRD, respectively (P <0·001) (Fig 4A). Likewise, considering the 59 children evaluated at post-HSCT3, the 38 patients with a negative MRD had an EFS probability of 84% (95% CI, 72-97), while EFS was 44% (95% CI, 19-69) for the 16 patients with MRD-low and 0% (95% CI, 0-67) for the 5 children with MRD-high (P <0·001) (Fig 4B).

The impact of MRD change from the pre-HSCT to post-HSCT is shown in Fig 4C. Patients with unchanged negative MRD (i.e., MRD-neg both before HSCT and at post-HSCT1 time point) had the best EFS probability, 80% (95% CI, 67-93). Children whose MRD decreased from pre-HSCT to post-HSCT1 had an EFS probability of 37% (95% CI, 21-52) if they reached MRD-neg and of 14% (95% CI, 0-40) if they only achieved a low level of positivity. Patients with unchanged positive MRD (i.e., a positive MRD before HSCT that remained at the same positivity level also at post-HSCT1) had an EFS of only 23% (95 CI, 0-46).

The effect of MRD variation from post-HSCT1 to post-HSCT3 is shown in Fig 4D. EFS was 88% (95% CI, 75-100) for patients with an unchanged negative MRD, 80% (95% CI, 45-100) for those with an unchanged low-positivity MRD and 100% for the 2 children whose MRD decreased from positive to negative (P = N.S.). In contrast, EFS was only 8% (95% CI, 0-24) for those whose MRD increased between post-HSCT1 and post-HSCT3 (P <0·001).

Multivariable analysis

Table VI presents the results of multivariable analysis of EFS. As expected, disease status at HSCT had a significant association with EFS probability. The risk ratio of treatment failure was 2·59 (95% CI, 125-536; P = 0·011) for CR2 patients belonging to the S3-S4 groups vs. CR1 patients, and 2·44 (95% CI, 1·00-5·91; P = 0·049) for other CR patients

vs. CR1 patients. Pre-HSCT MRD confirmed its strong predictive value also in multivariable analysis. The risk ratio of treatment failure was 2·18 (95% CI, 1·10-4·31; P = 0·025) for MRD-low vs. MRD-neg patients and 4·14 (95% CI, 1·84-9·32; P = 0·001) for MRD-high vs. MRD-neg patients.

Discussion

The probability of cure for children affected by ALL exceeds 80% with current front-line chemotherapy.[1,2] For this reason, the indication for allogeneic HSCT in CR1 has been progressively restricted and, nowadays, only patients with very high risk genetic features or those with suboptimal response to initial treatment are offered transplantation in CR1. Likewise, considering patients who experience leukaemia relapse, allogeneic HSCT is reserved for those with high-risk characteristics, namely those with BM relapse of B-cell precursor (BCP) ALL occurring within 6 months from treatment discontinuation or with T-cell ALL, or to children with standard risk disease, but with persistently positive MRD at the end of induction therapy. Unfortunately, despite the use of a fully myeloablative conditioning regimen, often including TBI, 20-40% of children given allogeneic HSCT ultimately relapse[6,18,23,29,35,36] , with disease recurrence remaining the most frequent cause of treatment failure.[5] Previous reports have shown that pre-transplant MRD level can predict the risk of post-transplant relapse of patients with ALL.[7,8,14–16,18,37] Pre-transplant intensification chemotherapy aimed at achieving MRD negativity or significant reduction has been suggested as a potential strategy in order to prevent leukaemia relapse after HSCT.[18] Furthermore, extensive clinical and experimental data support the concept of an immune-mediated graft-versus-leukaemia (GVL) effect after allogeneic HSCT,[38] suggesting that immunological interventions, such as less intensive GVHD prophylaxis, early discontinuation of immunosuppression or administration of DLI, could have an effect in preventing relapse and improving transplant outcome.[39–41]

In this study, we retrospectively evaluated the outcome of a large cohort of children and adolescents with ALL given allogenic HSCT in first, second or subsequent CR and correlated the outcome with pre- and post-transplant MRD. Overall, we observed an EFS probability of 50%, a value comparable to that of previous reports,[35,36,42] with a low NRM

of 9%. The cumulative incidence of relapse exceeded 40%, and disease recurrence was confirmed to be the most important cause of treatment failure. This high relapse rate was mainly due to the very poor outcome of high-risk patients, namely those transplanted in CR2 and belonging to the S3-S4 BFM Risk group (EFS = 9%) or those transplanted in more advanced disease (EFS = 18%). On the contrary, children in S1 and S2 risk groups who were transplanted in CR1 or in CR2 had an EFS probability of 60% and 76%, respectively.

We found a strong association between pre-transplant MRD and disease phase at transplantation, with the highest pre-HSCT MRD being observed in children transplanted in CR2 and belonging to the S3-S4 BFM risk group. Indeed, 42% of these patients had a MRD level $\geq 1 \times 10^{-3}$ at time of HSCT, as compared to less than 20% observed in the other subgroups. Our data confirm that the S3-S4 BFM relapse risk group has poorer molecular response to conventional chemotherapy and, to optimize the efficacy of transplantation as final consolidation treatment, patients in this risk group are candidates to new therapeutic approaches, including experimental immunotherapies based on the use of bispecific T-cell engager (BiTE) antibodies targeting the CD19 antigen ubiquitously present on Bcp-ALL.[43]

As expected, pre-transplant MRD was a strong predictor of outcome, thus confirming previously reported studies on the value of pre-transplant MRD in children affected by ALL.[7,8,14–16,18] We observed that the prognostic significance of pre-transplant MRD was consistent in all disease phases at HSCT. Nevertheless, a new and, in our opinion, important finding was that the level of MRD positivity had a different impact on EFS according to the disease phase at HSCT. In patients transplanted in CR1, only a high MRD ($\geq 1 \times 10^{-3}$) was associated with an increased risk of relapse. On the contrary, considering patients transplanted in CR2, a low-level MRD positivity ($< 1 \times 10^{-3}$) was also associated with a high relapse rate and poor outcome (Fig 3C). Our finding differs from the observation of Eckert *et al* (2015),[44] of a negative impact only of an MRD $\geq 1 \times 10^{-3}$ and supports the concept that, in contrast to CR1 patients, for those who relapse, low level MRD positivity also suggests an intrinsic resistance of the leukemic cells to chemo- and radiotherapy.

MRD was also evaluated during the first and third trimester after transplantation. Patients with a negative MRD early post-transplant had a good EFS probability, which was even better for those who were still negative at the third trimester assessment, although one relapse was observed in this subgroup at more than 4 years after transplantation. However, as previously suggested,[18,20] low level MRD positivity after transplantation was not invariably associated with relapse. Indeed, children with MRD $\leq 1 \times 10^{-3}$ at the first and third trimester post-transplant had an EFS of 30% and 44%, respectively. Conversely, only one out of the 4 patients with high MRD positivity at post-HSCT1 and none of the 5 with high MRD positivity at post-HSCT3 is surviving in remission.

Our data show that patients with pre-transplant low-level positive MRD and grade II-IV acute GVHD or chronic GVHD have a lower risk of relapse as compared to those without GVHD. For this reason, considering that this analysis was retrospective and that no clinical investigator received information regarding MRD results before transplantation or during the post-transplant follow-up, we believe that a low level MRD positivity can be controlled by the GVL effect of the transplant, while the finding of a high level MRD warrants a prompt and more aggressive intervention, such as the immediate discontinuation of all immunosuppressive therapy or the use of DLI. High-risk patients with early low-level MRD who are transplanted in CR2 may also benefit greatly from such prompt interventions of immune modulation, also considering that DLI did not seem to be associated with an increased rate of acute GVHD in a paediatric cohort treated with pre-emptive DLI for positive MRD after HSCT.[20]

Furthermore, adoptive cell therapy with chimeric antigen receptor (CAR) T cells[45,46] might be even more effective and with less severe side effects if used in patients with only MRD positivity post-HSCT, before progression to an over haematological relapse.

Our data also provide support to the results published by Bader *at al* (2015),[42] showing that MRD after HSCT is a dynamic process and that variations of MRD over time are important. In our experience, the change between pre-HSCT and post-HSCT1 enabled the identification of 3 categories of patients: those with good prognosis (unchanged negative MRD), those with poor prognosis (unchanged positive MRD or decreasing but still positive MRD) and those with an intermediate prognosis (MRD decreasing to negative or increasing from negative to low positive). The variation between post-HSCT1

and post-HSCT3 was even more important, identifying 2 subgroups with a dramatically different outcome: a first group of patients with very good prognosis (those with MRD remaining negative or decreasing from positive to negative and those with an unchanged low-level positivity, with a EFS probability ≥ 80%), and a group of patients with severe prognosis (those whose MRD increases between post-HSCT1 and post-HSCT3, who had an EFS probability of only 8%) (see Fig 5C, D).

In our study, the median time from transplant to morphological leukaemia relapse was 7 months, with a range between 1 and 68 months. Only 4 (8%) out of the 48 relapses were observed within the first 3 months, while 26 (54%) occurred between the months 3 and 9 and 18 (38%) after the third trimester. For this reason, the prospective evaluation of MRD after HSCT could identify, in advance, patients with the highest risk of relapse and with a strong indication for prompt immunological intervention, such as rapid tapering or discontinuation of the immunosuppressive treatment, infusion of DLI or other form of immune-therapy.[39–41,47,48] MRD must be cleared before the graft becomes tolerant toward the recipient.[42] Thus, it is reasonable to hypothesize that these interventions could be more effective if performed early after HSCT and, if possible, with the lowest MRD level.

