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ARTICLE

Methods and role of minimal residual disease after stem cell transplantation

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Abstract

Relapse is the major cause of treatment failure after stem cell transplantation. Despite the fact that relapses occurred even if transplantation was performed in complete remission, it is obvious that minimal residual disease is present though not morphologically evident. Since adaptive immunotherapy by donor lymphocyte infusion or other novel cell therapies as well as less toxic drugs, which can be used after transplantation, the detection of minimal residual disease (MRD) has become a clinical important variable for outcome. Besides the increasing options to treat MRD, the most advanced technologies currently allow to detect residual malignant cells with a sensitivity of 10^{-5} to 10^{-6}.

Under the patronage of the European Society for Blood and Marrow Transplantation (EBMT) and the American Society for Blood and Marrow Transplantation (ASBMT) the 3rd workshop was held on 4/5 November 2016 in Hamburg/Germany, with the aim to present an up-to-date status of epidemiology and biology of relapse and to summarize the currently available options to prevent and treat post-transplant relapse. Here the current methods and role of minimal residual disease for myeloid and lymphoid malignancies are summarized.

Acute myeloid leukemia

Because of the observed rate of relapses in AML in complete remission it is obvious that minimal residual disease is present though not morphologically evident. Complete remission in AML is defined as <5% blasts in bone marrow and recovery of peripheral blood count [1]. Conventional cytomorphological assessment may miss a level of residual leukemic cells which may cause subsequently clinical relapse. The detection and standardization of minimal measurable disease in patients with morphologically defined complete remission may have clinical impact with respect to outcome as well as regarding therapeutic interventions (see Table 1) [2, 3].

More recently the European LeukemiaNet published a consensus document which tried to standardize the methods of MRD measurement which will allow to design prospective clinical trials [4]. The increasing number of molecular mutations revealed by high-throughput sequencing such as FLT3-ITD, TP53, DNMT3A, IDH1 and 2, CBFB-MYH11, KIT, RUNX, NPM1, and others allow a more specific detection of MRD. There is an increasing number of references highlighting the importance of MRD detection in AML in the transplant as well as in the non-transplant setting [5–13]. Despite its clinical relevance the optimal method as well as standardization and timing still need to be determined in well-designed clinical studies.

Real time quantitative PCR (RQ-PCR)

RQ-PCR can be used to identify chimeric fusion genes such as PML-RARA or AML1-ETO, gene rearrangements or

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Table 1 Sensitivity of different MRD methods in AML

Method	Sensitivity	Variables with need for standardization
RQ-PCR	10^{-4}–10^{-6}	Bone marrow vs peripheral blood analytical cutoff
dPCR	10^{-4}–10^{-6}	Sample processing: bone marrow vs peripheral blood
MFC	10^{-3}–10^{-5}	Sample processing: selection of antigens, number of events
NGS	10^{-2}–10^{-4}	Blood form relevant gene selection, depth of coverage

alterations. Chimeric fusion genes represent about 25% of AML cases and RARA usually provides high sensitivity. Most studied mutation is NPM1 whose detection is associated with a higher risk of relapse [7, 8] though universally accepted cutoffs for MRD level are lacking. A reduction of NPM1 level to less than 4 log is associated with a high risk of relapse and reduced OS after stem cell transplantation [14]. Other studies confirmed a pre-transplant level of NPM1 > 1% resulted in poorer outcome post allo graft [15]. Also monitoring NPM1 post-transplant regarding clearance of MRD has been shown to be a reliable marker for subsequent clinical relapse and may be used for interventions such as donor lymphocyte infusions [16]. Other MRD marker such as FLT3-ITD have also been shown to be associated with high risk of relapse but the sensitivity is usually only 10^{-3}–10^{-4} [17]. Recently-described improved FLT3 internal tandem duplication PCR assay may predict better the risk of relapse after allo graft [18]. WT1 transcripts are over-expressed in the majority of AML cells but the lack of specificity questions the role of WT1 as MRD marker [19]. A more recent study however suggested a specificity and sensitivity for WT1 of 97 and 83%, respectively [20].

Zhu et al. reported on RUNX1/RUNX1T1 transcript level after second consolidation and described a better outcome in those MRD-positive patients who received an allogeneic stem cell transplantation [21]. Monitoring patients post transplantation allows to predict the risk of relapse and might be considered as a maker for donor lymphocyte intervention [22].

