CHAPTER 7

Summary, conclusions and future perspectives
The focus of this thesis is on the prognosis of patients with AML and the detection of leukemic cells that remain after treatment. The method that is used to detect these cells is immunophenotyping by FACS analysis. This technique allows to confirm lymphatic or myeloid origin of cells, as well as differentiation stage of cells, based on the expression of cell surface lineage markers. This is of importance not only at diagnosis, but also at relapse. Apart from that, aberrant expression of cell surface markers has been shown to be of importance in predicting prognosis of patients with AML, but also to discriminate normal from neoplastic cells [1]. Since AML is a very heterogeneous disease with respect to the cell surface marker “make up” of the cells, every newly diagnosed AML needs to be analyzed for aberrantly expressed marker combinations, to assess a unique immunophenotypic fingerprint. This unique fingerprint can then be used to detect leukemic cells among normal blasts in the bone marrow of patients treated with high dose chemotherapy. Minimal residual disease (MRD) is the term for the bulk of leukemic cells that remain in the bone marrow from patients who are morphologically in remission. Since MRD is the resultant of many resistance mechanisms, present both at diagnosis and revealed during treatment, it is generally anticipated that MRD may become the strongest predictor of relapse. During the last two decades there has been increasing interest in the role of the leukemic stem cell (LSC). It has been hypothesized that a primitive cell, resembling the hematopoietic stem cell, is at the origin of AML and, by virtue of inherent therapy resistance, is also responsible for relapse after treatment. Following our previous research, we have focused on the immunophenotypically defined (CD34+CD38-) stem cell [2;3], to further fine-tune this LSC compartment by using other flowcytometric parameters. By using a gating strategy that included all parameters, we assessed whether this compartment too has clinical impact in AML.

Chapter 1 provides a general overview of the literature regarding AML research. First, hematopoiesis and general features of AML are discussed as well as treatment and prognosis in AML. The last two sections are dedicated to MRD and leukemic stem cells. Here, technical aspects and possible clinical impact of MRD and leukemic stem cells in AML are discussed.

In chapter 2 we showed that immunophenotyping is of large value in acute leukemia, not only at diagnosis, but, since often lineage switches from AML to ALL or the other way around occur, also at relapse. Lineage switches can not always be detected by morphology. The patient described in chapter 2 showed a complete immunophenotypic switch from ALL to AML which was not easily recognized because morphology and chromosomal abnormalities were similar at relapse and diagnosis. This case is of importance, since treatment regimens had to be adapted accordingly. This case emphasizes the clinical importance of flowcytometric analysis at relapse, but preferably even earlier. Also, such immunophenotypic “switches” may be at the basis of false negative observations encountered in MRD studies.

CD25, the alpha chain of the IL-2 receptor, is a cell surface marker that can be detected by
flow-cytometry on leukemic blasts in the majority of AML patients. In chapter 3 we show that CD25 is of prognostic value: positivity for more than 10% at diagnosis predicts adverse outcome for AML patients below the age of 60, which was recently confirmed by others in a larger patient cohort [4]. The adverse prognosis was seen both in the whole young adult patient group and in the subgroup with intermediate cytogenetic risk, where the largest uncertainty is seen regarding the most optimal treatment. The expression of CD25 might aid here in clinical decision making. Since CD25 expression at diagnosis is also predictive for higher levels of MRD, as measured using other markers, the adverse prognosis might be due to higher levels of MRD. Although CD25 expression on AML blasts can be as high as 90%, CD25 turned out not the most suitable marker to discriminate normal from leukemic cells: at diagnosis expression on normal blasts may be 10%, while after hematopoietic reconstitution following treatment: CD25 is up-regulated on normal cells. Therefore MRD detection using this marker is not recommended.

Previous retrospective research by us and others [1;5-9] showed that MRD as detected using aberrant expression of a variety of cell-surface markers, is a strong predictor for clinical outcome. MRD is generally assessed in bone marrow (BM) aspirates and expressed as a percentage of total WBC count. In chapter 4 we show that peripheral blood (PB) contains five times lower MRD cell frequencies as compared to BM. We therefore hypothesized that MRD in BM can be under-estimated in cases with substantial contamination with PB. Hypothetically such can be avoided when considering primitive leukemic cells as a fraction of the total population of primitive cells. (aberrant primitive cells, aPC) instead of as a percentage of total WBCs (MRD). In the relative absence of normal primitive cells in PB at follow up, the aPC fraction in the BM may largely be independent of contamination with PB. We showed that this approach identifies not only MRD positive poor prognosis patients, but also poor prognosis patients who are MRD negative, but aPC positive. Since not all AML cases have primitive markers, we concluded that, in cases with a primitive marker present, relapse-free survival is best predicted when combining standard MRD percentage with aPC fractions.

Although the prognostic value of MRD in adults was shown in many retrospective studies, including our own, this had not yet been confirmed in using prospective studies. In chapter 5, we described the results of the first prospective study on the prognostic value of MRD, as defined by immunophenotyping, in adults under the age of 60. We used the results obtained from analyzing 517 patients enrolled in the HOVON/SAKK AML42a study. In total, 471 MRD time points, obtained from 389 patients, were evaluated (164 after induction cycle I, 183 after cycle II, and 124 after consolidation therapy), A cut-off of 0.1% was used to distinguish MRD positive (MRD+, >0.1%) from MRD negative patients (MRD-, ≤0.1%). After induction therapy, as well as after consolidation therapy, this cut-off distinguished between MRD- patients with relatively favourable outcome, and MRD+ patients with adverse outcome. Also in the clinically most interesting subgroup with intermediate risk, MRD cut-offs
distinguished two such patient groups. Since decisions about consolidation treatment have to be made after induction cycle 2, we investigated whether at this time point, MRD was an independent prognostic factor. Multivariate analysis after induction cycle II confirmed that high MRD values were associated with a considerably higher risk of relapse even after adjustment for consolidation treatment time-dependent covariate risk score and early or later achievement of CR.

