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Chapter 6

Minimal Residual Disease and Leukemic Stem Cells in Acute Myeloid Leukemia

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1. Introduction

Acute myeloid leukemia (AML) is the most common type of acute leukemia in adults. With current treatment strategies, almost 80% of AML patients (18-60 years) will achieve complete remission (CR). However, approximately 50% of these patients will experience a relapse, resulting in a five-year survival rate of only 35%-40% [1]. This implies that despite CR, in these patients a number of cancer cells survive treatment and can grow out to cause a relapse. Efforts towards development of more sensitive methods to accurately determine CR and detect residual cancer cells are necessary to improve risk-adapted management to eventually prolong overall survival rates.

2. Minimal residual disease and acute myeloid leukemia

In AML patients, morphologic assessment is performed to evaluate chemotherapy response and to define remission status. By definition, patients are in CR when less than 5% blast cells are present in the bone marrow (BM) concurrent with evidence of normal erythropoiesis, granulopoiesis and megakaryopoiesis. In addition, neutrophils and platelets in peripheral blood should be at least 1.0 x 10^9/l and 100 x 10^9/l, respectively [2]. Since about 50% of patients in CR will eventually experience a relapse, for prognostic purposes more precise assessment of the quality of CR is necessary. To this end residual disease detection could be of high importance. This so-called minimal residual disease (MRD) is thus defined as the persistence of leukemic cells after chemotherapy treatment and thought to be responsible for the emergence of relapse (Figure 1). Quantitative MRD frequency assessment could give important prognostic information after chemotherapy treatment. Two highly sensitive meth-

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ods for MRD detection in leukemia are multiparameter flow cytometry (MFC) and real-time quantitative polymerase chain reaction (RQ-PCR). Both methods and their clinical applications will be reviewed in this chapter.

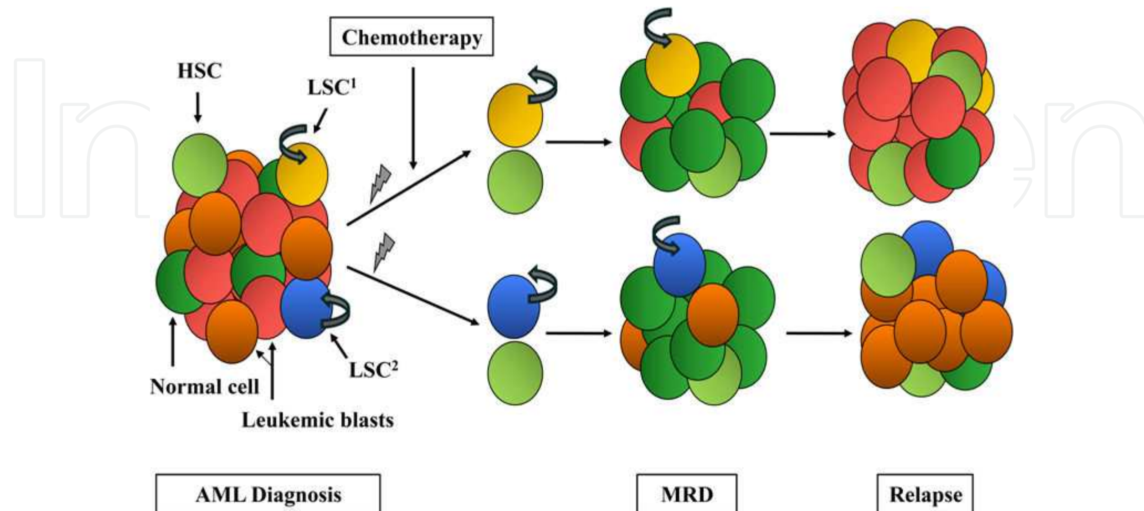

Figure 1. The role of minimal residual disease and leukemic stem cells in the emergence of relapse. HSC, normal hematopoietic stem cell, LSC, leukemic stem cell. At AML diagnosis a heterogeneous population of cells often coexist, including different subpopulations of LSCs. MRD frequency assessment focuses on the detection of leukemic cells present after treatment. Different subpopulations of chemotherapy resistant LSCs can grow out and cause relapse (discussed in section 3).

2.1. Immunophenotypic MRD detection

2.1.1. Principles of immunophenotypic MRD detection

One of the most frequently used techniques to assess MRD in leukemia is based on assessment of immunophenotypic aberrant antigen expression using flow cytometry. For practical purposes, in most cases, this approach is restricted to cell surface antigen expression. At diagnosis, so-called leukemia associated (immuno)phenotypes (LA[I]Ps, further referred to as LAPs) are determined. Such a LAP consists of (an) aberrantly expressed cell surface marker(s), usually combined with a myeloid marker (CD13/CD33) and with a normal progenitor antigen, i.e. CD34, CD117 or CD133. LAPs are grouped into (1) cross-lineage antigen expression (e.g. expression of lymphoid markers in myeloid blasts), (2) asynchronous antigen expression (co-expression of antigens that are not concomitantly present during normal differentiation), (3) lack of antigen expression and (4) antigen overexpression [3]. Such aberrancies can subsequently be used to detect MRD (Figure 2).

Due to large heterogeneity of immunophenotypes in AML, determination of LAPs has to be performed for each individual patient. These LAPs are not, or only in very low frequencies, present on normal BM cells in remission BM. Sensitivities have been reported to be in a range of 10^{-3} down to 10^{-5} (1 leukemic cell in 1,000 to 100,000 normal cells) [4-9]. Besides these relatively high sensitivities, it is also a very rapid technique. Main advantage of flow

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cytometric MRD assessment is its broad applicability: in 80%-95% of all AML patients one or more LAPs can be defined. [4,5,9-11]. There are, however, potential pitfalls/disadvantages that should be taken into account. *Firstly*, blast cells at diagnosis are often characterized by subpopulations with different immunophenotypes. For this reason a LAP defined at diagnosis, is often not a characteristic of the total population of leukemic blast cells. Since only the LAP positive (LAP$^+$) cells can be identified at follow-up, this may thus result in under-estimation of cell frequency of *all* leukemic blast cells, referred to as MRD. To approach the real MRD cell frequency, there is the possibility to correct LAP$^+$ frequency at follow-up for the LAP$^+$ frequency, as percentage of blasts, at diagnosis. *Secondly*, the presence of low percentages of normal cells that express a particular LAP may result in over-estimation of MRD cell frequency. This background staining may even lead to false-positive results. A relatively low background staining can be achieved by including a primitive marker in the definition of a LAP, since these cells are only present at low frequencies in normal BM. *Thirdly*, immunophenotypic shifts may occur in the course of treatment and result in false-negativity [6,12,13]. To avoid this, it is recommended to use multiple LAPs. *Finally*, due to the large number of different LAPs, MRD analysis is quite complex and needs vast experience in discriminating leukemic cells from cells with normal differentiation patterns.