Digital PCR (dPCR)

The development of digital PCR allows high sensitivity such as RQ-PCR but a better quantification of MRD and is studied currently in clinical trials [23].

Multiparameter flow cytometry (MFC)

Leukemia-associated immunophenotype (LAIP) is present in about 80–100% of AML patients [24] which allows detection of residual blasts by MFC and its prognostic impact has been shown in several studies [11, 24, 25] but the optimal cutoff is still under debate. Some authors proposed a threshold of 3.5×10^{-4} [26], others separate risk groups according to the detectable cells ($<10^{-4}$–$>10^{-2}$) [27]. The sensitivity of MFC in comparison to RQ-PCR is lower [28] but the more recent development of up to 10 color antibodies increases the sensitivity although this requires experienced investigations. Even if this method is increasingly used because of broader applicability methodological standardization across the laboratories are ongoing to provide comparable and reproducible results. Other drawbacks are the antigen switch in AML blasts at the time of relapse in comparison to time of diagnosis [29].

Next-generation sequencing (NGS)

Because of the well known clonal evolution and the phenotype switch in AML patients next-generation sequencing represents a useful tool to assess MRD in AML [30, 31]. The major drawback of NGS is the high sequencing error rate which impacts the sensitivity of the method [32]. Current new error-corrected read technologies and analytical techniques may allow us to increase the sensitivity [33].

Conclusion

MRD detection in AML has been shown to impact outcome after transplantation. The best method of MRD detection still needs to be determined and for each method, standardization and harmonization need to be done. Optimal timing and defining thresholds for MRD need to be evaluated. For specific MRD-based intervention well-designed prospective randomized trials are mandatory.

Today it is not clear if MRD-positive patients prior to transplantation should receive a more intensified conditioning regimen [34] or if post-transplant MRD positivity patients should receive donor lymphocyte infusion or pharmacological intervention e.g. with hypomethylating agents [35].

Multiple myeloma

Thanks to the major therapeutic improvements over the last 15 years, maximal cytoreduction is now frequently achieved in multiple myeloma (MM) [36]. This has boosted the interest in the evaluation of MRD in this neoplasm [37–39]. Unlike other lymphoid neoplasms where molecular methods for MRD detection clearly represent the "golden standard", in MM MRD evaluation has always been investigated using both molecular and

flow-cytometry-based approaches. Flow cytometry has broad applicability allowing MRD detection in nearly all MM patients and has been reliably capable to reach a sensitivity of 10^{-4} with possibly even better performances with the most updated >6 color or the so called "next generation" flow cytometry approaches [38, 40–42]. Molecular approaches relied for more than two decades on PCR-based amplification of clonal sequences from the IgH rearrangement and design of tumor-specific primers suitable for nested or real-time quantitative PCR [43]. This approach has excellent sensitivity (10^{-5}) and specificity and has been further improved by the development of "digital-droplet PCR" [44]. However, it is labor-intensive and in MM as opposed to other tumors it is severely limited by the failure of clonotype identification in approximately 40% of patients. The application of "Next generation sequencing" to molecular detection of MRD has substantially improved the rate of target identification and is able to provide quantitative MRD results which are comparable to those observed with PCR-based tools [45, 46]. Sensitivity might potentially be further increased provided that very large amounts of patient DNA are tested which is not always easily achieved in the clinical practice. The main limitation of NGS MRD is that the approach is currently provided as a service-based tool exclusively by one single private company. However, alternative methods including few developed in the context of academic consortia like Euroclonality-EuroMRD will become available in the future to the scientific community allowing a broader, less expensive and more investigator-controlled collection of results [39]. Finally hybrid approaches which employ NGS for target identification and real time quantitative or digital droplets PCR for MRD analysis on follow-up samples have been developed and might potentially become of clinical interest in some contexts.

The prognostic impact of MRD detection has been clearly established in multiple conditions and therapeutic contexts [39, 40, 47, 48]. Preliminary reports from the nineties indicated that autologous transplantation was unable to induce molecular remissions in MM as opposed to allogeneic transplantation, that allowed a proportion of patients to enter molecular remission [49]. On the other hand a number of reports proved the prognostic value of MRD evaluated by both molecular and flow cytometry-based approaches on multiple survival parameters [39, 40, 47, 48]. The substantial impact of the introduction of novel agents in MM on tumor clearance was clearly demonstrated by studies indicating that non-chemotherapeutic-based combinations were able to further decrease tumor burden after autologous transplantation allowing a proportion of patients to enter molecular remission [50]. Moreover MRD appeared to be an independent prognosticator in several large trials whose impact appeared independent from other important prognosticators such as cytogenetics.