In conclusion, we confirm that pre-transplant MRD allows early identification of patients at higher risk of relapse after allogeneic HSCT. The impact of pre-transplant MRD positivity is different in patients transplanted in first, second or subsequent CR. A prospective, longitudinal evaluation of post-HSCT MRD could provide accurate information to predict impending relapse, and thus represent a tool for implementing strategies of pre-emptive immunological intervention aimed at avoiding progression to frank relapse.

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Table I. Patient characteristics and transplant procedures.

Number of patients	119	(100%)
Gender:		
Male	74	(62%)
Female	45	(38%)
Median age at transplantation (years, range)	7	(1-18)
Immunophenotype:		
B-cell precursor ALL	105	(88%)
T-cell precursor ALL	14	(12%)
Cytogenetics:		
t(9;22)	17	(14%)
t(4;11)	3	(2%)
t(12;21)	4	(3%)
First-line chemotherapy protocol:		
AIEOP ALL 95	4	(3%)
AIEOP ALL 2000	97	(82%)
AIEOP ALL 2009	7	(6%)
EsPhALL	9	(8%)
Other	2	(2%)
Year of transplantation:		
2001-2005	15	(13%)
2006-2010	81	(68%)
2011-2014	23	(19%)
Disease phase at transplantation:		
CR1	43	(36%)
CR2 BFM S1-S2	39	(33%)
CR2 BFM S3-S4	26	(22%)
≥ CR3	11	(9%)
Donor:		
Matched family donor	45	(38%)
Unrelated donor	59	(49%)
Partially matched family donor	15	(13%)
Stem cell source:		
Bone marrow	77	(65%)
Peripheral blood	34	(28%)
Cord blood	8	(7%)
Conditioning regimen:		
TBI-based	113	(95%)
Busulfan-based	6	(5%)
Graft-versus-host disease prophylaxis:		
CsA	26	(22%)
CsA + MTX	20	(17%)
CsA + MTX + ATLG	52	(44%)
CsA + Steroids + ATLG	6	(5%)
Ex vivo T-cell depletion	15	(12%)

AIEOP, Associazione Italiana di Ematologia e Oncologia Pediatrica; ALL, Acute Lymphoblastic Leukaemia; BFM S1-S4, Berlin-Frankfürt-Mùnster standard risk groups; CR1, first complete remission; CR2, second complete remission; CR3, third complete remission; CsA, Ciclosporin A; EsPhALL, Safety and Efficacy of Imatinib Added to Chemotherapy in Treatment of Ph+ Acute Lymphoblastic Leukaemia in Children; MTX: short-term methotrexate; ATBI, Total Body Irradiation; ATLG: anti-T lymphocyte globulin.

Table II. Univariate analysis of event-free survival (EFS) according to patient and transplant characteristics.

Variable	N. of patients	N. of events	EFS %	(95% CI)	Log-rank P	Hazard ratio*	(95% CI)	P
Overall EFS	119	58	50%	(41-59)				
Gender:								
Male	74	39	46%	(35-58)	0.179			
Female	45	19	57%	(42-72)		0.69	(0.40-1.19)	0.182
Age at HSCT:**								
< 5 years	46	18	60%	(46-75)	0.083**			
5-9 years	36	15	58%	(42-74)		1.10	(0.56-2.19)	0.781
10-14 years	21	13	38%	(17-59)		1.78	(0.87-3.64)	0.114
≥ 14 years	16	12	25%	(4-46)		2.27	(1.09-4.71)	0.028
Phenotype:								
B cell precursor ALL	105	49	52%	(43-62)	0.413			
T cell precursor ALL	14	9	34%	(9-60)		1.34	(0.66-2.74)	0.416
t(9;22):								
No	102	50	50%	(40-60)	0.878			
Yes	17	8	53%	(29-77)		1.06	(0.50-2.24)	0.878
Disease phase at HSCT:								
CR1	43	17	60%	(45-75)	<0.001			
CR2 BFM S1-S2	39	9	76%	(62-90)		0.51	(0.23-1.14)	0.101
CR2 BFM S3-S4	26	23	9%	(0-21)		3.66	(1.93-6.93)	<0.001
Other CR	11	9	18%	(0-41)		3.92	(1.34-6.79)	0.008
Donor:								
MFD	45	21	53%	(38-67)	0.971			
UD	59	30	48%	(35-61)		1.07	(0.61-1.87)	0.809
PMFD	15	7	53%	(27-78)		1.04	(0.44-2.46)	0.921
Stem cell source:								
Bone marrow	77	36	53%	(42-64)	0.611			
Peripheral blood	34	19	42%	(25-59)		1.17	(0.67-2.05)	0.571
Cord blood	8	3	63%	(29-96)		0.66	(0.20-2.13)	0.482
Conditioning regimen:								
TBI	113	53	52%	(43-61)	0.036			
Chemotherapy	6	5	17%	(0-46)		2.59	(1.03-6.53)	0.043
GVHD prophylaxis:								
Cs-A	28	14	50%	(31-68)	0.803			
Cs-A + MTX	18	7	60%	(37-83)		0.63	(0.25-1.56)	0.318
Cs-A + MTX +ATLG	49	26	46%	(31-60)		1.00	(0.52-1.92)	0.994
Cs-A +Steroids+ATLG	9	4	56%	(23-88)		0.69	(.023-2.11)	0.520
Ex vivo T-cell depletion	15	7	53%	(27-28)		0.90	(0.36-2.23)	0.821
Acute GVHD								
Grade 0-I	62	32	48%	(35-60)	0.534			
Grade II-IV	57	26	53%	(40-67)		0.85	(0.51-1.42)	0.535
Chronic GVHD***								
Absent	94	43	53%	(43-64)	0.642			
Present	17	7	51%	(22-80)		0.83	(0.37-1.84)	0.642

95% CI, 95% confidence interval; ALL, acute lymphoblastic leukaemia; BFM S1–S4, Berlin-Frankfürt-Mùnster standard risk groups; CR, complete remission; CR1, first complete remission; CR2, second complete remission; CsA, ciclosporin A; EFS, event-free survival; GVHD, graft-versus-host disease; HSCT, haematopoietic stem cell transplantation; MFD, matched family donor; MTX, short-term methotrexate; APMFD, partially matched family donor; TBI, total body irradiation; TLG, anti-T lymphocyte globulin; UD, unrelated donor.

* The first value of each variable was considered as reference value to estimate the hazard ratio.

** Age at HSCT < 10 years vs. ≥ 10 years: Log-rank P = 0.014.

*** For chronic GVHD analysis, only the 111 patients surviving in remission at least 100 days post-transplantation were considered.

Table III. Association between disease phase at HSCT and pre-HSCT MRD level.

Disease phase at HSCT	Pre-HSCT MRD level			
	Neg	**Pos < 1 x 10^{-3}**	**Pos ≥ 1 x 10^{-3}**	***Total***
CR1	20 (47%)	19 (44%)	4 (9%)	*43 (100%)*
CR2 S1-S2	24 (61%)	10 (26%)	5 (13%)	*39 (100%)*
CR2 S3-S4	5 (19%)	10 (39%)	11 (42%)	*26 (100%)*
≥ CR3	2 (18%)	7 (64%)	2 (18%)	*11 (100%)*
Total	*51 (43%)*	*46 (39%)*	*22 (18%)*	*119 (100%)*

Chi-square P = 0.0009. BFM S1–S4, Berlin-Frankfürt-Münster standard risk groups; CR1, first complete remission; CR2, second complete remission; CR3, third complete remission; HSCT, haematopoietic stem cell transplantation; MRD, minimal residual disease.

Table IV. Impact of pre-transplant MRD on patient outcome.

Variable	N. of patients	N. of events	EFS %	(95% CI)	Log-rank P	Hazard ratio*	(95% CI)	P
EFS, all patients:								
MRD-negative	51	13	73%	(61-86)	<0.0001			
MRD-low	46	27	39%	(25-54)		3.04	(1.57-5.91)	0.001
MRD-high	22	18	18%	(2-34)		6.81	(3.29-14.09)	<0.001
Relapse, all patients:								
MRD-negative	51	10	20%	(11-35)	<0.0001			
MRD-low	46	22	50%	(37-67)		2.90	(1.39-6.05)	0.004
MRD-high	22	16	73%	(56-94)		6.97	(3.01-16.13)	<0.001
NRM, all patients:								
MRD-negative	51	3	7%	(2-21)	0.648			
MRD-low	46	5	11%	(5-25)		1.95	(0.47-8.03)	0.356
MRD-high	22	2	9%	(2-34)		1.58	(0.27-9.26)	0.614
EFS, CR1 patients								
MRD-negative	20	5	74%	(55-94)	<0.0001			
MRD-low	19	7	63%	(41-85)		1.29	(0.43-3.86)	0.643
MRD-high	4	4	0%	- -		75.69	(7.41-773.12)	<0.001
EFS, CR2 patients								
MRD-negative	29	6	78%	(62-94)	0.0001			
MRD-low	20	14	24%	(3-45)		5.10	(1.94-13.39)	0.001
MRD-high	16	12	25%	(4-46)		6.52	(2.41-17.63)	<0.001
EFS, CR2 S1-S2 patients								
MRD-negative	24	3	86%	(72-100)	0.115			
MRD-low	10	4	60%	(30-90)		3.95	(0.88-17.85)	0.074
MRD-high	5	2	60%	(17-100)		3.94	(0.66-23.71)	0.134
EFS, CR2 S3-S4 patients								
MRD-negative	5	3	30%	(0-77)	0.380			
MRD-low	10	10	0%	- -		2.26	(0.62-8.32)	0.219
MRD-high	11	10	9%	(0-26)		2.40	(0.65-8.93)	0.191
EFS, other CR patients								
MRD-negative	2	1	50%	(0-100)	0.131			
MRD-low	7	6	14%	(0-40)		4.21	(0.48-37.18)	0.196
MRD-high	2	2	0%	- -		12.65	(0.85-187.18)	0.065

95% CI, 95% confidence interval; CR1, first complete remission; CR2, second complete remission; CR3, third complete remission; EFS, event-free survival; HSCT, haematopoietic stem cell transplantation; MRD, minimal residual disease; MRD-high, MRD positive $\geq 1 \times 10^{-3}$;MRD-low, MRD positive $< 1 \times 10^{-3}$; MRD-negative, MRD negative; NRM, non-relapse mortality; S1–S4, Berlin-Frankfürt-Münster standard risk groups.