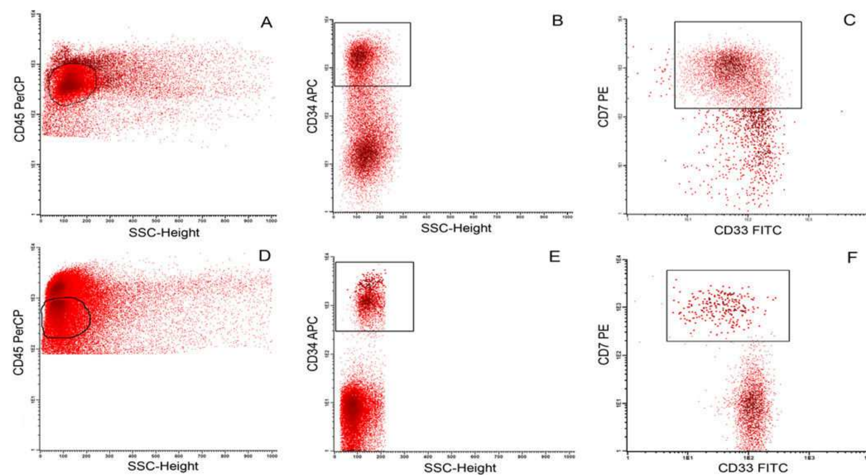

Figure 2. Example of MRD detection in BM using the aberrant phenotype of CD34$^+$CD7$^+$ cells at AML diagnosis (A-C) and during follow-up (D-F). Gating of the blast cells with CD45dim expression and low sideward scatter (SSC) (A, D), gating of the CD34 positive progenitors cells (B, E) and gating of the leukemic blast population with aberrant expression of CD7 on the myeloid progenitor cells (C, F). After chemotherapy treatment a residual population of leukemic blasts can be detected (F).

2.1.2. Prognostic value of immunophenotypic MRD in bone marrow

The likelihood of achieving CR after therapy and the duration of CR depend on different factors. Important prognostic risk factors available at diagnosis are: history of previous leukemia or myelodysplastic syndrome, age, white blood cell (WBC) count, percentage of BM blast cells and the presence of particular cytogenetic and/or molecular aberrancies [14]. Besides these pre-treatment prognostic factors, it is suggested that MRD detection in BM shortly after treatment offers an important post-treatment prognostic factor. To evaluate the impact of MRD fre-

quencies on relapse rate and overall survival (OS), MRD was related to outcome parameters using survival analyses such as Kaplan Meier curves. For these analyses, most studies set a threshold to define MRD negative (or low) and MRD positive (or high) patients. Different laboratories use different optimal cut-off values after both induction and consolidation therapy (Table 1). However, it should be emphasized that usually, it is not a single cut-off point, but a range of cut-off values that significantly predict clinical outcome.

Author	Patients (n)	Cut-off post-induction	Cut-off post-consolidation	Reference
San Miguel *et al.*	126	<0.01%, 0.01-0.1%, 0.1-1%, >/1%	not available	[15]
Feller *et al.*	52	0.14%	0.11%	[6]
Kern *et al.*	62	Log difference 1.70	Log difference 2.94	[5]
Maurillo *et al.*	142	0.035%	0.035%	[16]
Al-Mawali *et al.*	54	0.15%	0.15%	[10]

Table 1. Overview of studies in adult AML with cut-off values used for analyzing relapse free and overall survival.

San Miguel *et al*. were the first to show the prognostic impact of MRD in a group of 53 AML patients [4]. Later they extended the study to 126 patients. An overview of three-year relapse rates for three distinct patient risk groups with considerable patient numbers is given in Table 2. Univariate analysis of prognostic factors revealed five disease characteristics that had significant impact on relapse-free survival (RFS); these included cytogenetic abnormalities, number of chemotherapy cycles needed to achieve CR, WBC count, absolute peripheral blood (PB) blast cell count and MRD levels. In a multivariate analysis only cytogenetics (p =.03) and MRD levels (p =.002) were independent prognostic factors for RFS [15]. These results are in line with other studies. Results from Feller *et al.*, showed a relative risk of relapse of 3.4 after induction therapy (p =.003) and 7.2 after consolidation therapy (p =.004) in the patient group with high MRD levels [6].

MRD level	Patients (n)	Relapse rate ± standard error
< 0.1 %	16	9% ± 7%
0.1% - 1%	45	56% ± 9%
"/1%	21	83% ± 10%

Table 2. Overview of three-year relapse rates for three distinct risk groups based on MRD levels determined after induction therapy (p =.006) [15].

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Al-Mawali *et al.* demonstrated in a multivariate analysis that post-induction positive MRD status was an independent prognostic factor for both RFS and OS (p =.037 and p =.026, respectively) MRD positivity after induction therapy was also associated with increased risk of relapse (Hazard ratio [HR] 4.7, 95% confidence interval [CI] 1.1-20.5) [10]. Maurillo *et al.*, in a study following their original report [7], have reported similar results in a study of 142 AML patients. In a multivariate analysis of RFS, cytogenetics (p =.0001), multidrug resistance-1 phenotype (p =.03) and MRD positivity after consolidation therapy (p =.001) were independent prognostic factors. In multivariate analysis of OS, post-consolidation MRD positivity (p =.004) was the only independent prognostic factor [16]. Kern and colleagues, in an approach that established log reduction of blast cells as a measure for MRD, showed that after induction therapy < 1.7 log reduction (p =.006) and unfavorable karyotype (p =.0001) were independent prognostic factors for relapse [5]. After consolidation therapy < 2.94 log reduction (p =.006) and unfavorable karyotype (p =.015) were found to be independent factors for relapse. Although above-mentioned study results are promising and consistent, the clinical importance of MRD in adult AML still has to be validated in a prospective study. Terwijn *et al.* studied the value of MRD monitoring in a large cohort of 462 AML patients. Multivariate analysis, performed with conventional prognostic factors, demonstrated that MRD frequency was an independent prognostic factor for RFS after every cycle (first cycle p =.010, second cycle p<.00001 and consolidation p<.00001) and for OS after the first cycle (p =.023) and second cycle (p =.01). To our knowledge, this is the first study that demonstrates the importance of MRD monitoring in a prospective study [17]. Because of these prospective data, the next step would be to implement MRD status in clinical decision-making.

2.2. Molecular MRD detection

Although flow cytometry is an attractive technique for MRD detection, the limitations, including background staining, immunophenotypic switches, complexity of analysis and LAP expression on only part of the leukemic cells, give rise to alternative approaches for MRD detection, including molecular MRD monitoring using the Polymerase Chain Reaction (PCR) technique. This approach allows for the detection of mutations, translocations, inversions, deletions and polymorphisms. Real-time-(qRT-) PCR is the most sensitive technique for MRD detection: it allows detecting MRD with sensitivities that have been reported in a range of 10^{-4} to 10^{-6} [18-21]. QRT-PCR is now extensively being studied as approach for MRD detection. Common targets for molecular MRD monitoring, including fusion genes, overexpressed genes and gene mutations, will be reviewed in this section.

2.2.1. Fusion genes

Fusion genes are among the best potential targets for molecular MRD detection. In AML the most common chromosomal rearrangements, producing fusion genes, are t(8;21), t(15;17) and inv(16)/t(16;16). The corresponding fusion genes are *AML1-ETO*, *PML-RARα* and *CBFβ-MYH11*, respectively. Depending on geographics, these occur in about 15%-45% of all AML cases and are associated with favorable prognosis [22]. Molecular MRD studies performed for *AML1-ETO* and *CBFβ-MYH11* are relatively scarce and have included relatively few patients.

