CHAPTER 1

General introduction
1.1 Hematopoiesis

Hematopoiesis, or the production of blood cells, takes place in the bone marrow (BM) and is organized as a hierarchy. Hematopoietic stem cells (HSCs) are at the origin of the hematopoietic system and are the most immature blood cells. HSCs can divide to produce two daughter cells. One daughter becomes a more differentiated progenitor cell, while the other daughter remains an HSC. The latter process is referred to as self-renewal, which is one of the properties of stem cells. Progenitor cells, in their turn, differentiate further and form mature leukocytes, erythrocytes and thrombocytes (Figure 1). These cells have their function in immunity, oxygen transport and blood clotting, respectively. All these stages of differentiation can be analysed by morphology, where cells are stained and evaluated under a microscope. Besides morphology, the different stages of maturation can also be identified by the expression of cell surface antigens. The most primitive HSCs and progenitor cells express CD34, which is down regulated upon maturation of leukocytes. HSCs and progenitor cells can be distinguished based on the expression of CD38: HSC lack CD38 and as cells differentiate into more committed progenitor cells, CD38 is gained. The lymphatic cell lineage is characterized by the expression of CD19 on B-lymphocytes and CD3 on T-lymphocytes. Myeloid cells also show lineage restricted markers: CD13 and CD33 are expressed on all myeloid cells, while high CD14 expression characterizes monocytes and granulocytes are defined by a high expression of CD15.

![Figure 1](image-url) Normal hematopoiesis with animated morphology. Cell surface marker expression is shown for each cell type.
Since HSCs are relatively scarce and the production of new blood cells is completely dependent on HSCs, these cells are kept under special conditions. HSCs reside in specific locations in the bone marrow (BM) referred to as niches. In this microenvironment the process of differentiation and self-renewal is tightly regulated [1] by different cell types (macrophages, fibroblasts, osteoblasts and adipocytes) which produce signalling molecules, growth factors and cytokines.

### 1.2 Acute myeloid Leukemia

Acute Myeloid Leukemia (AML) is an aggressive malignant disease characterized by an arrest in differentiation of cells of the myeloid lineage. The differentiation stop leads to an accumulation of progenitor cells in the BM, which leads to a shortcoming of functional leukocytes, erythrocytes and platelets. Patients therefore present with symptoms of infections, anaemia and haemorrhage. Although impaired hematopoiesis in patients with AML is often attributed to the “crowding out” of normal progenitor cells, this is also seen in patients with a relatively low tumor burden. It has been shown that leukemic proliferation alters the stromal microenvironment and creates malignant niches that outcompete the niches of normal HSCs for CD34+ engraftment [2]. The aetiology of AML is largely unknown, however, the development of AML has been associated with several risk factors including genetic disorders such as Down syndrome as well as exposures to viruses, radiation, chemical or other occupational hazards, and previous chemotherapy [3-6]. AML presents at all ages, but the disease increases progressively with age and the highest incidence is found in patients above the age of 60. The incidence ranges from 0.7 to 3.9 cases per 100,000 between 0 and 60 years and from 6.7 to 19.2 cases per 100,000 above 60 years [7].

The diagnosis AML and prognosis of the disease is based on the outcome of different diagnostic investigations:

**Morphology**

The classical method to diagnose AML is by morphology. Blood and BM smears are examined under the microscope after a May-Grunwald-Giemsa or a Wright-Giemsa stain. It is recommended that at least 200 leukocytes on blood smears and 500 nucleated cells on bone marrow smears are counted [8]. A bone marrow blood blast count of a minimum of 20% is required for a diagnosis AML. There are, however, exceptions to this rule. When chromosomal aberrancies t(15;17), t(8;21), t(16;16) and inv(16) are detected, the diagnosis AML is set despite of the blast count. Cytochemistry can be used to identify lineage involvement by staining cells with myeloperoxidase (MPO) or Sudan Black. Positivity for MPO indicates an acute leukemia with myeloid differentiation. Absence of MPO, however, does not exclude myeloid leukemia since immature cells may lack MPO. Sudan Black staining parallels MPO but is less specific.
Immunophenotyping

Immunophenotyping is mainly used to determine lineage involvement in newly diagnosed acute leukemias. Multiparameter flowcytometry is used to evaluate the expression of cell-surface antigens on the malignant cell population. If a marker is expressed on 20% of the cells, the leukemia is considered positive for the marker, which aids in identification of the cell lineage assignment [9]. This is especially important when a mixed phenotype acute leukaemia (MPAL) is considered [10]. The amount of lineage specific markers and the level of expression is determinative whether a leukemia is of myeloid, B-lymphoid, T-lymphoid or combined origin. In Acute Lymphoid Leukemia (ALL), predominantly markers of the lymphoid lineage are found on the immature cells, like CD3 in the cytoplasm of immature T-cells in case of a T-ALL and often CD79a and CD10 are seen when it concerns a B-ALL. Predominantly myeloid markers are found in case of an AML. Quantification of lineage marker expression is also used to define Leukemia associated phenotypes (LAPs) for minimal residual disease (MRD) detection (see paragraph 3).

FAB en WHO classification

Since 1976 AML has been classified according to the French-American-British (FAB) group. Although this classification is not the common standard anymore, publications often still provide the FAB divisions. In 2001 the World health Organisation (WHO) introduced a new classification for leukemias and lymphomas. The most recent WHO classification in 2008 categorizes AML not only based on morphology and immunophenotype, but also based on cytogenetic and molecular abnormalities to create more homogeneous subgroups.

Cytogenetics

Cytogenetics is now a standard assay at diagnosis for the classification of AML [8]. Several cytogenetic aberrancies, including t(8;21) and inv(16) are considered sufficient to diagnose a patient with AML, even when the morphological blast count in BM is less than 20% [9]. Numerous cytogenetic aberrancies have been identified and many of them not only are diagnostic markers for specific AML subtypes but are also identified as independent prognostic factors for the achievement of CR and survival duration [11;12]. The karyotype of the leukemic cells is the strongest prognostic factor for response to induction therapy and for survival [13]. Based on their cytogenetic profile, patients are categorized into favorable, intermediate and adverse prognostic risk groups to guide treatment. Cytogenetics in AML and the impact on prognosis is discussed in more detail in paragraph 2.2. 40% to 50% of the AML patients do not show any chromosome abnormalities [14;15]. These patients are considered cytogenetically normal (CN) and are categorized into the intermediate-risk prognostic category because of their CR rate, relapse risk, and survival. However, this patient group is still very heterogeneous when regarding the molecular aberrancies. Indeed, during the last decade, several gene mutations and changes in gene expression have been discovered that strongly affect clinical outcome of CN AML patients.
Molecular analysis
Numerous gene mutations and deregulated gene expressions have been identified reflecting the enormous heterogeneity in cytogenetically defined AML subsets, especially for AML with normal karyotype [8]. The discovery of such mutations has lead to the re-classification CN-AML patients who formerly would have been grouped as intermediate risk. Molecular analysis in AML involves the detection of fusion genes like CBF-MYH11 and MLLT3-MLL, somatic mutations like in NPM1, FLT3, CEBPa and MLL and deregulated gene expression like in WT1 and EVI-1. These molecular aberrancies and their prognostic impact are discussed in more detail in paragraph 2.2. Because of their prognostic impact, it is now recommended to include molecular analysis in the initial work up of a patient with newly diagnosed AML.

