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Chapter

Molecular Monitoring in Acute Myeloid Leukemia Patients Undergoing Matched Unrelated Donor: Hematopoietic Stem Cell Transplantation

Irina Panovska-Stavridis

Abstract

Minimal residual disease (MRD) in acute myeloid leukemia (AML) is a complex, multi-modality assessment and much as its clinical implications at different points are extensively studied, it remains even now a challenging area. It is the disease biology that governs the modality of MRD assessment; in patients harboring specific molecular targets, high sensitivity techniques can be applied. In AML patients undergoing allogenic hematopoietic stem cell transplantation (alloHSCT), relapse in considered as leading cause for treatment failure. In post-transplant setting, regular MRD status assessment enables to identify patients at risk of impending relapse when early therapeutic intervention may be beneficent. We analyzed data of AML patients who underwent matched unrelated donor (MUD) HSCT since the introduction of this procedure in the Republic of North Macedonia. Chimeric fusion transcripts were identified in three patients; two of them positive for RUNX-RUNX1T1 transcript and one for CBFB-MYH11. One patient harbored mutation in the transcription factor CCAAT/enhancer binding protein α (*CEBPA*). Post-transplant MRD kinetics was measured by quantitative polymerase chain or multiplex fluorescent-PCR every three months after the transplantation during the first two years after the transplant. MRD negativity was achieved in three patients by the sixth month of HSCT, who were pre-transplant MRD positive. They sustained hematological and molecular remission for 19, 9 and 7 months, respectively. The forth patient died due to transplant-related complication. Our experience suggests, when molecularly-defined AML patients undergo HSCT, regular MRD monitoring helps predict impending relapse and direct future treatment strategies.

Keywords: minimal residual disease (MRD), molecular monitoring, matched unrelated donor (MUD) hematopoietic stem cell transplantation (HSCT), acute myeloid leukemia (AML)

1. Introduction

1.1 Acute myeloid leukemia (AML)

Acute myeloid leukemia (AML) is a heterogeneous malignant disease of the hematopoietic system, marked by mutational arrest of myeloid lineage precursor cells and limited myeloid differentiation capacity [1]. Theoretically, malignant transformation can arise at any level at which cell precursors are capable of self-renewal. However, the hematopoietic precursors are typically arrested in the earliest stages of myeloid maturation pathway - myeloblasts or promyelocytes. The malignant cells suppress the normal hematopoiesis by accumulating in the bone marrow and displacing the normal hematopoietic stem cells, resulting in depletion of normal blood cells. The presence of more than 20% myeloblasts in the bone marrow or peripheral blood, as assessed by morphological evaluation of blood smears and bone marrow aspirate smears is diagnostic for AML. Morphologically, AML cells resemble normal myeloblasts to some extent, although they are distinguished by specific features, such as Auer Bodies – crystalloid azurophylic granules or Auer Rods – needle-shaped conglomerates of granules. Immunophenotyping, cytogenetics and molecular genetics must be employed to confirm the diagnosis of AML and further characterize the AML subtype [1].

AML is one of the most common types of leukemia in adults, as stated by American Society of Cancer, the second most common, following chronic lymphocytic leukemia (CLL), but the leading cause of mortality of leukemic deaths [2]. The median age at diagnosis is approximately 70 years. The estimated annual age-standardized incidence rate is 4.3 per 100.000, more precisely, from 1.3 per 100.000 in patients younger than 65 years and 12.2 per 100.000 in patients older than 65 [2, 3].

The etiology of de novo cases of AML is quite obscure. Only a small portion of all AML cases - around 10%, are secondary AML, due to transformation of prior hematological malignancy, such as myelodisplastic syndrome (MDS) or myeloproliferative neoplasm (MPN). Around another 10% of all cases arise from suggested DNA damage of known previous factor, as prior therapy with alkylating agents or topoisomerases, or prior radiotherapy [4].

1.2 WHO classification of AML

The revised fourth revision to the World Health Organization classification of Tumors of Hematopoietic and Lymphoid Tissues, published in 2017, distinguishes six AML subtypes and it incorporates cytogenetic and molecular abnormalities into diagnostic algorithms, in contrast to previous classifications of AML, based on morphology and immunophenotype [5]. The entity "AML with recurrent genetic abnormalities" comprises 11 subcategories of AML, with acute promyelocitic leukemia (APL with PML-RARA) included. The abnormalities that are not included in this group are considered to be rare among adult population. Six well-defined recurrent balanced translocations and inversions and their variants are covered within this classification. Two entities included are new provisional subcategories: AML with *BCR-ABL1* fusion gene and AML with mutated *RUNX1*. Although a matter of long-time controversy, de novo BCR-ABL+ AML is now classified as distinct AML subtype. However, current data show that BCR-ABL occur primarily in AML with antecedent myeloid disorder, such as myelodysplasia-related changes [6]. The cytogenetic and molecular abnormalities included in this classification are summarized in **Table 1**.