In t(8;21) rearrangement, the AML1 gene on chromosome 21 fuses with the MTG8(ETO) gene on chromosome 8 to produce the fusion gene *AML1-MTG8*, also called *AML1-ETO* [23]. Tobal *et al.* studied 25 t(8;21) patients and demonstrated a 2 to 3 log reduction in the level of *AML1-MTG8* after induction chemotherapy and a further 2 to 3 log reduction after consolidation therapy. In all patients with durable CR (n = 20), *AML1-MTG8* transcripts levels in BM were ≤ 1 x 10^3 molecules/μg (median 227 molecules/μg). On the other hand, levels of ≥ 0.71 x 10^5 molecules/μg were predictive of relapse within 3 to 6 months (n = 5, median 1.49 x 10^5 molecules/μg) [24]. Krauter *et al.* retrospectively studied 37 AML patients with t(8;21) (n = 22) or inv(16) (n = 15) using RT-PCR. Levels of *AML1/MTG8* and *CBFβ/MYH11* were quantified relative to expression of a housekeeping gene. This resulted in significantly lower MRD levels in non-relapsing patients (median 0%, range 0%-1.5%) compared to patients who did experience relapse (median 0.14%, range 0%-15.6%, p<.01). Furthermore, RFS was significantly shorter in patients with high MRD levels (≥ 1% of the pre-treatment value) compared to patients without relapse (p<.001), with a similar trend for OS (p = 0.12) [25]. Guerrasio *et al.* retrospectively studied a cohort of 16 AML patients with *CBFβ/MYH11* rearrangements. Analysis in first CR revealed a significantly higher mean copy number of *CBFβ/MYH11* transcripts in patients who relapsed (mean 151) than in patients with stable remission (mean 9, p<.0001) [20]. Buonamici and colleagues also retrospectively studied 21 patients with inv(16) rearrangements and found that patients who relapsed always had *CBFβ/MYH1*:control ratios > 0.12% during CR (median 0.54%, range 0.12%-7.1%). Patients without subsequent relapse, on the other hand, always had ratios < 0.25% (median 0%, range 0%-0.25%), suggesting a cut-off point of 0.25% above which relapse is probable. Despite these promising results, two patients with MRD levels below the cut-off of 0.25% still experienced a relapse [26]. It thus seems that quantitation of both *AML1/MTG8* and *CBFβ/MYH11* can detect important changes in the level of fusion transcripts and that it can give prognostic information. However, it is important that these results still have to be confirmed in larger studies. Despite the advantages, MRD monitoring using *AML1/MTG8* and *CBFβ/MYH11* is possible in only a minority of AML patients.

More research has been done on the *PML-RARA* transcript in acute promyelocytic leukemia (APL). Although the outcome for APL patients has significantly improved with the development of targeted therapies, including all-trans-retinoic acid (ATRA) and arsenic trioxide (ATO), still 10%-15% of APL patients will suffer from relapse. Therefore MRD monitoring to predict clinical outcome could also be of importance in this subgroup of AML patients. In a large prospective study, including 406 APL patients, MRD, monitored by *PML-RARA* transcript analysis, was used to direct pre-emptive therapy with ATO and to guide use of transplantation. In this study, MRD was identified as most powerful prognostic factor for RFS (HR 17.87, 95% CI 6.88-46.41, p<.0001) in a multivariate analysis. Furthermore, increases in *PML-RARA* transcript levels were used to guide pre-emptive therapy and this resulted in a cumulative relapse incidence of only 5% at 3 years [27]. This showed that detection of APL fusion transcripts after chemotherapy treatment is a valid strategy for MRD guided pre-emptive therapy, a strategy that allows reduction of relapse rates. This approach, however, is only applicable in approximately 5%-30% of AML patients.

Mixed-lineage leukemia (*MLL*) fusion genes, which are the result of 11q23 rearrangements, occur in around 10% of both acute lymphocytic leukemia (ALL) and AML and are associated with adverse clinical outcome [28,29]. MRD detection using *MLL* transcripts is challenging, since up to 50 different translocations, resulting in different *MLL* fusion genes, have been described. Most common 11q23 abnormalities are t(9;11)(p22;q23), t(11;19)(q23;p13.3) and t(6;11)(q27;q23) [30]. Mittelbauer *et al.* studied 209 patients at AML diagnosis and detected *MLL* gene rearrangements in 27 patients (12.9%). The *MLL-AF6* fusion transcript, caused by t(6;11)(q27;q23), was detected by RT-PCR in 6 of those 27 patients. All 6 patients achieved hematological CR, however, only one patient achieved molecular CR and that patient was still in stable CR 33 months after diagnosis. The other 5 patients did not reach molecular CR and they all relapsed 2.6-8.3 months after achieving hematological CR. The authors suggest that a reduction of positive blasts below the RT-PCR detection limit of 10^{-5} to 10^{-6} seems to be a prerequisite for long term CR. Unfortunately the incidence of *MLL-AF6* was only 3% in the whole group of 209 patients, resulting in low applicability of this assay [21]. To summarize, levels of *MLL* fusion transcripts may be useful to evaluate treatment response and predict clinical outcome. However, large prospective studies have to be performed to confirm the clinical importance. A major drawback is the limited applicability due to the relatively low incidence of 11q23 rearrangements, also characterized by high numbers of different translocations.

2.2.2. Overexpressed genes

Since in only a small fraction of patients, fusion transcripts are present, overexpressed genes might offer a potential alternative target for molecular MRD monitoring. Such overexpressed genes are either silenced or expressed at very low levels in normal hematopoietic cells. Commonly overexpressed genes are *WT1*, *EVI1* and *PRAME.* In particular for *WT1,* which has originally been described in the development of Wilms tumor [31], multiple studies have been performed. This gene was shown to be also highly expressed in leucocytes of several hematopoietic malignancies, including AML [32,33]. Although the mechanisms of this overexpression are poorly understood, *WT1* overexpression could be a suitable candidate as molecular marker for MRD monitoring. However, the potential use of this marker may be hampered by the overexpression of *WT1* in normal regenerating BM [34-36]. In a large study by Cilloni *et al.,* 504 patients were studied at diagnosis and *WT1* was found to be overexpressed in 86% of the cases. Of these, 129 patients were analysed during follow-up and it was demonstrated that after the first chemotherapy cycle a larger than 2-log reduction in *WT1* levels was an independent prognostic factor for decreased risk of relapse (HR 0.54, range 0.36-0.83, p =.004). After consolidation therapy low *WT1* levels also predicted decreased risk of relapse (p =.004) [37]. Hämäläinen and co-workers analysed *WT1* expression at diagnosis in BM of 100 AML patients and found no prognostic significance as such (cut-off 9.7%, compared with K562 cell *WT1* gene expression). Although *WT1* expression levels were constantly detectable during the remission period, they nevertheless found that an increase in *WT1* expression levels may be a predictor for relapse [38]. Although these results show that *WT1* overexpression for MRD monitoring is a potentially useful marker that can

be used in the majority of AML patients, despite multiple studies having been performed, the clinical utility of *WT1* monitoring remains somewhat controversial [37,38].