1.3 Treatment

Treatment of AML is divided into 2 phases: induction therapy and consolidation therapy. The current “standard of care” for AML patients involves an initial phase of intense chemotherapy (induction) followed by post-remission treatment (consolidation), including additional chemotherapy cycles and/or auto/allogeneic stem cell transplantation. From a clinical perspective, treatment for adults with AML differs for younger patients (from 18 years to 60 years of age) and for older patients (from 60 years of age), since more and more evidence indicates that in older patients, the leukemia-specific differences account for reduces treatment outcome [16].

Induction therapy
The achievement of a complete remission (CR) of AML is a prerequisite for a good prognosis. Therefore, the first part of AML treatment, remission induction therapy, is aimed at inducing CR. CR has been traditionally defined as the presence of less than 5% abnormal blasts in the BM, the absence of circulating blast and extramedullary AML, recovery of neutrophils count to $1.5 \times 10^9/l$ and platelets recovery above $100 \times 10^9/l$ in the peripheral blood [17;18]. The traditional treatment for AML/high risk MDS patients is based on a combination of cytarabine (cytosine arabinoside or Ara-C) and an anthracyclin (daunorubicin or idarubicin [17;19]. Most centers apply treatment schedules based on these drugs, sometimes supplemented with etoposide. Instead of anthracyclines, induction therapy may include mitoxantrone and amsacrine. The dose of cytarabine that is administered to patients varies from low dose (100-200 mg per square meter body surface) to high dose (up to 3000 mg/m2). It has been recently published that no significant differences was found between patients receiving an intermediate-dose of cytarabine (400 mg/m2) and patients receiving a high-dose, with respect to CR rates, relative risk of relapse and event free survival at 5 years. With the most optimal induction therapy regiment, CR is currently achieved in 60% to 80% of the younger patients [18;20]. However, the majority of patients with AML are 60 years of
age or older, and in this age group, the CR rate is only around 50% [21]. Standard therapy for these older patients has been a combination of daunorubicin (45 to 50 mg/m2 for 3 days) plus cytarabine. Recent data shows that daunorubicin in a higher dose (90 mg/m2), with the entire dose administered in the first induction cycle, leads to a higher response rate than with the conventional dose, without additional toxic effects [22]. CR rates of 64% are now reached in this patient group.

**Consolidation therapy**

Induction therapy alone is usually not sufficient for the maintenance of CR. Without more treatment (consolidation therapy), residual leukemic cells in the bone marrow are likely to cause a relapse of the disease. After achieving CR through induction chemotherapy (either after one or two cycles), the next step is to consolidate this remission by eradicating potential residual leukemic cells by additional chemotherapy (high dose cytarabine), autologous hematopoietic stem cell transplantation (HSCT) or allogeneic HSCT [8]. Autologous HSCT as a postremission strategy is considered as an alternative option for postremission high dose chemotherapy for patients with adverse-risk or intermediate risk AML [23]. With this form of therapy, patients donate their own stem cells which are stored in a freezer. Subsequently, a high dose of chemotherapy and/or radiotherapy, is given with the intention of eradicating the malignant cell population, followed by a transplantation of the initial donated stem cells.

For high risk AML patients, allogeneic HSCT is the treatment option of first choice. The transplant is obtained from either an HLA identical sibling donor, or a matched unrelated donor. The immune-mediated graft-versus-leukemia effect of the transplant reduces the risk of relapse and improves relapse free survival [24]. However, higher mortality and morbidity due transplant-related complications as infection and graft vs host disease annul the benefits of a reduced risk of relapse. Therefore, allogeneic stem cell transplantation is usually not applied to patients with favourable prognostic cytogenetics. Once CR has been achieved, there is no standard post remission strategy for patients of 60 years and older.

**2 Prognostic factors in AML**

Predictors of response to therapy in AML include patient related factors, disease related factors and treatment dependent factors. Patient and disease related parameters are factors that are identified at diagnosis. Based on these parameters, patients can be classified an treated according to the risk of survival outcome. Treatment dependent factors are factors assessed after therapy, providing information on response to treatment.
2.1 Patient related parameters

Age
Although AML presents at all ages, it is mainly a disease of the elderly. The highest incidence is seen in patients above the age of 60 years. A report by the German AML Cooperative group evaluated a cohort of patients aged 16-85 years old. In a multivariate analysis of prognostic factors, age above the year of 60 was found to be an independent adverse prognostic factor with regard to the achievement of CR and OS and RFS [25]. There are multiple factors that might underly this adverse prognosis of the elderly patient. AML in older patients is more frequently preceded by a myelodysplastic phase and is associated with a multidrug resistant phenotype [26].Poorer outcome has been considered to be the result of concurrent comorbidities, a higher likelihood to of underlying hematopoietic disorders, and biologically poor risk disease. Older patients are more likely to have unfavourable cytogenetics [27]. Moreover, because of the poor performance status of the elderly patient, clinicians are less likely to treat these patients aggressively. Therefore, lower levels of aggressive treatment may compound underlying prognostic differences associated with patient factors and disease biology [28].

2.2 Disease related parameters

WBC count
In several large prospective studies of previously untreated patients with AML, the WBC count stands out as an independent prognostic factor. Patients presenting at diagnosis with WBC count of more than 100 x10^9/l who generally have a significantly reduced complete response rate and a greater rate of relapse [29].

