

# **PhD Thesis**

## **Leukemic stem cells and a novel method of minimal residual disease detection in acute myeloid leukemia**

submitted by

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for the academic degree of a

**Doctor of Philosophy**

at

**Medical University of Graz  
Division of Hematology  
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**2019**

## **Declaration**

I hereby declare that this thesis is my own original work and that I have fully acknowledged by name all of those individuals and organizations that have contributed to the research for this thesis. Due acknowledgement has been made in the text to all other material used. Throughout this thesis and in all related publications I followed the “Standards of Good Scientific Practice and Ombuds Committee” at Medical University of Graz, Austria.

Shruti Daga

04/05/2019

**I dedicate this PhD thesis to my parents, husband and  
grandmother, Late. Mrs. Meena Daga.**

## ACKNOWLEDGEMENTS

I am thankful to some amazing people for their support and help without which the project would have not been complete. To begin with, I would like to thank the Medical University of Graz for giving this chance to pursue my PhD through Molecular Medicine (MolMed) PhD program. This study was funded by the Austrian Research Promotion Agency (FFG) within the COMET program (CBmed) and support from the Oesterreichische Nationalbank (Anniversary Fund, project number: 15689 to AW). AW was also supported by an unrestricted grant from the Ingrid-Shaker- Nessmann Krebsforschungsvereinigung and Leukämiehilfe Steiermark.

I would like to express my heartfelt gratitude to my project supervisor ao.Univ.Prof. Dr. Albert Wölfler for giving me this opportunity to join his team as a PhD student. I wish to thank him not only for his constant support and willingness to share his immense knowledge but also for his advice and motivation as well. Without his supervision and insightful comments during the entire study period and writing of this thesis, the job on my hands would have been difficult. I cannot imagine having a better mentor for my PhD study.

In addition, I would like to thank members of my thesis committee Prof. Dr. Heinz Sill and Prof. Gerald Höfler for their valuable suggestions and feedback which helped me to improve my study.

A special thanks to Dr. Andreas Reinisch and Dr. Armin Zebisch for their critical comments and advices which helped me in the completion of this project.

My PhD would have not been complete without the support of my parents, grandparents, and my in laws. I am immensely grateful to my parents for encouraging me to continue my studies further. Regardless of the situation, you have been my strong pillars of support. I am forever indebted to you for your selfless love and sacrifices you made to shape me into the person I am today. I also extend my gratitude to my sister Trupti for her love and encouragement. I wish to thank my family for believing in me and most of all for their never-ending patience. I also wish to thank my husband, Hemang for his rock steady support, patience and for putting up with me when things got rough. I also extend my gratitude to my parents in laws for their persisting patience and motivation.

I wish to thank my colleagues Ms. Angelika Rosenberger, Ms. Daniela Thaler, Ms. Margret Frühwirth, Ms. Karin Lind, Ms. Sybille Hofer, Ms. Birgit Feilhauer and Ms. Carmen Egger for their help during the entire period. I also wish to thank my friends Dr. Ridhima Lal, Dr. Anita Sahu-Osen and Ms. Smriti Sharma for their constant encouragement and advices.

I also wish to acknowledge the contribution of the many patients without whose generosity; I would have not been able to complete this project. Finally, I acknowledge one and all who have contributed either directly or indirectly for the completion of my project.

## DISCLOSURES

Parts of my dissertation have already been published and I can confirm that all co-authors who have actively contributed to the results of the thesis have agreed to use their data in my thesis.

1. **Daga S<sup>1,2</sup>**, Rosenberger A<sup>1</sup>, Quehenberger F<sup>3</sup>, Krisper N<sup>2</sup>, Prietl B<sup>2</sup>, Reinisch A<sup>1</sup>, Zebisch A<sup>1</sup>, Sill H<sup>1</sup>, Wölfler A<sup>1,2</sup>. High GPR56 surface expression correlates with a leukemic stem cell gene signature in CD34-positive AML. *Cancer Medicine*. 2019 Apr; 8(4):1771-1778.
2. Gaksch L<sup>1</sup>, Kashofer K<sup>3</sup>, Heitzer E<sup>4</sup>, Quehenberger F<sup>5</sup>, **Daga S<sup>1,2</sup>**, Hofer S<sup>1</sup>, Halbwedl I<sup>3</sup>, Graf R<sup>4</sup>, Krisper N<sup>2</sup>, Hoefler G<sup>3</sup>, Zebisch A<sup>1</sup>. Residual disease detection using targeted parallel sequencing predicts relapse in cytogenetically normal acute myeloid leukemia. *American Journal of Hematology*. 2018 Jan; 93(1):23-30.
3. Combining flow cytometry-based leukemic cell enrichment and mutational analysis for detection of minimal residual disease in acute myeloid leukaemia: a pilot study. **Shruti Daga<sup>1,2</sup>**, Angelika Rosenberger<sup>1</sup>, Karl Kashofer<sup>3</sup>, Ellen Heitzer<sup>4</sup>, Franz Quehenberger<sup>5</sup>, Iris Halbwedl<sup>3</sup>, Ricarda Graf<sup>4</sup>, Nina Krisper<sup>2</sup>, Gerald Höfler<sup>3</sup>, Andreas Reinisch<sup>1</sup>, Armin Zebisch<sup>1</sup>, Heinz Sill<sup>1</sup> and Albert Wölfler<sup>1,2</sup>. **Manuscript in preparation.**

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## ABSTRACT

Acute myeloid leukemia (AML) is an aggressive malignant disorder of hematopoietic stem and precursor cells (HSPCs) characterized by an adverse clinical outcome due to frequent relapse after initial response to therapy. AML is driven by a minor fraction of leukemic stem cells (LSCs), which predominantly reside within the CD34<sup>+</sup>38<sup>-</sup> subpopulation and whose persistence is considered being the primary cause of disease relapse.

In the first part of this study we aimed to characterize surface markers to identify leukemia cells that reflect LSC activity at diagnosis. Among 16 markers analyzed by multicolour flow-cytometry (MFC), GPR56 and CLL-1 were found to be the most prominently differently expressed markers within the CD34/38 subcompartments. While GPR56 was highest expressed within the LSC-enriched CD34<sup>+</sup>38<sup>-</sup> population as compared to CD34<sup>+</sup>38<sup>+</sup> and CD34<sup>-</sup> leukemic bulk cells, CLL-1 expression was lowest in CD34<sup>+</sup>38<sup>-</sup> leukemic cells and highest on CD34<sup>-</sup> blasts. Furthermore, high GPR56 surface expression in CD34<sup>+</sup>38<sup>-</sup> leukemic cells correlated with a recently published LSC gene expression signature and was associated with decreased overall survival in patients receiving intensive chemotherapy. In contrast, CLL-1 expression correlated inversely with the LSC gene signature and was not informative on outcome. Our data therefore strongly support GPR56 as a promising clinically relevant marker for identifying leukemic cells with LSC activity at diagnosis in CD34-positive AML.

In the second part of study, we aimed to develop a highly sensitive and broadly applicable method for detection of minimal residual disease in AML by combining MFC-based leukemic cell enrichment followed by mutational analysis using either targeted deep sequencing or digital PCR. A combination of antibodies against CLL-1, TIM-3, CD123 and CD117 was identified to perform best for leukemic cell enrichment by enabling staining of >90% of leukemic cells in 134 of 146 diagnostic AML samples (91.7%). In dilution experiments using *NPM1*-mutated samples and normal BM, leukemic cell enrichment by these markers followed by mutational analysis showed a sensitivity of 10<sup>-5</sup> for residual disease detection. For validation, BM samples of 41 patients in complete remission (CR) after induction chemotherapy were prospectively collected and analyzed for MRD using this newly developed two-step detection method. In 39 samples DNA quality of sorted cells was sufficient for sequencing. Twenty-one samples tested MRD positive, whereas 18 were negative. With a median follow-up of 559 days 71% of MRD positive (15/21) and 28% (5/18) of MRD negative patients relapsed (p=0.0065). Accordingly, median relapse free survival was significantly shorter in MRD positive patients (283 vs. not reached, p=0.0031).

Furthermore, in multivariate analysis MRD positivity as detected by this method was the most informative parameter for cumulative incidence of relapse (hazard ratio 7.07).

In conclusion, MFC-based leukemic cell enrichment using antibodies against CLL-1, TIM-3, CD123 and CD117 followed by mutational analysis is feasible for MRD detection with high sensitivity and informative on relapse risk in AML patients. Further multi-center clinical trials to standardize and to compare this promising novel method to other approaches of MRD detection are warranted.

## Zusammenfassung

Die akute myeloische Leukämie (AML) ist eine aggressive maligne Erkrankung der hämatopoietischen Stamm- und Vorläuferzellen (HSPCs), die trotz initialem Ansprechen auf die Therapie häufig durch einen Krankheitsrückfall charakterisiert ist und damit mit einer schlechten Prognose verbunden ist. Eine meist geringe Anzahl an sogenannten leukämischen Stammzellen (LSCs) verursacht die Erkrankung und ist für die fortlaufende Bildung der aggressiven Leukämiezellen verantwortlich. LSCs befinden sich überwiegend in der CD34<sup>+</sup>38<sup>-</sup> Subpopulation der AML-Zellen und ihre Persistenz nach Therapie gilt als Hauptursache für das Krankheitsrezidiv.

Im ersten Teil dieser Studie zielten wir darauf ab, Oberflächenmarker zu charakterisieren, die es ermöglichen, jene Leukämiezellen zu identifizieren, die eine LSC-Aktivität zum Zeitpunkt der Diagnose zeigen. Unter 16 Markern, die mittels Mehrfarben-Durchflusszytometrie (MFC) analysiert wurden, zeigten die Oberflächenmarker GPR56 und CLL-1 die unterschiedlichste Expression innerhalb der einzelnen CD34/38-Subpopulationen der AML-Zellen. Während GPR56 im Vergleich zu den anderen Subpopulationen in den LSC-angereicherten CD34<sup>+</sup>38<sup>-</sup> Leukämiezellen am stärksten exprimiert war, war CLL-1 in den CD34<sup>+</sup>38<sup>-</sup> Zellen niedrig und in den CD34<sup>+</sup>38<sup>+</sup> und CD34<sup>-</sup> Leukämiezellen deutlich höher exprimiert. Darüber hinaus konnten wir zeigen, dass eine hohe GPR56-Oberflächenexpression in CD34<sup>+</sup>38<sup>-</sup> Leukämiezellen mit einer vor kurzem veröffentlichten LSC-Genexpression-Signatur korreliert. Zudem fanden wir heraus, dass eine hohe GPR56-Expression in AML-Zellen zum Diagnosezeitpunkt mit einem verringerten Gesamtüberleben bei Patienten, die eine intensive Chemotherapie erhielten, assoziiert ist. Im Gegensatz dazu korrelierte die CLL-1-Expression invers mit der LSC-Gen-Signatur und war nicht informativ für das klinische Outcome von AML-Patienten. Diese Daten zeigen, dass GPR56 ein vielversprechender, klinisch relevanter Marker zur Identifizierung von leukämischen Zellen mit LSC-Aktivität zum Diagnosezeitpunkt einer CD34-positiven AML ist.

Im zweiten Teil der Studie haben wir uns zum Ziel gesetzt, eine sensitive und breit anwendbare Methode zur Erkennung der minimalen Resterkrankung (MRD) bei der AML zu entwickeln. Dabei kombinierten wir eine Leukämiezellanreicherung mittels MFC gefolgt von einer Analyse von Leukämie-spezifischen Mutationen mittels DNA-Tiefensequenzierung bzw. digitaler PCR. Eine bestmögliche Leukämiezellen-Anreicherung erreichten wir durch eine Kombination von Antikörpern gegen CLL-1, TIM-3, CD123 und CD117. Diese Kombination ermöglichte eine Anreicherung von > 90% der Leukämiezellen in 134 von 146

diagnostischen AML-Proben (91,7%). Bei Verdünnungsexperimenten mit *NPM1*-mutierten Proben und normalem Knochenmark (KM) zeigte die Anreicherung der Zellen durch diese Marker gefolgt von einer Mutationsanalyse, eine Sensitivität von  $10^{-5}$  für die Erkennung von residualen Leukämiezellen. Das heißt, wir konnten damit eine Leukämiezelle unter 100.000 normalen Knochenmarkszellen erkennen. Zur Validierung dieser Methode wurden KM-Proben von 41 Patienten in kompletter Remission (CR) nach Induktionstherapie mit Hilfe dieser neu entwickelten Zwei-Stufen-Nachweismethode hinsichtlich ihrer MRD analysiert. In 39 Proben reichte die DNA-Qualität sortierter Zellen für die Sequenzierung aus. Einundzwanzig Proben testeten MRD positiv, während 18 negativ waren. Bei einer medianen Nachbeobachtung von 559 Tagen entwickelten 71% der MRD-positiven (15/21) und 28% (5/18) der MRD-negativen Patienten ein Rezidiv ( $p=0.0065$ ). Dementsprechend war das mediane Rezidiv-freie Überleben bei MRD-positiven Patienten (283 Tage vs. nicht erreicht,  $p=0.0031$ ) deutlich kürzer. Darüber hinaus war die MRD-Positivität, wie sie durch diese Methode festgestellt wurde, in der multivariaten Analyse der informativste Parameter für die kumulative Inzidenz eines Rezidivs (Hazard Ratio 7.07). Zusammenfassend kann gesagt werden, dass die hier entwickelte Messung der MRD, die auf Anreicherung von Leukämiezellen mittels MFC mit Antikörpern gegen CLL-1, TIM-3, CD123 und CD117 gefolgt von einer Mutationsanalyse basiert, eine hohe Sensitivität hat und eine gute Vorhersagekraft bezüglich des Rezidivrisikos bei AML-Patienten besitzt. Weitere multizentrische klinische Studien zur Standardisierung und zum Vergleich dieser vielversprechenden neuen Methode mit anderen Ansätzen der MRD-Messung bei AML sollten durchgeführt werden.