AML with recurrent genetic abnormalities adapted from 4th revised WHO classification 2017		**Risk group, as per 2017 ELN stratification**
Cytogenetic abnormality	**Molecular abnormality**	
t(8;21) (q22;q22.1)	RUNX1-RUNX1T1- AML	Favorable
inv(16)(p13.1q22) or t(16;16)(p13.1;q22)	CBFB-MYH11- AML	Favorable
t(9;11)(p21.3;q23.3)	MLLT3-KMT2A AML	Intermediate
t(6;9)(p23;q34.1)	DEK-NUP214 AML	Adverse
inv(3)(q21.3q26.2) or t(3;3)(q21.3;q26.2)*	GATA2; MECOM	Adverse
t(1;22)(p13.3;q13.3)	RBM15-MKL1	
	AML with mutated NPM1	*Favorable if without FLT-ITD or with FLT-ITD low Intermediate if FLT-ITH high*
	AML with biallelic mutations of CEBPA$	*Favorable*

**Inv(3)(q21.3q26.2) results in reposition of a distal GATA2 enhancer to activate MECOM expression, not a fusion gene.*
$Mutated CEBPA is associated with biallelic mutations of the gene, not a single mutation.

Table 1.
Recurrent cytogenetic and molecular abnormalities in AML (4th revision of WHO classification, published in 2017) and ELN risk categories.

1.3 AML risk categories

Current AML risk categorization follows the latest 2017 European LeukemiaNet (ELN) recommendations and is based on pretreatment genetic abnormalities. It is designed for risk-adapted treatment approach of patients with AML, conforming to their molecular profiles [7]. Three risk categories are recognized: favorable risk, intermediate risk and adverse risk group [7]. However, the prognostic significance of genetic abnormalities should be only analyzed in association/codependence with other patient-related or disease-related prognostic factors. Increasing age is correlated with poor prognosis for two reasons; not only the poorer performance status in older age groups and the increased risk of toxicity and treatment-related mortality, but also the increased probability of previous underlying malignancy such as MDS or MPN, associated with adverse cytogenetic and higher risk of treatment resistance. Furthermore, the presence of two genetic abnormalities simultaneously and their interactions can result in different prognostic impact, depending on the presence or the absence of another. The best studied example is the NPM1-FLT3-ITD interaction. As shown in **Table 1**, mutated NPM1 in the absence of a *FLT3*-ITD or presence of *FLT3*-ITD with a low allelic ratio is related to favorable prognosis, while the high allelic ratio of *FLT3*-ITD relocates it in the intermediate risk group. To the contrary, not mutated NPM1, or wild-type NPM1 in the absence of a *FLT3*-ITD or low allelic ratio *FLT3*-ITD is considered as intermediate risk group and finally, wild-type NPM1 plus high allelic ratio of *FLT3*-ITD carries adverse prognosis [8]. ELN risk categories are presented in **Table 1** in association with WHO classification of AML. In addition to those abnormalities, mutations in RUNX1, mutations in ASXL1 and in TP53 convey particularly poor prognosis [9]. In regards to AML-karyotype, complex karyotypes and monosomal karyotypes, specific aneuploidies, such as deletion of chromosome 5 and chromosome 7 or 5q, 7q deletion, predict adverse prognosis in AML patients. These are often associated with TP53 mutations [10].

2. The role of allogeneic hematopoietic stem cell transplantation (alloHSCT) in AML

Allogeneic hematopoietic stem cell transplantation plays a key role in the management of patients with AML, making it one of the commonest indications for alloHSCT. By overcoming the limitations of donor availability and the increasing pool of donors in the recent decades on one hand and improving the transplant procedures and post-transplant strategies on the other, alloHSCT evolved into a definitive curative option for a significant number of AML patients [11]. The major challenge remains the identifying the allo-mandatory patients who are likely to benefit from an allograft.

2.1 Matched related sibling and unrelated donor SCT in AML

The search for a compatible hematopoietic stem cell donor is based on the human leukocyte antigen (HLA) profile of the patient undergoing SCT. The preferred donor type for SCT is a matched sibling donor (MSD), bearing the most favorable outcome; however only about a third of all patients have an available sibling donor. Histocompatibilty antigens are co-dominantly expressed and are inherited following Mendelian rules of inheritance, which means there is only a 25% likelihood of a patient and their sibling inheriting the same parental haplotypes. Assuming an average of 2 to 3 children per family in European countries it has been estimated that a patient seeking a transplant has a 30% likelihood of having a matched sibling donor and therefore a 70% likelihood that the same patient will need a transplant from an unrelated donor. Since the world's first donor registry was founded in 1974, WMDA nowadays comprises of 75 hematopoietic stem cells donor registries from 53 countries, with more than 33.573.307 volunteer donors listed up to date. (https://statistics.wmda.info). The coordination between the transplant centers is facilitated through donor registries. The unrelated donor search procedure commences with a formal search request which is sent to the national registry, which further undertakes the responsibilities in all steps until the graft distribution [12]. The process of an unrelated donor search and activation is completed in 2 months on the average and up to 10 weeks.