Cytogenetics
Certain cytogenetic mutations have been associated with favorable, average, or adverse treatment outcome [11;12;30]. Based on cytogenetic aberrancies, three risk groups of AML patients can be distinguished: a group with favorable outcome, a group with intermediate prognosis and patients with adverse outcome [13;31]. Translocations t(8;21) and t(16;16)/inv(16), resulting in expression of the anti-differentiation fusion proteins AML1/ETO and CBFB/MYH11, respectively, are among the most common cytogenetic aberrations found in patients with AML and are associated with a favorable outcome [32]. This patient group shows a relatively low relapse rate (<25%) and a 4-year survival of at least 70% [18]. Patients with either 5 unrelated abnormalities, monosomy 5 or 7, deletions in the long arm of chromosome 5, or 3q abnormalities were defined as adverse prognostic [19]. This patient group shows a relapse rate of more than 70% and a 4 year overall survival rate of less than 20%. Patients with normal karyotype, or chromosomal abnormalities not enclosed in the
favourable or adverse risk groups, were defined as intermediate risk. The relapse probability in this group is around 50% and and 4-year overall survival is 40% to 50%. In 2008, patients with a monosomal karyotype were added as an extra risk category: patients with 2 or more autosomal monosomes or 1 autosomal monosomy in combination with at least 1 structural chromosomal abnormality showed a 4-year OS of only 4% and are therefore considered as having a very poor prognosis [33].

**Molecular analysis**

The fact that AML is a heterogenous disease is strongly reflected by the numerous different (combinations of) genetic mutations in cytogenetically normal AML (Figure 2). The most widely used molecular markers in relation to prognosis are discussed here.

**FLT3**

FMS-like tyrosine kinase 3 -internal tandem duplication (FLT3-ITD) is a type I transmembrane protein expressed on the surface of hematopoietic cells that stimulates growth of immature myeloid cells and stem cells. The internal tandem duplication of the FLT3 gene is one of the most common genetic abnormalities in AML, mainly found in CN-AML. The frequency of FLT3-ITD in adult AML is 24% [34]. The expression of FLT3 is a be significant
prognostic factor for poor outcome in patients less than 60 years old. In adults with AML from 16 to 60 years with otherwise normal cytogenetics, presence of FLT3-ITD significantly decreased the length of remission and overall survival [35], especially in the absence of an NPM1 mutation [36].

**NPM1**
Nucleophosmin-1 (NPM1) is a nucleocytoplasmic protein that shuttles between cytoplasm and nucleus. NPM1 mutations are found in 30% of the AML patients. In several studies, the presence of the NPM1 mutation in CN-normal AML has been associated with a higher CR rate and prolonged RFS [37;38]. Almost half of NPM1-mutated cases also have FLT3 mutation (33%). Despite this association, patients with an NPM1 mutation show favorable response to induction chemotherapy [39], which is most pronounced in AML patients with wildtype FLT3 [38].

**CEBPα**
C/CAAT enhancer binding protein alpha (CEBPα) is a transcription factor with a key role in hematopoiesis by regulating granulocytic differentiation. CEBPα mutations show a low incidence (approximately 10%). About one third of the AML patients with CEBPα mutations also have a FLT3-ITD. Data suggest that AML patients with a normal karyotype and a CEBPα mutation, lacking a FLT3-ITD is considered favorable risk, remaining cases should be categorized in the intermediate risk AML category [40;41].

**WT1**
The tumor suppressor gene WT1 (Wilms’ tumor gene) encodes for a zinc finger DNA-binding protein with predominantly transcription repressing properties. WT1 has been shown to be expressed in the vast majority of patients with acute myeloid leukemias (AML). Bergmann et al showed that high levels of the WT1 mRNA were associated with poor long-term outcome [42], while others did not find that correlation [43]. Mutations in WT1 were found in 12.6% of 617 younger adult AML patients, and were of prognostic in FLT3-ITD positive AML patients [44].

**EVI-1**
Overexpression of the oncogene EVI-1 (ectopic virus integration site 1) is found in the minority of AML patients, but identifies a distinct subgroup of patients with very poor outcome [45].

**Cell surface markers**
Several aberrant immunophenotypes are associated with the presence of genetic aberrations. CD34 negativity is common for NPM1 mutated AML together with a strong expression of CD33. The aberrant expression of CD19 and CD56 are often observed in AML with t(8;21)
and AML with t(15;17) shows the CD34- HLA-DR- phenotype. It is unknown whether the aberrant immunophenotypes are just bystander effect or offer a functional contribution to the observed poor or favourable prognosis. Several cell surface markers have shown to be of prognostic value.

**CD7**
CD7 normally expressed on T-cells and is often found as an aberrantly expressed cell-surface marker on AML, where it is associated with adverse outcome. In a recent study in normal karyotype AML, CD7 was expressed in 37% of cases and was associated with shorter DFS in a multivariate analysis [46]. Outcome was particularly worse when CD7 was combined with CD14 expression [47].

**CD25**
Expression of the interleukin-2 receptor alpha-chain was observed 63% of newly diagnosed AML. In a multivariate analysis CD25 expression proved to be an independent adverse predictor for OS and RFS [48], with the poorest outcome in patients also positive for a FLT3-ITD. CD25 expression identified a subgroup of patients with poor outcome within the intermediate cytogenetic risk group, indicating a possible role for CD25 in stratifying therapy. Recent research confirmed these findings, and showed that CD25 is correlated with stem-cell gene-expression signatures associated with adverse outcome in AML [49].

**CD34**
CD34 is expressed on immature white blood cells. By many groups, CD34 expression on leukemic blasts has been associated with poor prognosis, although no conclusive evidence for this was obtained in a large meta-analysis of over 2000 patients [50]. The studies evaluated in this analysis all use a relatively high cut-off for CD34 expression of 10-20%, which might conceal the true CD34 negative AML cases. The CD34 negative phenotype is often seen in NPM1 mutated AML [51;52]. Given that the cut-off levels for positivity usually were rather high (10–20%) [50] while NPM1 cases usually showed less than 5% CD34, has probably obscured the prognostic significance of CD34 expression. A lower cut-off in the range of 1-5% has been proposed to be a more predictive cut-off [53].

**CD56**
CD56 is the neural adhesion molecule, which is expressed on natural killer cells, but is absent on the myeloid lineage. Expression on AML blasts is associated with extramedullary leukaemia, multidrug resistance, reduced CR achievement and unfavorable prognosis [54]. In addition, CD56 expression in AML with t(8;21) is associated with significantly shorter CR duration and survival [55;56]. It thereby identifies a poor prognosis subgroup within an otherwise good prognostic patient population.
2.2 Therapy dependent parameters

Although highly significant in prognosis prediction at diagnosis, pretreatment factors cannot capture prognostically relevant variations in efficacy of antileukemic therapy consequent upon patient-related differences in drug metabolism, or provide any information on the patient's response to leukemia [57]. Therapy related factors are assessed after chemotherapy and therefore provide additional prognostic information.