*The first value of each variable was considered as reference value to estimate the hazard ratio.

Table V. Effect of acute and chronic GVHD on the cumulative incidence of relapse, stratified by pre-transplant MRD level.

Variable	N. of patients	N. of events	Cumulative Incidence %	(95% CI)	Log-rank P	Hazard ratio*	(95% CI)	P
Acute GVHD:								
All patients								
Grade 0–I	62	30	49%	(38-64)	0.054			
Grade II-IV	57	18	32%	(22-47)		0.57	(0.32-1.01)	0.053
MRD-negative								
Grade 0–I	26	5	20%	(9-43)	0.990			
Grade II-IV	25	5	20%	(9-44)		0.99	(0.29-3.38)	0.990
MRD-low								
Grade 0–I	24	16	67%	(50-88)	0.018			
Grade II-IV	22	6	27%	(14-54)		0.33	(0.13-0.85)	0.021
MRD-high								
Grade 0–I	12	9	75%	(54-100)	0.354			
Grade II-IV	10	7	70%	(47-100)		0.62	(0.24-1.61)	0.327
Chronic GVHD:								
All patients								
Grade 0–I	94	38	41%	(32-53)	0.646			
Grade II-IV	17	6	36%	(19-69)		0.82	(0.36-1.89)	0.640
MRD-negative								
Grade 0–I	44	8	19%	(10-35)	0.472			
Grade II-IV	6	2	33%	(11-100)		1.87	(0.43-8.20)	0.404
MRD-low								
Grade 0–I	34	18	55%	(40-75)	0.171			
Grade II-IV	8	2	27%	(8-89)		0.39	(0.09-1.58)	0.186
MRD-high								
Grade 0–I	16	12	75%	(57-100)	0.628			
Grade II-IV	13	2	67%	(30-100)		0.69	(0.18-2.58)	0.580

95% CI, 95% confidence interval; GVHD, graft-versus-host disease; MRD, minimal residual disease; MRD-high, MRD positive $\geq 1 \times 10^{-3}$; MRD-low, MRD positive $< 1 \times 10^{-3}$; MRD-negative, MRD negative.
*The first value of each variable was considered as reference value to estimate the hazard ratio.

Table VI. Results of multivariable analysis of pre-transplant patient characteristics and pre-transplant MRD on event-free survival (EFS).

Independent variable	Hazard ratio	(95% CI)	P
Age at HSCT:			
≥ 10 years vs. < 10 years	1.62	(0.94-2.78)	0.080
Disease status at HSCT:			
CR2 S1-S2 vs. CR1	0.57	(0.24-1.33)	0.195
CR2 S3-S4 vs. CR1	2.59	(1.25-5.36)	0.011
Other CR vs. CR1	2.44	(1.00-5.91)	0.049
TBI			
No vs. Yes:	1.29	(0.47-3.57)	0.618
Pre-HSCT MRD			
Pos < 1 x 10^{-3} vs. Neg	2.18	(1.10-4.31)	0.025
Pos ≥ 1 x 10^{-3} vs. Neg	4.14	(1.84-9.32)	0.001

95% CI, 95% confidence interval; CR, complete remission; CR1, first complete remission; CR2, second complete remission; HSCT, haematopoietic stem cell transplantation; MRD, minimal residual disease; S1–S4, Berlin-Frankfürt-Münster standard risk groups; TBI, total body irradiation.

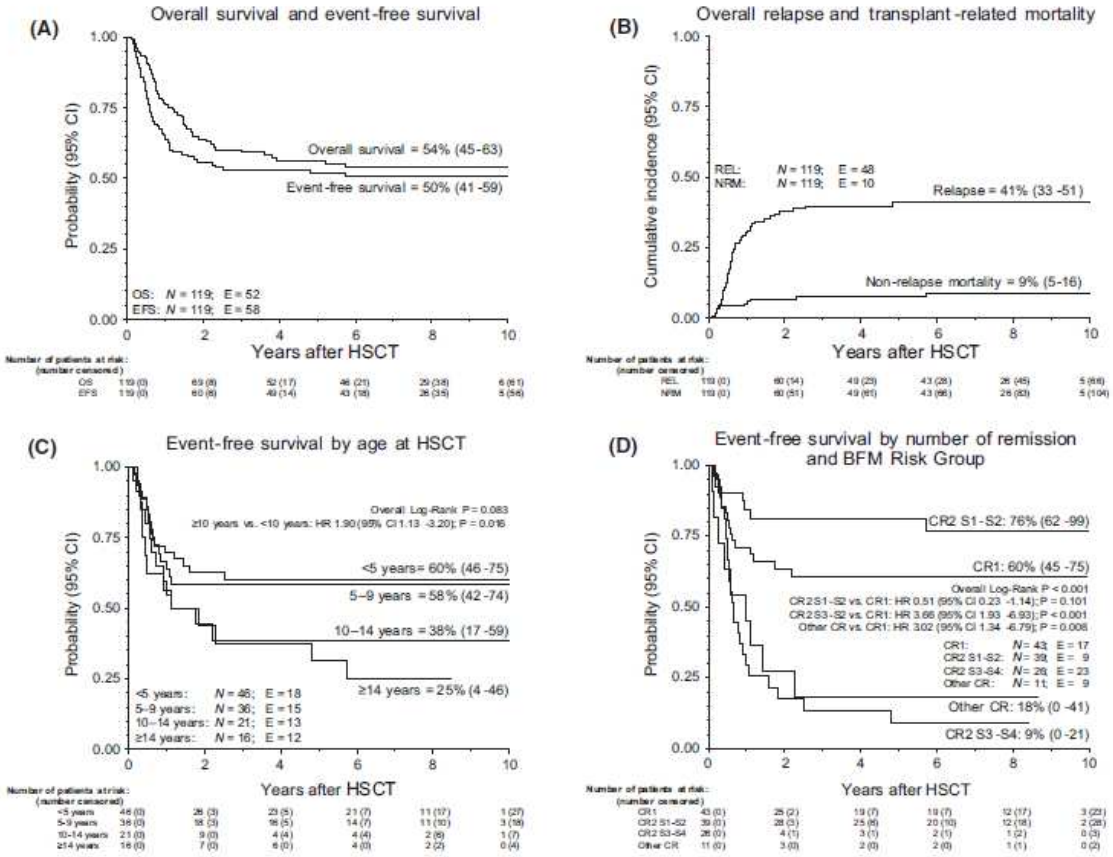

Fig 1. Probability curves of the study population by time from transplantation.
(A) Overall probability of survival (OS) and event-free survival (EFS). (B) Overall cumulative incidence of relapse and non-relapse mortality transplant-related mortality (TRM). (C) Overall probability of EFS according to age at transplantation. (D) Overall probability of EFS according to the number of remission and BFM risk group.

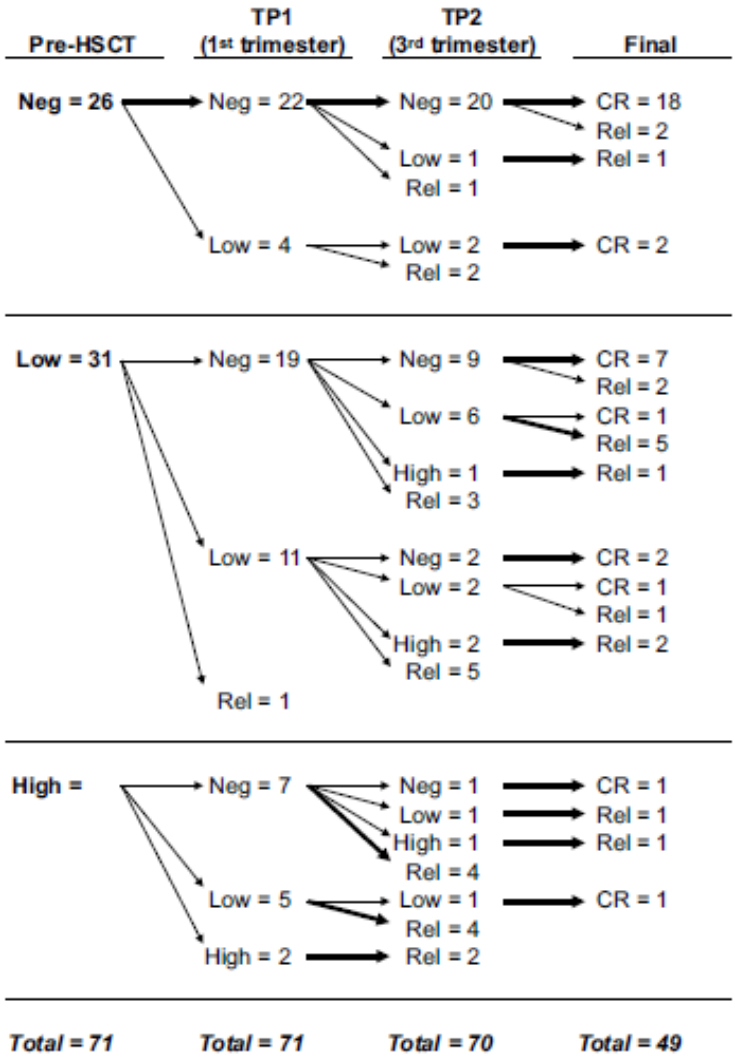

Fig 2. Prospective evolution of MRD before HSCT and at post-HSCT1 and post-HSCT2 time points. Only patients with MRD evaluated at least 2 time points (pre-HSCT and post-HSCT1) are included. The width of the arrows is proportional to the percentage of patients. CR, complete remission; HSCT, haematopoietic stem cell transplantation; Neg, negative; Rel, relapse; TP1, 1st trimester (first 3 months post-HSCT);TP2, 3rd trimester (month 7–9 post-HSCT).