Another potential marker is *PRAME*, a gene originally recognized in melanoma patients, which is expressed in 35%-64% of AML patients [39-41]. In a study by Qin *et al.*, using BM material from 204 newly diagnosed patients, *PRAME* overexpression was found in 55.4% of the cases. In follow-up patients who achieved continuous haematological CR (n = 20), *PRAME* levels had decreased but never reached the normal range in 6/20 patients. All of these patients ultimately relapsed. In seven patients *PRAME* levels decreased down to normal levels, but thereafter rose again above normal values; all of these patients eventually relapsed within 4 months. In the remaining patients (7/20) *PRAME* levels decreased down to the normal range and these patients remained in continuous CR. This suggests that the *PRAME* gene may be a useful marker for MRD monitoring [42]. Although these results are promising, a few potential pitfalls should be taken into account. Both *WT1* and *PRAME* are expressed in relatively high levels in normal hematopoietic cells, which result in high levels of background expression before and after treatment. *WT1* and *PRAME* are thus not highly specific markers, with risk on false-positive results [37,38]. To avoid this, PB may be used as an alternative source of cells, since both *WT1* and *PRAME* levels are much lower in circulating normal PB cells, than in normal BM cells [37,42]. Furthermore, a more general disadvantage for gene overexpression is the risk of RNA degradation during isolation procedures that might result in false-negativity. Since overexpression of *WT1* is more frequent in AML cells than *PRAME, WT1* is probably the most useful target for MRD monitoring.

The ecotropic virus integrations-1 (*EVI1*) proto-oncogene is associated with chromosome 3q26 rearrangements and high expression at diagnosis predicts poor clinical outcome [43]. *EVI1* overexpression has been demonstrated in approximately 8% of the AML patients [44]. To our knowledge, no MRD studies using *EVI1* overexpression have been performed thus far. At least 4 different splice forms have been identified (*EVI1-1A, -1B, -1C and -3L*) [45]. Seen the low frequency of cases with overexpression, *EVI1* based MRD detection would add significantly to MRD detection if all splice variants could be detected. Since *EVI1* positive patients have an extremely poor prognosis it may be suggested that MRD based pre-emptive therapy in *EVI1* positive patients would allow therapeutic intervention at an earlier time point and thereby possibly improve clinical outcome. Future studies will have to confirm if *EVI1* is indeed a useful and stable MRD marker.

2.2.3. Gene mutations

Since fusion genes are only present in 15%-45% of AML patients and overexpressed genes seem to be less specific MRD markers, gene mutations may offer another attractive group of targets for MRD monitoring.

A decade ago, fms-like tyrosine kinase 3 (*FLT3*) mutations were found to be present in approximately 30% of AML patients. Different *FLT3* mutations exist; however, the most common is the *FLT3*-internal tandem duplication (*ITD*) in the juxtamembrane region. This *ITD* results in an extra sequence that varies between 3 and 400 base pairs and is thought to cause a constant activation of the tyrosine kinase receptor, resulting in advantages for cell surviv-

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al. The *FLT3-ITD* occurs in approximately 23% of adult AML patients and is associated with poor prognosis [46-48]. Since *FLT3-ITD* is suggested as potential MRD marker, several studies have been performed to confirm this. Chou *et al.* demonstrated that both OS and disease free survival (DFS) were significantly longer in CR patients who obtained a > 3-log reduction compared to the CR patients with less reduction (OS not reached v. 14.7 months, p =. 016, DFS 7.5 v. 3.0 months, p<.001). Moreover, a > 3-log reduction of *FLT3-ITD* was an independent prognostic factor for DFS (HR 0.264, p =.002) with a trend for OS (HR 0.308, p =.057) [49]. Thus, MRD monitoring by *FLT3-ITD* can provide prognostic information [49-52]. However, a serious limitation of the use of a *FLT3-ITD*, is its instability during disease. In part of the AML samples that harbor a *FLT3-ITD* at diagnosis, it has changed or disappeared at relapse [49,53-55], which would result in false-negative MRD results. Furthermore, because of the heterogeneity in *FLT3-ITD* lengths and molecular sequence, no common qRT-PCR can be developed, which offers a serious problem in regular diagnostics [46,51,56].

Mutations in the nucleophosmin (*NPM1*) gene have also been identified as frequent genetic alterations, occurring in approximately 35% of all AML patients. This mutation occurs most frequently in exon 12 of the gene, resulting in loss of one or both C-terminal tryptophan residues leading to an aberrant localization of the protein in the cytoplasm [57,58]. The presence is strongly associated with a normal karyotype, where *NPM1* mutations occur in approximately 60% of patients [59]. When not accompanied by a *FLT3* mutation, it has been described as a favorable factor in patients with normal cytogenetics [60,61]. Quantitative monitoring of *NPM1* mutations after treatment has shown to give important prognostic information [50,62-66]. Although stability of this marker has been reported in several studies [50,62,63,65], loss of the *NPM1* mutation at relapse has also been found [67,68]. It has to be taken into account that more than 30 different types of *NPM1* mutations have been described, but fortunately two types (type A and type B) are by far the most common [57,58]. It can be concluded that MRD assessment by *NPM1* mutations is a suitable, stable and sensitive marker. However, more prospective studies are warranted to validate these results and to confirm stability of *NPM1* during disease.

CCAAT/enhancer binding protein alpha (*CEBPα*) is a transcription factor involved in the regulation of myeloid differentiation and cellular growth arrest [69]. *CEBPA* mutations have been reported in 8% to 19% of cytogenetically normal AML patients and are associated with favorable prognosis [70-72]. There are two major types of *CEBPA* mutations, including C-terminal mutations that occur in the bZIP domain and N-terminal mutations. Furthermore, some patients carry biallelic mutations, whereas others are heterozygous for different kind of mutations [72]. Although *CEBPA* is a potential suitable target for MRD monitoring, to our knowledge, no studies have been reported thus far. Seen the low frequency of cases with a *CEBPA* mutation, *CEBPA* based MRD detection would have limited applicability in AML patients. Future clinical trials have to demonstrate if *CEBPA* is indeed a suitable and stable marker for MRD detection.

2.3. Clinical applications of MRD

As discussed above, MRD frequency assessment using immunophenotypic and molecular parameters in patients with AML in clinical remission has important prognostic value and can predict forthcoming relapses. Therefore, it would be of potential importance to monitor MRD cell frequency for risk stratification. Current AML risk stratification is based on a number of parameters determined at diagnosis, including origin of leukemia (secondary AML, AML after myelodysplastic syndrome), age, WBC count, and presence of certain cytogenetic and/or molecular aberrancies [14]. Novel AML risk stratification should not only be based on risk assessment at diagnosis, but also on MRD cell frequency as a "response to treatment" parameter. Including MRD in AML risk stratification could help identify CR patients after induction therapy with increased MRD levels and therefore high risk of relapse. For instance, good risk patients with high MRD levels after induction therapy may benefit from allogeneic stem cell transplantation, while on the other hand intermediate risk group patients with low MRD levels could be spared from an allogeneic transplantation and the accompanying toxicity. Especially in this intermediate risk group, MRD monitoring would be of great help, since the prognosis of these patients is difficult to estimate. Therefore, MRD based clinical decision making after induction therapy may contribute to better RFS and OS rates.