2.1.1 The "ideal" unrelated donor profile

The general recommendation is selection of a 10/10 HLA-matched unrelated donor whenever possible (in loci HLA A, HLA B, HLA C, HLA DR and HLA DQ). The second best choice would be 9/10 identical unrelated donor. HLA typing is necessary to be performed at high-resolution level by using next-generation sequencing (NGS) or sequencing-based typing (SBT) as preferable typing method. As published by Lee et al. 2007, HLA mismatch in any of non-permissive or high-risk alleles: HLA A, B, C and DRB1 results in 10% decrease in survival probabilities for each mismatch in transplanted patients at early disease stage, and even worse at advanced disease stage [13]. Donor associated features may narrow down the choice of suitable unrelated donor. At the time of recruitment, donors are assessed in order to exclude medical conditions or habits that can possibly cause harm to transplant recipient, in particular – their history of infectious diseases, inherited, autoimmune and malignant diseases. According to WMDA recommendations a minimum donor blood-borne infectious disease markers testing is suggested, including serology for hepatitis B and C, HIV, syphilis and HTLV1/2. Those recommendations are adapted in line with local polices as additional endemic transmissible diseases may also be covered [14]. The donor's age is considered an important factor, probably the most

powerful one; younger donors are associated with better prognosis with a 5.5% increase in the hazard ratio for overall mortality for every 10-year raise in donor age [15]. Donor registries recruit donors between 16 and 55 years and almost half of the registered donors registered are younger than 35. Diverse data on the impact of sex mismatch and blood group incompatibility are been reported, emphasizing the correlation with other factors, such as conditioning regimen or stem cell source. Donor CMV serum-positivity is a negative prognostic factor for transplant outcome. It appears however, that not the CMV serum-negativity, but a matched patient/donor CMV serum status determines the transplant outcome more significantly [16].

2.2 Haploidentical related donors

The recent improvements in transplant technologies have led to the consideration of using a haploidentical related donor when HLA- matched sibling or HLA-matched unrelated donor is not available. It is estimated that around a half of the patients in need of a transplant have rare haplotypes and HLA-matched donor cannot be found in donor registries. In the past, the major inquiry in terms of haploidentical setting has been the expected high rate of GVHD. On the contrary, over the past few years, the use of post-transplant cyclophosphamide on day +3 and day +4 after the transplant, has significantly decreased the rate of acute and chronic GVHD and nowadays haploidentical HSCT is established transplant method for patients lacking HLA-matched donor [17]. Even more, numerous retrospective studies have shown similar outcomes for MUD – SCT and haploidentical HSCT [18].

2.3 Umbilical cord blood transplantation

The first umbilical cord blood transplantation (UCBT) was successfully performed in 1988, in a 5-year-old child, diagnosed with Fanconi Anemia, using HLA-matched sibling, an older brother. Subsequently, in the following years, UCB transplants were encouraged in the pediatric population for the treatment of malignant and non-malignant hematological diseases, using both related and unrelated donors. Therefore, data on UCBT mainly originate from procedures performed in children. The major limitations of this procedure are delayed engraftment and delayed immune reconstitution, leading to severe and often fatal infections [19]. However, during the past few years, as mismatched transplant activity increases, UCBT is progressively superseded by haploidentical HSCT.

3. The role of minimal residual disease (MRD) in AML

In AML patients, a complete hematological remission is defined as total recovery of blood counts, less than 5% blasts in bone marrow and recovery of heamatopoiesis of all cell lineages, as assessed by cytomorphological examination [20]. Morphological assessment of post-therapy disease status is limited to 100–400 nucleated cells and can identify the presence of leukemic cells to levels of 1:20 white blood cells (WBC). Besides the small number of analyzed cells another limitation of cytomorphology is the subjective component and the inaccuracy in distinguishing normal from leukemic myeloblasts [21]. Limitations of cytomorphological assessment were partially overcome by the introduction of highly sensitive methods able to detect the smallest residual leukemic cells populations or minimal (measurable) disease. *Minimal residual disease (MRD)* indicates presence of leukemic cells at levels of $1:10^4$ to $1:10^6$ WBC. MRD detection in AML is necessary for various reasons. Firstly, it is an objective, well-defined post-treatment method to establish

a remission status at deeper level. Secondly, it is a powerful tool for risk stratification of AML and guiding treatment approach. And finally it enables identifying impending relapse in AML patients in complete remission [22–24]. Several studies have so far reported a positive correlation between the MRD positivity and the risk of relapse and shorter survival rate, compared to MRD negative AML patients. This refers to MRD status during and after post-remission chemotherapy, as well as prior and after SCT [24–33]. However, AML is a malignancy with complex molecular landscape and despite the fact that genetic aberrations are shown to be powerful prognostic determinants none of them have been ascertained to accurately predict the outcome [9]. No guidelines or recommendations are available so far on when and how to implement MRD assessments and on how to apply the results to clinical practice. According to ELN recommendations, during the treatment phase MRD should at least be assessed at the following time points: at the time of diagnosis, after 2 cycles of standard induction/consolidation chemotherapy and after the end of the treatment. For patient candidates for allo-HSCT, an MRD assessment should be carried out after the last chemotherapy, not exceeding 4 weeks of the initiation of the conditioning regimen [22].

3.1 MRD in AML patients undergoing allo-SCT

In a post-transplant setting, the primary importance of MRD evaluation is to detect impending relapse and thus to identify patients who may benefit from early clinical intervention [22–24]. The continual death rate due to relapse after allo-SCT is discouraging, even despite the changing landscape of AML and novel treatment paradigms. Data from the Center for International Blood and Marrow Transplant Research (CIBMTR) report 63% mortality rate in patients undergoing unrelated donor HSCTs due to transplant-related events including graft-vs.-host disease, infection and other causes-organ toxicity or second malignancies, compared to 37% of deaths due to relapse [34]. This high relapse ratio suggests that there are residual leukemic cell populations that have survived therapy, capable of causing relapse, referred to as measurable or minimal residual disease. These cell subsets are believed to be present even up to several months before apparent morphological disease, at a time when they can be solely detected by high sensitivity methods.