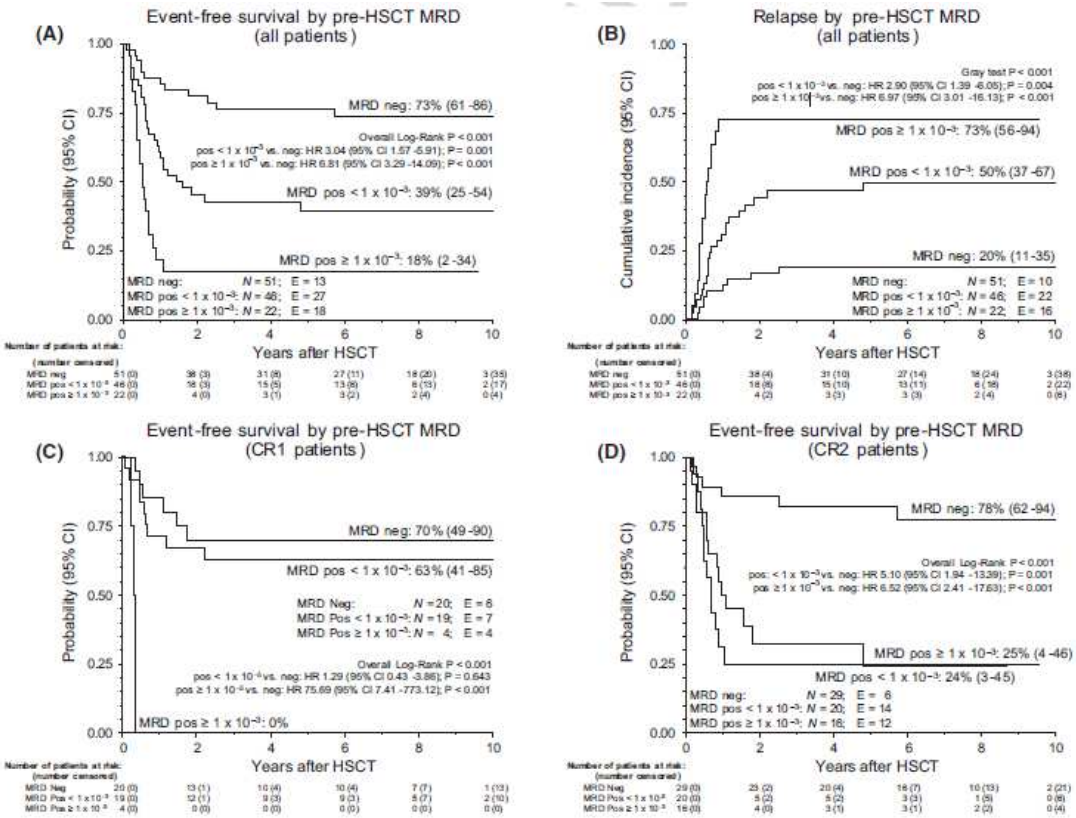

Fig 3. Prognostic significance of MRD levels before HSCT. Event-free survival (EFS) (A) and cumulative incidence of relapse (B) according to pre-HSCT MRD in the whole study population and EFS for patients transplanted in CR1 (C) or in CR2 (D). 95% CI, 95% confidence interval; CR1, first complete remission; CR2, second complete remission; HR, hazard ratio; HSCT, haematopoietic stem cell transplantation; MRD, minimal residual disease.

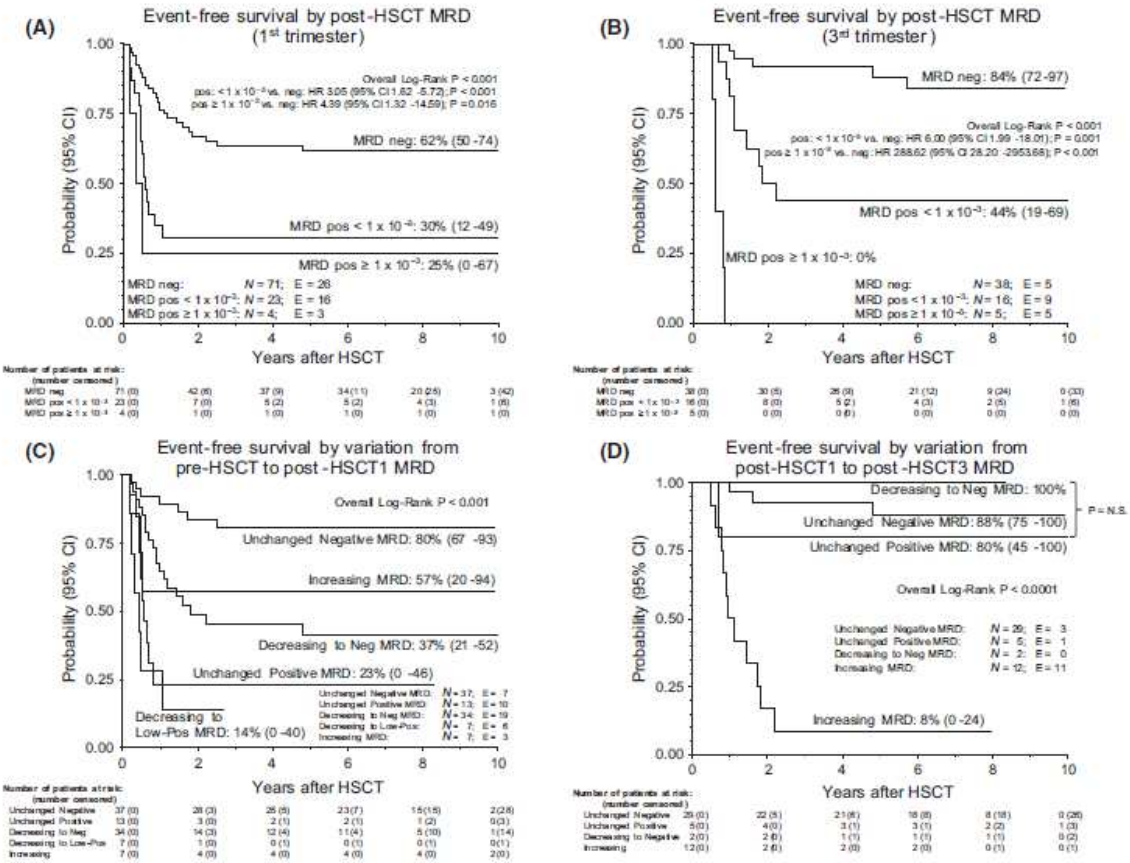

Fig 4. Event free survival (EFS) according to MRD post-transplantation. EFS according to post-transplant MRD level at post-HSCT1 (A) and post-HSCT3 (B) time points and according to the variation from pre-HSCT to post-HSCT1 time points (C) and from post-HSCT1 to post-HSCT3 (D). MRD variation is classified as unchanged negative (an already negative MRD that remains negative), unchanged positive (a positive MRD that remain positive at the same level), decreasing to negative (a positive MRD that becomes negative), decreasing to low-positive (a highpositivity MRD that becomes low-positive) and increasing (from negative to positive or from low-positive to high-positive). 95% CI, 95% confidence interval; HR, hazard ratio; HSCT, haematopoietic stem cell transplantation; HSCT1, 1st trimester (first 3 months post-HSCT); HSCT3, 3rd trimester (month 7–9 post-HSCT); MRD, minimal residual disease; N.S., not significant.

CHAPTER 2

Comparison of MRD detection by RQ-PCR and ddPCR in pediatric ALL before HSCT

2.1 INTRODUCTION

Detection of Minimal Residual Disease (MRD) is the most powerful prognostic factor in pediatric patients with Acute Lymphoblastic Leukemia (ALL)[1], as also discussed in the previous chapter. Due to its key role in *in vivo* response evaluation effectiveness to the induction and consolidation therapy, MRD monitoring was currently implemented into the major frontline and relapse treatment protocols for childhood ALL.[2, 3, 4, 5] MRD evaluation is used to stratify patients in different classes of risk and represents a leading guide in treatment decisions.

Also in the transplantation setting, as previously stated, the significance of MRD presence was explored: several studies reported the unfavorable prognostic significance of high MRD levels before HSCT (Haematopoietic Stem Cell Transplantation);[6,7, 8] other studies showed that detectable MRD after HSCT represents a substantial risk of post transplant relapse.[9, 10]

Various laboratory techniques are nowadays able to measure the MRD levels, quantitative flow cytometry or PCR-based molecular methodologies[11] and, currently, one of the most validated and internationally standardized tool to assess MRD in childhood ALL remains the real-time quantitative PCR (RQ-PCR).

