Chapter 1

General introduction
**Hematopoiesis**

Hematopoiesis, the process of the formation of all types of blood cells, takes place in the bone marrow (BM). Hematopoietic stem cells (HSCs) are at the origin of hematopoiesis. These HSCs are cells capable of both self-renewal and formation of daughter cells, so called progenitor cells. These progenitor cells ultimate give rise to effector cells present in the peripheral blood (PB), like erythrocytes, platelets and different kind of leukocytes (eg granulocytes, monocytes and lymphocytes). This process of hematopoiesis is under control of a complex mechanism of signal transduction in which growth factors and cytokines bind to receptors on the surface of cells. This process takes place in the so called bone marrow niche where HSCs are surrounded by other supporting cell sources formed by mesenchymal stem cells and where cellular processes like cell division or cell differentiation are activated. When errors occur in this process of normal hematopoeisis this can give rise to hematologic malignancies like leukemia.

**Acute myeloid leukemia**

**Diagnosis**

Acute myeloid leukemia (AML) is the most common type of acute leukemia in adults. Acute myeloid leukemia arises by an abnormal accumulation of leukemic blasts cells in the bone marrow by a differentiation block, resulting in a loss of normal functional blood cells. Since erythrocytes, platelets and leukocytes are important for oxygen transport, coagulation and immunity, respectively, patients with AML often present with symptoms like anemia, bleeding and infections. Leukemic blasts may also infiltrate organs, resulting in for example lymphadenopathy, splenomegaly, hepatomegaly and gingival hyperplasia. In Western Europe the estimated incidence of AML is 0.7-3.9 per 100,000 under the age of 60 and the incidence increases to 6.7-19.2 per 100,000 for people above 60 years.

The diagnosis of AML is classically made using morphology: using microscopy, stained BM cells are studied and cells present in all kind of different differentiation stages can be identified based on their morphologic characteristics. The French-American-British (FAB) classification was used to classify the different AML subtypes based on these characteristics. In this classification a distinction is made between myeloblastic (FAB MO, M1, M2), promyelocytic (M3), myelomonocytic (M4), monoblastic (M5a), monoblastic-monocytic (M5b), erythroleukemic (M6) and megakaryoblastic leukemias (M7). Flow cytometry (FACS, fluorescence activated cell sorting) can also be used to distinguish cell types from each other based on different proteins present intracellularly or on their cell surface. Hematopoietic stem cells, for instance, are characterized by cell surface expression of CD34, a marker characteristic for stem- and progenitor cells. More differentiated blood cells have lost their expression of CD34. The role of immunophenotyping in the determination of residual disease will be discussed later.
Furthermore, cytogenetic and molecular analyses nowadays give important additional information regarding the type of AML. Certain molecular and/or cytogenetic aberrancies are associated with certain subtypes of AML and which differ from the other subtypes in prognosis and/or preferable treatment.² The classification of the World Health Organization (WHO) that was recently updated (2016³) is nowadays used more often as compared to the FAB classification since this classification also takes into account immunophenotypic characteristics as well as cytogenetic and molecular aberrancies. Furthermore, earlier cytotoxic treatment or prior myelodysplastic syndrome and pre-existing germ line predisposition syndromes are included. Whole genome sequencing, in which the whole genetic profile of the leukemia can be analysed, is getting more important and it is expected that whole genome sequencing will play an important role in AML diagnostics in the near future.

Treatment²
The primary goal of induction chemotherapy treatment aims for the achievement of a hematological complete remission (CR). By definition, patients are in CR when less than 5% blast cells, assessed by morphology, are present in the 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 10⁹/l and 100 x 10⁹/l, respectively.⁴ Induction chemotherapy in HOVON protocols consists of two cycles of chemotherapy, consisting of cytarabine, idarubicine, daunorubicine or mitoxantrone. After achievement of CR post remission therapy is given to maintain the hematological remission of the disease. This consolidation therapy can consist of a third cycle of chemotherapy treatment, an autologous or allogeneic stem cell transplantation. For autologous stem cell transplantation, stem cells of the patient are harvested during remission and cryopreserved. After high dose chemotherapy, these cryopreserved autologous stem cells are re-infused to ascertain faster recovery from the cytotoxic damage. In allogeneic stem cell transplantation the stem cells of the patient are replaced by donor stem cells. To enable engraftment of the donor cells, chemotherapy and/or total body irradiation is given. After such an allogeneic transplantation graft versus host disease can occur in which donor cells attack healthy cells of the recipient. However, the associated graft versus leukemia response enables an immunologic response in which donor cells attack residual leukemic cells. Since an allogeneic stem cell transplantation is a treatment associated with morbidity and even mortality, strict consolidation therapy selection is necessary, implying that only patients with a high risk of relapse should be considered for allogeneic stem cell transplantation.