The role of MRD detection has been substantial also in the allogeneic transplantation field. Older studies clearly indicated that the graft vs myeloma effect was particularly effective in clearing residual tumor clones [49]. The use of reduced-intensity conditioning regimens had also intriguing effects on the kinetics of residual myeloma clones [51–53]. Interestingly a slower kinetic of tumor reduction was observed that nevertheless appeared able to obtain deep and sustained molecular responses, with substantial reduction of the tumor burden detected by RQ-PCR during the early post-transplant follow-up [51]. Moreover it was demonstrated that post-transplant treatment with donor lymphocyte infusions and/or novel agents had substantial impact on MRD kinetic and clinical outcome, suggesting that MRD might represent an attractive tool to guide post-transplant treatment in MM [54].

Based on the large bulk of results on MRD assessment in MM consensus criteria for response and minimal residual disease assessment in multiple myeloma have been recently developed which includes the definition of "flow-MRD negative", "sequencing-MRD negative", and "sustained MRD-negative" which are now routinely adopted in clinical trials addressing MRD as a secondary endpoint [55].

In summary, MRD evaluation is a critical instrument for response evaluation in MM. Its use allows to better define the clinical activity of novel regimens and might provide insights and potential comparisons of the depth of response between different regimens. However the most important goal would be to employ MRD to guide treatment decision and to offer intensification or de-intensification programs to selected patient populations. This possibility is under exploration in several lymphoid neoplasms and there is a clear need to explore its applicability also in MM through the development of clinical trials comparing standard vs tailored therapeutic schedules.

CLL and lymphoma

MRD can be assessed in chronic lymphocytic leukemia (CLL) using either standardized real-time quantitative PCR targeting the patient-specific immunoglobulin heavy chain rearrangement (ASO *IGH* RQ-PCR) [56] or specialized four- to eight-color flow cytometry (MRD flow) according to ERIC guidelines [57, 58]. EuroFlow technical guidelines [59] allow further improvements in standardization. Both MRD flow and RQ-PCR are equally suited [60] to reach the accepted sensitivity threshold for MRD positivity (10^{-4}) [61]. The PCR-based approach can detect, but often not quantify, MRD at ranges between 10^{-4} and 10^{-5} in a

Table 2 Studies on the significance of MRD after allogeneic stem cell transplantation

	Farina et al. (2009) [78]	Dreger et al. (2010) [63]	Richardson et al. (2013) [65]	Logan et al. (2013) [62]	Algrin et al. (2017) [79]
N @ landmark	29	38	26	31	37
Landmark @	6 months	12 months	9 months	12 months	12 months
MRD positive	44%	29%	23%	32%	46%
Relapse risk by MRD	7 vs 42% @ 4 y	HR 0.09	HR 0.08	HR 0.11	20 vs 45% @ 4 y
p Value	0.031	0.0052	0.003	<0.0001	0.089
Method for MRD monitoring	Nested ASO-PCR	MRD flow / IGH ASO-RQ PCR	MRD flow	NGS	MRD flow

MRD negativity was generally defined as $<10^{-4}$

HR hazard rate, *y* years

fraction of the cases. MRD flow is more broadly available, faster and less labor-intensive than PCR, but relies on the assessment of fresh samples. Provided sufficient total DNA is utilized. Next generation sequencing (NGS) MRD approaches [57, 62] show promise to achieve higher sensitivities of up to 10^{-6}. However, NGS technology currently lacks internationally accepted standardization. The vast majority of data demonstrating the importance of MRD both in transplanted and conventionally-treated CLL patients have been obtained by means of MRD flow.