In chapter 6, the role of leukemic stem cells (LSCs) in diagnosis and remission BM was investigated. The expression of CD34 and absence of CD38 was used to immunophenotypically define the leukemic stem cell. Since normal hematopoietic stem cells too show this immunophenotype, aberrantly expressed markers were used to discriminate between both cell types [3;10]. However, these markers do not cover LSCs in all AML cases, while, in addition, in individual cases, partial exression on the LSC compartment is also seen. This could lead to underestimation of LSC frequency in such cases. In this chapter, we describe a multi-parameter approach using different flowcytometric parameters to identify LSCs and which include marker aberrancies, cell size and granularity. Using this flowcytometric approach, LSC and HSCs can be discriminated at diagnosis, as we confirmed by molecular assays, and in vivo assays using NOD-SCID IL-2Rγ-/- mice. Putative HSCs, purified by FACS cell sorting, showed no molecular aberrancies and were capable of multilineage engraftment in mice, while purified LSCs showed molecular aberrancies and caused leukemic engraftment. By using this gating strategy, LSC could not only be detected at diagnosis, but also in remission BM. LSC frequencies provided robust prognostic information, potentially valuable for risk stratification-based treatment: high LSC percentages led to higher relapse rates as compared to patients in whom no or very low levels of LSCs were detected.

**Future perspectives**

The prospective study showing the prognostic value of MRD described in chapter 5 paves the way for implementation of MRD in clinical AML studies. Besides prognostic factors defined at diagnosis AML, based on MRD percentages, clinical decisions (i.e. whether to perform an allogeneic stem cell transplantation) can also be made depending on the MRD status of patients after induction therapy. This is especially important for patients with favorable and intermediate risk based on the molecular and cytogenetic profile as assessed at diagnosis. MRD positive patients in this group might benefit from allogeneic stem cell transplantation to improve survival. Thus based on our findings, MRD measurements should be implemented in AML treatment. It also could be used in allogeneic transplantation, e.g. in timing of immune-suppression or donor lymphocyte infusions. Lastly, it may serve as a surrogate endpoint in trials investigating new drugs. In chapter 4, we showed that MRD can be measured in peripheral blood in lower quantities as compared to BM. Monitoring MRD in PB as an alternative source to BM has previously
been proposed by Maurillo et al. [11]. Similar to our data, in their study too the levels of leukemic cells were lower in PB, but with a good correlation between BM and PB. In addition, in that study both BM-MRD and PB-MRD inversely correlated with relapse-free survival. These data are very promising, since PB is more easy accessible as compared to BM, allowing multiple sampling time points with much less discomfort for patients. In addition, the background levels of immature cells are close to zero in normal PB, which makes interpretation of acquired data much easier and better reproducible.

The bulk of MRD cells is composed of a heterogeneous population of cells, often largely differing in levels of differentiation. For MRD assessment, extensive experience in interpreting normal BM differentiation patterns is required. It has been hypothesized that only the most primitive cells within the blast fraction are at the basis of outgrowth to relapse. We showed that monitoring LSCs in the CD34+CD38- compartment, as compared to the total malignant blast population, indeed offered an accurate prognostic marker. This indirectly confirms that the CD34+CD38- compartment is an important leukemia initiating compartment. The latter is important since other compartments (CD34+CD38+, CD34-) have also shown to contain leukemia initiating ability in animal experiments [12]. Related to this, compared to MRD monitoring, CD34+CD38- monitoring is only possible in patients with a CD34+ AML containing CD38 negative cells. It is likely that in other cases, CD34+CD38+ and CD34-LSCs have to be considered.

Compared to total blast MRD, an advantage of LSC monitoring is its specificity. By using a backbone of markers (CD45, CD34 and CD38) only one lineage marker or aberrantly expressed marker needs to be added when combined with scatter parameters to distinguish these cells from HSCs. A disadvantage of “LSC-MRD” is that very low frequencies, which demand more cells to be analyzed and extra statistical considerations. The combination of MRD and LSC monitoring may offer the most optimal parameter to guide future intervention therapies. The prognostic value of LSC detection should anyhow first be proven in a prospective setting.

In addition to prognostic relevance, a better characterization of the cell surface antigen make up of the LSC might provide new leads for LSC therapy. The study described in chapter 6 reveals that individual LSC markers found to be expressed in individual AML cases are absent in other cases or may miss substantial portions of LSCs present in a particular AML case. Although with the use of additional markers and additional parameters this may not be an objection for specific detection of LSCs, it nevertheless highlights that antibody-mediated LSC directed therapy targeting a specific antigen, will not cover all AML cases and, moreover, often will not target every LSC present in a single AML case. The prospective isolation of both LSCs and HSCs from one patients’ BM will provide the opportunity to further explore the possibilities for a broader applicable antibody-mediated therapy. Moreover, it may lead to discovery of other LSC-specific targets. The ultimate goal would be to use this knowledge to develop highly specific anti-LSC therapies that eradicate LSCs, while sparing HSCs.
Reference List