Measurable residual disease detection

Measurable residual disease and acute myeloid leukemia
With current treatment strategies, almost 80% of AML patients (18-60 years) will achieve CR. However, approximately 50% of these patients will experience a relapse, resulting in a five-
year survival rate of only 35%-40%. As described above, morphologic assessment is currently the golden standard to evaluate chemotherapy response and to define remission status. 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, nowadays also referred to as measurable residual disease (MRD), is thus defined as the persistence of leukemic cells after chemotherapy treatment and is thought to be responsible for the emergence of relapse (Figure 1). Quantitative MRD frequency assessment could give important prognostic information after chemotherapy treatment. Two sensitive methods 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.

![Diagram of MRD and Leukemic Stem Cells](image)

**FIGURE 1 | The role of MRD 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 later).

**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 immunophenotypes (LAIPs) are determined. Such a LAIP 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. LAIPs are grouped into (1) cross-lineage antigen expression (e.g. expression of lymphoid markers on 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. Such aberrancies can subsequently be used to detect MRD (Figure 2).
FIGURE 2 | Example of MRD detection in BM using the aberrant phenotype (asynchronous type) of CD34+CD7+ cells at AML diagnosis (A-C) and during follow-up (D-F). Gating of the blast cells with dim expression of CD45 and low sideward scatter (SSC) (A, D), gating of the CD34 positive progenitors cells (B, E) and gating of the leukemia blast population with aberrant expression of CD7 on the myeloid CD34+ progenitor cells (C, F). After chemotherapy treatment a residual population of leukemic blasts can be detected (F).

Due to large heterogeneity of immunophenotypes in AML, determination of LAIPs has to be performed for each individual patient. These LAIPs 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). Besides these relatively high sensitivities, it is also a very rapid technique. Main advantage of flow cytometric MRD assessment is its broad applicability: in 80%-95% of all AML patients one or more LAIPs can be defined. 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 LAIP defined at diagnosis, is often not a characteristic of the total population of leukemic blast cells. Since only the LAIP positive (LAIP^+) cells can be identified at follow-up, this may thus result in under-estimation of cell frequency of all MRD leukemic blast cells. To approach the real MRD cell frequency, there is the possibility to correct LAIP^+ frequency at follow-up for the LAIP^+ frequency, as percentage of blasts, at diagnosis. Secondly, the presence of low percentages of normal cells that express a particular LAIP 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 LAIP, since these cells are only present at low frequencies in normal BM. A complicating factor is that different LAIPs have different specificities, allowing different sensitivities to be reached for different LAIPs. Thirdly, immunophenotypic shifts may occur in the course of treatment and result in false-negativity. To avoid this, it is recommended to use multiple LAIPs. Finally, due to the large number of different LAIPs, MRD analysis is quite complex and needs vast experience in discriminating leukemic cells from cells with normal differentiation patterns.
An alternative approach to detect residual leukemic cells is the so-called different-from-normal (DfN) approach. In this approach aberrant maturation and differentiation patterns are used to detect cells that are different from normal and presumed leukemic.16,17 Using this DfN approach besides the earlier described LAIP approach, could significantly improve MRD detection since this approach can also be used when no LAIPs or no BM material is present at time of diagnosis. Nevertheless, this DfN approach whereby maturation and differentiation patterns are studied still demands expertise.

**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.18 Besides these pre-treatment prognostic factors, it has become clear that MRD detection in BM shortly after treatment offers an important post-treatment prognostic factor. To evaluate the impact of MRD frequencies 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, often ranging between 0.01% to 0.2%.19 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. Despite different methods to discriminate patients with high MRD levels from patients with low MRD levels, all studies found MRD to be a significant predictor for patient outcome.10-13,20,21 Recently more studies including large numbers of AML patients have been published22,23 under which our own study by Terwijn et al that described the prognostic relevance of MRD in a a large cohort of AML patients. Therefore in recent/current studies MRD is implemented in clinical decision-making (GIMEMA study groups, MRC AML trials, HOVON/SAKK 132 study).