Sequential MRD assessments were instrumental in elucidating the mode of action of allogeneic SCT and in demonstrating the chance for cure afforded by this treatment modality. A delayed, but long-lasting induction of MRD negativity occurring only upon immunosuppression tapering could be observed in about 40% of all transplanted patients [63], thus corroborating the existence of GVL effects. The relapse risk might be lower in patients in whom MRD clearance coincided with the withdrawal of the immunosuppression compared to patients who became MRD-negative directly after transplantation [64]. Decreasing MRD levels in temporal relationship with an activation of the graft immune system are therefore thought to mirror efficacious GVL. High GVL activity in turn likely causes a sustained and profound reduction of CLL tumor load beyond the limit of detection of sensitive MRD methods. MRD negativity is logically regarded a necessary prerequisite of cure. Considering delayed reduction of MRD levels a surrogate for GVL activity the prognostic impact of a negative MRD result shortly after withdrawal of the immunosuppression is comprehensible. MRD negativity at landmarks 6–12 months after transplantation was indeed associated with a very low long-time relapse risk in several studies (Table 2), in particular, if sensitive and quantitative methods for MRD monitoring were applied [62, 63, 65]. The eradication of measurable MRD using DLI was consequently attempted even in patients without any other sign of leukemia [64–66]. Of note, allogeneic SCT was demonstrated to induce sustained MRD negativity beyond 10 years after transplantation.

A comparison of the clinical significance of MRD assessments after allogeneic stem cell transplantation to its impact on outcome after conventional treatment further elucidates fundamental differences between both the therapeutic strategies. Published analyses from a total of 12 major CLL trials and series using standardized MRD have proven its significance to predict PFS and OS after (immuno-)chemotherapy. That body of evidence includes MRD data from phase 2 [67, 68] as well as 3 [69, 70] trials and currently comprises assessments from 1729 patients. MRD was shown to be predictive for clinical outcome in both partial and complete responders [71]. The vast majority of patients who received induction immunochemotherapy experienced MRD recurrences during follow-up which could be delayed, but not prevented by antibody maintenance therapy [72]. The observation of rising MRD kinetics over time demonstrate that conventional treatment lacks curative potential eventhough it is very effective and can often provide long-lasting disease control. Whereas data from the non-allogeneic setting thus corroborate that MRD is more important than clinical responses and help validating the methods utilized for MRD detection, MRD kinetics over time also demonstrates profound differences of the mechanism of action. It is noteworthy that the sensitivities of MRD detection in the transplant vs. non-transplant setting were comparable (typically 10^{-4}), meaning that the achievement of the same MRD level carries a different long-term prognosis depending on the treatment modality.

In contrast to CLL, molecular methods are the mainstay for MRD assessments in follicular and mantle cell lymphomas. Currently, most patients with mantle cell lymphoma can be monitored using ASO *IGH* RQ-PCR [56], whereas the disease specific t(11;14) rearrangement represents a suitable additional PCR target in about 30% of cases only. Both molecular methods reach a sensitivity of up to 10^{-5} and a typical quantitative range of 10^{-4}. MRD is measurable in

about 50% of all follicular lymphoma cases by means of an RQ-PCR assay targeting the t(14;18) translocation [73]. This method achieves a sensitivity of up to 10^{-5}. Recently Droplet Digital PCR has been introduced into MRD monitoring in mantle cell and follicular lymphomas showing promise of increased applicability and ease of use [44]. Comparative analyses between RQ-PCR and NGS in both the lymphoma entities generally showed good correlation, but also cases with discrepant results [45], mandating additional evaluations of the novel technology. Ample published evidence exists for the prognostic impact of MRD in follicular [73] and mantle cell [74, 75] lymphomas in the non-transplant setting, whereas the utility of MRD after allogeneic transplantation remains anecdotal to date [76]. The detection of circulating tumor DNA as an innovative tool to assess MRD in lymphomas (including diffuse large B-cell lymphomas) is still in its infancy [77]. It therefore cannot be recommended for routine clinical use yet [78, 79].

In summary, MRD has contributed to our current understanding of the mechanism of action of allogeneic stem cell transplantation in CLL and has been used to individually tailor immunological intervention after transplantation. Given the availability of equally sensitive methods for MRD detection in lymphomas, experiences from CLL might be transferred to this group of diseases.

Acute lymphoblastic leukemia

Acute lymphoblastic leukemia (ALL) is well suited for MRD assessment by molecular methods because both B-cell and T-cell ALL undergo rearrangements in immune globulin (IgH) and T-cell receptor (TCR) genes early in development, prior to leukemic changes. Thus, all leukemia from the founder B or T cell clone shares the same gene rearrangement that can then be tracked. Using standardized PCR methodologies, detection of levels at or below 10^{-4} can be routinely achieved and used for risk classification [80]. Newer methods of next generation sequencing (NGS) of IgH or TCR loci that improve sensitivity to 10^{-5}–10^{-7} have been published recently [81, 82]. A challenge associated with molecular methods is the need for ALL blast DNA to define a leukemic clone. This is labor intensive and is unsuccessful in approximately 3–5% of patients. To avoid this issue, other cooperative groups have developed methods of identifying definitive blasts by multi-parametric flow cytometry (FC) [83]. While this method gives results more quickly, it requires technical expertise and consistency in approach.