3.2 Methods for MRD assessment

A number of methods are employed for MRD measurement but is the biology of the disease itself that governs the modality of MRD assessment. The complexity of AML, the myriad of genetic aberrations and the diversity of immunophenotypes restrain the recognition of uniform approach for MRD detection. In addition, as per Butturini A, the of MRD detection in AML is altered by the size of the tested sample, sample source (blood, bone marrow) and the time point of sample obtaining rather than the sensitivity of the employed method for MRD assessment, due to the heterogeneous distribution of residual leukemic cells and the fluctuating expression of the MRD target [24]. In general, two methods are commonly incorporated into clinical routine: multiparameter flow cytometry (MFC) which allows detection of aberrant immunophenotypes and molecular methods by using tumor-specific molecular primers, such as RT-qPCR or multiplex fluorescent- [22, 24]. MFC affords relative sensitivity of $1{:}10^{-3}$. The main constrains of this method are that not all leukemia cells present aberrant immunophenotypes and that the initial phenotypes may change through disease evolution and clone selection [35].

3.2.1 Molecular MRD in AML patients

Two approaches are available for molecular MRD monitoring. The first one is real-time PCR-based and the second is by using sequencing techniques. PCR-based MRD assessment affords sensitivity of $1:10^{-5}$ to $1:10^{-6}$, which means 100–1000 fold greater than other methods applied. Therefore, it is the ELN-recommended platform for molecular monitoring in AML due to the established high sensitivity [22, 24]. However, considering the molecular heterogeneity of AML, it is restricted to less than half of patients (35% in older patients as their frequency decreases with age); those harboring specific molecular targets that can be tracked for MRD monitoring, including mutations, translocations, inversions, deletions and polymorphisms. More precisely, PCR-based MRD monitoring is proposed for AML with validated molecular markers, such as mutations in the gene encoding nucleophosmin (NPM1) and the chimeric fusion genes RUNX1- RUNX1T1, CBFB-MYH11 and PML-RARA [24, 25]. For these mutations, standardized PCR-assays are employed with well-defined threshold levels [36]. In contrast, the use of the mutations in FLT3-ITD, FLT3-TKD, NRAS, KRAS, DNMT3A, ASXL1, IDH1, IDH2, MLL-PTD, EVI1 and WT1 as single MRD markers is not recommended because of frequent losses or gains of certain mutations at relapse. These markers could be used in combination with a second MRD marker if present [22].

ELN defined the molecular responses for patients in complete hematological remission after completing chemotherapy or after a performed transplant procedure [22]. *Complete molecular remission* is defined by two successive MRD negative samples in an interval of a minimum of 4 weeks. *Molecular persistence at low copy numbers* is defined as presence of 100–200 copies/10^4 ABL copies corresponding to <1–2% of target to reference gene or allele burden; and a copy number or increase of more than 1 log between 2 MRD positive samples. *Molecular progression* is defined as an increase of MRD copy numbers >1 $\log_{10}$ between 2 positive samples. And finally, *molecular relapse* is an increase of MRD copy numbers >1 $\log_{10}$ between 2 positive samples in a patient who previously achieved MRD negativity.

In our practice, MRD assessment, using RT-PCR is routinely performed in AML patients with genetic aberrations. MRD is measured during and after post-remission chemotherapy, and in patients undergoing SCT prior the transplant procedure and at précised time points during the post-transplant period. However, in this paper, we focus on the molecular monitoring in patients undergoing MUD – HSCT, diagnosed with AML with specific genetic aberrations. We present here our findings at four AML patients and our initial experiences. Specific recommendations for molecular follow-up in AML-patients harboring these aberrations and the clinical implication of MRD status in post-transplant period will be discussed in line with our results.

4. Molecular monitoring in patients undergoing allo-SCT: single center experience

4.1 Case definition

Since the introduction of MUD-HSCT in the Republic of North Macedonia in November 2018, 10 AML patients underwent MUD HSCT until June 2020. Of those ten, molecular markers were identified in a total of 4 patients; two patients were positive for RUNX-RUNX1T1 transcript, 1 patient for CBFB-MYH11 transcript and 1 patient had mutation in CEBPA gene. The medical records of these patients were

reviewed for initial findings, clinical manifestations, clinical course, treatment regimen and outcome. Patients' individual characteristics are summarized in **Table 2**. Two patients were diagnosed with "de novo" AML and the other two patients had secondary AML evolving from antecedent myelodysplasia and myeloid sarcoma, respectively. All patients were transplanted in first complete hematological remission. Three patients received conditioning regimen considered myeloablative [37] with Bu-Cy + ATG and the one patient received reduced intensity chemotherapy regimen with Bu-Flu + ATG [37]. In all patients, peripheral blood stem cells were used as graft source. The patients underwent HSCT between November 2018 and January 2020. During the post-transplant period, bone marrow samples for MRD monitoring were obtained at scheduled time points - the first one within two months of HSCT and thereafter at +3, +6, +9 and + 12 months of HSCT [38] (**Figure 2**). The cut-off date for follow-up was June 30, 2020. Median follow-up time was 8 months (range: 3–19 months).

4.2 Samples

Samples of 5–10 ml of bone marrow aspirate in EDTA were used for PCR based analyses. PCR analyses were performed at the Center for Biomolecular Pharmaceutical Analyses - UKIM-Faculty of Pharmacy – Skopje.