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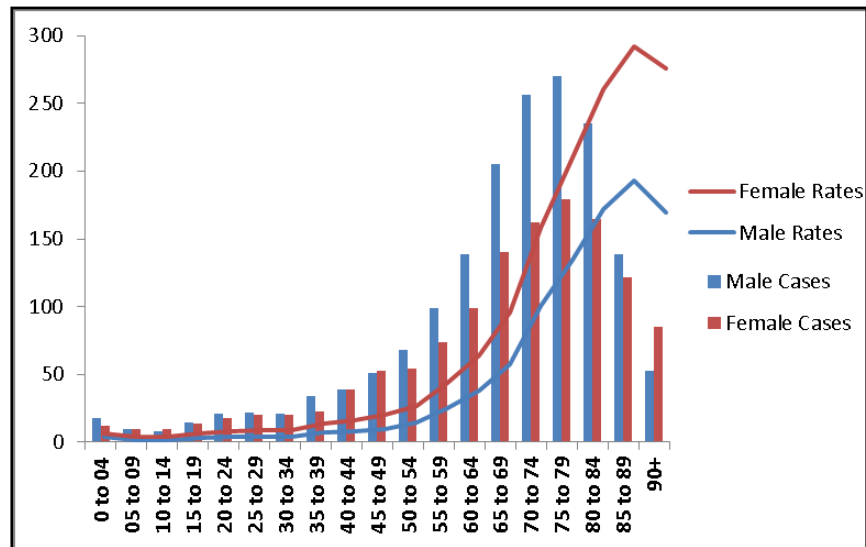
## 1. INTRODUCTION

The burden of cancer is on a constant rise globally due to changing lifestyle. Globocan estimates show approximately 14.1 million new cancer cases and 8.2 million deaths due to cancer occurred in 2012 worldwide (Miller, Siegel et al. 2016). Acute myeloid leukemia (AML) represents about 1.3% of all new cancer cases. In 2017, an estimated 21,380 new cases of acute myeloid leukemia were diagnosed and an estimated 10,590 people died of this disease (Khan, Orchard et al. 2019). AML is a heterogeneous clonal disorder characterized by an excessive accumulation of immature leukemic cells known as blasts, which lose their ability to differentiate. Blast cells accumulate in the bone marrow, blood, and organs leading to hematopoietic insufficiency (Deschler and Lübbert 2006). AML can progress rapidly and if left untreated can be fatal in weeks or months.

AML accounts for approximately 25% of all leukemias, making it the most frequent type of myeloid leukemia. AML is characterized by the presence of  $\geq 20\%$  blasts in bone marrow or peripheral blood (Estey and Döhner 2006). AML can originate in a previously healthy individual and is then known as de novo AML. AML could emerge as a consequence of treatment with cytotoxic agents referred to as therapy-related AML (tAML) (Sill, Olipitz et al. 2011). Transformation to AML from a preceding clonal illness such as myelodysplastic /myeloproliferative neoplasms (MDS/MPN) is referred to as secondary AML (sAML) (Szotkowski, Rohon et al. 2010).

### 1.1 INCIDENCE

The prevalence is 3.8 cases per 100,000 in adults'  $\leq 65$  years. The prevalence rises to 17.9 cases per 100 000 in adults'  $\geq 65$  years (De Kouchkovsky and Abdul-Hay 2016). AML, therefore primarily is a disease of later adulthood. The median age at presentation is about 70 years (Forman, Stockton et al. 2003). Incidence rates escalate gradually from age 40-44, more steeply from age 60-64, with the highest incidence in the age group 85-89 in males, and  $\geq 90$  in females (Ries, Eisner et al. 2007) (Figure 1).



**Figure 1:** Incidence of AML is strongly related to age and is found to increase with age. The figure displayed has been originally created using statistics from Cancer Research UK, Accessed May 2018.

## 1.2 CLASSIFICATION OF AML

The two frequently used systems in the classification of AML are the French-American-British (FAB) system and the World Health Organization (WHO) system. The FAB system was the first attempt on distinguishing the various types of AML and it is based on morphology and cytochemistry (Bennett, Catovsky et al. 1976). 8 subtypes of AML are recognized under the FAB classification (Table 1).

**Table 1:** FAB classification of AML. (Kumar 2011)

| FAB subtype | Morphological classification                        | % of all AML cases |
|-------------|---|--------------------|
| AML-M0      | Undifferentiated acute myeloblastic leukemia        | 5                  |
| AML-M1      | Acute myeloblastic leukemia with minimal maturation | 15                 |
| AML-M2      | Acute myeloblastic leukemia with maturation         | 25                 |
| AML-M3      | Acute promyelocytic leukemia                        | 10                 |
| AML-M4      | Acute myelomonocytic leukemia                       | 20                 |
| AML-M4eos   | Acute myelomonocytic leukemia with eosinophilia     | 5                  |
| AML-M5      | Acute monocytic leukemia                            | 10                 |
| AML-M6      | Acute erythroid leukemia                            | 5                  |
| AML-M7      | Acute megakaryoblastic leukemia                     | 5                  |

In order to incorporate new developments made in the diagnosis and management of AML, the World Health Organization (WHO) introduced a new classification system in 2001, which was first revised in 2008 and later in 2016. The WHO classification aims to combine genetic information with morphology, immunophenotyping and clinical presentation (Vardiman, Harris et al. 2002, Arber, Orazi et al. 2016). The WHO classification identifies six major classes (Table 2).

### **1.3 AETIOLOGY**

Studies have shown a number of causes that could predispose to AML such as (a) congenital abnormalities (b) environmental factors.

#### **1.3.1 Congenital Abnormalities**

Children with Down syndrome have a 10 to 20 fold increased likelihood of developing acute leukemia (Fong and Brodeur 1987). Other inherited diseases associated with AML include Klinefelter syndrome, Li-Fraumeni syndrome, Fanconi anemia, and neurofibromatosis (Pötsch, Voigtländer et al. 2002) (Table 4).

#### **1.3.2 Environmental Factors**

Exposure to ionizing radiation has been associated to AML. An increased incidence of AML was witnessed amongst survivors of the Japan atomic bomb explosions, with a peak at 5 to 7 years after exposure (Preston, Kusumi et al. 1994). Chronic exposure to certain chemicals also shows an increased risk for the development of AML. Benzene is the best studied and most widely used potentially leukemogenic agent (Savitz and Andrews 1997). Persons exposed to embalming fluids, ethylene oxides, and herbicides also appear to be at increased risk. Furthermore, smoking has been debated to be associated with an increased risk of developing AML (particularly of FAB subtype M2), especially in persons older than 60 years (Pogoda, Preston-Martin et al. 2002).

**Table 2:** WHO classification of AML. (De Kouchkovsky and Abdul-Hay 2016)

| <b>FAB subtype</b>                                     | <b>Morphological classification</b>   |
|--|---|
| <b>AML with recurrent genetic abnormalities</b>        | AML with t(8;21)(q22;q22)/RUNX1-RUNX1T1   |
|  | AML with inv(16)(p13.1q22)<br>AML with t(16;16)(p13.1;q22)/CBFB-MYH11                             |
|  | APL with PML-RARA   |
|  | AML with t(9;11)(p21.3;q23.3)/MLLT3-KMT2A   |
|  | ML with t(6;9)(p23;q34.1)/DEK-NUP214  |
|  | AML with inv(3)(q21.3q26.2)<br>AML with t(3;3)(q21.3;q26.2)/GATA2, MECOM                          |
|  | AML with t(1;22)(p13.3;q13.3)/RBM15-MKL1<br>(megakaryoblastic)                                    |
|  | AML with BCR-ABL1 (provisional entity)  |
|  | AML with mutated <i>NPM1</i>  |
|  | Provisional entity: AML with mutated <i>RUNX1</i><br>AML with biallelic mutations in <i>CEBPA</i> |
| <b>AML with myelodysplasia-related changes</b>         |   |
| <b>AML not otherwise specified</b>                     | AML with minimal differentiation  |
|  | AML without maturation  |
|  | AML with maturation   |
|  | Acute myelomonocytic leukemia   |
|  | Acute monoblastic/monocytic leukemia  |
|  | Acute erythroid leukemia  |
|  | Pure erythroid leukemia   |
|  | Acute megakaryoblastic leukemia   |
|  | Acute basophilic leukemia   |
| Acute panmyelosis with myelofibrosis                   |   |
| <b>Myeloid sarcoma</b>                                 |   |
| <b>Myeloid proliferations related to Down syndrome</b> | Transient abnormal myelopoiesis   |
|  | ML associated with Down syndrome  |
| <b>Therapy-related myeloid neoplasms</b>               |   |
| <b>Myeloid neoplasms with germline predisposition</b>  |   |
| <b>Acute leukemias of ambiguous lineage</b>            | Acute undifferentiated leukemia   |
|  | Mixed phenotype acute leukemia ,B/myeloid,NOS<br>Mixed phenotype acute leukemia ,T/myeloid,NOS    |
|  | Natural killer cell (NK) lymphoblastic leukemia/lymphoma<br>(Provisional entity)                  |

## 1.4 CYTOGENETICS AND MOLECULAR GENETICS IN AML

### 1.4.1 Chromosomal Aberrations

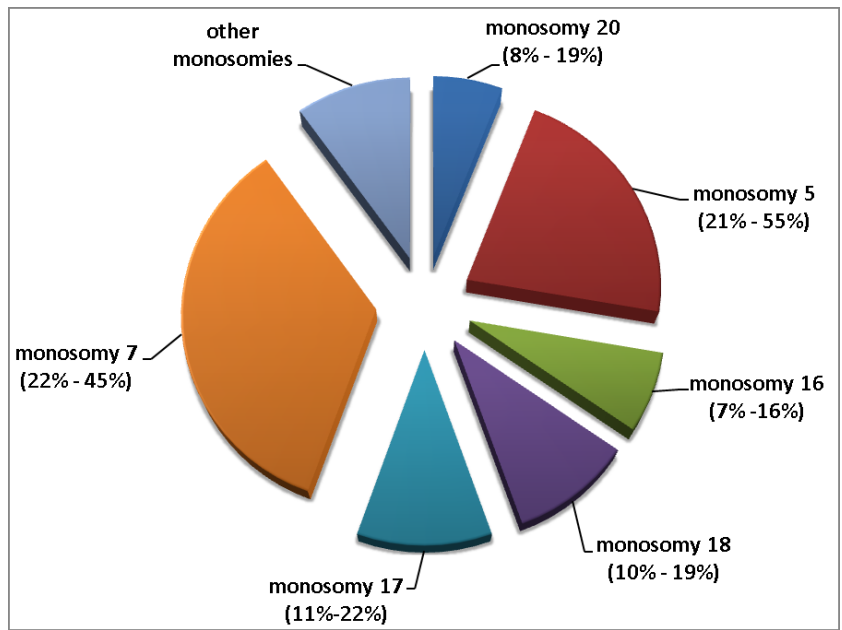
Chromosomal abnormalities can be found in about 50% of AML cases, while approximately 40%–50% of the AMLs diagnosed are cytogenetically normal (CN-AML). Several identified chromosomal aberrations have not only been proven to be important mutational drivers of AML development, but also to carry significant prognostic information concerning disease outcome.

#### a) Deletions & Duplications

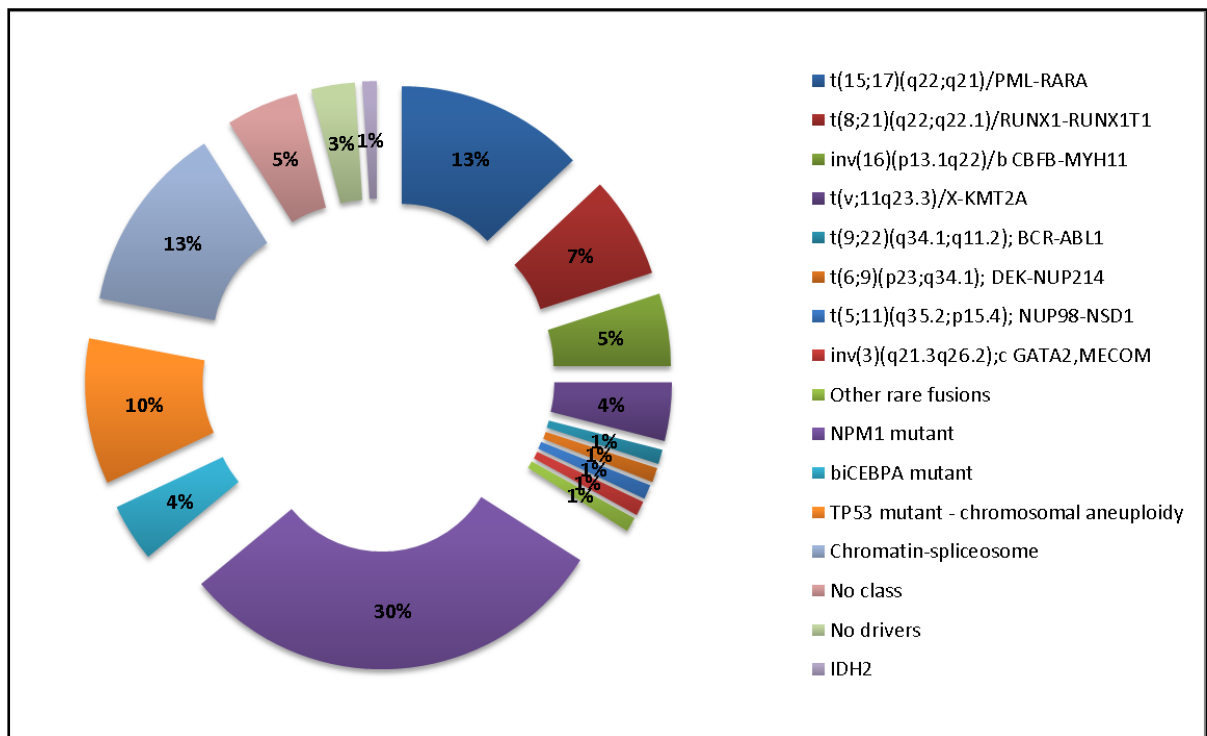
Commonly found abnormalities are deletions of chromosomal arms, such as del (5q), del (7q) or loss of whole chromosomes, such as chromosome 7, 5, 9 and Y followed by monosomies 17, 18, 16, 5, and 3 (Mrózek, Heinonen et al. 2001, Voutiadou, Papaioannou et al. 2013, Anelli, Pasciolla et al. 2017) (Figure 2). Studies have also shown that any type of monosomy in AML was associated with a poor outcome (Medeiros, Othus et al. 2010, Voutiadou, Papaioannou et al. 2013). Trisomies 8 and 21 have also been known to occur in AML cells.