Both PCR and FC MRD approaches are well-established in the care of children and adults undergoing chemotherapy for ALL, but these techniques have also been shown to be predictive of outcome both before and after hematopoietic cell transplantation (HCT). Studies outlined below focus on

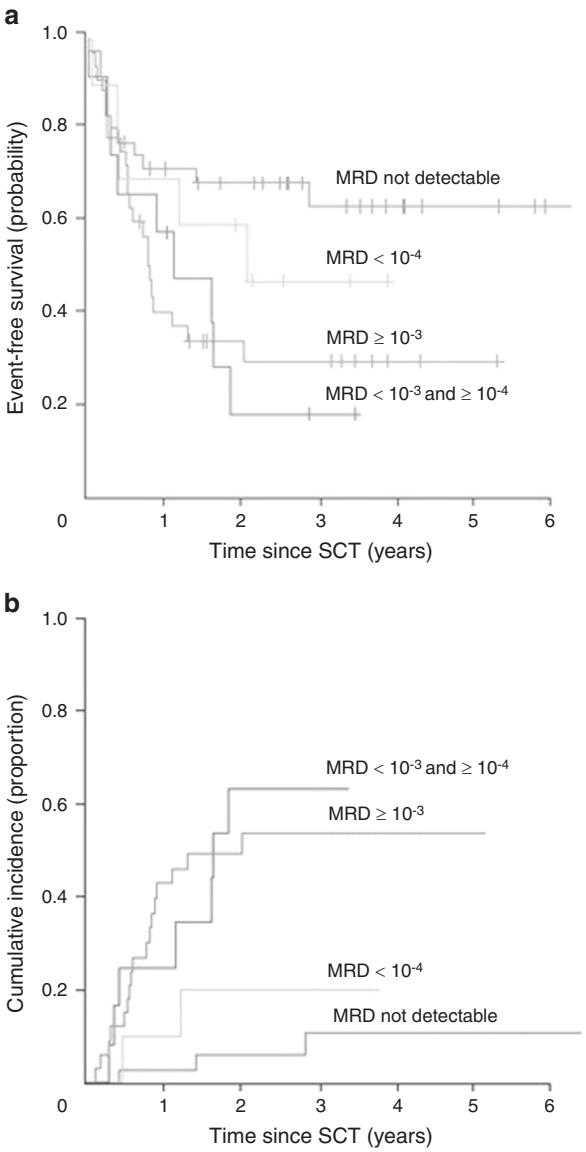

Fig. 1 Event-free survival (**a**) and cumulative incidence of relapse (**b**) of children with ALL with varying levels of MRD pre-HCT (from Bader J Clin Oncol. **27**:377–384)

pediatric work, but similar outcomes have been noted in adult trials [84, 85].

Early work by European groups firmly established that PCR detection of MRD at $>10^{-4}$ pre-HCT led to worsened outcome, whereas, detectable MRD below that amount leads to outcomes similar to patients who have no detectable MRD (Fig. 1) [86, 87]. Patients with levels of MRD > 10^{-4} have been noted to have relapse rates approaching 60% or more. This work has been confirmed in multiple settings and using multiple stem cell sources [88–90]. FC approaches have shown similar poor outcomes in patients with MRD > 0.1% [91, 92]. These findings have prompted clinicians to give additional cycles of therapy prior to transplant in order to decrease MRD burden [93], although