This molecular technique measures MRD levels by using, as PCR target of the leukemic clone, the patient-specific junctional regions of immunoglobulin (IG) and T-cell receptor (TCR) gene rearrangements. These leukemia-specific "fingerprints" are identified at diagnosis of ALL patients, by PCR amplification, clonality and sequencing analysis. Today, over the 95% of pediatric ALL patients can be successfully MRD monitored by RQ-PCR, during their therapy and follow-up periods, using at least two leukemic clonal markers.[12, 13]

Measuring MRD means to detect levels of leukemic cells in bone marrow (BM) aspirate not identifiable by morphological examination (<5% of leukemic cells); so it is necessary to use a highly specific and sensitive assay: RQ-PCR allows to reach a sensitivity of 10^{-4} - 10^{-5}, or rather it is able to detect one single tumor cell on 100.000 normal leukocytes.

However, RQ-PCR presents some limits. Quantification in each assay is rely on the building of a calibration standard curve with the DNA collected at diagnosis of patient;

this is a relative quantification, in which standard curve is necessary to compare the resulted value (unknown) with the tumor load (known) of diagnosis and to extrapolate MRD value of each follow-up sample. In other words, RQ-PCR is strongly dependent on the availability of diagnostic material, which can limit the feasibility of the experiment. In absence of enough diagnostic material, it becomes difficult to continue to monitor over time high-risk patients, who, instead, need of a greater surveillance, both for HSCT planning and monitoring of molecular remission after transplantation.

In each RQ-PCR experiment, parallel amplification of reference gene (albumin gene) is required to normalize quantitative obtained data and to verify the quality and the amplificability of DNA samples. In addition, quantitative PCR is unable to provide reliable target quantification for a substantial proportion of samples that have a tumor burden between the sensitivity and the quantitative range of the method [definition of sensitive range (SR) and quantitative range (QR) are reported in the subparagraph 2.3.6]; this window of inadequate quantification might range up to two logs. Samples falling in this window are sometimes difficult to categorize for clinical purposes and are usually defined as positive not-quantifiable (PNQ).[12]

Droplet Digital PCR (ddPCR) is a new approach for detection and quantification of nucleic acids that offers an alternate method to conventional quantitative PCR. System, workflow, operating characteristics and analytical performances of this third-generation PCR technology have been described in several publications.[14, 15, 16, 17, 18, 19]

The ddPCR system combines water-oil emulsion droplet technology with microfluidics and it works by partitioning a sample of nucleic acid into 20.000 uniform nanoliter-sized droplets in which DNA molecules are randomly distributed. At the start, a mixture of DNA sample, master mix, and TaqMan Assay reagents are loaded in each well, and the use of a nanofluidic chip provides a convenient and straightforward mechanism to run thousands of PCR reactions in parallel. So, in each droplet an indipendent PCR reaction occurs[20] and a single molecule can be amplified a million-fold or more. During amplification, TaqMan chemistry with dye-labeled probes is used to detect sequence-specific targets. When no target sequence is present, no signal accumulates. Following PCR analysis, droplets are individually analyzed to detect the presence (positive) or absence (negative) of the signal. Furthermore, setting a fluorescence threshold for each

detection channel, the method affords a digital system of droplet classification (hence the name "digital" of method)[14] and calculates the average number of copies per droplet based on the fraction of positive droplets. To account for wells that may have received more than one molecule of the target sequence, a correction factor is applied using the Poisson model.

Thus, an absolute count of the exact number of target molecules in the sample is generated using the fraction of positive reactions, without the need for standards or endogenous controls. On the contrary of relative quantification by RQ-PCR, digital PCR allows for absolute quantification and this represents the main advantage of the digital method.

Digital PCR has become one of the most accurate and reliable tools for the examination of genetic alterations in a wide variety of cancers due to its high sensitivity and specificity. Indeed, it is currently being applied for absolute allele quantification, rare mutation detection, analysis of copy number variations, DNA methylation, and gene rearrangements in different kinds of clinical samples.[21]

Most applications of ddPCR in cancer are focused on liquid biopsies, including cell-free DNA as well as circulating tumor cells. Mononuclear cells obtained from peripheral blood and BM aspirate could also be, in some way, considered liquid biopsies that have been investigated by ddPCR. ddPCR has already been compared to quantitative PCR for assessing MRD using patient-specific IGH rearrangements and the BCL2/IGH MBR translocation in patients with multiple myeloma, mantle cell lymphoma, and follicular lymphoma, showing similar sensitivity, reproducibility and accuracy.[20, 22]

Antigen receptor gene rearrangements have also been measured by ddPCR for MRD monitoring in adult ALL.[23] In these malignancies, ddPCR offers several advantages over the standard qPCR-based methods, mainly that ddPCR allows an absolute quantification of target sequences without the need for standard reference curves, in a more applicable, less labor-intensive, and more cost-effective manner.[20]

Since studies concerning ddPCR analyses in pediatric ALL are still missing, we sought to investigate the applicability of ddPCR for MRD detection in childhood ALLs.

In this preliminary study, ddPCR analyses are performed on BM aspirates collected at pre-HSCT of 65 transplanted pediatric patients. The study aimed to experience the

feasibility of digital method within ALL pediatric transplantation setting, to understand if possible quantitative advantages could be taken compared to conventional RQ-PCR.

2.2 PURPOSES OF THE RESEARCH

The ddPCR technology has been recently applied to various medical fields, but its use in MRD detection is under investigation. Thus, we purpose to verify the feasibility of digital assay in the context of pediatric ALL, in terms of sensitivity and reproducibility of results.

Overall, we performed ddPCR analyses on BM samples of 65 pediatric ALL transplanted patients. At a first level of study, we analyzed 23 BM aspirates at pre-HSCT whose MRD analyses resulted positive but not-quantifiable (PNQ) by RQ-PCR. With these preliminary analyses, we attempted to define if the same reaction conditions for MRD assessment by both methods were operable; moreover, we attempted to understand if a subsequent comparison between RQ-PCR and ddPCR could be feasible.

Patients with PNQ-MRD values by RQ-PCR method require special attention in ALLs, being difficult to clinically characterize. Thus, we performed ddPCR analyses on these samples also with the aim to assess whether not-quantifiable MRD valuated by RQ-PCR can be quantified by ddPCR.

Subsequently, we performed ddPCR analyses on 42 BM aspirates at pre-HSCT of patients transplanted in first complete remission (1CR), comparing head-to-head ddPCR results with RQ-PCR results. The aims of this comparison were: 1) to evaluate if results obtained with the two methods reached a good degree of concordance; 2) to evaluate if ddPCR was able to identify low MRD positivity, in contrast to PNQ value detected by RQ-PCR; 3) to compare the prognostic impact of MRD assessed by the two methods on transplanted patients, evaluating if ddPCR was able to identify in advance relapsed patients.

2.3 PATIENTS AND METHODS

2.3.1Patients

The present study included 65 pediatric patients affected by ALL, with age between 1 and 18 years, in first, second, or subsequent complete morphological remission (1CR, 2CR or other CR) and given allogeneic HSCT in one of the following AIEOP (Associazione Italiana di Ematologia e Oncologia Pediatrica) transplant centers: Padua, Pavia and Turin. Transplants were performed between January 2001 and June 2014.

Inclusion criteria were: 1) availability of DNA extracted by BM aspirates collected at diagnosis/relapse and immediately before HSCT; 2) complete morphological remission at time of HSCT, defined as less than 5% blasts by morphological examination; 3) allogeneic HSCT from a matched sibling donor (MSD), a matched family donor (MFD), a matched unrelated donor (MUD) or a partially matched (haploidentical) family donor (PMFD).

In all donor-recipient pairs, histocompatibility was determined by high-resolution molecular typing of HLA-A, B, C (Human Leucocyte Antigens) and DRB1 loci. An informed consent was obtained from parents for all patients, according to Institutional Guidelines.

Of 65 ALL pediatric patients enrolled, a subgroup of 23 pre-HSCT BM samples, resulted PNQ by RQ-PCR, was analyzed by ddPCR. In the second part of study ddPCR analyses were performed on 42 pre-HSCT BM samples of patients transplanted in first CR.

Details on clinical characteristics of patients enrolled in the study are reported in Table I and II.

Table I. Clinical characteristics of 23 patients resulted positive but not quantifiable by RQ-PCR at pre-HSCT.

Number of patients	23	(100%)
Gender:		
Male	14	(61%)
Female	9	(39%)
Median age at transplantation (years, range)	8	(1-18)
Immunophenotype:		
B-cell precursor ALL	19	(83%)
T-cell precursor ALL	4	(17%)
Cytogenetics:		
t(9;22)	3	(13%)
t(4;11)	1	(4%)
t(12;21)	0	(0%)
None	19	(83%)
First-line chemotherapy protocol:		
AIEOP ALL 2000	16	(70%)
AIEOP ALL 2009	1	(4%)
EsPhALL	2	(9%)
AIEOP ALL R2006	4	(17%)
PCR-MRD risk class:		
SR	0	(0%)
MR	12	(52%)
HR	10	(43%)
No data	1	(4%)
Final MRD risk class:		
SR	0	(0%)
MR	1	(4%)
HR	11	(48%)
No data	11	(48%)
Disease phase at transplantation:		
CR1	12	(52%)
CR2	8	(35%)
≥ CR3	3	(13%)
Donor:		
Matched sibling donor (MSD)	1	(4%)
Matched family donor (MFD)	6	(26%)
Unrelated donor (UD)	11	(48%)
Partially matched family donor (PMFD)	5	(22%)

Table II. Clinical characteristics of 42 patients transplanted in first complete remission.