#### b) Translocations

Cytogenetic abnormalities including t(8;21)(q22;q22), t(15;17)(q22;q12), inv(16)(p13.1;q22) and alterations of 11q23 are the four most common translocations occurring each in 3% to 10% of AML cases, while for others such as t(16;16)(p13.1;q22), t(9;11)(p22;q23), t(1;22)(p13.3;q13.3) the prevalence is significantly less (Stasi, Del Poeta et al. 1993, Martens and Stunnenberg 2010). Abnormalities such as t(6;9)(p23;q34.1), inv(3)(q21.3q26.2), t(3;3)(q21.3;q26.2) are also known in AML (Grimwade 2001). The translocation t(15;17) is associated with a particular subtype of AML, acute promyelocytic leukemia (APL). Translocations t(8;21), inversion (16) and t(16;16) constitute the core binding factor (CBF) leukemias (Kumar 2011) (Figure 3). Table 4 shows the most frequent chromosomal aberrations and their corresponding fusion genes in AML.



**Figure 2:** Frequency of autosomal monosomies in AML. The figure displayed has been originally created using statistics from Anelli, Pasciolla et al. 2017.



**Figure 3:** Molecular classes of AML in patients aged up to 65 years. The figure displayed has been originally created using statistics from Rowe et al, 2014.

**Table 3:** Selected risk factors associated with AML. (Deschler and Lübbert 2006, License number: 4605900391982)

|                                 |   |
|---------------------------------|---|
| Genetic disorders               | Down syndrome, Klinefelter syndrome, Ataxia telangiectasia, Patau syndrome Shwachman syndrome, Kostman syndrome Neurofibromatosis, Fanconi anemia, Li-Fraumeni syndrome |
| Physical and chemical exposures | Benzene, Embalming fluids   |
|                                 | Drugs such as pipobroman  |
|                                 | Pesticides & Herbicides, Cigarette smoking  |
| Radiation exposure              | Nontherapeutic and therapeutic radiation  |
| Chemotherapy                    | Alkylating agents   |
|                                 | Topoisomerase-II inhibitors   |
|                                 | Anthracyclines and Taxanes  |

**Table 4:** Oncofusion proteins associated with AML. (Martens and Stunnenberg 2010, License number: 4605901195140)

| Translocations | Oncofusion protein | Frequency of occurrence (% of AML) | Prognosis    | FAB            |
|----------------|--------------------|------------------------------------|--------------|----------------|
| t(8;21)        | AML1-ETO           | 10%                                | favorable    | M2             |
| t(15;17)       | PML-RAR $\alpha$   | 10%                                | favorable    | M3             |
| inv(16)        | CBF-MYH11          | 5%                                 | favorable    | M4             |
| der(11q23)     | MLL-fusions        | 4%                                 | Variable     | M4/ M5         |
| t(9;22)        | BCR-ABL1           | 2%                                 | adverse      | M1/M2          |
| t(6;9)         | DEK-CAN            | <1%                                | adverse      | M4/M2          |
| t(1;22)        | OTT-MAL            | <1%                                | Intermediate | M7             |
| t(8;16)        | MOZ-CBP            | <1%                                | adverse      | M4/ M5         |
| t(7;11)        | NUP98-HOXA9        | <1%                                | intermediate | M4/M2          |
| t(12;22)       | MN1-TEL            | <1%                                | variable     | M4/M7          |
| inv(3)         | RPN1-EVI1          | <1%                                | adverse      | M1/M2/M4/M6/M7 |
| t(16;21)       | FUS-ERG            | <1%                                | adverse      | M1/M2/M4/M5/M7 |

## 1.4.2 Gene Mutations

Next generation sequencing (NGS) has now allowed us to better define the genetic landscape of myeloid neoplasms and nearly 30 recurrently mutated genes have been identified. The Cancer Genome Atlas Research Network (TCGA) evaluated the genomes of 200 AML patients and found that an AML genome on an average harbors 13 mutations with 2-5 genes being recurrently mutated (Network 2013).

### i. Nucleophosmin 1 (*NPM1*)

The most frequently detected mutation is a heterozygous mutation of the *NPM1* gene (Kühnl and Grimwade 2012). It can be found in about one-third of AML cases. Several *NPM1* mutations have been defined, all being frameshift mutations by the insertion of 4 bases. In majority of *NPM1* mutated AML, insertions are found at exon-12 (rarely at exon-9 and exon-11) (Falini, Mecucci et al. 2005, Albiero, Madeo et al. 2007). This results in a longer protein with a different C-terminal amino acid sequence. This change in C-terminal leads to cytoplasmic localization of the *NPM1* protein, thus impairing the protein's nuclear shuttle function, which is essential for activation and stabilization of tumor suppressors such as p53 and ARF (Grummitt, Townsley et al. 2008).

### ii. CCAAT Enhancer Binding Protein $\alpha$ (*CEBPA*)

The *CEBPA* gene is mutated in approximately 5–10% of AML patients (Leroy, Roumier et al. 2005). About 70% of cases show a normal karyotype and about 25% carry concomitant *FLT3-ITD* mutations (Preudhomme, Sagot et al. 2002). Additionally, some cases of familial AML have been found to be associated with germline *CEBPA* mutations (Klein and Marcucci 1993). Mutations in *CEBPA* promote proliferation and inhibit differentiation of myeloid lineage (Pabst, Mueller et al. 2001, Bereshchenko, Mancini et al. 2009). The two most commonly occurring mutations are: a) N-terminal frame-shift mutations leading to expression p30, a truncated isoform of *CEBPA* and loss of p42, the full length protein and b) C-terminal in-frame insertions or deletions that affect DNA binding (Pabst and Mueller 2009). Interestingly, the majority of patients harboring *CEBPA* mutations, display biallelic mutations, combining an upstream mutation in one allele with a downstream mutation in the other.

**iii. Fms-Like Tyrosine Kinase 3 (*FLT3*)**

Mutations in the *FLT3* gene are found in 15–35% of AML patients (Yokota, Kiyoi et al. 1997). The most frequently occurring *FLT3* mutation in AML is an internal tandem duplication located within the intracellular domain of this receptor tyrosine kinase (*FLT3*-ITD). The duplication of a fragment within exons 14 and 15, that code for the juxtamembrane domain, results in constitutive activation of *FLT3* thereby promoting uncontrolled proliferation of leukemic cells (Nakao, Yokota et al. 1996).

Missense mutations have also been defined in the activation loop domain of the tyrosine kinase of *FLT3* (*FLT3*-TKD). These mutations are found in 5-10% of AML and rarely coexist with *FLT3*-ITD (Thiede, Steudel et al. 2002). The *FLT3*-TKD mutations commonly occur in codon 835 leading to a change of an aspartic acid to tyrosine (D835Y), however, other point mutations, deletions, and insertions within codon D835 and its adjacent codons have also been found (Abu-Duhier, Goodeve et al. 2001, Yamamoto, Kiyoi et al. 2001).

**iv. *RAS***

The isoforms *N-RAS* and *K-RAS* are mutated in up to 25% of de novo AML patients (Bos 1989). Activating mutations are typically found in codons 12, 13, and 61 of the *RAS* proto-oncogenes (Ritter, Kim et al. 2004). *HRAS* mutations are not so common in myeloid leukemia (Neubauer, Dodge et al. 1994). *NRAS* mutations are often identified in patients with *inv*(16) or *t*(16; 16) (Coghlan, Morley et al. 1994).

**v. Isocitrate dehydrogenase (*IDH*)**

Recurrent mutations in *IDH1* and *IDH2* are described in about 30% of CN-AML (Rakheja, Konoplev et al. 2012). *IDH1* mutations occur at position R132 while in the *IDH2* gene mutations occur at position R140 and R172 (Paschka, Schlenk et al. 2010, Chotirat, Thongnoppakhun et al. 2012). All these positions define highly conserved amino acids at the catalytic active site of these enzymes. The mutated proteins therefore show an altered function resulting in an unusual accumulation of the oncometabolite 2-hydroxyglutarate (2-HG) (Dang, White et al. 2009).

**vi. Ten–Eleven Translocation 2 (*TET2*)**

The *TET2* gene is mutated in 10–20 % of AML cases (Gaidzik, Paschka et al. 2012). *TET2* mutations are very diverse and spread throughout the entire coding region (Delhommeau, Dupont et al. 2009) . The majority of cases harbor nonsense and frameshift mutations resulting truncated translation (Abdel-Wahab, Mullally et al. 2009, Chou, Chou et al. 2011). The TET2 protein catalyzes the conversion of 5-methylcytosine to 5-hydroxymethylcytosine which results in demethylation of DNA (Ito, Shen et al. 2011) and *TET2* mutations disrupt this enzymatic function.

**vii. Mixed Lineage Leukemia (*MLL*)**

The mixed lineage leukemia (*MLL*) gene is located on chromosome 11q23 and chromosomal translocations and mutations containing the *MLL* gene define a distinct set of acute leukemia. Partial tandem duplications of the *MLL* gene (*MLL*-PTD) have been found to occur in 3–10% of adult AML cases (Krivtsov and Armstrong 2007). *MLL*-PTD involves an in-frame duplication of the 5' segment of the *MLL* gene (Caligiuri, Strout et al. 1998). *MLL*-PTD occurs with higher incidence in cases with CN-AML (up to 8%) (Steudel, Wermke et al. 2003) but is highest in trisomy 11 (up to 25%) (Rege-Cambrin, Giugliano et al. 2005).

**viii. Additional sex comb-like 1 (*ASXL1*)**

The *ASXL1* gene is mutated in 5%–11% of AML cases (Pratcorona, Abbas et al. 2012). It is suggested that *ASXL1* protein may play a role in epigenetic regulation (Chou, Huang et al. 2010). Interestingly, *ASXL1* mutations are five times more common in elderly patients (16.2%) as compared to younger patients (3.2%) (Metzeler, Becker et al. 2011).

**ix. DNA Methyl transferase 3A (*DNMT3A*)**

*DNMT3A* mutations occur in approximately 14–18% of AML cases, including 20–35% with normal karyotype (Thol, Damm et al. 2011). Many loss-of-function mutations have been described in all exons of *DNMT3A*, with a missense point mutation at amino acid R882 occurring most frequently (Ley, Ding et al. 2010). Interestingly, knockout of *DNMT3A* by itself was insufficient to initiate leukemia (Challen, Sun et al. 2012).

x. **TP53**

*TP53*, a tumor suppressor gene is one of the recurrently mutated genes in AML (Cancer Genome Atlas Research Network 2013, Rucker, Schlenk et al. 2012). While *TP53* mutations are detected at low frequencies in de novo AML, they can be detected at higher frequencies of up to 30% in tAML (Renneville, Roumier et al. 2008). Deletions or point mutations, usually missense mutations, frequently comprising exons 4–8, result in inactivation of the *TP53* gene.

## **1.5 DIAGNOSIS**

### **1.5.1 Morphology**

According to the WHO classification, AML is confirmed when the blood (PB) or bone marrow (BM) contains  $\geq 20\%$  blasts except for AML with t(8;21), t(15;17), t(16;16) or inv(16) (Vardiman, Harris et al. 2002). Morphological examination of bone marrow (BM) and peripheral blood (PB) smears are performed using a Wright-Giemsa or May-Grünwald-Giemsa stain. At least 200 WBCs on PB smears and 500 nucleated cells on BM smears should be counted (Döhner, Estey et al. 2010).

### **1.5.2 Immunophenotyping by flow cytometry**

Immunophenotyping using multiparameter flow cytometry (MFC) is used to characterize leukemia and lymphoma subtypes (Vardiman, Harris et al. 2002). Expression of several cluster differentiation (CD) markers on the cell surface or in the cytoplasm can be semi-quantified by measuring the binding of fluorescently labeled antibodies and detected using MFC. Binding of these antibodies establishes an immunophenotype, which is unique for a specific cellular lineage, maturational stage, and/or state (Campos, Guyotat et al. 1989, Baumgarth and Roederer 2000).

At least 4-colour MFC is recommended to ensure precise lineage involvement and identify the degree of differentiation (Craig and Foon 2008, Kalina, Flores-Montero et al. 2012). Generally,  $\geq 20\%$  leukemic cells expressing a particular marker are considered to be positive for that marker. For certain markers such as CD34, cytoplasmic CD3, and CD117 etc. a lower cutoff of 10% has been applied (Bene, Castoldi et al. 1995, Kalina, Flores-Montero et al. 2012). CD marker profiles can be applied to any fluid such as PB, cell suspension from lymph node and BM aspirate. Within minutes, thousands of cells from a sample can be analyzed. Table 5 provides a list of markers useful for diagnosis of AML.