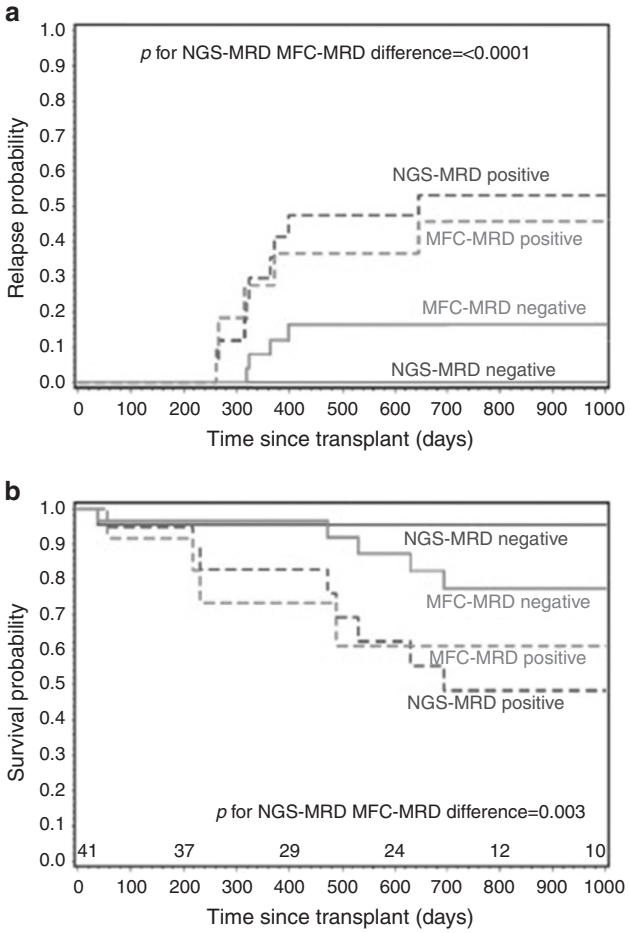

Fig. 2 Probability curves of patients who were MRD-negative or positive by flow cytometry or deep sequencing. **a** Risk of relapse. **b** Overall survival. (from Pulsipher, Blood. 2015 May 28;125(**22**):3501–8)

more studies verifying the survival benefit of this approach are needed.

Recently, MRD assessment post-HCT has been shown to be a strong predictor for outcome [92–95]. Both PCR and FC methods have been noted to (1) identify patients at high risk of relapse if disease is detected at $>10^{-3}$ for PCR and >0.1% for FC at any point after HCT, and (2) predict with increasing accuracy risk of relapse as patients with lower levels of detectable MRD get further from transplant. Additionally, patients who test MRD positive either before or after transplant who go on to develop acute and/or chronic GVHD have been shown to have decreased relapse compared to patients who do not develop GVHD [92]. Also, studies of early intervention using withdrawal of immune suppression +/− donor lymphocyte infusions have suggested some patients who would have relapsed can be salvaged [96].

With the development of less expensive sequencing platforms, a number of laboratories have developed NGS-MRD detection techniques that can markedly increase sensitivity [82]. A few studies have investigated NGS-MRD for risk determination as part of standard ALL chemotherapy approaches [97, 98]; further study is needed to discern whether this technique is better or whether it is too sensitive. In contrast, early data suggest a distinct advantage to NGS-MRD techniques compared to FC for HCT patients [99, 100]. A Pediatric Blood and Marrow Transplant Consortium (PBMTC)/Children's Oncology Group (COG) study of 56 children undergoing HCT showed statistically superior ability to predict relapse and survival using NGS-MRD compared to flow ($p < 0.0001$). Notably, patients who were NGS-MRD negative had 0% relapse vs. 16% in children negative by FC ($p = 0.02$). 2-year OS was 96 vs. 77% for patients who were NGS- and FC-MRD negative ($p = 0.003$; Fig. 2). Of note, the presence of NGS-MRD at any level post-HCT was highly predictive of relapse, even as early as 30 days after the procedure, a marked improvement over FC testing early post-HCT.

In summary, patients with ALL undergoing HCT can be risk classified by either PCR or FC-based MRD methodology used just prior to or after HCT. Interventions based upon MRD both prior to and after HCT are being used to attempt to change the natural history of patients identified as high risk of relapse, and well-planned studies to define whether outcomes of these patients can truly be modified are needed. Finally, NGS-MRD measurement before and after HCT appear to offer an advantage both in determining risk of relapse and chances of success, however, studies to date are small and much additional studies are needed to confirm these early observations.

Conclusion

There is increasing evidence that measureable residual disease prior and after autologous and allogeneic stem cell transplantation has a major impact on clinical relapse and survival after transplantation in hematological malignancies. The methods of detection of MRD has remarkably improved regarding specificity and sensitivity, and different methods such as multiparametric flow cytometry, PCR or NGS are currently used in clinical practice. While improvement in standardization of the different methods were done, a specific threshold for intervention has not been defined. Therefore well-designed clinical studies are required to reduce the risk of relapse and improve overall survival.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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