**Complete Remission**
Achieving complete remission (CR) in patients with acute myeloid leukaemia (AML) is the primary endpoint for the evaluation of induction treatment and treatment strategies. Achievement of CR is a well known prognostic factor for AML, and is used as a response criterion for therapy evaluation [58;59]. Although CR with incomplete platelet recovery and CR with incomplete blood count recovery have also been introduced as response criteria [60], CR was found to be the most important factor [59]. In addition, early clearance of circulating peripheral blasts during induction therapy is also an independent factor for RFS [61].

**Minimal residual disease**
The outcome of induction therapy is monitored by morphology, where a level of less than 5% blasts in the bone marrow is considered a successful response to therapy. However, morphological assessment is quite a blunt method to monitor treatment response, as it is insensitive and subjective (with marked inter-observer variation). A more sensitive and objective method for the detection of residual disease would be helpful to separate the “real” responders from those patients who have residual disease at a level below the detection of cytomorphology but who are likely to relapse. The detection of minimal residual disease (MRD) by PCR and flowcytometry will be discussed.

3 Minimal residual disease

To date, cytomorphology is the most commonly used technique to monitor response to therapy. However, this method is not considered very sensitive and is mainly used to determine whether a state of complete remission (CR, less than 5% blasts in the bone marrow) has been achieved. However, even in a state of CR, it is assumed that a significant residual disease burden remains that is too small to be detected by standard cytomorphologic analysis. This so-called minimal residual disease (MRD) is considered to be the initiator of relapse. It has been widely reported that MRD is a strong predictor for survival [62-67]. Therefore, detection of MRD is becoming increasingly important in risk stratification and early detection of relapse in patients with AML. In addition, MRD can also be used as a tool to monitor the response to new therapies. Established MRD assays are based on either polymerase
chain reaction (PCR) amplification of genetic abnormalities or flowcytometric detection of abnormal immunophenotypes. One of the major difficulties in MRD detection in AML is the enormous heterogeneity of the disease in both immunophenotypic and molecular appearance of the cells. This is not only observed between AML patients, but also within a single AML cell can show different phenotypes. Therefore, there is no particular single molecular marker or cell surface marker that can be used to identify leukemic cells in a state of complete remission.

### 3.1 PCR-based MRD

This technique is based on the detection of genetic aberrations, such as fusion genes and mutations specific for that particular AML. Real time quantitative reverse transcription PCR (RQ-PCR) is the most sensitive method for the detection of MRD in AML, since 1 leukemic cell can be identified amongst 105–106 normal cells. The disadvantage of this technique, however, is that the genetic aberrancies have only been identified in subgroups of AML. For many years, molecular MRD has been restricted to the major recurrent translocations (PML-RARA, AML1-ETO, CBFB-MYH11 [68]. In this setting, RQ-PCR has minimal background, as these aberrant transcripts are completely absent in normal cells. RQ-PCR may also be used in patients without fusion transcript, but with other mutant phenotypes, e.g. FLT3-ITD, partial tandem duplication of MLL (MLL-PTD), and NPM1 mutations [69;70]. A disadvantage of FLT3-ITD as a target is that it is not stable during the course of the disease in about 4% of patients, and levels of transcript per cell may increase during disease progression [71;72]. In addition, detection of transcripts that are abnormally overexpressed in AML (e.g., WT1 and PRAME) has been utilized for MRD assessment but these transcripts are not leukemia specific, and are therefore limited in their utility [73].

### 3.2 Flowcytometry-based MRD

With an ability to detect 1 malignant cell in 10,000 normal cells, flowcytometry proved to be also a solid and sensitive tool for the detection of MRD. The major advantage of using flowcytometric MRD detection over PCR-based MRD lies in the fact that this method is applicable to virtually all patients [57]. Immunophenotypic analysis of leukemic cells is an attractive approach for MRD investigation, due to the low cost, relative simplicity and speed. The main disadvantage of flowcytometric detection of MRD is that leukemic cells do not express well-characterized AML-specific cell surface antigens. The ability to detect MRD by flowcytometry therefore relies on the identification of cell surface markers or combinations of markers that are absent on normal immature WBCs. AML progenitor cells often express a combination of cell surface antigens that is not found on normal cells. The abnormal phenotype of leukemic blasts is specific for each AML and this forms its signature, a leukemia
associated phenotype (LAP). Since LAPs are absent or very infrequent on normal BM progenitor cells, malignant cells can be identified amongst normal blasts even in a state of CR [74]. The presence of LAPs on AML cells is therefore basis of flowcytometric monitoring of MRD in AML [64, 75].

**Leukemia associated antigens**
Five different types of LAPs are used to indentify leukemic cells in AML: Cross lineage expression, overexpression of antigens, lack of antigen expression, asynchronous expression of antigens and aberrant light scatter patterns [76]. Cross lineage expression defines the expression of a lymphoid marker on a myeloid cell. A common example of this is the expression of the T-cell marker CD7 combined with CD13 or CD33, which are both common myeloid markers. Over expression of a commonly expressed antigen on myeloid cells is also used as an aberrancy. The lack of expression is seen when, for example, CD13 is expressed and CD33 expression is lacking. Asynchronous expression is the combination of a mature myeloid marker with a primitive myeloid marker, for example CD15 combined with CD34, a combination that is not found on normal progenitor cells. The quality of a LAP depends on the following parameters: Specificity, sensitivity, and stability. Specificity of a LAP is measured by the background expression: the percentage of LAP expression on normal BM cells. To determine the amount of background expression of the different LAPs on normal cells, normal BM cells from healthy donors are used. However, such analysis can still produce misleading results. In clinical practice, samples that contain residual leukemic cells are not compared with steady-state healthy BM. Instead, bone marrow samples are used that are regenerating after heavy chemotherapy. The expression of presumed leukaemia-specific phenotypes can be very different in such samples compared to steady-state healthy bone marrow [77]. The specificity can be increased by using more markers to form a LAP. A LAP is considered highly specific when the background expression is less than 0.1%. Low specific LAPs are mostly those without a primitive marker in the marker combination (CD34, CD117, CD133). The higher the specificity of a LAP is, the better the quantification of MRD at lower levels can be. Currently, this accurate quantification is limited below 0.01%. The sensitivity of a LAP depends on the percentage of leukemic blasts that are positive for the LAP at diagnosis. Due to the phenotypic heterogeneity of AML, it is often not possible to include all the AML cells into one LAP. The part of the cells that cannot be covered by a LAP is then excluded from analysis, since these cells cannot be discriminated form normal progenitors in regenerating BM after chemotherapy. Monitoring MRD with LAPs requires a stable expression between diagnosis and relapse. In AML, phenotypic switches between diagnosis and relapse have been known to occur [78, 79]. Certain LAPs are known for their instability. CD19 expression is known to disappear during the course of the disease, which makes using this LAP sensitive to false negative MRD results. To avoid false-negative results, it is therefore strongly recommended to use as many independent LAPs as possible. This can be accomplished since often more than one LAP is present at diagnosis [64].
Different methods have been described to assess a LAP for the purpose of MRD detection. The method of Feller et al. is a double step procedure [64]. First, a standard screening panel of different monoclonal antibodies (mAbs) (6 tubes each with four colors) are used to determine the immunophenotype of the leukemic cells. From this, one or more putative 4-color LAPs are defined, always including CD45 as a WBC marker. Using CD45 allows to easily detect blasts, since there is a clear differential expression seen as compared to other cell populations. If possible, a primitive marker is included in the LAP as well. Second, the validity of putative LAPs is confirmed in a second step where four markers are combined within one tube (so-called second run). The LAP positive population should be at least 10% of all diagnosis leukemic blasts in order to reach a minimal sensitivity. In follow-up material, the established LAPs are used with the same combinations of antibodies as used in the second run to determine the possible presence of MRD. Other groups have shown similar methods. The group of Venditti also use a two step method to assess a LAP, but only LAPs were used if present on at least 50% of the cells at diagnosis [67;80]. One LAP consisted of a minimum of 3 colors and at least two LAPs were selected for each case. San Miquel et al. described in their manuscript in 2001 a large 3 color screening panel also followed by a patient-specific LAP formation. However, the colors used in the LAPs vary widely [66]. Kern et al. also used a two-step panel to form a LAP. In this paper was described that a minimum of one LAP