### 1.5.3 Cytogenetics and molecular cytogenetics

Karyotyping is also included in the diagnostic work-up and in risk assessment of an alleged AML. About 50% of newly diagnosed AML patients present with cytogenetic abnormalities (Mrózek, Heinonen et al. 2001). Cytogenetics is one of the key prognostic factors to predict overall survival and remission rates. Several chromosomal abnormalities such as monosomies, translocations etc. are common in AML (Stasi, Del Poeta et al. 1993, Grimwade 2001). These aberrations can be detected with karyotypes established on metaphase-blocked cultured cells. In order to diagnosis a normal karyotype, at least 20 metaphase cells from BM need to be analyzed.

Specific chromosomal alterations such as balanced and unbalanced translocations, amplifications, inversions, or deletions of genes or genomic sections can also be visualized using fluorescence in situ hybridization (FISH) (Döhner, Estey et al. 2010). Besides confirmation of t(15;17) and its variants, detection of MLL gene fusion partners requires FISH analysis.

### 1.5.4 Molecular genetic testing

Presence or absence of specific gene mutations have facilitated further classification of AML cases and are known to influence patients' prognosis (Network 2013, Lindsley, Mar et al. 2015). This is mainly relevant for cytogenetically normal AML (CN-AML) patients.

AMLs with mutations in *NPM1* or *CEBPA* have been included as provisional entities in the WHO classification. Three molecular markers – *FLT3* internal tandem duplications, *CEBPA* and *NPM1* have been established in clinical practice already for more than a decade (Vardiman, Harris et al. 2002). Currently, the European Leukemia Net (ELN) recommends screening for the following genes during diagnostic workup:

- a. Mutations in *CEBPA*, *NPM1* and *RUNX1* genes since they define disease groups
- b. Mutations in *FLT3* (both for ITDs and TKD mutations); activating mutations of *FLT3* are not only prognostic, but could beneficially be affected by pharmacological inhibition of tyrosine kinase activity
- c. *ASXL1* and *TP53* gene mutations as they have been consistently shown to confer poor prognosis.

The prognostic significance of other gene mutations (e.g., *IDH1*, *IDH2*, *DNMT3A* etc.) is not well defined (Döhner, Estey et al. 2017)

**Table 5:** Cell-surface and cytoplasmic markers useful for the diagnosis of AML. (Cruz et al, 2017, License number: 4605911208232)

|                         |  |
|-------------------------|--|
|                         |  |
| Precursor stage markers | CD34, CD38, CD123, CD117, HLA-DR                                 |
| Granulocytic markers    | CD13, CD15, CD16, CD33, CD65, cytoplasmic myeloperoxidase (cMPO) |
| Monocytic markers       | CD11c, CD14, CD64, CD4, CD11b                                    |
| Megakaryocytic markers  | CD41 (glycoprotein IIb/IIIa), CD61 (glycoprotein IIIa), CD42     |
| Erythroid marker        | CD235a (glycophorin A), CD36                                     |
| Lymphoid markers        | CD2, CD5, CD7, CD19, CD56  |

**Table 6:** Molecular markers in AML. (Ferrara and Schiffer 2013, License number: 4605910032138)

|                  | Frequency in de-novo AML | Frequency in CN-AML | Strong associations                            | Not recorded with               | Outlook  |
|------------------|--------------------------|---------------------|--|---------------------------------|--|
| <i>NPM1</i>      | 35%                      | 50%                 | CN-AML, DNMT3A, FLT3-ITD, FLT3-TKD, IDH1, IDH2 | <i>CEBPA</i> double mutant      | Favorable in CN-AML patients and older patients  |
| <i>CEBPA</i>     | 7%                       | 8–19%               | CN-AML, FLT3-ITD                               | <i>NPM1</i>                     | Favourable if biallelic in CN-AML patients   |
| <i>FLT3-ITD</i>  | 20–25%                   | 30–35%              | CN-AML, APL, t(6;9), NPM1                      | -                               | Adverse in CN-AML patients; might differ with allelic burden; uncertain effect in APL patients |
| <i>FLT3-TKD</i>  | 5%                       | 14%                 | CN-AML, NPM1                                   | -                               | Debatable  |
| <i>KIT</i>       | -                        | 25%                 | CBF leukemia                                   | Most other karyotypes           | Adverse in adults with CBF leukemia  |
| <i>TET2</i>      | 8–12%                    | 23%                 | Probably CN-AML                                | <i>IDH1, IDH2</i>               | Debatable  |
| <i>DNMT3A</i>    | 14–22%                   | 20–33%              | CN-AML, NPM1, FLT3                             | CBF leukemia, <i>CEBPA, MLL</i> | Probably adverse in patients with CN AML   |
| <i>IDH1/IDH2</i> | 8–16%                    | 30%                 | CN-AML, NPM1, FLT3                             | <i>TET2, WT1</i>                | Debatable  |
| <i>ASXL1</i>     | 5–30%                    | About 10%           | Rarely with NPM1 & FLT3                        | <i>CEBPA</i>                    | Adverse in patients with CN-AML & elderly patients; more common in older patients              |

## **1.6 PROGNOSIS/RISK STRATIFICATION**

Over the years, prognostic factors in AML have remarkably evolved. Several clinical and biological features have been identified to predict the probability that a patient will achieve remission and eventually cure in response to treatment. Patient-related as well as disease-related factors influence prognosis of AML.

### **1.6.1 Patient-related factors**

#### **1.6.1.1 Age**

One of the strongest patient-related prognostic factors in AML is age of the patient at diagnosis. In the elderly patients, many factors contribute to a poor prognosis: comorbidities and contraindications against intensive cytotoxic treatment, decreased performance status, increased incidence of adverse cytogenetic abnormalities, former myelodysplastic syndrome (MDS) or cytotoxic treatment. Despite taking into account all these factors, age is an independent prognostic factor associated with inferior outcomes (Buchner, Berdel et al. 2009, Krug, Berdel et al. 2009). Regardless of the cytogenetic risk, patients in the age group  $\geq 65$  years have a dismal prognosis even after intensive chemotherapy (Schoch, Kern et al. 2004, Appelbaum, Gundacker et al. 2006). Nonetheless, age should not be the only basis of treatment decisions.

#### **1.6.1.2 Performance status**

Performance status (PS) is another powerful predictor of outcome, particularly in elderly patients. Patients with a performance status  $>2$  exhibit a poorer prognosis independent of age (Juliussen, Antunovic et al. 2009). Additionally, a combination of performance status and age in elderly patients is highly predictive for early death post induction treatment (Appelbaum, Gundacker et al. 2006).

### **1.6.2 AML-related factors.**

AML-related factors include white blood cell (WBC) count, existence of former MDS, previous cytotoxic therapy for another disorder and cytogenetic and molecular genetic changes in the leukemic cells at diagnosis.

### 1.6.2.1 Cytogenetics

Genetic aberrations are strong prognostic factors. Almost 50% of adults present with cytogenetic abnormalities at diagnosis. Based on the prognosis associated with known translocations and mutations, the ELN classified AML into favorable, intermediate and adverse groups. Earlier ELN used a 4-group system, which comprised of the intermediate I and intermediate II categories. The two categories were distinguished based on genetic characteristics, rather than on prognostic stratification. However, recent studies proved no significant prognostic difference in patients who primarily compose these two groups (Döhner, Estey et al. 2017). Therefore in 2017 the panel decided to simplify the ELN system by using a 3-group classification system (favorable, intermediate, and adverse).

The favorable risk group comprises of CBF-leukemia patients exhibiting balanced translocations  $t(8;21)$ ,  $t(15;17)$ , or  $inv(16)$ . Over 60% of these patients achieve long-lasting complete remission with high-dose cytarabine-based therapy alone (Byrd, Ruppert et al. 2004). Patients with CN-AML harboring *NPM1* mutations without FLT3-ITD as well as biallelic *CEPBA* mutations also belong to the favorable risk group and demonstrate higher CR and better OS rates (Döhner, Schlenk et al. 2005, Fasan, Haferlach et al. 2014).

The intermediate risk group comprises AML patients with *NPM1* mutations along with FLT3-ITD<sup>-low</sup>. Another subgroup in the intermediate risk group is AML patients with no mutations in *NPM1* but with low FLT3-ITD mutational load. Concerning cytogenetic findings, AMLs with  $t(9;11)(p21.3; q23.3)$  involving the *MLLT3* and *KMT2A* genes and other cases where cytogenetic findings are neither categorized as favorable nor adverse also belong to the intermediate risk category.

The adverse risk group comprises AMLs with complex or monosomal karyotypes. The following cytogenetic anomalies are classified as high-risk:  $abn(5q)/del(5q)$ ,  $-5$ ,  $-7$ ,  $abn(7q)/del(7q)$ ,  $t(11q23)$ ,  $-17$ ,  $abn(17p)$  and are thought to be unfavorable markers even in patients who receive allogeneic stem cell transplantation. Monosomal karyotypes are associated with the worst prognosis and very poor overall survival (Orozco and Appelbaum 2012). Complex karyotypes occur in 10% to 12% of AML cases. Patients with complex karyotype also exhibit a very dismal outcome (Mrózek, Marcucci et al. 2012). Complex karyotype is defined as the presence of 3 or more chromosome abnormalities without  $t(8;21)$ ,  $inv(16)$  or  $t(16;16)$ , and  $t(15;17)$ . Chromosomal loss at 17p with or without *TP53*

mutations are seen in about two-thirds of AMLs with complex karyotypes (Haferlach, Dicker et al. 2008, Rucker, Schlenk et al. 2012).

**Table 7:** Risk stratification in AML. (Cruz et al, 2017, License number: 4605911208232)

| Risk category       | Genetic abnormalities  |
|---------------------|--|
| <b>Favorable</b>    | <p>t(8;21)(q22;q22.1)/<i>RUNX1-RUNX1T1</i><br/>           inv(16)(p13.1q22) or t(16;16)(p13.1;q22)/<i>CBFB-MYH11</i><br/>           Mutated <i>NPM1</i> without FLT3-ITD/FLT3-ITD<sup>low</sup><br/>           Biallelic mutated <i>CEBPA</i></p>  |
| <b>Intermediate</b> | <p>Mutated <i>NPM1</i> and FLT3-ITD<sup>high</sup><br/>           Wild-type <i>NPM1</i> without FLT3-ITD or with FLT3-ITD<sup>low</sup><br/>           (without adverse risk genetic lesions)<br/>           t(9;11)(p21.3;q23.3)/<i>MLLT3-KMT2A</i><br/>           Cytogenetic abnormalities not classified as favorable or adverse</p>   |
| <b>Adverse</b>      | <p>t(6;9)(p23;q34.1)/<i>DEK-NUP214</i><br/>           t(v;11q23.3)/<i>KMT2A</i> rearranged<br/>           t(9;22)(q34.1;q11.2)/<i>BCR-ABL1</i><br/>           inv(3)(q21.3q26.2) or t(3;3)(q21.3;q26.2); <i>GATA2</i>, <i>MECOM</i>(<i>EV11</i>)<br/>           -5 or del(5q); -7;-17/abn(17p)<br/>           Complex karyotype, monosomal karyotype<br/>           Wild-type <i>NPM1</i> and FLT3-ITD<sup>high</sup><br/>           Mutated <i>RUNX1</i><br/>           Mutated <i>ASXL1</i><br/>           Mutated <i>TP53</i></p> |

### 1.6.2.2 Molecular Genetics

Lately, in addition to cytogenetics, gene mutations have also been recognized as risk factors. Somatic mutations have been identified in numerous genes, the frequencies of which differ among the cytogenetic groups (Mardis, Ding et al. 2009). While some mutations can be identified in combination with two or three other mutations, others are mutually exclusive (Table 6).

Presence of the FLT3-ITD in CN-AML cases is associated with poor outcome (Thiede, Steudel et al. 2002). Numerous studies show that CN-AML harboring *NPM1* mutations have improved CR rates and better RFS and event-free survival (EFS). Interestingly, patients with *NPM1* mutations also harbor FLT3-ITD (40%) and FLT3-TKD (15%) mutations (Marcucci, Haferlach et al. 2011). The FLT3-ITD mutations negate the beneficial effects of *NPM1* and the outcome is far worse than in presence of a mutated *NPM1* and wildtype *FLT3* (Döhner, Schlenk et al. 2005, Schlenk, Döhner et al. 2008). Presence of the *CEBPA* mutations in CN-AML is associated with a favorable prognosis (Taskesen, Bullinger et al. 2011, Fasan, Haferlach et al. 2014). Retrospective studies have showed that the presence *KIT* mutations in favourable risk patients with t(8;21) or inv(16)/t(16;16) confers an inferior outcome (Nanri, Matsuno et al. 2005).

Mutations in the runt-related transcription factor 1 (*RUNX1*) occur concomitantly with unfavorable factors like older age, antecedent myeloid disorder, and associated gene mutations (eg: *SRSF2*, *ASXL1*) and are independently linked to poorer outcome (Tang, Hou et al. 2009, Mandler, Maharry et al. 2012). *ASXL1* mutations are associated with dismal survival in with CN-AML cases (Pratcorona, Abbas et al. 2012, Schnittger, Eder et al. 2013) as well as in elderly patients with CN-AML and favourable genetic risk (Metzeler, Becker et al. 2011)

Mutations in *TP53* gene are concomitant with complex karyotype, monosomal karyotype, and specific chromosomal aneuploidies (del (5/5q), del (7/7q) (Haferlach, Dicker et al. 2008, Bowen, Groves et al. 2009). In AML patients presenting with a complex karyotype, TP53 alterations are an independent adverse prognostic factor for survival. (Rücker, Schlenk et al. 2012). Mutations in several other genes have been identified; however, the prognostic effect of most of these findings is unclear, with inconsistent results from various groups (Table 6).