Number of patients	42	(100%)
Gender:		
Male	23	(55%)
Female	19	(45%)
Median age at transplantation (years, range)	9	(1-18)
Immunophenotype:		
B-cell precursor ALL	32	(76%)
T-cell precursor ALL	10	(24%)
Cytogenetics:		
t(9;22)	12	(29%)
t(4;11)	3	(7%)
t(12;21)	0	(3%)
None	27	(64%)
First-line chemotherapy protocol:		
AIEOP ALL 2000	23	(55%)
AIEOP ALL 2009	5	(12%)
AIEOP ALL R2006	7	(17%)
EsPhALL	6	(14%)
Other	1	(2%)
PCR-MRD risk class:		
SR	0	(0%)
MR	8	(19%)
HR	26	(62%)
No data	8	(19%)
Final MRD risk class:		
SR	0	(0%)
MR	1	(2%)
HR	34	(81%)
No data	7	(17%)
Donor:		
Matched sibling donor (MSD)	7	(17%)
Matched family donor (MFD)	13	(31%)
Unrelated donor (UD)	19	(45%)
Partially matched family donor (PMFD)	3	(7%)

2.3.2 Treatment protocols

All patients were enrolled in one of the following first-line treatment protocols: AIEOP-BFM ALL 2000, AIEOP-BFM ALL R2006, AIEOP-BFM ALL 2009 or EsPhALL (as shown in Table I and II). Eligibility criteria for transplantation are reported in Chapter 1, subparagraph 1.1.3.

Patients with a first leukemia relapse were stratified according to the Berlin-Frankfurt-Munster (BFM) relapse risk stratification[24] and treated according to the AIEOP LLA REC 2003 protocol. Re-induction treatment for patients who presented a second relapse before HSCT varied for single patient.

2.3.3 DNA samples

The diagnosis of ALL was established based on morphological, cytochemical and immunological criteria according to the French-American-British (FAB) and World Health Organization (WHO) classifications.[25, 26]

BM aspirates, obtained at diagnosis or at relapse and in the thirty days before transplantation, were collected in sodium citrate tubes and maintained at room temperature for a few hours until further processing. For molecular evaluation, mononucleated cells (MNC) were separated from BM samples by lymphoprep-based gradient centrifugation (Axis-Shield, Oslo, Norway). Genomic DNA was extracted using the Puregene DNA purification kit (Gentra System, Minneapolis, MN). DNA quality and concentration was estimated by NanoDrop ND-1000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, USA) before experimental use.

2.3.4 Ig/TcR gene rearrangements detection

Genomic DNA samples obtained at diagnosis were screened by PCR amplification using the BIOMED-1 primer set for Ig kappa deleting element gene rearrangements

IGK-Kde (IGKV-Kde, intron-Kde), complete and incomplete TRD (TRDV-(TRDD)-TRDJ1, TRDD2-TRDJ1, TRDV2-TRDD3, TRDD2-TRDD3) and TRG (TRGV-TRGJ1.3, TRGV-TRGJ2.3, TRGV-TRGJ1.1, TRGV-TRGJ2.1) gene rearrangements.[27] Complete and incomplete IGH rearrangements (IGHV-(IGHD)-IGHJ, IGHD-IGHJ) were also identified using 5 IGHV and 7 IGHD family primers in combination with one JH consensus primer according to BIOMED-2.[28] Also for complete and incomplete TRB (TRBV-TRBD-TRBJ and TRBD-TRBJ) gene rearrangements, the respective BIOMED-2 multiplex PCR primer sets were used. TRD/A (TRDV2-TRAJ) gene rearrangements are identified using multiplex PCR primer sets.[29]

A new PCR screening was needed for patients in second or other CR, if gene rearrangements of diagnosis were not confirmed at relapse.

The PCR reactions with a positive amplification signal were subjected to homo/heteroduplex analysis, to discriminate between amplifications derived from monoclonal (omoduplex PCR product) or polyclonal (heteroduplex PCR product) lymphoid cell populations.[30, 31]

2.3.5 Gene analyses and primers design

The PCR products resulted omoduplex were directly sequenced using the Big Dye Terminator Cycle Sequencing Reaction Kit and analyzed using an automatic ABI PRISM 310 Genetic Analyzer (Life Technologies, Carlsbad, CA, USA). Instead, biclonal or biallelic PCR products were separated by DNA cloning before the successive sequencing. TopoTA cloning kit (pCR2.1-TOPO vector) (Invitrogen, Paisley, UK - Life Technlogies) is used.

Patients-specific junctional regional sequences of potential PCR-MRD targets were evaluated by the Immunoglobulin Basic Local Alignment Search Tool (IgBlast, http://www.ncbi.nlm.nih.gov/igblast/, National Cancer for Biotechnology Information, Bethesda, MD) and the international ImMunoGeneTics information system (IMGT, http://www.imgt.org, Initiator and Coordinator: Marie-Paule Lefranc, Montpelier, France).

Sequence data of the junctional region were used to design allele-specific oligonucleotide primers (ASO-primers) for each PCR target by using Primer Express (Life Technologies) program.

2.3.6 RQ-PCR assay

All the designed ASO-primers were tested in combination with germline primers and TaqMan fluorescent probes by RQ-PCR, using the 7900HT Sequence Detection System (Life Technologies).[32, 33]

MRD PCR targets were tested for specificity and sensitivity to select two targets for each patient, one of them with at least a sensitivity of 10^{-4} and a quantitative range of 10^{-4}. The "quantitative range" (QR) represents the part of the standard curve amplification in which the MRD levels can be quantified reproducibly and accurately, whereas the "sensitivity" ("sensitivity range" or SR) reflects the lowest MRD level that still can be detected, although not reproducibly and accurately.[12]

To perform RQ-PCR analysis, 500 ng of DNA (100ng/µl) per 25 µl of volume reaction was used for each reaction and 3 replicates are performed for each time point. To define the background amplification (or rather the non-specific amplification of non-leukemic DNA), 6 replicates of polyclonal DNA were used. Polyclonal DNA was obtained from mononuclear cells (MNC) from a pool of five healthy donors and also used to build a standard curve of calibration to allows for quantification of follow-up sample; the diagnostic DNA specimen was serially diluted in polyclonal DNA. The serial dilutions ranged from 10^{-1} to 10^{-5} and were tested in triplicate. For normalization of the quantitative results, a reference gene (albumin gene) was always amplified in parallel reaction.

2.3.7 Interpretation of MRD RQ-PCR results

RQ-PCR results were interpreted according to the guidelines developed by the European Study Group on MRD Detection in ALL (EuroSG MRD ALL).[12]

For each experiment a logarithmic value of QR and SR were assigned. MRD negativity was established when there was absence of specific amplification or amplification within 1 threshold cycle (Ct) of the background or amplification with a distance of more than 20 Ct's (ΔCt) from the undiluted diagnostic sample; in this study, samples with these characteristics were defined as qNEG (negative by quantitative PCR). MRD positivity was considered if follow-up sample showed a specific amplification product of more than 1 Ct lower than the background and separated less than 20 Ct's (ΔCt) from the undiluted diagnostic sample. MRD positivity values were defined quantifiable if the sample gave a Ct within the QR and the ΔCt between replicates was less than 1.5 (reproducibility criterion); these samples were here defined as qPQ (positive and quantifiable by quantitative PCR). If the above reported conditions of positivity are not achieved, MRD values were defined positive not quantifiable and samples were here classified as qPNQ (positive not quantifiable by quantitative PCR).

2.3.8 ddPCR assay

Since the limited availability of some diagnostic or follow-up DNA, one PCR target for patient was analyzed by ddPCR; the target with the highest QR was chosen between the two target analyzed by RQ-PCR. ddPCR assays were performed with the same primers and TaqMan fluorescent probes used for RQ-PCR, with identical nucleotide sequences. Reference gene, as control for DNA input, was amplified in same reaction with target gene, to minimize variability.

The QX200TM Droplet Digital TM PCR System (BioRad Laboratories, Hercules, CA) was used according to the manufacturer's instructions. Each DNA sample (100ng/µl) was loaded in sestuplicate. Final volume of 20µl ddPCR reaction contained 3µl of DNA (300 ng), 10µl of 2X ddPCR Supermix (BioRad Laboratories), 2µl of 20X primers and 0.8µl of 20X probe (final concentrations of 500 nM and 200 nM, respectively), 1µl of 5 U/µl of Hind III restriction enzyme and 1µl of ALB assay (BioRad Laboratories) containing forward and reverse primers for albumin gene and a specific HEX fluorescent probe. Droplets were generated by a QX200 Droplet Generator, loading reaction mixtures

together with 70 µl of droplet generation oil. Droplets were transferred into a 96-well PCR plate by aspirating 40 µl from the DG8 cartridge into each well. All samples were amplified on the conventional Bio-Rad T100 Thermal Cycler. After amplification, the plate was subsequently loaded into the QX200 Droplet Reader and analyzed by QuantaSoft software.

Standard reagents and consumables supplied by Bio-Rad were used, including, cartridges and gaskets, droplet generation oil and droplet reader oil.

2.3.9 Interpretation of MRD ddPCR results

In absence of standardized guidelines to interpret ddPCR analyses and according to the manufacturer's applications guide, we considered acceptable only experiments that brought a number of droplets ≥ 10.000/replicate. (Figure 1)

Figure 1. Total number of events droplets generated by ddPCR assay (each column represents a single reaction-well).