**Molecular MRD detection**

Although flow cytometry is an attractive technique for MRD detection, the limitations, including background staining, immunophenotypic switches, complexity of analysis and LAIP 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 in a range of 10^{-4} to 10^{-6}.24-27 QRT-PCR is now extensively being studied as approach for MRD detection. Common targets for molecular MRD monitoring include fusion genes (AML1-ETO28, PML-RARα29 and CBFβ-MYH1126), gene mutations (FLT3- ITD30-33 and NPM1), and over-expressed genes (WT34,35 and PRAME36). NPM1 mutations are considered to be very suitable because of high stability and sensitivity.31,37-40 A major drawback in molecular MRD detection is the fact that only part of
the patients harbours a specific mutation (in the case of NPM1 about 35%). Similar to flow cytometry, some aberrancies are quite unstable during disease, e.g. FLT3ITDs, which limits usefulness for molecular MRD monitoring. Over-expressed genes are not recommended for MRD monitoring. Recently, there has been more interest in next generation sequencing (NGS) for the detection of MRD. Although NGS also requires diagnosis material to be able to monitor and track mutations, NGS is highly sensitive and residual mutations as detected with NGS are associated with relapse.41-43 Moreover NGS provides important additional information regarding the clonal architecture and the existing of multiclonality in AML.

**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.18 In the most recent AML HOVON/SAKK H132 study, MRD cell frequency is included as a “response to treatment” parameter in the study risk scheme. 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 assessment 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.44-46 Therapeutic options in the case of post-transplant relapse consist of withdrawal or decrease of dose of immune-suppressive drugs, or immunotherapeutic intervention with donor lymphocyte infusion. As these approaches intend to boost the graft versus leukemia effect, they are most effective when the leukemic cell load is low. 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 relapse47,48, patients with full chimerism (> 95% donor cells) can still suffer from relapse.48 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 levels before49-51 and after52,53 an allogeneic transplantation indeed correlates with clinical outcome and identifies patients who are likely to relapse. 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 and prolong OS. To confirm this hypothesis, large prospective studies with MRD based clinical intervention in the post-remission phase are essential.

However, BM aspiration is often a burden for patients and therefore PB would be an attractive alternative source for MRD detection. In acute lymphoblastic leukemia different studies using either molecular assays\textsuperscript{54-56} or flow cytometry\textsuperscript{57} have shown that PB can replace BM as a source for MRD detection. In AML, a correlation was found between BM and PB for mutant NPM1 levels\textsuperscript{58} and WT1 expression.\textsuperscript{59} Maurillo and colleagues were the first to show that immunophenotypic MRD levels in PB indeed have prognostic relevance in AML.\textsuperscript{60} However, future larger studies should confirm if PB could indeed be used for immunophenotypic MRD detection in AML.

**Leukemic stem cells and acute myeloid leukemia**

**Definition of leukemic stem cells**

Already decades ago, 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.\textsuperscript{61,62} These cells are now referred to as leukemic stem cells (LSCs) or leukemia initiating cells. It is assumed that similar to normal hematopoiesis, leukemia is hierarchically structured.\textsuperscript{63} 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.\textsuperscript{64} Furthermore, the immunophenotype of LSCs resembles the immunophenotype of normal HSCs. The majority of HSCs are present in the CD34\textsuperscript{+}CD38\textsuperscript{−} immunophenotypic compartment\textsuperscript{65,66}, and initial AML studies demonstrated leukemia initiating capacity also to be in the CD34\textsuperscript{+}CD38\textsuperscript{−} compartment.\textsuperscript{65} This small subpopulation of CD34\textsuperscript{−}CD38\textsuperscript{−} cells was able to engraft and cause leukemia in non-obese diabetic/severe combined immune-deficient (NOD/SCID) mice. These cells were present at a frequency of only 0.2 to 100 cells per 10\textsuperscript{6} mononuclear cells.\textsuperscript{65} Nowadays it is known that AML LSCs can also reside within the CD34\textsuperscript{+}CD38\textsuperscript{−} or the CD34 immunophenotypic compartment.\textsuperscript{67-71} 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.\textsuperscript{72} 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 (Figure 1).\textsuperscript{68,73,74} In CD34 positive patients often both CD34\textsuperscript{+}CD38\textsuperscript{−} cells, CD34\textsuperscript{−}CD38\textsuperscript{−} and CD34\textsuperscript{−} 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\textsuperscript{+}CD38\textsuperscript{−} compartment has been shown to be less immunogenic compared
to the other compartments\textsuperscript{75}, which may explain why it was almost exclusively the CD34$^+$CD38$^-$ compartment that engrafted in NOD/SCID mice with only residual immunity\textsuperscript{63}, 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.\textsuperscript{67} 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 \textit{in vitro}\textsuperscript{75} and \textit{in vivo}.\textsuperscript{76} 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.\textsuperscript{73}

Besides the ability of LSCs to initiate and sustain the initial AML, 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.