## 1.7 THERAPY of AML

### 1.7.1 Induction therapy

Currently the standard for induction therapy is the “3+7” regimen. The purpose of induction is to achieve complete remission (CR) and includes three days of an anthracycline such as daunorubicin ( $\geq 60 \text{ mg/m}^2$ ) and seven days of continuous cytarabine intravenously ( $100\text{-}200 \text{ mg/m}^2$ ). CR is defined by presence of  $\leq 5\%$  blasts in the bone marrow, recovery of absolute platelet and neutrophil counts with no sign of extramedullary AML (Cheson, Bennett et al. 2003, Showel and Levis 2014). As soon as the diagnostic work-up has been completed, induction therapy should be started without further delay, since retrospective studies have shown that treatment delay beyond 5 days adversely affects the outcome. Please insert corresponding citation?

Efforts to increase CR rates by using a different anthracycline such as mitoxantrone, idarubicin (Arlin, Case et al. 1990), high-dose cytarabine (Bishop, Matthews et al. 1996), additional cytotoxic agents (etoposide, fludarabine, topotecan) (Estey, Thall et al. 2001), modulators of multidrug resistance, or growth factors (Lyman, Dale et al. 2010) have mostly been unsuccessful. Treatment for APL patients differs from other AML subtypes. The combination of the vitamin A derivative all-trans retinoic acid and arsenic trioxide allows chemotherapy-free treatment resulting in high remission and overall survival rates (Sanz and Lo-Coco 2011).

### 1.7.2 Consolidation Therapy

The main aim of the consolidation is designed to eliminate residual leukemia cells that persist despite achieving CR after induction therapy. Consolidation therapy either consists of chemotherapy or allogeneic hematopoietic stem cell transplantation (HSCT) (Estey and Döhner 2006, Ferrara and Schiffer 2013). The choice of therapy depends on the relapse-risk of the leukemia and the age of the patient, existing comorbidities as well as the availability of an appropriate donor for HSCT.

High-dose cytarabine is used as consolidation in CBF-AML patients and patients carrying either *NPM1* without FLT3-ITD or biallelic *CEBPA* mutations, which all belong to the favorable relapse-risk group according to current ELN guidelines. For other patients fit for intensive treatment the most preferred consolidation therapy is 2-4 cycles of intermediate dose cytarabine (Döhner, Estey et al. 2017), but relapse risk still remains high. Patients having an unfavorable risk profile, most likely do not benefit from such chemotherapy.

Therefore, such patients as well as patients with an intermediate-risk profile and a suitable matched donor should undergo allogeneic stem cell transplantation (Döhner, Weisdorf et al. 2015). If no donor is available, patients with an unfavourable risk profile are recommended investigational treatments.

### **1.7.3 Allogeneic Stem Cell Transplantation**

HSCT is recommended for patients responding poorly to chemotherapy alone. Such patients may benefit from allogeneic stem-cell transplantation with matched sibling or alternative donors (De Kouchkovsky and Abdul-Hay 2016). At present, HSCT is considered appropriate in patients having an intermediate or unfavorable risk profile. CBF-AML patients carrying *KIT* mutations could also be considered for HSCT (Majhail, Farnia et al. 2015). However, HSCT has a treatment-related mortality of 10-25% because of graft versus- host disease and substantial adverse effects on quality of life (Cornelissen, Van Putten et al. 2007). Therefore, risks and benefits of HSCT need carefully assessed and balanced (Cornelissen et al., 2015).

### **1.7.4 Treatment options in older patients**

Non-intensive therapies such as hypomethylating agents, like decitabine or azacitidine (Deschler and Lübbert 2006) are given to older patients unable to withstand intensive treatments. Superior survival and quality-of-life is seen in patients treated with these hypomethylating agents (Dombret, Seymour et al. 2015).

### **1.7.5 Additional treatment options**

To improve the prognosis of AML patients numerous new-targeted treatments have been developed. Multi-kinase inhibitors such as midostaurin and sorafenib are being studied in patients carrying *FLT3* mutations. Monoclonal antibodies and chimeric antigen receptor T-cells (CAR T-cells) targeting cell surface markers such as CD33 and CD123 are currently in early clinical studies (Stein and Tallman 2016, Döhner, Estey et al. 2017).

### **1.7.6 CURRENT TREATMENT OUTCOMES**

In patients  $\leq 60$  years, about 60-85% enter complete remission (CR) after induction treatment while only 45-60% of elderly patients treated with intensive therapy attain CR (Burnett, Wetzler et al. 2011, Döhner, Weisdorf et al. 2015). Though induction therapy results in high remission rates in young patients, about half of these patients eventually relapse and die without HSCT (Gerstung,

Papaemmanuil et al. 2017). Even with HSCT, about one third will relapse (Rowe, Li et al. 2005, Cornelissen, Van Putten et al. 2007). Thus, AML can only be cured in 35-40% of adult patients who are  $\leq 60$  years and in 5-15% of patients older than 60 years. In a large epidemiological study the reported relative 5 year OS rates for patients below 45, below 65, and above 65 were 41%, 30%, and 4%, respectively (Showel and Levis 2014).

## **1.8 MINIMAL RESIDUAL DISEASE**

Recent evidence suggests that the presence of the residual therapy-resistant cells during remission drive leukemia relapse and make the cure of AML challenging. At the time of diagnosis, the tumor load is 90-100% with few or no normal cells in BM of AML patients. After undergoing therapy, the numbers of leukemic cells decrease below 5% in the BM and the patient is in CR. However, a few leukemic cells still persist which eventually give rise to relapse. These small numbers of cancer cells that persist in a patient during or after treatment, even though clinical and microscopic examinations confirmed CR is termed as minimal residual disease (MRD) (Figure 3). These persisting residual cells cannot be detected by conventional microscopic techniques (Benton and Ravandi 2017) . Several sensitive methods have been developed in recent years for MRD detection based on the detection of cancer-specific deoxyribonucleic acid (DNA), ribonucleic acid (RNA) or proteins – to measure minute levels of cancer cells in blood or bone marrow samples. Thus, MRD indicates the presence of leukemic cells detected at levels of 1 in  $10^2$  to  $10^6$  normal bone marrow cells, in comparison to 1 in 20 cells with morphology-based evaluation (Schuurhuis, Heuser et al. 2018).

## **1.9 TECHNIQUES FOR MRD DETECTION**

### **1.9.1 Molecular methods for MRD detection**

Molecular MRD is based on detecting a leukemia-specific DNA or messenger RNA (mRNA) sequence through the use of real-time polymerase chain-reaction (PCR)-based approaches, since cytogenetic techniques such as karyotyping and/or FISH lack the sensitivity required for MRD monitoring (Schuurhuis, Heuser et al. 2018). Based on the presence of a persisting PCR-positive leukemia-specific signal after therapy, relapse risk can be estimated in individual patients.

Approximately 15–20% of AML harbor recurrent chromosomal translocations, including  $t(8;21)(q22;q22);RUNX1-RUNX1T1$ ,  $inv(16);CBF-MYH11/t(16;16)$ ;  $DEK-CAN$  and  $t(15;17)(q22;q21)$ ;  $PML-RARA$  (Van Dongen, Macintyre et al. 1999). Monitoring these gene fusion transcripts by qPCR at diagnosis and follow-up can serve as surrogate markers for residual disease. Clinically established DNA or RNA based PCR tests are available for patients harboring the translocations  $t(8;21)$ ,  $t(15;17)$ ,  $t(6;9)$  and  $inv(16)$  (Guerrasio, Pilatrinio et al. 2002, Zhu, Zhang et al. 2013). Due to their rather low incidence, use of these markers is limited to a minor group of AML patients.

Recurrent somatic mutations and overexpressed genes can also serve as a candidate for MRD detection by quantitative PCR. Probable targets for MRD detection are mutations in *NPM1*, *MLL*, *FLT3*, and overexpression of *PRAME*, *WT1*, and *EVII*. While MRD detection based on qPCR assays targeting mutated *NPM1* or *WT1* overexpression is clinically established, the utility of the other potential targets is currently being explored.

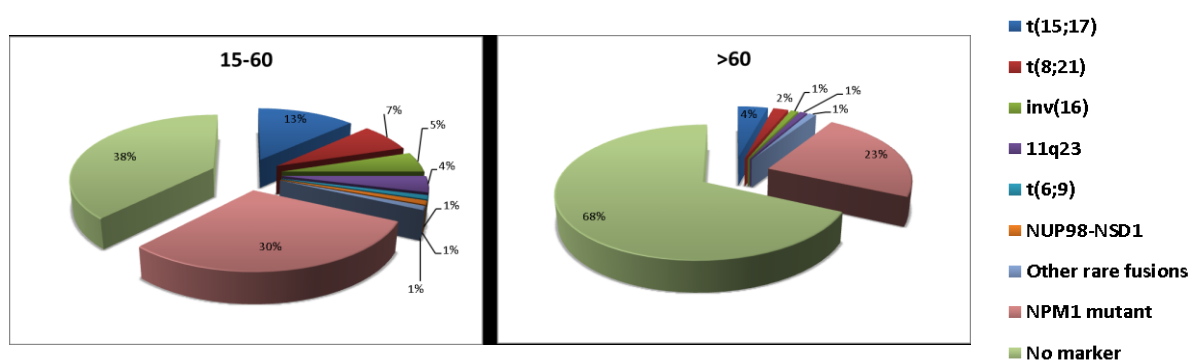
#### **1.9.1.1 *NPM1* Mutations**

*NPM1* mutations, usually 4-nucleotide frameshift insertions in exon 12 are found in about 30% of of AMLs and can be effectively traced using RT-PCR (Schnittger, Kern et al. 2009). *NPM1* is one of the most clinically relevant markers for molecular monitoring in CN-AML. An allele-specific qPCR test of mutated *NPM1* was first developed in 2006 (Gorello, Cazzaniga et al. 2006). The mutated *NPM1* transcript is frequently highly expressed, affording a sensitivity of 1 in  $10^5$  when using RNA-based assays (Falini, Nicoletti et al. 2007). Owing to its reliability and stability throughout disease *NPM1* serves as a good target for molecular MRD. Recent studies suggest that qPCR assay for *NPM1* mutations is suitable for monitoring and quantifying MRD in patients with AML (Krönke, Schlenk et al. 2011; (Hubmann, Köhnke et al. 2014, Balsat, Renneville et al. 2017). Indeed, these studies revealed that patients with persisting mutated *NPM1* transcripts in PB after two cycle of chemotherapy were at an increased risk of relapse. Monitoring both PB and BM samples serially showed that analysis of BM affords a median 1-log increment in sensitivity thereby increasing the MRD detection rate. The ELN MRD Working Party recommends monitoring *NPM1* transcripts in PB as well as BM (Schuurhuis, Heuser et al. 2018).

### 1.9.1.2 *WT1* Overexpression

The gene, Wilms tumor 1 (*WT1*), although expressed in about 90% of AMLs is often overexpressed to a level that it can be used as a MRD test in about 50% of cases (Cilloni, Renneville et al. 2009). However, the significance of MRD detection using *WT1* overexpression is currently under debate due to its rather limited specificity and sensitivity.

Even though sensitivity of the PCR-based techniques is high (up to 1 in  $10^5$  cells in some studies), it cannot be applied to the majority of patients. Altogether about 60% of AMLs in adults  $\leq 60$  years can be covered (Grimwade, Jovanovic et al. 2009, Grimwade and Freeman 2014) (Figure 4). Moreover, RNA is a much less stable target for diagnostics than DNA and requires careful handling and processing.



**Figure 4:** Proportion of leukemia specific targets for MRD detection by RT-PCR according to age. The figure displayed has been originally created using statistics from Grimwade and Freeman 2014.

### 1.9.2 Flow cytometry-based tests

MRD detection by MFC can rely on two approaches: either on the detection of leukemia-associated immunophenotypes (LAIPs), which is based on identified LAIPs at diagnosis and their tracing at later time points (Al-Mawali, Gillis et al. 2009). The second possibility is to recognize cells, which are different from normal in the remission bone marrow. This latter approach can also be used, if there is no initial phenotype known. Leukemic cells typically can show four kinds of immunophenotypic abnormalities: absence of expression, cross-lineage antigen expression, asynchronous expression and abnormal overexpression of an antigen (Table 5) (Al-Mawali, Gillis et al. 2008, Kern, Bacher et al. 2010).

The advantage of using MFC for MRD detection is its applicability in a majority of AML patients (>80%) and a quick turnaround. Many antibody panels have been recommended by experts or validated in clinical practice (Freeman, Terwijn), but testing still remains to be standardized. The ELN MRD Working Party has started attempts for standardization and currently recommends using an adequately large panel of antibodies (8 colors) for detection of MRD by flow cytometry (Schuurhuis, Heuser et al. 2018). One of the limitations of MFC is that immunophenotypic shifts may occur over the course of disease making it difficult to detect MRD in patients with a different LAIP when they relapse (Oelschlägel, Nowak et al. 2000, Langebrake, Brinkmann et al. 2005). Furthermore, a high level of expertise is required to interpret the data proficiently when it comes to MRD detection. The sensitivity for the detection of malignant cells varies according to the type of leukemia, the panel of antibodies used, the number of cells analyzed and the expertise of the laboratory. Currently, sensitivities of 0.1 to 0.01% (1 in  $10^3$  to  $10^4$ ) can be reached with MFC (Del Principe, Buccisano et al. 2016, Ravandi, Jorgensen et al. 2017).