As shown in Figure 2, to correctly quantify each analysis, we set the threshold value by manual bending with a sufficient distance from the background to ensure suitable sensitivity and specificity, as reported by the application guide.

To perform a comparative analysis between ddPCR and RQ-PCR, each experiment included a positive control sample (dilution 1:10 of diagnostic DNA) in single replicate and two negative control samples: polyclonal DNA performed in 6-fold and no template control (NTC) performed in 2-fold at least. (Figure 2) As for RQ-PCR assays, for ddPCR analysis polyclonal DNA was also used to define background amplification.

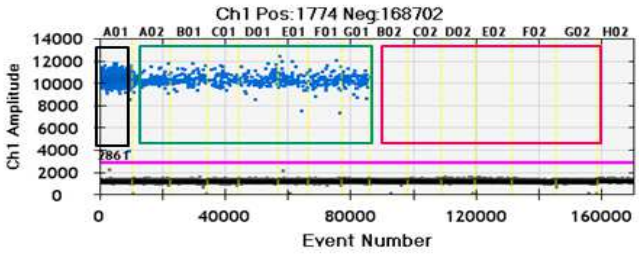

Figure 2. 1D-plot of ddPCR assay, with data output (relative specific droplets) of diagnostic sample (black box), pre-HSCT BM sample (green box) and polyclonal DNA (red box).

To analyze the results, all samples were quantified using the following ratio: [copies/µl MRD pre-HSCT sample] / [copies/µl MRD diagnostic sample]. In particular, to calculate copies/µl MRD of both follow-up and diagnostic samples, copies/µl of target DNA were previously normalized on copies/µl of albumin DNA, to align the comparison of results and, finally, logarithmic values are compared.

In each experiment, sample was tested according to the following criteria: MRD negativity was established if no positive droplets were observed or if positive droplets were below the background; in this study samples with these characteristics were defined as dNEG (negative by ddPCR). MRD positivity was considered quantifiable when a reproducible number of positive droplets were observed (at least 3) in the total number of replicates and the difference between positive and background droplets was ≥ 3; samples with these characteristics were defined as dPQ (positive and quantifiable by ddPCR). If no reproducible number of positive droplets were observed (< 3) and if the number of positive droplets was < 3 in presence of background amplification, samples were defined as dPNQ (positive but not quantifiable by ddPCR).

The data were added up for the replicates.

2.3.10 Statistical analysis

Quantitative variables were reported as median value and range, while categorical variables were expressed as absolute value and percentage. Demographic and clinical characteristics of patients were compared using the Chi-square test or Fisher's exact test

for categorical variables, while the Mann-Whitney rank sum test or the Student's t-test were used for continuous variables as appropriate. Survival analyses were performed according to Kaplan-Meier method;[34] differences were compared by the Log-Rank test.[35] All P values were two sided and had a type I error rate fixed at 0.05. Follow-up was censored on January 31st, 2016. Statistical analysis was performed by using the SAS statistical program (SASPC, version 9.3; SAS Institute, Cary, NC).

2.4 RESULTS

The whole cohort of 65 patients included in this study was considered evaluable for MRD analysis at pre-HSCT and all the pre-HSCT BM samples were successfully analyzed by both RQ-PCR and ddPCR.The MRD-target used to monitor each patient by ddPCR was the same used to monitor them by RQ-PCR at diagnosis or at relapse (for patients at second or other CR). The logarithmic range of sensitivity of ddPCR assays was between $5.5x10^{-5}$ and $1.1x10^{-6}$, being highly comparable in terms of sensitivity of assays with RQ-PCR; in addition, the ddPCR assay showed a good level of reproducibility, also with regard to the number of positive droplets between the different replicates.
A subgroup of 23 pre-HSCT qPNQ samples was analyzed by ddPCR; then, ddPCR analyses were performed for 42 transplanted in 1CR patients. A comparison between ddPCR and RQ-PCR MRD results was successfully done.

23 qPNQ samples. The 23 BM samples were resulted PNQ by RQ-PCR: 8/23 cases resulted qPNQ for presence of the background amplification, while 15/23 cases for not to be reproducible (the ΔCt between replicates was > 1.5). As shown in Table III, 13/23 samples were resulted PQ by ddPCR, 7/23 were resulted dNEG and only 3/23 samples were resulted PNQ also by ddPCR analysis; for 2/3 dPNQ samples the difference between positive and background droplets was < 3, while for 1/3 dPNQ samples no reproducible number of positive droplets was observed (only one droplet).
After allogeneic HSCT, 13/23 qPNQ patients had the relapse event, as also reported in Table III. Six of 13 relapsed children resulted dPQ, 5/13 resulted dNEG and 2/13 resulted dPNQ, by ddPCR analyses.

Table III. Results of ddPCR analyses on 23 pre-HSCT BM samples PNQ by RQ-PCR and distribution of ALL patients who relapsed after HSCT.

		RQ-PCR
		PNQ
ddPCR	PNQ	3 (2/3 Rel)
	PQ	13 (6/13 Rel)
	NEG	7 (5/7 Rel)
	Total	23 (13/23 Rel)

42 samples of 42 1CR transplanted patients. The MRD results for this subgroup of patients were: by RQ-PCR analyses, 19/42 samples were resulted qNEG, 7/42 qPQ and 16/42 qPNQ; by ddPCR analyses, 16/42 samples resulted dNEG, 19/42 dPQ and 7/42 dPNQ. A significant concordance was observed between RQ-PCR and ddPCR MRD results (Pearson $r = 0.98$, $P < 0.0001$), as shown in Figure 3.

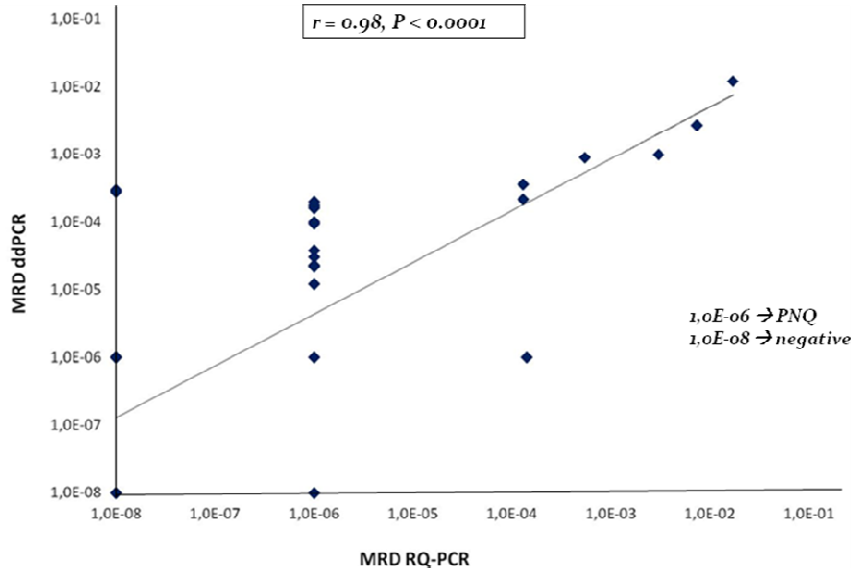

Figure 3. Concordance analyses between results obtained by both methods on 42 pre-HSCT BM samples of ALL patients transplanted in 1CR.

Results obtained by the two techniques were fully concordant for 19/42 cases: for both techniques 12/19 samples resulted NEG, 6/19 PQ and 1/19 PNQ. In contrast, 23/42 cases were discordant. Indeed, 7/23 discordant samples were qNEG, while were resulted dPNQ for 5 of them (5/7) and dPQ for 2 of them (2/7). Fifteen of 23 discordant samples were qPNQ, while resulted dPQ for 11 of them (11/15) and dNEG for 4 of them (4/15). The remaining discordant sample was PQ by RQ-PCR but resulted PNQ by ddPCR. (Table IV)

After allogeneic HSCT, 15/42 ALL patients transplanted in 1CR experienced a relapse event. Six of 15 relapsed patients resulted qPNQ and dPQ, 3/15 resulted NEG for both methods, 3/15 resulted PQ for both methods, 1/15 results qNEG and dPNQ, 1/15 results qNEG and dPQ, 1/15 results qPNQ and dNEG. (Table IV)

Table IV. Results of ddPCR analyses on 42 pre-HSCT BM samples of ALL patients transplanted in 1CR and distribution of ALL patients who relapsed after HSCT.