![Figure 3. MRD assessment in AML. This patient showed the aberrant expression of CD7 the blasts at diagnosis, as defined in a first run screenings panel. Since the blasts were positive for CD33 (normal myeloid marker) and CD34 (marker on primitive cells) a LAP was composed that consisted of CD7-CD33-CD45-CD34, a combination known for a stable expression and a low background expression on normal CD34+ cells. 22% of the blasts showed expressed the LAP at diagnosis. After first cycle of chemotherapy, 1.27% AML blast remained in the BM and after second cycle, this was 0.36%. This patient relapsed showing the same immunophenotype as was detected at diagnosis.](image-url)
was identified in every patient [65]. The LAPs used consisted of three colors, without CD45 present in every LAP [81]. LAPs were used that were present on at least 5% of the cells at diagnosis. In childhood AML MRD research, different methods were reported. Sievers et al described a standard panel of 8 tubes with 3 colors, including a CD45 backbone. Langebrake et al used a 6 tube panel with a CD34 and CD33 backbone [82] to evaluate the presence of MRD in every case of AML, without screening for the presence of a specific LAP at diagnosis first. With the exception of the group of Kern et al. all groups defined MRD as a percentage of total WBC. Kern et al used a log reduction of leukemic blasts to quantify MRD [65].

The analysis of the acquired flowcytometric data to assess the level of MRD is a complex task. Detailed knowledge of normal immunophenotypes is required to identify LAPs and is in daily practice performed by experienced researchers. An example of MRD assessment is shown in Figure 3. Standardisation of MRD detection is not yet accomplished an might be difficult since the selection of antibody combinations and methods of LAP assessment varies between laboratories.

**Future prospectives**

The group of Venditti et al showed that, besides using BM as a source to monitor MRD, also peripheral blood (PB) can be used [62;83]. After both induction therapy and after consolidation therapy, MRD percentages obtained from BM showed a significant correlation with the data obtained from PB, although the cut-off used to predict outcome was lower as compared to BM. The main advantage of using PB over BM would be that PB is easier to obtain less invasive for the patient. It would be possible to obtain many cells for analysis, which is not always possible for BM cells. In addition, the frequency of time points to monitor MRD could be higher, which leads to more information regarding a possible upcoming relapse. Another advantage would be the analysis of MRD. In BM, the technician needs to have extended experience with maturation patterns of normal cells, since these cannot be mistaken for malignant blasts. Since PB cells are more mature and in general do not contain progenitor cells, MRD analysis in PB might be easier to perform. Whether PB can replace BM for MRD analysis in the future remains to be elucidated. In a state of clinical CR, the BM is recovering from chemotherapy treatment and can be quite empty. The consequence when only little BM cells are available for MRD analysis is that not every LAP that was detected at diagnosis can be analysed at follow-up. The use of multiple colours which are combined in one tube can overcome this problem. Due to a wide availability of different fluorochromes, multicolour flowcytometry can currently be performed with up to 8 colours per tube, which enables to combine LAPs, leading to a reduction of tubes, cells, cost and time. In addition, Voskova et al showed that multicolour significantly improved the sensitivity and accuracy of MRD analysis [84]. Together with the improvements in analysis software, this development promises improvement for MRD assessment in future studies.
4 Leukemia Stem cells

Although cancer in general is regarded an uncontrolled proliferation of neoplastic cells with impaired differentiation, a heterogeneity and hierarchy has been identified in cells isolated from solid tumors and in leukaemia cells. In 1994, Lapidot and colleagues disassociated immature cells from the bulk of leukemic cells obtained from AML patients [85]. These cells were capable of initiating leukaemia in immune deficient mice. These leukemia initiating cells or leukemic stem cells (LSCs) resided in a subpopulation of cells with the CD34+CD38-immunophenotype and were only a small part of the total tumor burden. Since then, it has been hypothesized that only a minor subpopulation of cells is responsible for maintenance of the tumor burden and that only the eradication of these cancer stem cells will lead to a complete cure of the disease [86].

Figure 4. Mature blood cells arise from a hematopoietic stem cell (HSC) that differentiates to progenitor and mature cell populations (in this example, cells of the myeloid lineage are shown). HSC also have the capacity to self-renew (indicated by the curved line). Leukemic stem cells arise by means of a mutation in either a normal stem cells or in a progenitor cell, and subsequently grow and differentiate to create primary tumors. LSCs also have self renewal capacity. To date, it is unclear whether LSCs arise from accumulation of mutation is HSCs, or in more committed progenitors. Figure adapted from Jordan et al. [109].
LSCs have been defined on the basis of their ability to induce leukaemia in animal hosts, to self renew and to give rise to progenitor cells that lack these properties. The number of LSCs varies widely between patients [87]. The burden of LSCs present at the time of diagnosis of AML shows prognostic impact: AML patients whose mononuclear cell fraction at initial diagnosis fails to engraft NOD/SCID mice (suggesting low LSC content) show superior long-term survival [88]. In addition, high frequency of CD34+CD38- cells at diagnosis (suggesting high LSC content), correlates with MRD and inferior survival in adults [89] and is also of prognostic relevance in childhood AML [90].