Also after consolidation therapy, MRD based clinical intervention is promising. Even after an allogeneic transplantation, still a proportion of 20%-40% of the patients will relapse [73-75]. Therapeutic options in the case of post-transplant relapse consist of withdrawal or decrease of dose of immune-suppressive drugs, or immunotherapeutic intervention with a donor lymphocyte infusion. As these approaches intend to boost the graft versus leukemia effect, they are most effective when the leukemic cell load is small. Therefore early detection of impending post-transplant relapses is essential and would allow immunotherapeutic intervention at a low leukemic burden. The current standard to guide post-transplant treatment is the level of donor chimerism. This refers to the percentage of donor cells in PB or BM and it can be determined using short tandem repeat (STR)-PCR. Although mixed chimerism (< 95% of donor cells) has been associated with a higher incidence of relapse [76,77], patients with full chimerism (> 95% donor cells) can still suffer from relapse [77]. Additional monitoring of MRD levels in these transplanted patients could improve successful prediction of relapse, since MRD analysis directly detects the neoplastic part of the patient cell population, while STR analysis reflects total donor and total patient populations. Multiple studies have shown that MRD monitoring after an allogeneic transplantation indeed correlates with clinical outcome and identifies patients who are likely to relapse [78-81]. Therefore, it can be suggested that MRD based pre-emptive immunotherapy after transplantation could reduce relapse and improve survival. Standardization of treatment, based on MRD and chimerism analysis in the post-transplant period, seems therefore warranted. In conclusion, since MRD frequency assessment gives important prognostic information after both induction and consolidation therapy, it seems likely that using MRD for therapeutic intervention in the post-remission phase might reduce relapse rates en prolong OS. To con-

firm this hypothesis, large prospective studies with MRD based clinical intervention in the post-remission phase are essential.

2.4. Improvement of and alternatives for bone marrow MRD detection

2.4.1. Improvements for immunophenotypic and molecular MRD detection

Although flow cytometric MRD monitoring has many advantages, one of the difficulties is the complexity of MRD analysis. Nowadays, more advanced data analysis programs, that aid to distinguish between normal and malignant cells, are available [82]. This might simplify the analysis and result in more objective results. Notwithstanding the high prognostic value of MRD monitoring, in almost all studies 20%-40% of the patients with immunophenotypic defined low MRD levels still suffer from a relapse [5-7,10,16]. There are several potential reasons for missing these MRD cells. Normal BM cells express LAPs at low frequencies. Counting these cells as leukemic might result in false-positivity. This background expression thus seriously hinders specific identification of leukemic cells. On the other hand, subtracting background levels might under-estimate MRD frequencies and this could result in false-negatives. High specificity and thereby high sensitivity can be achieved when only the most specific immunophenotypic aberrancies are used, i.e. with no expression in normal cells. Inclusion of markers/marker combinations that allow excluding non-specific events in a multi-color approach may increase specificity. This is already shown for the transition of a four to five-color flow cytometric approach [83]. Another explanation for MRD misclassification is low sensitivity of the aberrant immunophenotype. Marker expression may be highly heterogeneous in an AML sample: LAPs may thereby often not be expressed on the total population of blast cells, thereby, at follow up, preventing the identification of all leukemic cells. Improvements can only be expected with the discovery of new aberrancies that cover larger parts of diagnosis blast cells. At present, with the large differences in specificity and sensitivity of LAPs the level of detection of MRD varies between patients: 1:10,000 or even better may be reached in one patient, while in another patient 1:1,000 may be the best attainable. Besides misclassification, immunophenotype shifts can also contribute to false-negative observations. To reduce this, it is recommended to use multiple LAPs for MRD monitoring [6,12,13]. Recently, it has become clear that such shifts may occur through clonal selection: while major molecular clones may disappear upon therapy, minor diagnosis clones may survive chemotherapy treatment, and grow out to relapse [84]. This may be accompanied by immunophenotype changes [84]. More efforts towards recognition of minor clones at diagnosis, that potentially can expand to cause relapse, may identify emerging molecular clones and immunophenotypes instead of disappearing molecular clones and immunophenotypes only. For molecular MRD, in fact most of the pitfalls for immunophenotypic MRD hold here as well. Similar to MFC, multiple molecular MRD studies have reported patients with low molecular MRD levels that still suffer from relapse [25,26,37,38,42]. Underlying causes may include 1) as argued earlier for different LAPs, Q-PCRs for different mutations and fusion genes reach different sensitivities as well; 2) part of the blasts may only be characterized by the molecular aberrancy of interest; and 3) molecular clone shifts occur between diagnosis and relapse. To avoid these false-negative results

different molecular markers, if present in the patient, could be quantified for MRD monitoring. There are no real solutions for these problems unless more generally applicable, specific and stable markers are discovered. Until then, combining as many molecular and immunophenotypic targets may contribute to accurate MRD based prediction of relapse. Another possible explanation for finding false-negative MRD results is the fact that it may not only be the number of leukemic blasts that determines the chance for relapse, but more specifically the number of leukemic stem cells (LSCs). These LSCs can cause tumor outgrowth, thereby leading to MRD and finally resulting into overt disease relapse [85]. Although these stem cells are much less frequent than whole blast MRD, LSC frequency assessment may offer an additional specific and biologically relevant determinant of risk on relapse. In section 3 the role of leukemic stem cells in acute myeloid leukemia will be further discussed.

2.4.2. Alternative parameters for risk stratification

Perhaps the conceptually simplest method to evaluate treatment response is calculating the decrease rate of peripheral blasts shortly after treatment. As shown previously for childhood acute lymphocytic leukemia [86], this may directly reflect the chemosensitivity of individual patients. The big advantage would be that it is applicable independent of the initial immunophenotype of the blasts. To accurately calculate such a blast cell decrease rate, blast frequencies have to be measured every day of chemotherapy treatment. Lacombe *et al.* reported two different modalities to evaluate blast cell decrease: 1) calculation of the blast cell decrease slope by linear regression between day 0 and the first day when at least 90% of the initial blast load has disappeared, 2) assessment of the total time period needed to reach 90% blast decrease. All patients (n = 74) who reached a 90% blast reduction within 6 days achieved CR. The authors also showed a strong correlation for both modalities with patients' clinical outcome [87]. Since leukemic blasts at diagnosis in most cases are present in the PB too, it has been proposed that PB may represent an alternative specific source for immunophenotypic MRD detection. Since aspiration of BM is an invasive procedure, MRD detection in the PB would offer significant advantages over BM-MRD both for patients and physicians. Furthermore, the BM contains immature normal populations that resemble LAPs, while these are thought to be largely absent in PB. The latter would clearly have advantages for the easiness of interpreting MRD. Although MRD frequencies in PB are lower than in BM, PB-MRD frequencies correlated with BM-MRD frequencies and turned out to have prognostic value [88]. Once the value of PB-MRD is validated in other studies and once it has been confirmed that BM-MRD is positive in all PB-MRD positive cases, PB-MRD may replace BM-MRD, provided that PB is MRD positive. In case of PB-MRD negativity, it will probably remain necessary to perform BM acquisition, since BM-MRD is more sensitive. Another alternative parameter for risk stratification is the presence of B-lymphocyte precursors in AML BM. A high level of B-lymphocyte precursors after first CR thereby predicts for DFS [89]. Furthermore, an abnormal high $CD34^+$ myeloid / $CD34^+$ lymphoid ratio ($\geq$ 10) is associated with worse outcome [90]. The development of an algorithm including not only BM-MRD, but also other parameters, including PB-MRD, blast reduction rate, $CD34^+$ myeloid/ lymphoid ratio and the percentage of B-lymphocyte precursors, may contribute to improved accurate prediction of relapses.