\section*{Identification of leukemic stem cells}

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.\textsuperscript{72,75} Since both HSCs and LSCs reside within this compartment, discrimination between CD34$^+$CD38$^+$ HSCs and LSCs, with the aim to assess LSC load and, for new target finding, 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.\textsuperscript{77} 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$^+$, CD33 CD13$^+$, and CD15$^+$HLA-DR$^+$. Besides these lineage markers, a growing number of other markers are now available to discriminate between LSCs and HSCs. These include CLEC12A/CLL-1, CD25, CD32, CD33, CD44, CD47, CD96, CD123, IL1-RAP, and TIM-3 (an example of identification of marker positive LSCs and marker negative HSCs is in Figure 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). Expression of CLEC12A/CLL-1 on CD34⁺CD38⁻ cells. Two populations of stem cells are shown: a CLEC12A negative stem cell population, turning out to contain the HSCs and the CD34⁺CD38⁻ cells with expression of CLEC12A. These stem cells with aberrant expression of CLEC12A turned out to represent the LSC compartment (F).

An overview of LSC markers is given in Table 1. 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.

TABLE 1 | Overview of stem cell markers

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Function</th>
<th>Reference</th>
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<tbody>
<tr>
<td>CLEC12A</td>
<td>C-type lectin-like molecule-1</td>
<td>78</td>
</tr>
<tr>
<td>Lineage markers</td>
<td>Lymphoid lineage and myeloid lineage markers</td>
<td>77</td>
</tr>
<tr>
<td>CD25</td>
<td>Interleukin-2 receptor α-chain</td>
<td>79</td>
</tr>
<tr>
<td>CD32</td>
<td>Fc fragment of IgG, low affinity IIα receptor</td>
<td>79</td>
</tr>
<tr>
<td>CD33</td>
<td>Myeloid marker</td>
<td>80</td>
</tr>
<tr>
<td>CD44</td>
<td>Receptor for hyaluronan</td>
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</tr>
<tr>
<td>CD47</td>
<td>Integrin associated protein</td>
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</tr>
<tr>
<td>CD96</td>
<td>T cell-activated increased late expression protein</td>
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<td>CD123</td>
<td>Interleukin 3 receptor alpha chain</td>
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<td>TIM-3</td>
<td>T-cell Ig mucin-3</td>
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</tr>
<tr>
<td>IL1-RAP</td>
<td>Interleukin 1 receptor accessory protein</td>
<td>86</td>
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</table>
FIGURE 4 | Heterogeneity in expression of different stem cell markers in one AML case at diagnosis. CD34-CD38 population was analysed for the expression of six LSC markers: CD2 (A), CLEC12A (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. In this particular example CD123 was the best marker.
Leukemic stem cell targeted therapy

Apart from determining the LSCs load, characterization of these malignant cells may offer 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. CLEC12A, CD123 and CD33 are examples. CLEC12A is a very promising anti-tumor target since, unlike CD123 and CD33, this antibody is not expressed on normal hematopoietic stem cells. Recently a bispecific antibody that binds CLEC12A and CD3 has been shown to target AML cells in animal models. Future clinical studies, including the Merus clinical trial (Clinical trial number NCT03038230) in which VUMC AML patients are included, should reveal if this treatment is indeed effective in humans. CD123 is also expressed in a large proportion of AML patients and an anti-CD123 antibody has shown promising results in mice and human. Recently a novel CD123 agent combined with CD3 has been proven to specifically kill AML blasts via a T-cell-CD123 specific response cascade (T-cell activation and proliferation). Further clinical studies are needed to determine the efficacy and safety 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 (GO) has been studied in several trials and, after initial disappointment relating to toxicity, new studies with altered treatment schedules suggest that GO is beneficial in certain subgroups of AML patients, including patients with favorable cytogenetics. In recent studies it has been shown that CD33 expression on blasts is a predictive factor for the effect of GO. Recently, Vadalustimab, a CD33-directed antibody was found to be safe in AML patients when combined with standard chemotherapy treatment and promising results are reported. However, it is important to emphasize that no studies so far determined the correlation between the efficacy of GO and the presence of CD33 positive LSCs. It may be speculated that subgroups of patients with CD33 positive LSCs may benefit more from this additional therapy. Further clinical trials will have to determine if other stem cell markers are potential targets as well. Finally, the introduction of chimeric antigen receptor T-cells (CAR-T) enables redirecting T-cell specificity making use of a leukemia associated antigen. Although promising, more clinical trial results are needed to further evaluate both the incidence of side effects and the anti-leukemia effect.