### 4.1 NOD/SCID assay

Conclusive evidence for the existence of LSC came from the identification of a very rare population of cells, that were able to repopulate in mice [85;87]. The mouse model used was a SCID model: severe combined immune deficient mice, which indicates that these mice have no innate immunity and are capable of accepting a human graft. Injected human AML cells are capable of homing to the BM microenvironment of the mice, where engraftment and outgrowth of human leukemia takes place. Because of this feature, LSCs are also referred to as SCID leukemia initiating cells [91]. It has been shown that LSCs not only reside in BM, but also in PB from AML patients. Cheung et al. [92] showed an even better homing and engraftment of PB CD34+ cells as compared to BM CD34+ cells. Prior to stem cell transplantation, the mice undergo sublethal total body irradiation of 350 cGy. As a result of the irradiation, SDF-1 levels are increased by proliferating osteoblastic progenitors which supports a better homing and engraftment of the transplanted cells [93-95]. SDF-1 acts as a chemoattractant for stem cell homing to the BM through CXCR4 expressed on surface of the stem cells [96-98]. The NOD/SCID model lacking the gamma part of the IL2-receptor is an even more susceptible mouse for human cells and is currently the golden standard for the validation of LSCs in vivo [99]. In this model it was shown that injected LSCs home and engraft within the osteoblast-rich area of the BM [100].

Current research showed that approximately 50% of the AML samples is able to engraft NOD/SCID mice [88]. The lack of engraftment seen in certain samples was not due to AML LSC frequency, immune rejection, homing or tissue source. It was hypothesized that engrafters in the NOD/SCID assay at 6 weeks may represent diseases driven by potent leukemia-initiating cells with stem cell-like self-renewal and proliferation abilities, whereas nonengrafters may involve less potent leukemia-initiating cells with more restricted progenitor-type self-renewal and proliferation abilities.

A fundamental question remains whether immunocompromised mice are reliable models for studying human cancers. The ideal animal model would accurately represent LSC biol-
ogy as it occurs in humans. However, even highly immunocompromised mice like NOD/SCID IL-2R gamma -/- mice may underestimate the frequency of human cancer cells with tumorigenic potential due to differences between the mouse and human tissue microenvironment or due to residual immunocompetence in these animals.

Self renewal capacity of LSCs was shown by applying serial transplantation into NOD/SCID mice [91]. After injection and leukemic outgrowth in the primary recipient, cells were collected from the BM and injected into a secondary recipient. When the criterion of self-renewal is met, LSCs initiate leukemia again in this second mouse. In addition, some LSCs emerged only in secondary or tertiary recipients, indicating they divided rarely and underwent self-renewal rather than commitment after cell division within primary recipients [91].

4.2 Cell of origin

With the identification of the LSC came the question from what normal cell the LSC arises. On that topic, there has been some confusion: LSCs are not necessarily addressed as "stem cell" because they arise from a normal HSC, but because LSCs meet all the criteria that also defines normal stem cells: LSCs are relatively rare, are able to undergo self-renewal, have a quiescent cell cycle status and give rise to a progenitor population [101]. Despite many studies in the recent years, the origin of LSC remains unclear. During normal maturation from stem cell to progenitor cell to a mature functional cell, mutations may potentially occur at any point during this process and give rise to a malignant cell (Figure 4). One hypothesis is that a mutation in a HSC directly gives rise to a LSC. Since HSCs already contain stem cell properties like self-renewal and proliferative capacity, this theory seems plausible. And since usually multiple mutations are required for malignant transformation, the most logical candidate for this transformation is the cell with the longest life-span: the HSC. However, there is experimental evidence which suggests that mutations in a more committed progenitor cell, that has lost most properties of a true stem cell, can also initiate and maintain AML. For example, using an AML mouse model, it was shown that purified populations of common myeloid progenitors (CMP) or granulocyte/macrophage progenitors (GMP) transduced with a retroviral vector encoding the MLL-ENL translocation generated AML in these mice [102]. Interestingly, purified HSCs, CMP, and GMP generated similar disease upon MLL-ENL transformation, although the disease occurred less frequently when derived from the CMP and GMP populations. These findings suggest that, at least for certain types of mutations, it may be possible to generate LSCs from progenitor cells rather than the more primitive HSC [103;104]. In addition, for the oncogene MOZ-TIF2, it was demonstrated that not only HSCs, but also downstream myeloid progenitors could be the targets of transformation and give rise to identical leukemias [105]. From an immunophenotypical point of view, the observation that CD13 and CD33 were expressed on most LSCs also suggested that LSCs arise from myeloid committed progenitors. However, the fact that these markers are now also found
expressed on HSCs made the hypothesis that LSCs arise from HSCs more viable [106-108]. This hypothesis is strengthened by the fact that both LSCs and HSCs share the CD34 positive and CD38 negative phenotype.

4.3 Stem cell immunophenotype

In human LSC analysis, different immunophenotypically and functionally defined compartments have been described to harbour stem cells: LSCs as well as HSCs.

CD34+CD38-

The CD34+CD38- immunophenotype is currently the main characteristic used to define stem cells. Dick en Bonnet described already in 1997 that a hierarchy existed in the bulk of AML cells, with CD34+ CD38- cells as the most primitive [87]. This was shown by injecting these cells into immunocompromised mice, leading to human AML. Later, serial transplantation of these leukemic cells into secondary and even tertiary animals showed that also the criterion of self renewal was fulfilled: cells that already induced leukemia could do the same when these were injected into the BM of the secondary mouse. In addition, HSCs have also been described as CD34+CD38low, while CD34+CD38+ cells identified only short term repopulating cells and progenitors [110]. It was the group of Dr Bonnet that published in 2008 about the possible inhibitory role of the CD38 antibody [111]. Incubating cells with CD38 showed less engraftment, which suggested that the hierarchy that was shown before, was induced by antibody toxicity. Since approximately 20% of the AML cases are CD34negative (<1% CD34 expression) and this population has been shown to be of normal origin [112], these AML harbour the LSCs in a population other than CD34+CD38-.

SP

The Side Population (SP) represents a rare fraction of cells that is detected by dual-wavelength flowcytometry based on their ability to efflux fluorescent Hoechst 33342 dye [113;114]. Stem cells express the ATP-binding cassette (ABC) transporter breast cancer resistant protein 1 (Brcp1) that regulates this dye efflux. In normal BM, HSCs were identified in this population and in AML this population harbours LSCs [115]. The SP cells obtained from AML BM showed expression of aberrant (lineage) markers (these markers will be discussed in paragraph 4.4.) which emphasised their malignant origin [116]. LSC within the SP fraction could therefore be discriminated from normal SP stem cells based on expression of CLL-1 and lineage markers. However, also in this compartment the question is raised on the reliability of the marker used. Cells that do not completely efflux Hoechst 33342 might lack engraftment capability due to the toxic dye, and not due to the lack of stem cell properties.
**CD34**

Recently, the CD34- compartment was reported to contain LSC activity. This was shown for AML subtypes that harbor the NPM1 mutation, which is associated with a CD34- phenotype. Approximately 50% of the AML cases where CD34- cells were sorted and injected into immunocompromised mice showed the presence of LSCs [52]. In CD34 negative AML, it was shown before that LSC resided in the SP fraction, with the CD34-CD38+ phenotype, while HSC SP-cells were CD34+CD38- [53]. This indicated that the immunophenotype of AML LSC is more heterogenous and varies between different AML samples.