### **1.9.3 Next Generation Sequencing (NGS)**

Although PCR based techniques of MRD detection are both specific and sensitive, they can be applied only to <50% of AML cases. With growing understanding of the genetic landscapes in AML, NGS has been increasingly considered for MRD detection (Patel, Gönen et al. 2012, Network 2013). The DNA fragments of the target of interest are amplified by PCR, are then sequenced in parallel and compared to the reference sequence. The percentage of a particular aberrant sequence among the total number of sequences for the target region is usually expressed as the variant allele fraction (VAF) and is a direct measure of underlying disease (Vedula and Lindsley 2017, Zhou and Wood 2017). The advantage of NGS based MRD testing is that NGS allows parallel testing of several genetic mutations thereby not only defining which mutations are present but also enumerating the proportion of cells that carry every mutation. Furthermore, because disease-specific multigene panels cover the vast majority of recurrent mutations in AML nearly, there is no need for patient-specific assays. The major limitation of NGS based MRD detection is the current base error rate, which allows only for a low sensitivity of about 0.5 to 1% (Schmitt, Kennedy et al. 2012, Gaksch, Kashofer et al. 2018).

### 1.9.4 Digital PCR (dPCR)

dPCR permits absolute quantification of a target sequence at a very high sensitivity (Hindson, Chevillet et al. 2013). Instead of the bulk-reaction used in RT-PCR, the dPCR reaction is performed by dividing a PCR reaction into hundreds of thousands (or greater) of individual droplet “compartments,” each with, at most, 0-1 copy of the region of interest before amplification. This permits digital “counting” of positive droplets that corresponds to the number of targets present in a sample (Huggett, Cowen et al. 2015). Although presently the most sensitive technique for detection of MRD, dPCR is laborious to validate and needs to be personalized making it suitable only for a single gene of interest (Grimwade and Freeman 2014, Buccisano, Hourigan et al. 2017).

**Table 8:** Pros and cons of methods used to detect MRD in AML. (Cruz et al, 2017, License number: 4605911208232)

| Technique | Sensitivity            | Pros   | Cons   |
|-----------|------------------------|--|--|
| MFC       | $10^{-3}$ to $10^{-4}$ | <ul style="list-style-type: none"> <li>• wide applicability (&gt;90%)</li> <li>• relatively quick results</li> <li>• high specificity when using defined LAIP</li> <li>• can distinguish between live and dead cells</li> <li>• ease of data storage</li> <li>• provides information about whole sample cellularity</li> </ul> | <ul style="list-style-type: none"> <li>• challenging with subjective interpretation</li> <li>• sensitivity dependent on antibody panel used</li> <li>• limited harmonization and standardization across laboratories</li> <li>• leukemic phenotype not necessarily stable over time</li> </ul> |
| NGS       | $10^{-2}$ to $10^{-3}$ | <ul style="list-style-type: none"> <li>• relatively easy to perform</li> <li>• wide applicability</li> </ul>   | <ul style="list-style-type: none"> <li>• limited standardization</li> <li>• error rate leads to low sensitivity of mutated sequences</li> </ul>  |
| RT-qPCR   | $10^{-3}$ to $10^{-5}$ | <ul style="list-style-type: none"> <li>• high sensitivity</li> <li>• well standardized</li> </ul>  | <ul style="list-style-type: none"> <li>• applicable to about 50% of all AML cases and 35% in older patients</li> </ul>   |
| dPCR      | $10^{-3}$ to $10^{-5}$ | <ul style="list-style-type: none"> <li>• high sensitivity</li> </ul>   | <ul style="list-style-type: none"> <li>• limited standardization</li> <li>• labor intensive</li> <li>• limited applicability</li> </ul>  |

## **1.10 CLINICAL SIGNIFICANCE OF MRD**

Once chemotherapy-induced complete remission has been achieved, MRD testing permits to determine whether the tumor has been likely eradicated or minimal amounts of leukemic cells still persist. This in turn helps to assess relapse risk and ideally would allow physicians to identify patients who should receive intensified therapies such as HSCT (Wood 2016, Schuurhuis, Heuser et al. 2018). Thus, MRD-guided treatment could allow personalizing therapy for AML patients.

### **1.10.1 Prognostic impact of MRD detection**

Several studies comprising adult and pediatric cohorts have established that, irrespective of the technique used (MFC, NGS or RT-PCR), presence of MRD is associated with higher relapse risk and shorter survival in AML (Hourigan, Gale et al. 2017, Gaksch, Kashofer et al. 2018, Jongen-Lavrencic, Grob et al. 2018)

Two large prospective, multicenter studies showed MRD detection by MFC as an independent prognostic factor in AML patients. Usually, a cutoff of 0.1% is used to distinguish the patient groups referred to as “MRD positive” and “MRD negative” (Schuurhuis, Heuser et al. 2018). Patients with a MRD-positive status had inferior outcome in multivariate analyses (Freeman, Virgo et al. 2013, Terwijn, van Putten et al. 2013). However, unlike MFC, MRD detection using molecular assays is achievable only in a minority of patients. The validated molecular targets for MRD detection in AML currently include the t(15;17), in APL, CBF-translocations, and mutations in *NPM1* (Grimwade, Jovanovic et al. 2009, Yin, O'Brien et al. 2012, Zhu, Zhang et al. 2013). MRD status in *NPM1* mutated AML was identified as the only independent prognostic indicator for death in a multivariate analysis in several studies (Krönke, Schlenk et al. 2011, Ivey, Hills et al. 2016).

### **1.10.2 MRD detection and its impact in HSCT**

The prognostic utility of MRD testing at the time of HSCT has been extensively explored. Accumulating evidence indicates that presence of MRD detected by MFC prior to HSCT is a strong, independent factor for post-transplant outcomes (Buckley, Wood et al. 2017). Patients

with a MRD positive status prior to HSCT had a poorer OS and higher cumulative incidence of relapse (Buccisano, Hourigan et al. 2017). In a recent study evaluating the prognostic significance of MRD measured by MFC before and after allogeneic HSCT, patients with a MRD- positive status during allogeneic HSCT had significantly higher rates of relapse (66 vs. 15%) and worse OS (52 vs. 83%) after one year in comparison to MRD-negative patients (Walter, Othus et al. 2015, Araki, Wood et al. 2016). Unfortunately, transformation from a MRD-positive status before transplantation to MRD-negative status after myeloablative conditioning did not improve OS or relapse rate greatly (Zhou, Othus et al. 2016).

### **1.10.3 MRD detection as a surrogate endpoint**

MRD measurements may gradually be used to optimize efficacy of treatment and enable drug development. Recently, two studies strongly recommended that MRD could be used as an alternative end point in clinical studies. Superior clinical outcomes with increased dose of daunorubicin was found to be associated with MRD negativity in CBF-AML (Prebet, Bertoli et al. 2014) while in another study improved OS with the supplementation of gemtuzumab ozogamicin to standard induction chemotherapy correlated with MRD status (Lambert, Lambert et al. 2014). However, additional data is necessary to establish the utility of MRD as a surrogate end point for AML clinical trials.

### **1.10.4 Choice of the test**

Efforts for MRD detection in AML began in the 1990s (Campana, Coustan-Smith et al. 1990), however its clinical significance has gained extensive recognition for the past two decades (Paietta 2002). Accordingly, the 2017 guidelines from the European Leukemia Net recommend that the term “CR” should be separated into CR-MRD positive and CR-MRD negative subgroups, with the former carrying a much higher risk of relapse (Döhner, Estey et al. 2017). Which technique is the best to measure MRD is still a matter of intense debate (Schoorhuis, Heuser et al. 2018.) MRD detection using NGS and dPCR are not yet ready for routine application outside of clinical trials. Therefore, currently recommended MRD detection is based on the use of MFC and PCR-based assays in suitable patients.

## **1.11 LEUKEMIC STEM CELLS (LSCs)**

Experiments using lethal irradiation in mice led to the discovery of a rare subset of cells with the ability to replenish the depleted bone marrow, now recognized as hematopoietic stem cells (HSCs) (Till and McCulloch 1961). HSCs are essential for the constant replacement of blood cells during the lifetime of an organism and are placed at the helm of the hierarchically organized hematopoiesis (Bacelli and Trumpp 2012). Normal HSCs possess the life-long ability to generate a new stem cell after cell division (self-renewal) and give rise to progenitor cells to all blood lineages (Wilson, Laurenti et al. 2008, Seita and Weissman 2010).

In AML John Dick and colleagues showed that also only a small fraction of leukemic cells was able to initiate leukemia in immunodeficient severe combined immunodeficiency (SCID) mice immunophenotypically similar to the donor (Lapidot, Sirard et al. 1994). It was then shown that similar to normal hematopoiesis, AML is structured in a hierarchical fashion with “stem cell-like” leukemic cells at top of tumor hierarchy (Bonnet and Dick 1997). Based on these observations, the “leukemic stem-cell” (LSC) or, more general, the “cancer stem-cell” hypothesis was established. In the meantime several lines of experimental evidence support this hypothesis showing that the majority of tumors and leukemia are indeed sustained by a minor population of stem-like cancer cells or LSCs, respectively (Reya, Morrison et al. 2001). In the next chapter properties and features of LSCs are described in more detail.

### **1.11.1 Universal LSC features**

#### **1.11.1.1 LSCs are at the helm of leukemia hierarchy:**

Located at the top of the tumor hierarchy, LSCs can self-renew and also generate non-LSC progeny, which form the tumor bulk (Reya, Morrison et al. 2001, Nguyen, Vanner et al. 2012). Hierarchical organization of tumors, governed by tumor stem cells has also been described in acute lymphoblastic leukemia (ALL) (Castor, Nilsson et al. 2005) breast (Al-Hajj, Wicha et al. 2003, Ginestier, Hur et al. 2007), brain (Singh, Hawkins et al. 2004), colon (Dalerba, Dylla et al. 2007) and pancreatic (Hermann, Huber et al. 2007, Li, Heidt et al. 2007) cancers.

#### 1.11.1.2 LSCs have limitless self-renewal potential:

Unlike the differentiated descendants, LSCs are capable of indefinite self-renewal (Brummendorf, Dragowska et al. 1998, Ting, Deneault et al. 2012). Usually, the LSCs are identified in a leukemic cell population by serial transplantation of tumor cells in immunodeficient mice. Therefore, upon transplantation into immunodeficient mice, LSCs can be functionally differentiated from non-LSCs by their ability to initiate a similar tumor *in vivo*, and to regrow after re-transplantation into a secondary recipient (self-renewal) and even in a tertiary recipient (Reinisch, Chan et al. 2015).

#### 1.11.1.3 LSCs predominantly remain in a quiescent state

Similar to HSCs, LSCs are mainly quiescent and not actively cycling (Wilson, Laurenti et al. 2008). However, when required the quiescent LSCs, like HSCs, can return to active cycling stages (Essers and Trumpp 2010). The presence of quiescent LSCs has also been described in several mouse models developing AML due to AML-associated gene fusions (Ishikawa, Yoshida et al. 2007).

#### 1.11.1.4 LSCs display resistance to chemotherapy

Quiescence may render LSCs unresponsive to therapy, since chemotherapeutic drugs in most instances only target actively cycling cells. Flow cytometric measurement of DNA and RNA content revealed that the majority (96%) of LSCs was in the G<sub>0</sub> phase of the cell cycle (Clarkson, Ohkita et al. 1967, Guan, Gerhard et al. 2003). LSCs were found to be resistant to *in-vitro* treatment with 5-fluorouracil and did not incorporate tritiated thymidine as a sign of cell proliferation (Terpstra, Ploemacher et al. 1996). Additionally, resistance to therapy might also be caused by increased expression of multidrug resistance transporters resulting in higher drug efflux (Dean, Fojo et al. 2005).

### 1.12 IDENTIFICATION OF LSCs

Initial studies showed the existence of LSCs in the Lin<sup>-</sup>CD34<sup>+</sup>38<sup>-</sup> compartment of AML as demonstrated by engraftment in NOD/SCID mice (Bonnet and Dick 1997, Dick 2008). Subsequent work employing both intravenous and intrafemoral transplantation as well as

improved, more immunodeficient mouse models, such as the NOD/SCID/IL2R-g null (NSG) mice, revealed that LSCs could also be detected (although at lower frequencies) in the CD34<sup>+</sup>38<sup>+</sup> and CD34<sup>-</sup> subpopulations (Sarry, Murphy et al. 2011) of CD34<sup>+</sup> AML patient samples. In the recent past, the CD34<sup>+</sup> LSCs have been further refined and a coexistence of two distinct LSC populations in AML patients has been shown: a CD34<sup>+</sup>38<sup>-</sup> fraction resembling the normal lymphoid-primed multi-potent progenitors (LMPP-like LSCs; Lin<sup>-</sup>CD34<sup>+</sup>38<sup>-</sup>CD90<sup>-</sup>CD45RA<sup>+</sup>) and a CD34<sup>+</sup>38<sup>+</sup> fraction similar to the granulocyte-macrophage progenitors (GMP-like LSCs; Lin<sup>-</sup>CD34<sup>+</sup>38<sup>+</sup>CD123<sup>+</sup>CD45RA<sup>+</sup>) (Goardon, Marchi et al. 2011). Interestingly, both populations coexisted indicating a hierarchical relationship wherein the LMPP-like LSCs could give rise to the GMP-like population, but not vice versa in almost 80% of patients studied (Goardon, Marchi et al. 2011). Furthermore, in an another recent study four leukemic populations sorted based on expression of CD34/CD38 were injected into mice and analyzed for their leukemia-initiating capacity (Ng, Mitchell et al. 2016). This approach demonstrated that LSC activity could be detected in some patient samples in all fractions, but with the majority of LSCs found within the CD34<sup>+</sup> population, particularly CD34<sup>+</sup>38<sup>-</sup>, and a minority of LSC in the CD34<sup>-</sup> population. Moreover, there were hardly any cases wherein LSCs originated from CD34<sup>-</sup> and/or CD34<sup>+</sup>38<sup>+</sup> fractions without concomitant activity in the CD34<sup>+</sup>38<sup>-</sup> subpopulation suggesting that the CD34<sup>+</sup>38<sup>-</sup> fraction contains LSCs, which are on the top of the leukemic cell hierarchy.