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3. Leukemic stem cells and acute myeloid leukemia

3.1. Definition of leukemic stem cells

It was hypothesized that a small population of cells, distinct from the bulk of tumor cells, is responsible for tumor initiation and growth in various cancers, including AML [91,92]. These cells are referred to as leukemic stem cells (LSCs) or leukemia-initiating cells (LICs). It is assumed that similar to normal hematopoiesis, leukemia is hierarchically structured. In many respects LSCs resemble normal hematopoietic stem cells (HSCs). Similar to HSCs, LSCs are defined by their ability to undergo self-renewal and the capacity to differentiate to a limited, although highly variable, extent [93,94]. Furthermore, the immunophenotype of LSCs resembles the immunophenotype of normal HSCs. The majority of HSCs are present in the $CD34^{+}CD38^{-}$ immunophenotypic compartment [95,96] and initial AML studies demonstrated leukemia initiating capacity also to be in the $CD34^{+}CD38^{-}$ compartment [97]. This small subpopulation of $CD34^{+}CD38^{-}$ cells was able to engraft and cause leukemia in non-obese diabetic/sever combined immune-deficient (NOD/SCID) mice. These cells were present at a frequency of only 0.2 to 100 cells per 10^{6} mononuclear cells [97]. Nowadays it is known that AML LSCs can also reside within the $CD34^{+}CD38^{+}$ or the $CD34^{-}$ immunophenotypic compartment [98-102]. There is growing evidence that the transformation of a normal human cell into a LSC not only can occur in a normal HSC, but also in a normal progenitor cell [103]. Mutations in a normal progenitor cell may confer self-renewal properties to progenitors. A recent study demonstrated that $CD34^{+}CD38^{-}$ LSCs, despite the immunophenotypic similarities with normal HSCs, are most related to normal progenitors instead of normal stem cells [102]. In addition, it has been demonstrated that within a patient, the pool of LSCs at diagnosis is often largely heterogeneous. This implies that different subpopulations of LSCs often coexist at diagnosis [84,101] (Figure 1). In CD34 positive patients often both $CD34^{+}CD38^{-}$ cells, $CD34^{+}CD38^{+}$ and $CD34^{-}$ cells are present and all are able to show leukemic engraftment when infused separately in NOD/SCID mice. However, no information exists on possible competition between these compartments in leukemogenesis. Moreover, the $CD34^{+}CD38^{-}$ compartment has been shown to be less immunogenic compared to the other compartments [104], which may explain why it was almost exclusively the $CD34^{+}CD38^{-}$ compartment that engrafted in NOD/SCID mice with residual immunity [97], while in the severely immunocompromised later mouse models, the other compartments engrafted as well. In CD34 negative AML by definition, the $CD34^{-}$ compartment and in particular the $CD34^{-}CD38^{+}$ compartment contain LSCs [100]. For clinical treatment and patient survival it is important to know which putative LSC will survive therapy. In that respect it is important to realize that the $CD34^{+}CD38^{-}$ compartment has been shown to be most therapy resistant *in vitro* [104]. In line with this, it has been reported that in CD34 positive relapsed patients a $CD34^{+}CD38^{-}$ subpopulation is most likely to survive chemotherapy treatment and expand towards development of relapse [84].

In the course of time other compartments enriched for LSCs have been identified. These are based on functional properties and include aldehyde dehydrogenase (ALDH) activity and drug efflux (Hoechst) capacity. ALDH is a group of cytosolic enzymes that catalyze the oxi-

dation of aldehydes. It plays an important role in the retinoid metabolism, since it is required for the conversion of retinol (Vitamin A) to retinoic acids. For maturation, loss of quiescence and differentiation of HSCs, these retinoic acids are important [105,106]. Furthermore, ALDH activity is supposed to protect cells from the toxic effects of cyclophosphamide and therefore high ALDH expression in leukemic cells may play a role in chemotherapy resistance [107,108]. Recently it has been shown that leukemic cells and normal hematopoietic cells differ in ALDH activity. Normal stem- and progenitor cells have high ALDH expression [109-112]. It has to be emphasized that it has recently been demonstrated that the population of cells with intermediate ALDH activity appeared to be enriched for leukemic $CD34^+CD38^-$ cells [113-115]. Several authors have confirmed the leukemia initiating capacity of these cells in NOD/SCID mice [116-118].

Another functional stem cell compartment is the so-called side population (SP). These SP cells are primarily defined by their capability of efficient Hoechst 33342 dye efflux and especially by the way in which fluorescence emission of Hoechst is recorded. In normal BM a population of $CD34^+CD38^-$ cells was found in the SP [119,120]. In AML, it has been demonstrated that the SP compartment contains a heterogeneous population of cells, containing HSCs, LSCs, LSC progenitors and early lymphocytes [121]. AML SP cells have shown to be able to initiate acute leukemia in NOD/SCID mice [122,123]. All these immunophenotypic and functional findings are important for gaining insight in the process of leukemogenesis and especially for the development of new therapies aiming at eradication of LSCs.

Besides the ability of LSCs to initiate and sustain the initial AML, there is increasing evidence pointing towards the importance of LSCs in the occurrence of MRD and the emergence of a relapse. LSCs are thought to be more resistant to standard chemotherapy compared to the total population of malignant blast cells and therefore these LSCs are able to escape apoptosis. Other essential LSC features are their acquired capacity for self-renewal and proliferation. Such properties allow LSCs to survive chemotherapy treatment, to divide and to grow out and cause a relapse (Figure 1). Consequently, identification and characterization of LSCs is fundamental to gain insight in the mechanisms that underlie relapse and how to evade relapse.

3.2. Identification of leukemic stem cells

Since the assumed role of LSC in the emergence of an AML relapse, identification of these probably most malignant cells becomes imperative. The hypothesis would thus be that quantitation of LSCs in AML patients would give important information about treatment response and risk of relapse. Similar to MRD identified by flow cytometry, LSCs in BM can be identified using cell surface antigen expression. As mentioned before, LSCs can reside in different immunophenotypic compartments, but, as argued before, the $CD34^+CD38^-$ defined LSCs may be most malignant/resistant [84,104]. Since both HSCs and LSCs reside within this compartment, discrimination between $CD34^+CD38^-$ HSCs and LSCs is challenging. Immunophenotypic LSC detection is often possible making use of the fact that the lineage marker combinations used for MRD detection, are frequently aberrantly expressed on $CD34^+CD38^-$ cells too [124]. These lineage markers include CD2, CD7, CD11b, CD13, CD15, CD19, CD22

CD33, CD56 and HLA-DR. Combinations of lineage markers could also be used, like CD33+CD13- and CD15+HLA-DR-. Besides these lineage markers, a growing number of other markers is now available to discriminate between LSCs and HSCs. These include CLL-1 CD25, CD32, CD33, CD44, CD47, CD96, CD123 and TIM-3 (Figure 3). An overview of LSC markers is given in Table 3.