**ALDH**

Aldehyde dehydrogenase (ALDH) is an intracellular detoxifying enzyme expressed in the liver and is responsible for oxidizing aldehydes to carboxylic acids. Inhibition of ALDH using DEAB (dimethylaminobenzaldehyde) as an inhibitor, promotes HSC self renewal via the reduction of retinoic acid activity. This demonstrates that ALDH is a key regulator of HSC differentiation [117]. A method has been developed for the assessment of ALDH activity in viable cells. With this non-toxic method it was shown that normal HSCs could be identified from cord blood cells by their ALDH bright expression pattern [118]. ALDH bright cells obtained from AML samples contained both HSCs as LSCs [118,119]. A common feature of AML cases was that the HSCs show a higher ALDH activity as compared to LSCs [120,121]. This feature makes ALDH a suitable marker for LSC detection as well as a possible target for LSC specific therapy.

**SLAM**

In mice, stem cells could be detected using three cell surface markers : CD150, CD48 and CD244 from the so-called SLAM family of proteins [122]. The entire HSC population was contained by the immunophenotype CD150+, CD244- and CD48-.

### 4.4 LSC cell surface markers

In addition to studies aiming to define the immunophenotype of stem cells in general, other studies have focussed on the expression of cell-surface markers which can discriminate between LSCs and HSCs. Preferably, these markers are expressed on the LSC and are absent on the normal HSC which makes these markers excellent targets for therapy using monoclonal antibodies.

**CD44**

CD44 is a ubiquitously expressed transmembrane glycoprotein that is extensively alternatively spliced, leading to production of many variant isoforms (CD44v) [123]. Elevated CD44v expression on AML cells has been reported and the expression of certain variants has been associated with poor prognosis in AML. CD44 mediates adhesive cell-cell and cell-extracel-
lular matrix interactions through binding to its main ligand, hyaluronan, a glycosaminoglycan highly concentrated in the endosteal region. Since CD44 is also expressed on normal progenitors and more mature cells, this marker might not be specific enough to distinguish between normal and malignant cells.

**CD44v6**

CD44 has many splice variants, which can be detected with specific antibodies. Approximately 12% of the normal CD34+ cells shows CD44v6 expression. CD44v6 expressing leukemic cells were detected in 69 of 95 patients[124]. In AML CD44v6 is associated with adverse prognosis.

**CLL-1**

C-type lectine like molecule-1 (CLL-1) is an adhesion molecule of unknown function. In the CD34+CD38—defined stem cells compartment, CLL-1 is preferentially expressed on LSCs [125]. In normal bone marrow, CLL-1 is expressed on CD38+ myeloid progenitors, but not on the most primitive CD34+CD38- cells, making it an attractive therapeutic target. Although antibodies directed to CLL-1 do not seem to block engraftment or impair viability of LSCs, at least some anti-CLL-1 antibody is internalized following cell-surface binding [126], raising the possibility that they could be coupled to toxins like calicheamicin to produce a cytotoxic response [127].

**CD123**

CD123 is the alpha chain of the IL-3 receptor. It is widely reported to be over-expressed on AML blasts and LSCs in comparison with normal HSCs [128;129]. The over expression of CD123 provides an advantage for the leukemic cells: there is a correlation with the proportion of cycling cells, an apoptosis-resistant phenotype and it is clinically associated with higher blast count and adverse prognosis [130]. Although normal HSCs also express CD123, the over expression on LSCs might be sufficient for proper distinction. For targeted therapy, however, the expression on HSCs might have adverse consequences.

**CD47**

This transmembrane ligand for the signal regulatory protein alpha (SIRPa) receptor is normally found on macrophages and dendritic cells. The interaction of CD47 with its receptor results in an inhibitory effect on macrophage phagocytosis. Although CD47 is also found on HSCs, LSC were found to upregulate CD47 [131]. Inhibition of CD47 binding by using a neutralizing antibody resulted in an increased phagocytosis of AML LSCs, but not of HSCs. Furthermore, LSC showed less engraftment potential when injected in immunodeficient mice. In addition, CD47 expression on AML blasts predicted an adverse prognosis.
CD96
The transmembrane glycoprotein CD96 is a member of the Ig gene superfamily. It was identified by a signal sequence trap PCR method to be selectively expressed on AML-LSC [132]. It was demonstrated by the group of Weissman that CD96 was expressed on the majority of AML-LSCs. In addition, LSC activity was highly enriched in the CD96+ AML fraction. Normal HSCs did not express CD96, suggesting that CD96 is a marker of a candidate therapeutic target [133].

TIM3
The cell surface marker T-cell Ig mucin-3 (TIM3) was most recently described as a unique marker for LSCs [134]. TIM3 is a negative regulator of Th1-T-cell immunity and is also expressed on innate immune cells like dendritic cells, monocytes and macrophages. Animal experiments showed that NBM stem cells do not express TIM3, while LSCs showed expression of high levels of TIM3, which makes this a candidate target for monoclonal antibody therapies directed at the LSC [135].

Myeloid lineage markers
CD13 and CD33 are common markers of the myeloid lineage. Besides the presence on mature myeloid cells, expression of CD13 and CD33 has also been described for LSCs [107]. Despite the fact that HSCs also express CD13 and CD33, discrimination between HSCs and LSCs may still be possible, since CD33 levels are lower on HSCs in the context of AML [106].

4.5 LSC specific therapy
The chemotherapeutic agents used today effectively eradicate the bulk of blast cells in the majority of patients. However, drugs based on targeting actively proliferating cells may not be very effective in targeting the LSC. The vast majority of LSCs are in the G0 phase of the cells cycle, which might explain their relative resistance to chemotherapy [136].