### **1.13 CLINICAL IMPACT OF LSCs**

The burden of CD34<sup>+</sup>38<sup>-</sup> LSCs in AML patients is of strong prognostic significance. CD34-negative status, defined by the absence of CD34<sup>+</sup> cells, was found to be an independent prognostic factor to identify patients with better prognosis in AML in comparison to patients with CD34<sup>+</sup>38<sup>-</sup> LSCs (Hanekamp, Denys et al. 2015). Furthermore, patients with low CD34<sup>+</sup>38<sup>-</sup> frequencies at diagnosis had low minimal residual disease rates after chemotherapy and a median relapse-free survival of 16 months, in comparison to 5.6 months in those with CD34<sup>+</sup>38<sup>-</sup> frequencies higher than 3.5% (van Rhenen, Feller et al. 2005). It has also been hypothesized that persistence of quiescent LSCs could induce leukemia relapse, which frequently occurs years after completion of therapy (Aguirre-Ghiso 2007). Using paired diagnostic and relapse samples from AML patients after conventional therapy, Ho et al, showed a 10 to 100 fold increase in LSC frequency at relapse (Ho, LaMere et al. 2016). For these reasons, persisting LSCs are currently thought to be responsible for the relapse of

the disease. In addition, studies exploring the association of typical LSC gene expression signatures in diagnostic AML samples with clinical outcomes in large cohorts of patients provided strong evidence for the link between frequency of LSCs and clinical outcomes (Eppert, Takenaka et al. 2011, Ng, Mitchell et al. 2016). Furthermore, from a clinical perspective, the cancer stem cell model implies that LSC eradication is crucial to achieve long-term remissions and to cure leukemia. Since LSCs and HSCs share similar phenotypes, the quest for differentially expressed cell surface markers is appealing.

#### **1.14 MEMBRANE MARKERS OF LSCs**

Several cell surface markers have been proposed to be useful in LSC phenotyping. Aberrant expression of a certain cell surface marker characteristic for LSC should allow distinguishing them from normal HSCs but also non-LSC bulk leukemic cells. Many of the LSC markers have been identified based on their expression on AML cells that engraft immunodeficient mouse models or by sequencing RNA from sorted cell fractions.

CD123, the high-affinity receptor for interleukin-3 was found to be higher expressed on CD34<sup>+</sup>38<sup>-</sup> LSCs when compared to normal HSCs in 18 AML patients. In addition, CD34<sup>+</sup>123<sup>+</sup> cells showed engraftment in mice unlike CD34<sup>+</sup>123<sup>-</sup> cells (Jordan, Upchurch et al. 2000, Florian, Sonneck et al. 2006). C-type lectin-like molecule-1 (CLL-1) was found to be expressed on leukemic cells in 92% of patients analyzed (Bakker, van den Oudenrijn et al. 2004). CD96, a member of the Ig gene family was found to be highly expressed on the CD34<sup>+</sup>38<sup>-</sup> LSCs in comparison to HSCs in 65% of AML patients (Hosen, Park et al. 2007). The ‘don’t eat me signal,’ CD47, was found to be highly expressed on the CD34<sup>+</sup>38<sup>-</sup> LSCs which protects the LSCs from being phagocytosed by macrophages and dendritic cells. Expression of CD47 was found to be associated with poor OS (Majeti, Chao et al. 2009, Galli, Zlobec et al. 2015). Through transcriptomic analysis TIM-3, a negative regulator of Th-1 T cell immunity, was found to be expressed on CD34<sup>+</sup>38<sup>-</sup> fraction of AML cells. Subsequent studies showed that TIM-3 expression enabled separation of LSC from HSCs in AML patients (Kikushige, Shima et al. 2010, Jan, Chao et al. 2011). A complementary study identified IL1RAP, which was found to be highly expressed on the surface of CD34<sup>+</sup>38<sup>-</sup> cells of AML patients with the 7/7q deletion, normal karyotype and high-risk MDS (Barreyro, Will et al. 2012, Ågerstam, Karlsson et al. 2015). CD44, the receptor for hyaluronan acid, was found to be co-expressed on CD123<sup>+</sup> LSCs (Zöller 2015). Treatment of mice

transplanted with CD34<sup>+</sup>CD38<sup>-</sup> LSCs with an anti-CD44 antibody resulted in significantly longer survival of mice. Microarray analysis of LSCs identified CD25 to be expressed on 34.4% of LSCs isolated from 61 AML patients (Saito, Kitamura et al. 2010). CD25<sup>+</sup>CD34<sup>+</sup>CD38<sup>-</sup> cells were able to engraft immunodeficient mice and induce AML. CD99, a transmembrane glycoprotein was found to be expressed in 43% of AMLs, predominantly in M1/M2/M3 subtypes (Zhang, Barcos et al. 2000). Interestingly, novel markers such as JAM-C (De Grandis, Bardin et al. 2017) and GPR56 (Saito, Kaneda et al. 2013) have also been proposed to identify LSCs able to induce AML in immunocompromised mice.

Although there is currently no consensus, which markers are best suited for identification of LSCs in AML, the identification of such markers has paved the way for therapeutic targeting of LSCs using antibody and chimeric antigen receptor (CAR)-T cell based approaches. Monoclonal antibodies targeting antigens such as CD123, CD47 and CD44 have shown to be effective against LSCs in xenotransplantation studies (Majeti 2011). Since GPR56 and CLL-1 were in the specific focus of this project, both markers are described here in more detail.

#### **1.14.1 C-type lectin-like molecule-1 (CLL-1, CLEC12A, CD371)**

CLL-1, containing a C-type lectin-like domain, belongs to the type II transmembrane receptor family. Members of this family share a common protein fold and are involved in various functions like cell signaling, cell adhesion, inflammation and immune response. Expression of CLL-1, which is also known as CLEC12A and CD371, is restricted to cells of the myeloid lineage. The CLL-1 protein is a negative regulator of granulocyte and monocyte function. Using a combination of phage display technology with flow cytometry, CLL-1 was identified as a novel surface marker on AML cells (Bakker, van den Oudenrijn et al. 2004). Further studies found CLL-1 to be expressed in 92% of AMLs (Bakker, van den Oudenrijn et al. 2004, Larsen, Roug et al. 2012). CLL-1 expression was found on CD34<sup>+</sup>CD38<sup>-</sup> cells in 87% of AML cases but no expression was found on normal HSCs. Interestingly CD34<sup>+</sup>CLL-1<sup>+</sup> leukemic cells were capable of engrafting immunodeficient mice, indicating that these cells possess functional properties of LSCs (van Rhenen, van Dongen et al. 2007, Darwish, Sudha et al. 2016). Moreover, enrichment of LSCs was observed in the side-populations isolated from AML patients (Moshaver, van Rhenen et al. 2008). These LSCs concomitantly expressed CLL-1. Given these observations, efforts have been made to develop agents targeting CLL-1 expressing AMLs. Two monoclonal antibodies targeting CLL-1 display cytotoxicity against AML cell lines and primary AML blasts in vitro as well as show reduced

engraftment of HL-60 cells in nude mice (Zhao, Singh et al. 2010, Lu, Zhou et al. 2014). In addition, CLL-1 is currently being explored as a potential target for CAR-T cell therapy. First results with CAR-T cells targeting CLL-1 displayed therapeutic potential against CLL-1<sup>+</sup> AML cells in vitro as well as in vivo while sparing normal HSCs (Wang, Chen et al. 2018).

#### **1.14.2 G-Protein Coupled Receptor, 56 (GPR56)**

GPR56, an orphan adhesion G-protein coupled receptor belongs to the secretin family and is characterized by the presence of a large extracellular domain with several adhesive folds, coupled to a seven-span transmembrane domain and an intracellular domain. Adhesion GPCRs are known to play a role in regulation of cell migration, adhesion, guidance and polarity (Aust 2010, Langenhan, Aust et al. 2013). GPR56 was initially associated with stem cell biology in the neural system, wherein neural stem and progenitor cells express high levels of GPR56 (Bai, Du et al. 2009) . The role of GPR56 in cancer was first identified in 1999. GPR56 was found to be downregulated in numerous highly metastatic melanoma cell lines (Liu, Parker et al. 1999). Since then GPR56 expression has been described in other types of cancers such as pancreatic (Huang, Fan et al. 2008) and lung cancer (Kausar, Sharma et al. 2011) as well as glioblastoma (Shashidhar, Lorente et al. 2005). Transient apoptosis can be induced by knockdown of GPR56 in several cell lines derived from melanoma (M14), cervix (HeLa) and colon cancer (HCT116) suggesting that GPR56 may be essential for cell survival and has a pro-tumorigenic role (Ke, Sundaram et al. 2007). Knocking down GPR56 expression in ecotropic viral integration site-1 high (EVI-1<sup>high</sup>) leukemia cell lines AML1 and HNT34 resulted in decreased cell adhesion and induction of apoptosis (Saito, Kaneda et al. 2013). Very recently, GPR56 expression was shown to identify leukemic subpopulations with high repopulating capacity, a characteristic attributed to LSCs (Pabst, Bergeron et al. 2016). High GPR56 expression was also significantly associated with high-risk genetic subgroups and poor outcome. Furthermore, blocking GPR56 with a monoclonal antibody in the AML cell line MV4-11 significantly impaired its engraftment in NSG mice (Daria, Kirsten et al. 2016).

## 1.15 AIMS OF THE THESIS

In AML persisting LSCs may lead to minimal residual disease (MRD) and subsequently to relapse. Given this importance, a detailed characterization of the surface immunophenotype of LSCs to discriminate them from bulk leukemic blasts has been of great interest. Moreover, identification and targeting of these LSCs could contribute to improved risk stratification and patient outcomes. In the first part of this study, we therefore performed a detailed analysis of sixteen putative LSC markers in CD34/38 leukemic sub-compartments. The most differentially expressed markers were then selected to determine a possible correlation of their expression with a recently published LSC gene signature (Ng, Mitchell et al. 2016). Finally, expression levels of these surface markers were analyzed for their impact on survival in AML patients receiving intensive chemotherapy.

MRD monitoring in AML patients with CR helps to identify those with high risk of relapse. MRD monitoring has therefore entered clinical practice and may potentially guide therapy in the future. However, each detection method currently in use has clear limitations in sensitivity and/or specificity to accurately predict MRD. Moreover, due to the lack of a broad applicability of a single technique there is still a matter of intense debate which technique is the best to measure MRD (Schuurhuis, Heuser et al. 2018.). In the second part of this study we thus aimed to establish a novel method for monitoring MRD in AML with broad applicability by combining flow cytometry based leukemic cell enrichment and parallel sequencing of recurrently mutated genes using NGS. We hypothesized that application of parallel sequencing to identify the recurrently mutated genes in an enriched residual leukemic compartment should display a high sensitivity as well as specificity for monitoring MRD. Furthermore, we aimed to validate this novel method of MRD detection as a tool for the early detection of impending relapse.

## **2. MATERIALS & METHODS**

### **2.1 Patient cohorts and sample preparation**

#### **2.1.1 Clinical Samples**

Bone marrow (BM) or peripheral blood (PB) samples with blast counts >20% were collected from a total of 150 adults diagnosed with AML according to WHO criteria at Department of Hematology, Medical University of Graz, Austria. In 25 cases, samples were also available at relapse. To assess the normal CD34<sup>+</sup> hematopoietic stem and progenitor cell (HSPC) compartments normal bone marrow samples (NBM) were obtained from 16 lymphoma patients without any evidence of disease in the bone marrow. For validation of our newly developed method for detection of MRD in AML, BM samples were prospectively collected from a total of 41 patients in complete remission (CR) after one or two cycles of intensive chemotherapy at the Department of Hematology, Medical University of Graz, Austria. Information on clinical data such as white blood cell counts, cytogenetic and molecular risk stratification, treatment as well as outcome parameter were collected from medical records and the electronic documentation program MEDOCS (Medical Documentation and Communication System, SAP Germany). Informed consent was obtained from all patients and the research project was done in accordance to the Helsinki Declaration of 1975, which was revised in 1983. The study has been approved by the Institutional Review Board of Medical University Graz, Austria (Protocol Nr. 26-050 ex 13/14 and 29-499 ex 16/17).

#### **2.1.2 Preparation of bone marrow samples**

After erythrocyte lysis using lysing buffer (Beckman Coulter, USA) BM cells were washed with phosphate-buffered saline (D-PBS; Life Technologies, Breda, Netherlands) to remove any traces of lysis buffer and counted on the C-Chip disposable hemocytometer (Bioswisstec, Switzerland). Based on the number of cells, samples were appropriately aliquoted in sterile freeze medium (RPMI media+10% DMSO+10%FCS) and stored in liquid nitrogen until use.

#### **2.1.3 Preparation of mononuclear cells from peripheral blood samples**

Mononuclear cells (MNCs) were isolated from the peripheral blood samples using Lymphoprep (Stemcell Technologies, Vancouver, Canada). The blood was diluted with PBS (ratio 1:2, total volume 35 ml), carefully layered on 15 ml of Lymphoprep and centrifuged for 25 minutes at 25°C at 2500 rpm without brake. Upon centrifugation MNCs were isolated from the buffy layer using a pipette. The MNCs were then washed with D-PBS to remove

any remaining Lymphoprep and counted using a disposable hemocytometer. MNCs were appropriately aliquoted in sterile freeze media and stored in liquid nitrogen until use.