Outline of the thesis

Although most of the AML patients will achieve hematological remission after induction therapy, the majority of the AML patients will experience a relapse. In this thesis the focus is on minimal residual disease, and leukemic stem cells herein, in better predicting an AML relapse. Moreover, the role of MRD and LSC frequency in clinical decision-making is evaluated.

In chapter two we investigate the prognostic role of the immunophenotype of the AML blasts at time of diagnosis. To investigate this possible prognostic role we look into the existing of associations between molecular and cytogenetic aberrancies and the AML immunophenotype. Taking into account these associations, we aimed to find out if the immunophenotype of the bulk of blasts cells harbors independent prognostic information at time of diagnosis. If so, already at time of diagnosis this could lead to further refinement of currently used risk
schemes.

To investigate if the LSC frequency harbours prognostic information that can be used for AML relapse prediction, in chapter three we outlined the prognostic importance of CD34+CD38-LSCs in CD34-positive patients. At time of diagnosis and in remission bone marrow the prognostic relevance of the LSC frequency is shown. Moreover the prognostic relevance of the combination of the LSC frequency with MRD levels is retrospectively investigated.

Although the majority of AML patients is considered CD34-positive, implying that (at least part of) the leukemic blasts express CD34 on their cell surface, a small part is considered CD34-negative. In chapter four we re-identified CD34 negativity as the complete absence of leukemic CD34+ blasts, with only normal CD34 cells present. Consequently, no CD34+CD38- LSCs are present in this subgroup of AML patients. We found that the prognostic importance of CD34 status at time of diagnosis is defined by the presence of the group of CD34-negative leukemias.

In chapter five we prospectively defined LSC frequencies at time of diagnosis and at follow-up. In this large HOVON102 patient cohort, CD34-negative patients were also included. In the survival analysis LSC results are combined with MRD results as defined after chemotherapy treatment to find out if determination of the LSC frequency at follow-up has additional prognostic value as compared to MRD.

To enable specific determination of leukemic stem cells in an easy user-friendly assay, chapter six provides data in which we aimed to create a broadly applicable immunophenotypic detection tool for LSC detection both at diagnosis and follow-up. To find out which immunophenotypic markers are most useful for specific LSC detection, marker expression in AML of 15 different markers was compared with expression in healthy controls, with emphasis on levels of expression, expression on HSCs, redundancy of markers and stability of expression during therapy.

Chapter seven provides data concerning the applicability of peripheral blood cells as an alternative source for MRD detection. Since the collection of bone marrow cells is often a burden for the patient, peripheral blood may offer an attractive alternative source for immunophenotypic risk assessment. In this chapter MRD results as defined in BM and PB were compared in paired AML patients to study if PB can replace BM as a source for MRD assessment.

False negative MRD and LSC results remain an important concern whereby improvement of these immunophenotypic assays is warranted to further improve AML risk prediction. In chapter eight points of interest for future MRD and LSC studies are reviewed whereby especially the role of tumor instability on MRD detection is explored. Such tumor heterogeneity, in which the immunophenotypic, molecular and/or cytogenetic profile of the AML cells can change during the disease, can have important consequences for MRD and LSC detection. In this chapter we aim to give insight in how to account for this tumor heterogeneity in future MRD and LSC assessment.

Finally, in the last chapter (chapter nine) we describe results of this thesis and which implications these results may have for future AML risk group profiling and relapse prediction. Furthermore, future perspectives in this field of interest are discussed.
References

40. Kristensen T, Møller MB, Fris L, et al. NPM1 mutation is a stable marker for minimal residual disease monitoring in acute myeloid leukaemia patients with increased sensitivity compared to WT1 expression. Eur J Haematol 2011;87(5):400–8.