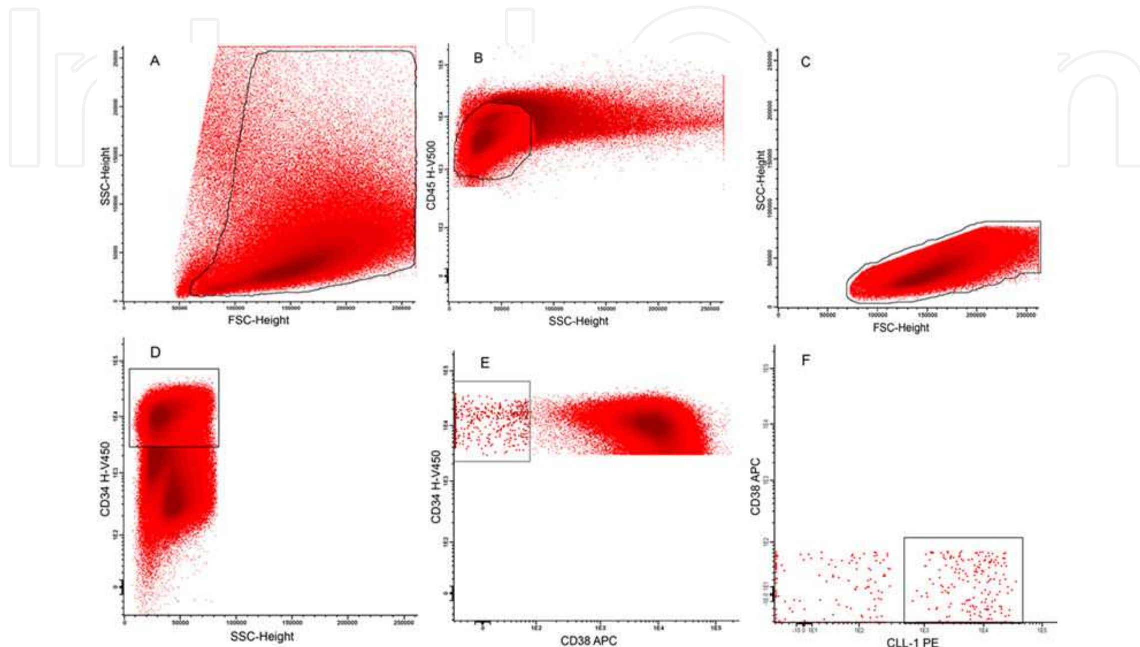

Figure 3. Gating strategy for CD34+CD38- LSC detection at diagnosis in AML bone marrow. Gating of viable white blood cells (A). Gating of blast cells with CD45dim expression and low sideward scatter (SSC) (B, C). CD34 positive progenitors cells (D). Gating of the CD34 positive and CD38 negative blasts (E). The CD34+CD38- cells gated against CLL-1. Two populations of stem cells are shown: a CLL-1 negative stem cell population, containing the HSCs and the CD34+CD38- cells with positive expression of CLL-1. These stem cells with aberrant expression of CLL-1 are defined as LSCs (F).

It is important to realize that there is a large heterogeneity in marker expression. This implies that marker expression differs between AML patients and even within an individual patient different stem cell markers are often differentially expressed (Figure 4). Thus, none of the individual markers are expressed in all AML cases. For accurate LSC detection, high specificity of stem cell markers is essential. Both CLL-1 and lineage markers have proven to be highly specific, since these are present on leukemic CD34+CD38- cells in a substantial part of the AML patient population, but absent on normal CD34+CD38- cells, also after chemotherapy [124,125]. For the other stem cell markers high specificity and stability during treatment/disease still have to be confirmed. The established differences in ALDH activity between CD34+CD38- LSCs and CD34+CD38- HSCs were confirmed using this aberrant marker approach [114,115], thereby strengthening that the functional ALDH assay offers an alternative tool for CD34+CD38- LSC identification, which importantly, could be applied in absence of aberrant antigen expression. In contrast, the SP phenotype does not discriminate between HSCs and LSCs since both may be present in the SP compartment. Here the immunophenotypic marker approach is necessary to discriminate between LSCs and HSCs [121].

Both ALDH and SP assays not only identify leukemia initiating cells with the $CD34^{+}CD38^{-}$ immunophenotype, but also other cell types, like $CD34^{+}CD38^{+}$ progenitors or $CD34^{-}$ cells [114,115,117].

Although functional assays, like ALDH and SP, are complex and time-consuming compared to standard immunophenotypic LSC detection, they may offer promising alternatives for $CD34^{+}$ AML patients without detectable $CD34^{+}CD38^{-}$ cells, as well as for AML patients who are defined as CD34 negative. The latter patients usually have less than 1% expression of CD34 on the leukemic blast cells which all are of non-neoplastic origin [133]. However, also for cases with $CD34^{+}CD38^{-}$ LSCs present, these functionally defined compartments may be important: since the frequency of SP cells is far lower compared to the frequency of $CD34^{+}CD38^{-}$ stem cells [121], an interesting possibility would be that combination of both assays may narrow the real stem cell compartment [121]. In contrast to the immunophenotypic definition, ALDH activity and dye efflux ability are likely directly related to drug response and in that sense may predict which stem cells will survive therapy. Together with the observations that immunophenotypically defined $CD34^{+}CD38^{-}$ cells are *in vitro* therapy resistant too [104] and that most likely a $CD34^{+}CD38^{-}$ subpopulation grows out to relapse [84], the possibility that relapses are caused by functionally defined subpopulations of the $CD34^{+}CD38^{-}$ compartment can be suggested.

Antigen	Function	Reference
CLL-1	C-type lectin-like molecule-1	[125]
Lineage markers	Lymphoid lineage and myeloid lineage markers	[124]
CD25	Interleukin-2 receptor α-chain	[126]
CD32	Fc fragement of IgG, low affinity IIa receptor	[126]
CD33	Myeloid marker	[127]
CD44	Receptor for hyaluronan	[128]
CD47	Integrin associated protein	[129]
CD96	T cell-activated increased late expression protein	[130]
CD123	Interleukin 3 receptor alpha chain	[131]
TIM-3	T-cell Ig mucin-3	[132]

Table 3. Overview of stem cell markers.

Seen the large clonal heterogeneity at diagnosis [84,101], and the possibility that not just the major clone at diagnosis, but often low-frequency $CD34^{+}CD38^{-}$ clones may grow out [84], this suggests that identification of functionally defined minor subpopulations present at diagnosis may offer clues how to predict relapse in a very early stage and thereby ultimately how to circumvent such relapses.

$CD34^+CD38^-$ population was analyzed for the expression of six aberrant markers: CD2 (A), CLL-1 (B), CD22 (C), CD96 (D), CD123 (E), CD11b (F). Expressions percentages for marker positive and marker negative $CD34^+CD38^-$ cells are shown for each marker.

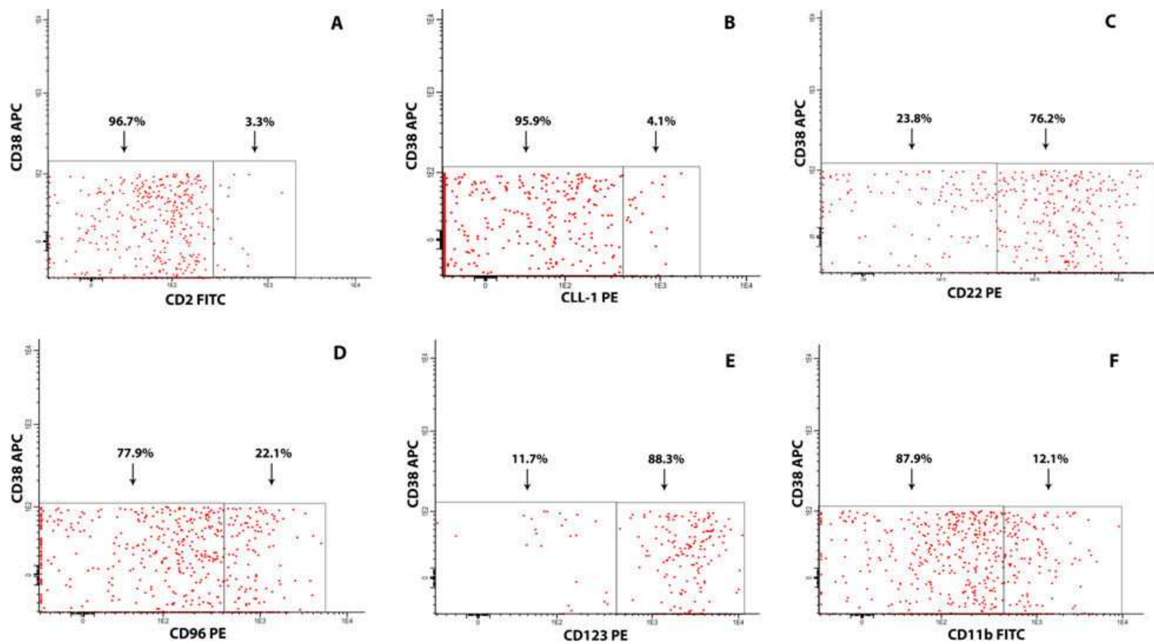

Figure 4. Heterogeneity in expression of different stem cell markers in one AML case at diagnosis.