4.2.1 Molecular PCR based methods (RT-PCR and multiplex fluorescent PCR)

Post-transplant MRD kinetics was evaluated by using quantitative polymerase chain reaction (RT-qPCR) or multiplex fluorescent-PCR every three months after the transplantation. Molecular analyses were performed at the Center for Biomolecular Pharmaceutical Analyses, UKIM-Faculty of Pharmacy, Skopje in bone marrow aspirates. RT- PCR is a high sensitivity method to detect the presence of leukemia cells down to levels of $1{:}10^{-5}$ to $1{:}10^{-6}$ white blood cells (WBC) [22, 36]. Mononuclear cells (MNCs) were isolated by Ficoll density gradient. Detailed procedures for MRD

	Patient 1	**Patient 2**	**Patient 3**	**Patient 4**
Age at diagnosis	22	21	58	37
Sex	male	male	female	Female
Comorbidities	no	no	no	No
2017 ELN risk stratification[16]	favorable	favorable	favorable	favorable
Molecular marker	CBFB-MYH11(inv16)	RUNX-RUNX1T1	5 bp deletion in CEBPA	RUNX-RUNX1T1
Number of induction therapies	2	2	3 (2 + 1)	2
Number of consolidation therapies	2	2	2	2
Time to HSCT	6 months	7 months	15 months	6 months
Disease status prior HSCT	CR1	CR1	CR1	CR1
MRD status prior HSCT	MRD + (0.15%)	MRD + (0.20%)	MRD +	MRD + (0.09%)

Table 2.
Individual and clinical pre-HSCT characteristics of analyzed AML patients.

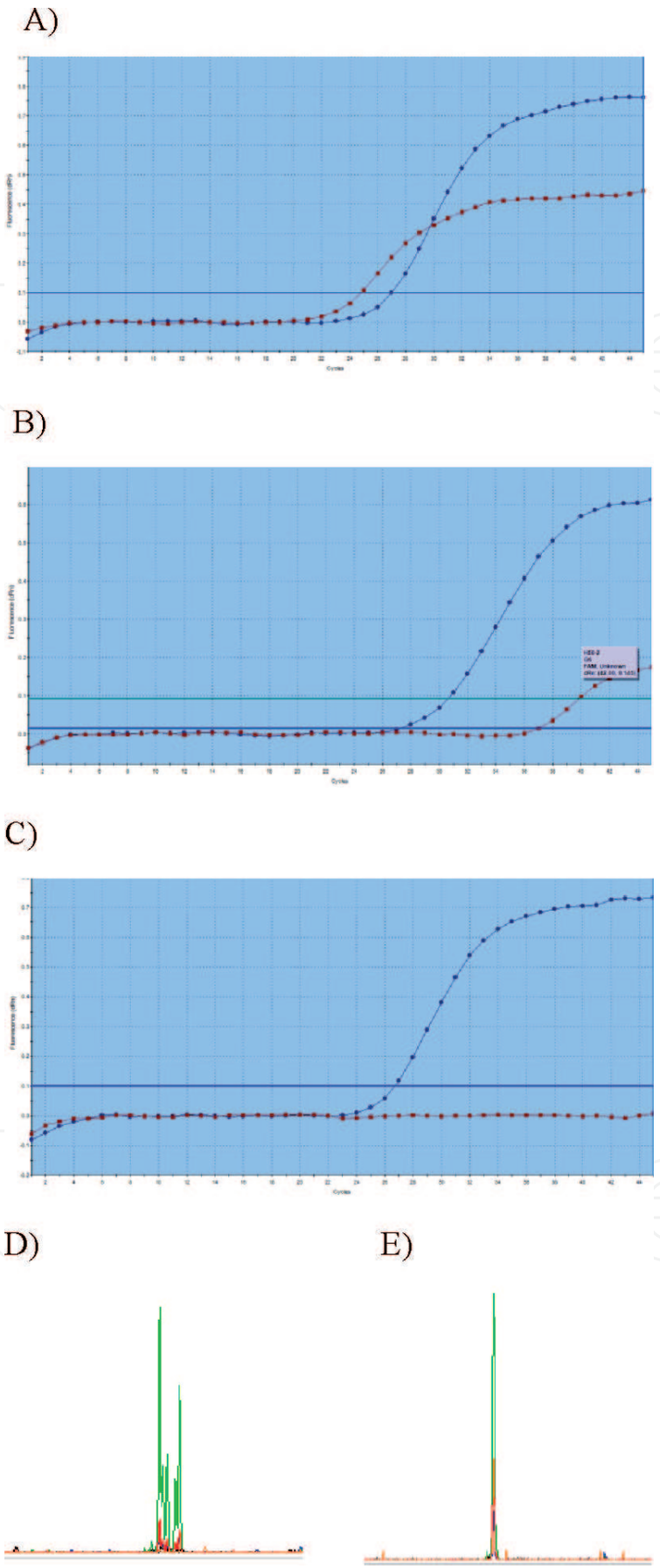

Figure 1.
MRD analyses using the AML-ETO hybrid transcript (A, B, C) or CEBPA mutation (D, E) molecular markers performed at diagnosis (a and D), pre-transplantation (B) and one month after transplantation (C and E). The blue circles and red squares in (A), (B) and (C) indicate the strength of the fluorescent signal generated during the RT/PCR amplification of the internal control ABL and hybrid AML-ETO transcripts, respectively. The arrow in (D) indicates the detection of the 5 bp-del mutant allele in the CEBPA gene.