		RQ-PCR			
		PNQ	**PQ**	**NEG**	**Total**
ddPCR	**PNQ**	**1**	**1**	**5**	**7**
				(1/5 Rel)	**(1/7 Rel)**
	PQ	**11**	**6**	**2**	**19**
		(6/11 Rel)	**(3/6 Rel)**	**(1/2 Rel)**	**(10/19 Rel)**
	NEG	**4**	**0**	**12**	**16**
		(1/4 Rel)		**(3/12 Rel)**	**(4/16 Rel)**
	Total	**16**	**7**	**19**	**42**
		(7/16 Rel)	**(3/7 Rel)**	**(5/19 Rel)**	**(15/42Rel)**

For the subgroup of 1CR transplanted patients, the prognostic impact of pre-HSCT MRD levels was also evaluated, comparing MRD measurements by both relative and absolute technologies. In parallel, and according to MRD results, patients were stratified in 3 classes: MRD-NEG (19 by RQ-PCR and 16 by ddPCR), MRD-PQ (7 by RQ-PCR and 19 by ddPCR) and MRD-PNQ (16 by RQ-PCR and 7 by ddPCR). When groups of patients (NEG, POS and PNQ, distinctly for RQ-PCR and ddPCR) were stratified according to MRD levels by RQ-PCR or ddPCR, differences among event-free survival (EFS) of groups were not statistically significant (p=0.16 and p=0.33, respectively). The EFS of patients with MRD NEG was 68% by both the two methods and the EFS of patients with MRD PQ were very similar: 43% and 47% by RQ-PCR and ddPCR, respectively. However, the EFSp of MRD PNQ patients was 56% by RQ-PCR and 71% by ddPCR. (Figure 4)

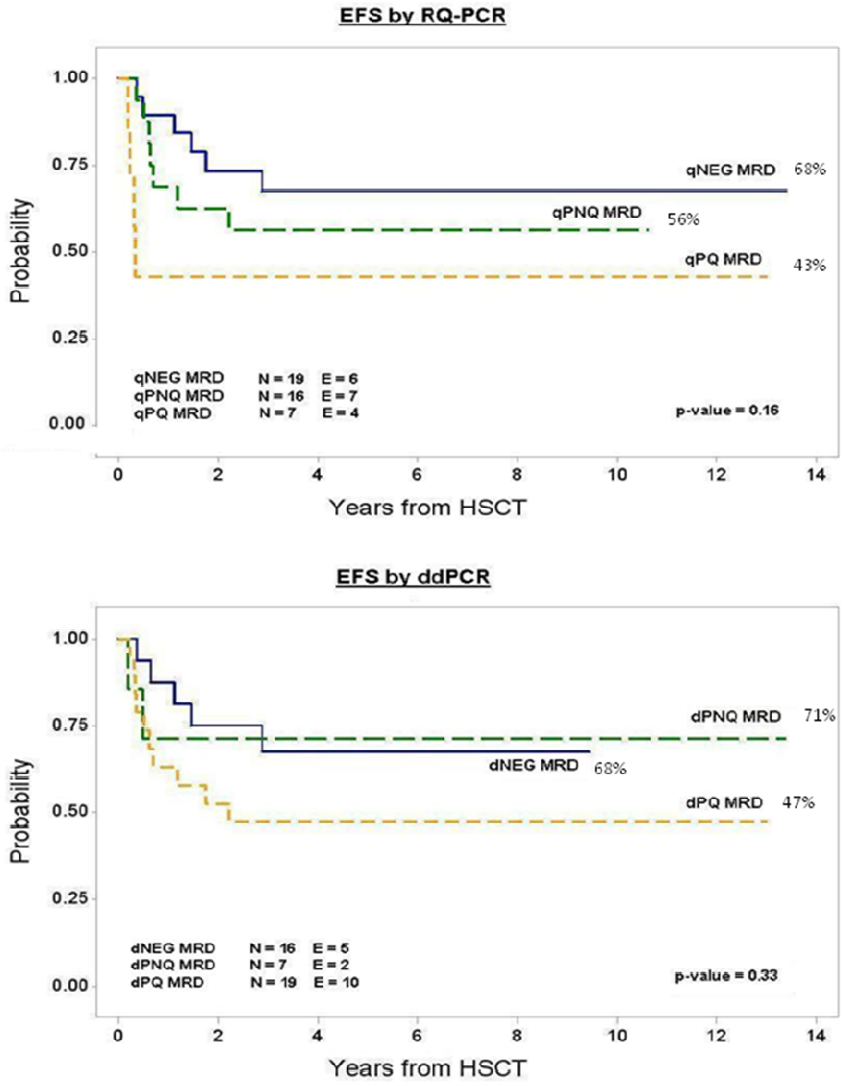

Figure 4. EFS probability of ALL pediatric patients stratified according to MRD levels at pre-HSCT, detected by RQ-PCR (upper panel) and ddPCR (lower panel).

2.5 DISCUSSION

One of the most relevant predictor of outcome in childhood ALL, to date, is the MRD monitoring, that has become part of diagnostic patient care. Most of recent protocols for ALL treatment, both for new diagnosis and ALL recurrences, rely widely on MRD evaluation as optimal reflection of the *in vivo* response to treatment in each individual patient, to use for accurate risk-group assignment with risk-adapted treatment.[11] MRD data gives the clinician a better knowledge and control of the clinical course in individual patients.[5]

With real-time quantitative PCR detection of Ig and TCR gene junctional regions, precise quantification of MRD levels is routinely achievable.[36, 32, 37, 38, 39, 40] Quantitative PCR technology well fulfills all the requirements as easy implementation, applicability in the vast majority of patients (90-95%), sufficient sensitivity (quantitative range of $\leq 10^{-4}$), speed and affordability.

However, RQ-PCR may result inadequate in situations of limited availability of DNA samples. Quantitative analysis employs a relevant amount of diagnostic DNA for each experiment and assessing MRD for a long time is crucial, particularly for patients classified as high-risk of relapse at first-line protocols or for patients who have been transplanted.

The recent introduction of Droplet Digital PCR has already led to several research breakthroughs in fields such as genomic alterations, gene expression, infectious diseases and important cancer biomarker discovery. Since ddPCR consistsof a massive sub-partitioning of the PCR mixture into thousands of nanoliter droplets through a water-in-oil emulsion, it represents an enrichment strategy that allows for the detection of low-level mutations by amplification of single DNA molecules.[21]

The digital PCR concept has many potential advantages over real-time quantitative PCR, including the capability to obtain absolute quantification without external references and robustness to variations in PCR efficiency.[41] Recent reports evaluated the applicability of ddPCR in adult lymphoproliferative disorders, such as lymphomas[20, 22] and acute lymphoblastic leukemia[23], showing a good correlation of results between RQ-PCR and ddPCR for all these studies.

With these premises, the rational of this preliminary research project was to investigate the feasibility of ddPCR application to MRD assessment in pediatric ALL patients transplanted in complete remission, with the main purpose to evaluate if, compared to conventional RQ-PCR, some benefits might be achieved, particularly in terms of quantification of samples.

In the study, the analysis of pre-HSCT BM samples resulted positive not-quantifiable by RQ-PCR, showed that ddPCR has sensitivity and reproducibility at least comparable with RQ-PCR. Surprisingly, 20 samples of 23 qPNQ, were instead resulted quantifiable by ddPCR, showing also a good accuracy for quantification. Furthermore, 13/20 quantifiable samples resulted positive by ddPCR; 6 of these 13 dPQ samples presented a relapse event after allogeneic transplantation.

The reaction condition settings turned out to be also good for the execution and the next interpretation of obtained results.

Even the study of bone marrow aspirates of children transplanted in first complete remission suggests that ddPCR may be more accurate in MRD quantification, since 11/15 pre-HSCT BM samples qPNQ resulted PQ by ddPCR and 6 of 11 dPQ patients experienced an event.

Overall, we can draw the conclusion that for 12 ALL patients who relapsed after HSCT (6 patients in each of the 2 ddPCR analyzed subgroups), RQ-PCR technique failed to identify relapse in advance, while ddPCR was able to measure a positive quantifiable value.

MRD analyses performed by the two molecular techniques, in subgroup of 1CR transplanted patients, show a good level of concordance between results ($P < 0.0001$) with 19/42 cases resulted fully concordant for both methods. Importantly, among the 23 discordant cases, 15 were PNQ by RQ-PCR, but were quantified by ddPCR; so this apparent discrepancy between the two molecular techniques actually favors further the ddPCR.

Statistical analyses for EFS probability showed no statistically significant differences among groups, when patients were stratified according to MRD levels by ddPCR compared to MRD levels by RQ-PCR ($p=0.33$ and $p=0.16$, respectively). Also with regard to PNQ MRD detected by ddPCR is not likely to impact on ALL patients'

outcome, since EFS of dPNQ patients was very similar to that of dNEG patients (71% vs 68%, respectively).

Overall, we can assert that the applicability of digital PCR was successfully tested. However this methodology still has some limitations that need to be addressed. For example, the set threshold of 10.000 droplets per well has not been far exceeded by all the replicates in some experiments and this fact may represent a cause to consider the entire assay not evaluable, with consequent loss of DNA sample. Thus, in future applications with ddPCR, we might suggest to reduce the threshold to 9.000 droplets per replicate, always allowing for the MRD quantification.

Moreover, false-negative as well as false-positive results, although relatively infrequent, have not been fully eliminated from ddPCR assays, raising concerns about the risk of making clinical decisions exclusively based on this methodology.

However, we can confirm that digital PCR offers advantages over the standard quantitative PCR methods, mainly that ddPCR allows an absolute quantification of target DNA without the need for standard reference curves, in a more applicable, less labor-intensive, and more cost-effective manner.[20] Furthermore, our results confirm the additional advantage of amplifying in duplex reaction the reference gene, to validate the quality of DNA samples.

In conclusion, no statistically significant difference emerges in prognostic impact on outcome (EFS) of ALL patients, according to the comparison of MRD levels resulted with the two molecular approaches. Concordance analyses of all evaluated pre-HSCT BM samples highlighted a good level of correspondence between results, also with an increased level of MRD quantification by digital approach. Full automation and multiplexing are major current and future challenges of ddPCR with regard to achieving maximal implementation into the clinical routine.

Our study suggests that digital method could be an accurate and applicable tool for MRD evaluation also in the context of pediatric ALL clinical trials, where MRD monitoring plays a key role, primarily for high-risk patients and for patients undergoing HSCT.

Our preliminary data also confirm the importance of extending the ddPCR analysis on other retrospectively collected cases of childhood ALLs, with the future aim to better

define the prognostic value of MRD levels by ddPCR for a prospective evaluation of outcome in pediatric ALL patients.

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