It would be more efficient to develop novel molecularly targeted therapies if the molecular genetic abnormalities in AML cells were simple and consistant. Unfortunately, this is not the case. AML is immunophenotypic as molecularly a very heterogeneous disease, with, in the most aggressive cases, highly complex genetic and molecular abnormalities. What further complicates this disease, is that affected genes do not always play a key role in the leukemogenic process. LSC-targeted therapy might therefore become patient-tailored. The increasing understanding in the difference between normal HSCs and LSCs will aid in the design of those therapies. In line with this thesis, this paragraph will focus on targeting LSC surface molecules.
CD33
The presence of CD33 on LSCs enables therapy directed against this antigen. Gemtuzumab ozogamicin (Myelotarg) is a monoclonal antibody to CD33 linked to a cytotoxic agent, calicheamicin. Since CD33 is not only present on LSCs, but also on normal HSCs [107;108], the disadvantage of this drug is that it also affects normal hematopoiesis, which leads to prolonged neutropenia and thrombocytopenia [137;138].

CD44
Using a mouse models, it was shown that CD44 is of major importance in engraftment of LSCs and by using a monoclonal antibody against CD44 [139], leukemic progression could be suppressed [123]. Jin et al. showed the relevance of the CD44 antibody for LSC fate by treating LSC injected mice with the CD44 specific mAb H90 [123]. H90 mediated ligation of CD44 efficiently and selectively eradicated AML LSCs in vivo by blocking LSC trafficking to supportive microenvironments and by altering their stem cell fate. Demonstrating that CD44 is required for maintenance of AML LSCs indicates that LSCs are niche dependent and provides a therapeutic approach for AML.

CD123
Jin et al showed recently that both CD34+CD38- cells as unsorted cells incubated with the CD123 neutralizing antibody 7G3 reduced engraftment of the cells [129]. Mice with AML that were treated with 7G3 showed a decrease in AML load and cells transplanted into secondary mice showed less engraftment, indicating that the LSC is directly targeted [129].

CD47
Anti-CD47 monoclonal antibodies have been shown to contain specific antileukemic effect without apoptotic effects on normal hematopoietic stem and progenitor cells [140]. The group of Weissman recently showed in mice that CD47 preferentially enabled phagocytosis of AML LSC and inhibited their engraftment in vivo [131]. Normal HSCs were not depleted using this therapy. Whether these findings also apply for humans remains to be established.

CLL-1
Monoclonal antibodies directed against CLL-1 mediate a dose-dependent complement-dependent cytotoxicity and antibody-dependent cellular toxicity against AML derived cell lines [141]. Mice with tumors induced by this cell-line were treated with anti-CLL-1 which led to reduced tumor size. Furthermore, anti-CLL-1 was reported to be effectively internalised by leukemic cells, which also makes toxin-conjugated antibody therapy a possibility for treatment [141]. In 2010, it was reported that drug-conjugated CLL-1 antibodies resulted in specific cell kill of AML cells positive for CLL-1 [142].
5 Outline of the thesis

Although current chemotherapy regimens induce complete remission in the majority of AML patients, still a large part of these patients eventually relapse. Minimal residual cells and in particular, leukemic stem cells that remain in the bone marrow after therapy are responsible for the outgrowth of this relapse. In this thesis the focus is to identify leukemic cells and particular to distinguish leukemic cells from normal progenitor cells using immunphenotyping.

The importance of immunphenotyping in acute leukemias is shown in chapter 2. While morphology is a reliable diagnostic method for newly diagnosed and relapsed leukemias, lineage switches, although not very common, cannot always be detected. Therefore, immunphenotyping should be used to reliably assess the lineage of origin of the cells.

In our search for novel cell surface markers to discriminate between normal and leukemic cells, we investigated the expression of CD25 in AML, which is the alpha-chain of the IL-2 receptor and usually is expressed on T-lymphocytes (chapter 3). Unfortunately, we found that normal CD34+ cells also showed CD25 expression, which decreases the specificity for this marker to detect MRD cells in remission bone marrow. In addition, CD25 is upregulated on normal cells after chemotherapy, which makes this marker not usable for MRD detection. However, CD25 detected on AML blasts at diagnosis is a relevant prognostic marker in AML and correlates with MRD frequency.

MRD frequency is normally assessed as a percentage of total WBC. We hypothesized that peripheral blood in bone marrow aspirates can dilute the samples, possibly leading to false-negative MRD percentages. The results in chapter 4 show that when residual malignant cells are assessed as a percentage of only the total primitive cells fraction, MRD negative patients can still be identified as adverse prognostic.

The prognostic impact of immunphenotypically defined MRD has been shown in many retrospective studies [143;144] and in two prospective studies in childhood AML [145;146]. In order to use MRD as a tool for the guidance of risk-based therapy regimens, MRD detection in a prospective study is required. In chapter 5, we describe the results of the first prospective study in AML patients below the age of 60. The prognostic impact of MRD is described after different cycles of treatment as well as in different risk groups based on cytogenetics and molecular data assessed at diagnosis.

After the achievement of clinical remission, the presence of remaining, drug-resistant LSCs can initiate relapse of the disease, which shows the need for LSC specific therapies. This
requires the need for LSC detection, preferable in parallel to HSCs detection. Well-know LSC-specific markers (like CLL-1 and aberrantly expressed lineage markers) aid in the discrimination between LSCs and normal HSCs, but additional parameters are required in those cases where LSCs lack this expression.

In chapter 6 we show methods to detect both HSCs and LSCs in the same AML BM at diagnosis, using aberrantly expressed markers to identify HSCs, but also light scatter parameters to identify HSCs. In addition, LSC detection and monitoring in BM obtained after chemotherapy treatment provides prognostic information necessary for risk-based treatment schemes.

In the last chapter, the results in the preceding chapters are summarized and future perspectives in this field of research are discussed.
Reference List

30. Schlenk RF, Dohner K, Krauter J et al. Muta
37. Schnittger S, Schoch C, Kern W et al. Nucleo
41. Renneville A, Boissel N, Gachard N et al. The favorable impact of CEBPA mutations in patients with acute myeloid leukemia is only observed in the absence of associated cytogenetic ab-
61. Elliott MA, Litzow MR, Letendre LL et al. Early peripheral blood blast clearance during induc-


77. Steinbach D, Debatin KM. What do we mean by sensitivity when we talk about detecting minimal residual disease? Leukemia 2008;22:1638-1639.


81. Kern W, Schoch C, Haferlach T, Schnittger S. Monitoring of minimal residual disease in acute


88. Pearce DJ, Taussig D, Zibara K et al. AML engraftment in the NOD/SCID assay reflects the outcome of AML: implications for our understanding of the heterogeneity of AML. Blood 2006;107:1166-1173.


122. Kiel MJ, Yilmaz OH, Ishawita T et al. SLAM fam-
ably receptors distinguish hematopoietic stem and progenitor cells and reveal endothelial niches for stem cells. Cell 2005;121:1109-1121.