assays detecting RUNX1-RUNX1T1, CBFB-MYH11 and CEBPA gene mutations have been published by the Europe Against Cancer Initiative [22]. The samples were run in triplicate. The molecular response was expressed as log reduction of transcript levels. MRD positivity was defined according to the Europe Against Cancer Program Criteria (amplification in at least 2 out of 3 replicates with cycle-threshold values of 40 or less, using a threshold setting of 0.1) [22]. The presence of mutations in the CEBPA gene was evaluated by multiplex fluorescent PCR analysis covering the coding region of the CEBPA gene and the exact molecular defects of all additional fragments was analyzed by Sanger sequencing [23]. Representative results of these analyses for the detection of the AML-ETO hybrid transcript and the mutation in the CEBPA gene are shown in **Figure 1**.

4.3 Pre-transplant MRD status

Molecular screening at the time of diagnosis is carried out in almost all our patients with newly diagnosed AML. Bone marrow samples are routinely investigated for RUNX1-RUNX1T1, CBFB-MYH11 and PML-RARα fusion transcripts, as well as single gene alterations, such as insertions in exon 12 in NPM1, FLT3 tandem duplication in exon 12, and deletions/insertions in CEBPA gene. All here presented patients were tested for the initial aberration prior to transplant procedure (median time = 13 days; range: 9–14 days). All patients were MRD positive (0.15%; 0.20% and 0.09% in patient 1, 2 and 4 respectively). In patient 3, presence of 5 bp deletion in CEBPA gene was confirmed.

4.4 Clinical histories

4.4.1 Case I

A young man at the age of 22 was diagnosed in March 2018 with myeloid sarcoma after surgically resection of the intestinal tumor mass. Three months later, the disease progressed in AML and standard induction chemotherapy regimen 3 + 7 DA (anthracycline plus ARA-C) was initiated. Molecular evaluation of the bone marrow showed expression of the CBFB-MYH11 inv.(16) (p13;22) fusion transcript. Remission was achieved after second induction and two consolidation therapies with high dose ARA-C were administered [1, 7]. The patient had a HLA haploidentical sibling. Unrelated 10/10 HLA matched donor, registered to PL-DKMS (Fundacija DKMS) was activated and allo-SCT was performed in November 2018 [14–16]. The patient received myeloablative conditioning regimen with Bu/Cy + ATG and conventional immunosuppressive therapy with cyclosporine and methotrexate was applied [33]. Peripheral blood stem cells (PBSC) were used as graft source at a total dose of $4{,}6 \times 10^6$ CD34+ cells/ kg. In post – transplant period, CBFB-MYH11 inv.(16) (p13;22) was used as a molecular MRD target [22]. On day +45 of HSCT, an increased MRD load, compared to that of pre-transplant MRD was documented. Consequently, the initial dose of 100 mg was reduced to 75 mg per day. On day +90 of HSCT, molecular MRD negativity was confirmed and continuously preserved up to the last evaluation at +15 months after HSCT. Immunosuppression was discontinued in December 2019, after completing one year of HSCT. Molecular MRD kinetics of CBFB-MYH11 inv. (16) (p13;22) in this patient is shown in **Figure 2**. Complete donor chimerism was first documented on day +90 and maintained to follow-up cutoff date. As this patient initially manifested extramyeloid presentation of AML, we did a PET scan assessment, at 12 months after HSCT and no pathological accumulation or activity were observed.

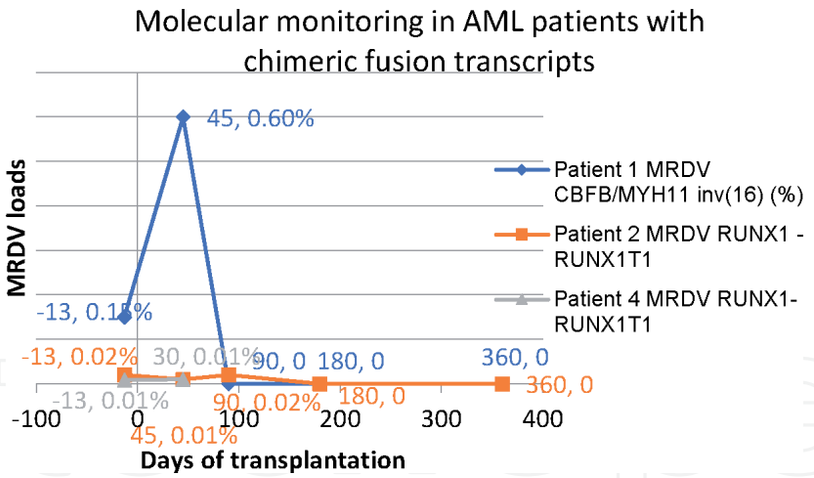

Figure 2.
Molecular MRD monitoring of patients with identified chimeric fusion transcripts. MRD kinetics of patients 1, 2 and 4 is marked with blue, orange and gray curve, respectively.