3.3. Prognostic value of LSC frequency

Since it has been hypothesized that the subpopulation of chemotherapy resistant LSCs is responsible for relapse, LSC frequency, similar to MRD frequency, should have direct prognostic impact.

Van Rhenen *et al.* were the first to study the correlation between the frequency of $CD34^+CD38^-$ cells at diagnosis and clinical outcome in 92 AML patients. In a multivariate analysis, including known risk factors, the frequency of the $CD34^+CD38^-$ compartment (% of blast cells) turned out to be an independent prognostic factor for RFS ($p = 0.004$) and DFS ($p = 0.05$) [134]. In a small group of pediatric AML patients, Witte *et al.* found that the $CD34^+CD38^-$ subpopulation was significantly lower in patients with 5-year DFS ($n = 8$) compared to patients with relapse and/or death ($n = 9$) (0.45% ± 0.61% v. 1.52% ± 1.52%, $p = 0.04$) [135]. Moreover, Hwang and colleagues have demonstrated, in a group of 54 AML patients, that the proportion of $CD34^+CD38^-$ cells at diagnosis was significantly lower in patients achieving CR compared to patients who did not achieve CR (median 0.7% v. 6.9%, $p = 0.006$) [136]. Lastly, using the ALDH activity assay, Ran *et al.* have shown a significant difference in OS and RFS between patients with high and low LSC frequencies (OS, $p = 0.04$, RFS, $p = 0.01$). Multivariate Cox regression for OS showed LSC frequency and WBC count at diagnosis to be the only significant prognostic factors (HR 10.5 for LSCs, $p = 0.05$), with borderline significance for RFS (HR 3.8, $p = 0.05$) [118]. In our recent study we refined the definition

used in aforementioned papers by including markers that enabled to discriminate LSC from HSC [137]. In total, 101 patients were monitored for LSC frequency. Again, differences in prognosis were found between patients groups defined by different cut offs (Table 4.)

	Cut-off	Number of patients above cut-off	Relative risk of relapse	95% C.I.
First cycle	5×10^{-6}	14	5.0	1.8-14.0
Second cycle	5×10^{-6}	18	4.7	2.2-10.1
Consolidation	2×10^{-6}	14	8.5	1.8-41.4

Table 4. Relative risk of relapse defined by LSC frequency [137].

All together, several studies showed $CD34^{+}CD38^{-}$ LSC frequency to be an independent prognostic risk factor. Important to emphasize, however, is that these studies focus on LSC detection and quantification at AML diagnosis. Because LSCs are hypothesized to be chemotherapy resistant and to grow out after treatment and then cause a relapse, it would be of utmost importance to study the frequency of these LSCs during follow-up. For the first time we also demonstrated that the frequencies of LSCs after different courses of therapy significantly correlated with clinical outcome [137]. More effort is needed to identify LSCs and their prognostic value in immunophenotypic compartments other than $CD34^{+}CD38^{-}$, like the $CD34^{+}CD38^{+}$ and $CD34^{-}$ compartment using the ALDH and SP assay. Ultimately, when the clinical importance of different stem cell compartments have been prospectively confirmed, this, together with MRD based strategies, should offer new diagnostic tools to guide clinical intervention and to monitor effectiveness of therapy and, moreover, to design new therapies that specifically target LSCs while leaving the normal HSCs intact.

3.4. Leukemic stem cell targeted therapy

Apart from the clinical application of LSCs, characterization of these malignant cells offers the design of new therapies that specifically target LSCs while leaving the normal HSCs intact. The most direct example of such therapy is the application of antibodies that are used to specifically discriminate between LSC and HSC. CD123 and CD33 are examples. It has been reported, using NOD/SCID mice, that treatment with the anti-CD123 antibody 7G3 improved mouse survival [138]. A humanized version of the anti-CD123 antibody (CLS360) has been studied in a phase 1 study in relapsed, refractory and high-risk AML patients. Interim analysis showed no treatment related toxicity, besides two mild infusion reactions and one infection possibly related to the treatment. Of eight patients treated with CLS360, one CR had been observed [139]. Further clinical studies are needed to determine the efficacy of this antibody in AML patients.

CD33 is expressed on leukemic blasts in 85%-90% of AML patients and therefore, already years ago, it had been suggested as a potential target for anti-AML therapy. The CD33 immunoconjugate gemtuzumab ozogamicin (Mylotarg) has been studied in several trials and,

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after initial disappointment relating to toxicity, new studies with altered treatment schedules suggest that Mylotarg is beneficial in certain subgroups of AML patients, including patients with favorable cytogenetics [140]. However, it is important to emphasize that no studies so far determined the correlation between the efficacy of Mylotarg and the presence of CD33 positive LSCs. It may be speculated that subgroups of patients with CD33 positive LSCs may benefit from this additional therapy. Further clinical trials will also have to determine if other stem cell markers are potential targets as well.

4. Conclusions and future perspectives

MRD frequency assessments by RQ-PCR and MFC in AML patients are more sensitive methods to define remission status compared to current morphologic assessment. Although RQ-PCR is in general the most sensitive technique, MFC is applicable in almost all AML patients. Since the importance of flow cytometric MRD detection has now been validated in a first prospective study, it is of utmost importance that, when these data are confirmed in other prospective studies, MRD status will be implemented in clinical decision-making. We have described that alternatives for BM MRD may include MRD assessment in peripheral blood and blast reduction, frequency of B-lymphocytes precursors and $CD34^+$ myeloid/lymphoid ratios. It thus seems that development of algorithms including all such parameters may ultimately contribute to improved detection of residual therapy resistant cells and early and accurate prediction of relapses. Also, based on the observation of immunophenotypic and molecular shifts, occurring between diagnosis and relapse, a new issue in MRD research may be that not only disappearing phenotypes, but also emerging "new" phenotypes have to be monitored. An alternative, probably more specific method to predict clinical outcome is LSC frequency assessment. Results so far on the clinical importance of LSCs are limited, but very promising, especially since for the first time the correlation between the presence of LSCs after treatment and clinical outcome has been reported. When the value of LSC assessment is confirmed in other retrospective and eventually prospective studies, it may be hypothesized that in the future, not only MRD, but also LSC frequency assessment may be implemented in clinical decision-making.

Hopefully, using the suggested approaches in this chapter, it will become possible to significantly improve clinical outcome of acute myeloid leukemia patients.

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