4.4.2 Case II

A previously healthy young man at the age of 21 was diagnosed with AML in January 2019. Initial findings of bone marrow revealed presence of RUNX1-RUNX1T1 fusion transcript. Remission was achieved after two cycles of standard DA (7 + 3) induction regimen and two consolidation therapies with high dose ARA-C were administered [1, 7]. MUD HSCT was performed in August 2019 after conditioning with Bu/Cy + ATG regimen [37]. Unrelated HLA 10/10 identical donor, signed to the German National Bone Marrow Donor Registry (DE-ZKRD), was activated [14–16]. A total of 5,2 × 10^6 peripheral blood stem cells/ kg were infused. MRD was measured by using RT- PCR, first on day +45 and thereafter at every three months. Up to the sixth month of HSCT, relatively steady kinetics of transcript levels was noted as shown at **Figure 2**. By gradual reduction of immuno-supression dose, molecular remission was documented at +6 month of HSCT and at +9 months consecutively. Complete donor chimerism was first documented on day +90 and maintained to the final evaluation at +9 months.

4.4.3 Case III

A 58-year-old woman was diagnosed with refractory anemia-myelodysplastic syndrome (MDS) in July 2018 and rapid progression into overt acute leukemia was demonstrated 4 months later. Initial molecular analysis detected 5 bp deletion in CEBPA gene. Induction chemotherapy (DA 7 + 3) was initiated and bone marrow evaluation showed no signs of remission after two cycles induction chemotherapies [1, 7]. Remission was achieved after one cycle salvage chemotherapy with FLAG-Ida regimen [7, 11]. Two consolidation therapies with high dose ARA-C were administered. MUD HSCT was performed in November 2019. Due to the patient age, reduced intensity chemotherapy (RIC) regimen was preferred, consisting of Busulfan and Fludarabin + ATG (fludarabine 30 mg/m^2 i.v for 5 days – from −8 to −4 and Busulfan 3.5 mg/kg/day for 2 days: day −5 and day −4) [37]. Unrelated HLA 10/10 identical donor from The Italian Bone Marrow Donor Registry (IBMDR) was activated [14–16]. Peripheral blood stem cells were used at a dose of 6,9 × 10^6 CD34+ cells/kg. MRD was first assessed on day +60 and the next one on day +120 with the last one assessed on day +150. Absence of previously detected deletion was confirmed on the first assessment, coupled with complete donor chimerism.

4.4.4 Case IV

A 37-year-old woman was diagnosed with RUNX1-RUNX1T1- mutated AML in July 2019. Treatment was initiated with DA (7 + 3) induction regimen and complete remission was established after two induction cycles [1, 7]. Two consolidation therapies with high dose ARA-C were applied afterwards. Unrelated HLA 10/10 identical donor, recruited through the German National Bone Marrow Donor Registry (DE-ZKRD) was activated and MUD HSCT was performed in January 2020 [14–16]. Peripheral blood stem cells were used as a source and a high number of HSC were harvested - 9×10^6 CD34+ cells/kg. Myeloablative regimen with Bu/Cy + ATG was used [37]. Conventional immunosuppressive therapy with cyclosporine and metothrexate was used. No significant complications were observed in the early post-transplant period [38]. Bone marrow evaluation was performed at +1 month of HSCT when complete donor chimerism was documented. She was MRD positive with MRD loads, nearly equal to those measured in pre-transplant evaluation (MRDv = 0.1% and MRDV = 0.09% respectively). On day +38 of HSCT, she appeared with symptoms of lower gastrtointestinal acute GvHD grade III, subsequently confirmed histologically. Immunosuppressive therapy with high dose methylprednisolone was immediately started. Three days later, she manifested acute severe respiratory symptomatology, leading to respiratory insufficiency and fatal outcome on day +45 of HSCT.

4.5 Post-transplant molecular monitoring

During the post-transplant period, in line with EBMT recommendations [38, 39], chimerism and molecular MRD status were assessed every three months and up to one year of HSCT, starting within two months of HSCT (45 days median time) [7, 24, 25]. In patient 1, a significantly increased MRD load was observed on day +45 of HSCT, compared to pre-transplant MRD load (0,6% and 0,15% respectively) and we reduced the immunosuppression dose. Immunosuppressive therapy is designed to prevent GvHD, but it also inhibits graft-versus leukemia effects (GvL). This patient had no previous signs of GvHD, thus he was carefully monitored for possible occurrence of new ones. Our objective was to enhance GvL effects, without causing serious GVHD, which resulted in achieving MRD negativity at +90 days of HSCT and maintaining it for a year so far in absence of GvHD manifestations. In patient 2, molecular loads kinetics showed a relatively steady curve, almost identical MRD values were measured until the sixth month of HSCT, when molecular negativity was documented. Patients 1, 2 and 3 had been followed up for 19 months of HSCT, 9 and 8 months respectively. They are in complete hematological and molecular remission for 13, 3 and 6 months, as defined by International Working Group for Diagnosis, Standardization of Response Criteria, Treatment Outcomes, and Reporting Standards for Therapeutic Trials in Acute Myeloid Leukemia: completely recovery of peripheral blood cells, <5% blasts in bone marrow, disappearance of the cells with previously documented leukemic immunophenotype and disappearance of previously detected molecular mutation [39]. Patient 4 died on day +45 of HSCT due to transplant-related complication. MRD status was assessed on day +30 of HSCT, when she was MRD positive with low MRD loads, equal to those prior to HSCT. Chimerism analyses in all patients, including patient 4 on day +30 of HSCT, documented early complete donor chimerism, which remained sustained during the follow-up evaluations in the first 3 patients.

5. Discussion

5.1 RUNX1-RUNX1T1- mutated AML

Chimeric fusion genes CBFB-MYH11, RUNX1-RUNX1T1, including PML-RARα, represent about 25% of AML cases, so called core-binding AML. As stated before, *RUNX1-RUNX1T1-mutated AML* is stratified into favorable risk group and therefore, patients harboring this mutation often do not undergo HSCT in first CR. Results from AML Study group show that half of these patients relapse very soon, almost all during the first year of completion of therapy [31]. In comparison, according to a single center study, among transplanted patients with RUNX1-RUNX1T1- AML, only 10–20% are expected to experience relapse [29]. RUNX1-RUNX1T1 transcript is well established powerful marker to predict risk of post-transplant relapse and direct future clinical interventions. In line with the same study [29, 33], RUNX1-RUNX1T1 levels kinetics can accurately predict forthcoming relapse, but not late relapse, due to the narrow time lag from molecular to morphological relapse. For this reason, time intervals between MRD assessments in these patients should not exceed 3 months.

5.2 CBFB-MYH11 - mutated AML

Acute myeloid leukemia (AML) with inv.(16)/ *t*(16,16), leading to specific *CBFB-MYH11* fusion transcript formation is also considered as favorable subtype [32, 33]. Therefore, in terms of transplantation, the same views are held as for RUNX1-RUNX1T1- AML [29–31]. Due to the general low incidence of this subtype, as well asa lower transplant rate in this group, all data originate from small sample-studies. Such limited data suggest that post-transplant MRD is predictive of relapse in contrast to pre-transplant MRD. The strongest predictive value is seen at +3 months of HSCT and it is thought that this period of time reflects the sensitivity of leukemia cells toward the transplant [32]. In regards to the optimal time intervals between MRD assessments in these patients, according to some published data [33], CBFB-MYH11 AML relapses appear to be generally indolent, with the longest delay of 8 months from molecular relapse to hematological evident relapse. However, these findings cannot be taken for granted, as the study involved non-transplanted patients.

5.3 AML with CEBPA aberrations

CEBPA aberrations can be found in up to 10% of patients with AML. Apart from AML, these mutations and deletions can also occur in MDS, multiple myeloma and non-Hodgkin's lymphoma (NHL) patients. CEBPA mutations result in functional block in myeloid differentiation and turning toward the erythroid lineage, with consequent erythroid hyperplasia or dysplasia, as was the case with our patient at the time of initial hematological assessment [40]. The most cases of *CEBPA*-mutant AML are double-mutated and exhibit two mutations and less than one third are single mutants. As per previously published data, double mutants have favorable prognosis, while the prognostic significance of single mutations is still unclear and it is codependent of the presence of additional gene mutations, such as FLT3-ITD and NPM1 and it is influenced by the karyotype [23, 40].

5.4 MRD and chimerism

In addition, in malignant diseases, chimerism kinetics seems to be remarkably correlated to MRD kinetics. Though, chimerism itself cannot be considered as an

indirect marker for post-transplant MRD monitoring, it serves more likely as prognostic factor for impending relapse. Therefore, chimerism analyses in bone marrow samples should be combined with MRD assessment in order to optimize the predictive value. As per EBMT recommendations, chimerism status should be evaluated at the same time points as MRD status during the post-transplant follow-up, or more precisely, within the first month of HSCT and at every three months during the first and the second year of HSCT [38, 39].

MRD status can be used to guide future clinical interventions in the post-transplant period. The presence of post-transplant MRD can identify those patients who are unlikely to benefit from re-application of similar therapies, because of selection and expansion of therapy-resistant clones. Different therapy strategies may be adopted in an attempt to eliminate MRD, varying from watchful waiting, through withdrawal of immunosuppression to more aggressive clinical interventions, distinguishing two general approaches - *immunomodulation* and *chemotherapeuteic agents* [41]. Immunomodulation includes donor lymphocyte infusion (DLI), natural killer (NK) cell infusion, both focused on enhancing GvL effects and chimeric antigen receptors (CAR) T-cells. In addition, emerging new chemotherapeutic agents such as DNA hypomethylating agents and targeted therapies could potentially eradicate MRD positivity [42].

6. Conclusion

Since major advancement had been achieved in the field of molecular monitoring, molecular MRD analyses became widely incorporated into the clinical routine and the decision making-process. PCR is currently used in our clinical practice in patients expressing specific molecular targets undergoing both autologous and allogeneic HSCT. Post-transplant molecular monitoring is of twofold significance: predicting impending relapse and guiding future MRD-based decisions and treatment strategies. In patients undergoing allogeneic HSCT, MRD should be evaluated within a month prior to the start of the conditioning regimen. During the follow-up period, MRD should be monitored every three months in a BM sample for at least two years and according to individual risk thereafter. MRD status itself is not a conclusive or a sufficient criterion to decide to intervene therapeutically. The main inquiry remains whether, when and at what thresholds a clinical intervention is required. Well-designed prospective clinical trials are needed to provide answers to these questions and establish MRD- guided clinical protocols.

Author details

Irina Panovska-Stavridis
University Clinic of Hematology, Skopje, Republic of North Macedonia

*Address all correspondence to: dr_irina@yahoo.com

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