## **2.2 Flow cytometry analysis**

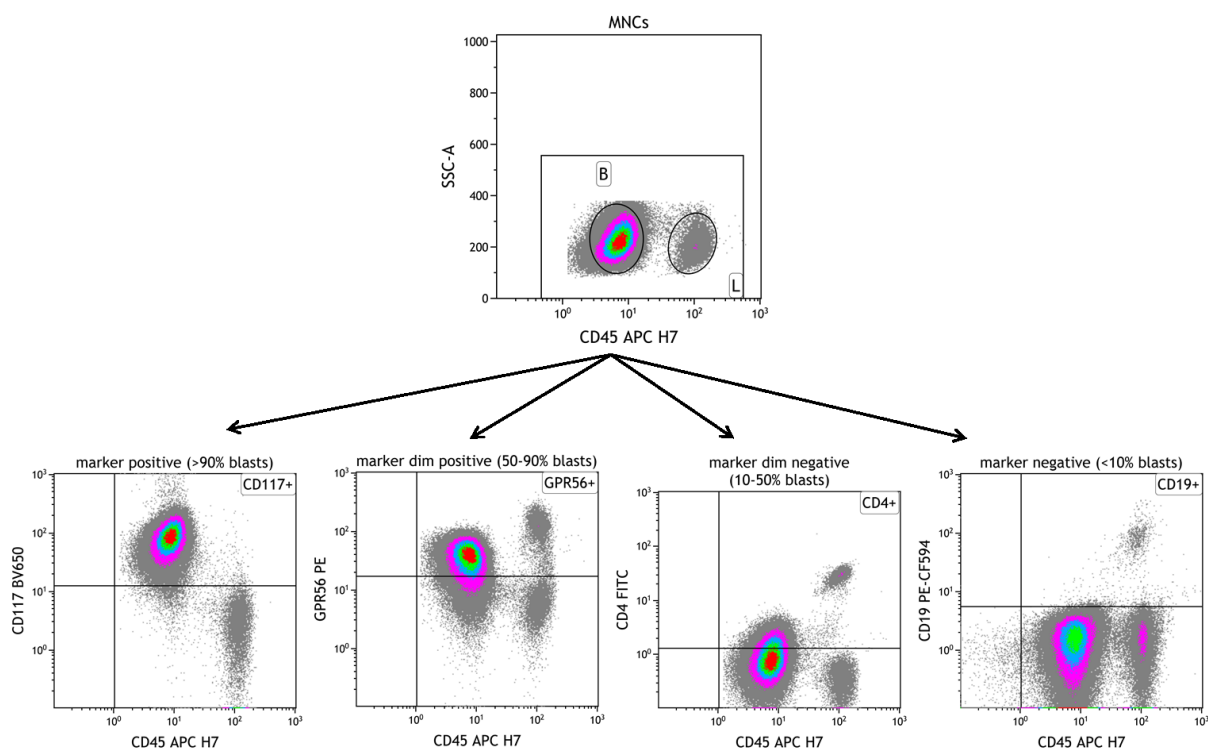
### **2.2.1 Sample preparation and instrument settings for flow cytometry**

12-color multiparameter flow cytometry (MFC) was performed using 4-laser Fortessa cytometer (Becton Dickinson; BD; San Jose, CA, USA). At time of analysis, the cryopreserved cells were thawed, washed with D-PBS. To prevent non-specific binding 1% sheep serum (Sigma-Aldrich) was added and incubated for 7 minutes followed by staining with the appropriate antibodies. The cells were incubated for 18 minutes in dark at room temperature and washed with D-PBS to remove any unlabeled antibodies. Table 9 shows the all the antibodies used. While for surface marker analysis at least  $2 \times 10^5$  events were recorded, at least  $1 \times 10^6$  cells were recorded for MRD detection using flow cytometry. Instrument settings and data acquisition were performed following the specific EuroFlow SOP (Kalina et al., 2012) using BD™ Cytometer Setup and Tracking (CS&T) beads (BD Biosciences) and Rainbow beads (Spherotech, Lake Forest, IL, USA). Specific compensation settings for each fluorochrome conjugate were used, whenever necessary. Flow cytometry data were analyzed using the Kaluza® (Beckman Coulter, USA) and Infocyt® software (Cytognos, Salamanca, Spain). The latter software also allowed simultaneous analysis of all markers by merging the three panels based on expression of backbone markers CD34, CD38 and CD45. Appropriate isotype controls were used to determine the level of background staining.

### **2.2.2 Flow cytometry data analysis**

After application of a harmonized flow cytometry analysis as outlined above a strict gating strategy was established to reliably determine surface marker expression. Leukemic blasts were identified based on low expression of CD45 and low side scatter. The cellular compartments of CD34-positive AMLs were defined by expression of CD34/CD38 and the expression of all the markers was analyzed on these individual cellular compartments (Figure 5). In CD34-negative AML samples expression of surface markers was analyzed on the entire blast population. The expression of single markers was either recorded using the arithmetic mean of the fluorescence intensity (MFI) or as the percentage of cells positive for the respective markers (Daga, Rosenberger et al. 2019). When recording the expression of the single markers using MFI, in order to distinguish unspecific background staining from

specific antibody staining, the arithmetic mean of MFI for the isotype control was deducted from the MFI value for expression of the single markers. For identification of surface markers for an optimal enrichment of leukemic cells for MRD detection we used the percentage of cells expressing the respective markers. For this, we applied a scoring system ranking each sample either positive (>90% leukemic cells express the marker), dim positive (50-90%), dim negative (10-50%) to negative (<10%) for a respective marker (Figure 5). Based on the scores the best marker(s) were defined in each AML sample. This standardized analysis protocol was applied to all patient as well as NBM samples.



**Figure 5:** Gating strategy to determine which markers were suitable for identifying and defining leukemic stem cells. Blasts (B in figure) were identified using SSC low and CD45 low. The expression of the 23 markers was analysed on the bulk leukemic cells. The scoring system ranked from positive (>90% cells express the marker), dim positive (50-90% cells express the marker), dim negative (10-50% cells express the marker) and negative (<10% cells express the marker).

**Table 9:** Antibody panels used in 12 color multi-parameter flow cytometry (Daga, Rosenberger et al. 2019).

| Tube # | CD Marker     | Clone       | Fluorophore | Company          | Volume( $\mu$ l) per million cells |
|--------|---------------|-------------|-------------|------------------|------------------------------------|
| T1     | CD34          | 581         | BV421       | BD               | 1.5                                |
|        | CD45RA        | HI100       | BV510       | BD               | 1.5                                |
|        | CD96          | 6F9         | BV711       | BD               | 3                                  |
|        | CD47          | B6H12       | BV786       | BD               | 3                                  |
|        | CD371 (CLL-1) | 50C1        | FITC        | BD               | 1.5                                |
|        | 7AAD          | -           | PerCP       | BD               | 5                                  |
|        | CD366 (TIM3)  | 7D3         | PE          | BD               | 2                                  |
|        | CD123         | 7G3         | PE-CF594    | BD               | 1.5                                |
|        | CD38          | HB7         | PE-Cy7      | BD               | 3                                  |
|        | IL1RAcP       | 89412       | APC         | R&D Systems      | 3                                  |
|        | CD25          | 2A3         | APC-R700    | BD               | 1.5                                |
|        | CD45          | 2D1         | APC-H7      | BD               | 2                                  |
| T2     | CD34          | 581         | BV421       | BD               | 1.5                                |
|        | CD33          | WM53        | BV510       | BD               | 1.5                                |
|        | CD117         | 104D2       | BV650       | BD               | 3                                  |
|        | CD99          | TU12        | FITC        | BD               | 12                                 |
|        | CD44          | G44-26      | PerCP-Cy5.5 | BD               | 1                                  |
|        | CD180         | G28-8       | PE          | BD               | 10                                 |
|        | CD49f         | GOH3        | PE-CF594    | BD               | 2                                  |
|        | CD38          | HB7         | PE-Cy7      | BD               | 3                                  |
|        | CD305         | DX26        | Alexa 647   | BD               | 3                                  |
|        | CD56          | NCAM16.2    | APC-R700    | BD               | 3                                  |
|        | CD45          | 2D1         | APC-H7      | BD               | 2                                  |
| T3     | CD34          | 581         | BV421       | BD               | 1.5                                |
|        | CD11b         | ICRF-44     | BV510       | BD               | 3                                  |
|        | CD14          | M $\phi$ P9 | BV711       | BD               | 3                                  |
|        | CD10          | HI10A       | BV786       | BD               | 3                                  |
|        | CD4           |             | FITC        | Beckmann Coulter | 1.5                                |
|        | GPR56         | 4C3         | PE          | Biolegend        | 5                                  |
|        | CD19          | HIB19       | PE-CF594    | BD               | 3                                  |
|        | CD38          | HB7         | PE-Cy7      | BD               | 3                                  |
|        | Jam C         | SHM33       | APC         | Biolegend        | 5                                  |
|        | CD7           |             | Alexa 700   | Beckmann Coulter | 3                                  |

### **2.2.3 Flow cytometry-based cell sorting**

Cell sorting was performed under sterile conditions using a BD Aria II cell sorter. The cells were stained with appropriate antibodies (Table 10), incubated for 18 minutes in dark at room temperature and washed with D-PBS to remove any unlabeled antibodies. For analysis of gene expression in GPR56/CLL-1 high versus low AML cells, CD34<sup>+</sup>/CD38<sup>-</sup> AML cells were sorted based on expression of GPR56 and CLL1 into four fractions as follows: CD34<sup>+</sup>38<sup>-</sup>GPR56<sup>hi</sup>; CD34<sup>+</sup>38<sup>-</sup>GPR56<sup>lo</sup>; CD34<sup>+</sup>38<sup>-</sup>CLL-1<sup>hi</sup> and CD34<sup>+</sup>38<sup>-</sup>CLL-1<sup>lo</sup> (Figure 6). For flow cytometry-based enrichment of residual cells, a single tube was designed by combining the chosen enrichment markers which included CLL-1, TIM-3, CD117 and CD123, in the phycoerythrin (PE)-fluorescence channel along with the backbone markers CD45, CD34 and CD38. Table 11 shows the all the antibodies used. After excluding monocytes and basophils (by using CD14 and CD203c, respectively) mononuclear cells were sorted into the PE positive and PE negative fractions. Purity of the sorted fractions was more than 95%. Figure 7 shows the detailed cell sorting strategy used.

For qPCR and digital PCR (dPCR) cells were sorted into 100µl D-PBS, while for parallel sequencing cells were sorted into 120µl PAXgene tissue fix (Qiagen, Hilden, Germany).

## **2.3 Analysis of gene expression**

### **2.3.1 Isolation of RNA**

FACS-sorted leukemic cells were centrifuged at 10000g for 1 minute and the supernatant was discarded. The pellet was resuspended in RLT buffer and stored at -20°C overnight. High-quality RNA was extracted using the RNeasy Micro Kit (Qiagen) as per the manufacturer's instructions and eluted in 20µl of RNase free water. The RNA obtained was quantified using Nanodrop (Thermofisher Scientific). A 260/280nm ratio was used as a parameter to evaluate the purity with a ratio of 1.80 regarded highly pure.

### **2.3.2 Reverse transcription PCR and gene expression analysis**

cDNA was synthesized from 35ng RNA using the Reverse transcription kit (Applied Biosystems, Foster City, CA) according to manufacturer's instructions. The reagents for the master mix are shown in Table 12. The PCR was run using program A.

Quantitative PCR (qPCR) was performed using the ABI Prism 7700 Sequence Detector (Applied Biosystems). All PCR reactions were performed in triplicates using TaqMan Gene expression mastermix (Table 13; Applied Biosystems). The PCR was run using program B.

GAPDH and RPL13A were used as internal control. Taqman Probes for following human genes were purchased from Applied Biosystems: GAPDH (Hs04194366\_g1); RPL13A (Hs02786624\_g1); CD34 (Hs02576480\_m1); GPR56 (Hs00938474\_m1); ZBTB46 (Hs01008166\_m1); MMRN1 (Hs01113299\_m1); CPXM1 (Hs00219709\_m1); DPYSLR3 (Hs00181668\_m1); SMIM24 (Hs00415400\_m1); BEX3 (Hs00276273\_s1); DNMT3B (Hs00171876\_m1); CDK6 (Hs01026371\_m1); SOCS2 (Hs00919620\_m1); AKR1C3 (Hs00366267\_m1); ARHGAP22 (Hs01098342\_m1); LAPTM4B (Hs00363282\_m1); EMP1 (Hs00608055\_m1); KIAA0125 (Hs00796164\_s1); NYNRIN (Hs00394058\_m1).

A threshold value of 0.1 was used and baseline was set from 10 to 20. The  $\Delta C_t$  values were calculated for every gene after normalization with internal control. A ratio of expression levels of genes in GPR56<sup>hi</sup> vs. GPR56<sup>lo</sup> cells as well as CLL-1<sup>hi</sup> vs. CLL-1<sup>lo</sup> cells was calculated (Daga, Rosenberger et al. 2019).

## **2.4 Mutational analysis**

### **2.4.1 Isolation of DNA**

High-quality DNA for dPCR was isolated from the FACS sorted cells using the QIAamp DNA Micro Kit (Qiagen) as per the manufacturer's instructions and eluted in 20 $\mu$ l of distilled water. The DNA obtained was quantified using Qubit Fluorometer (ThermoFisher Scientific, Waltham, USA). For parallel sequencing DNA from FACS-sorted cells was isolated using Ion AmpliSeq™ Direct FFPE DNA Kit (ThermoFisher Scientific). The PAXgene fixative was diluted by addition of 900 $\mu$ l PBS. The cells were then centrifuged at 10000g for 2 minutes and the supernatant was carefully discarded. 25  $\mu$ l of Direct Reagent from the Ion AmpliSeq™ Direct FFPE DNA Kit was added to the original tube and incubated for digestion for 60 min at 65 °C in a thermomixer. After, digestion, the tubes were allowed to cool down at room temperature for at least 20 minutes and DNA was stored at -70°C until use.

### 2.4.2 Digital PCR

The FACS sorted cells from 14 patients positive for *NPM1* mutation W288fs\*12 were analyzed by digital PCR for presence of mutation at remission. dPCR was performed in cooperation with the Institute of Human Genetics, Medical University of Graz, Austria.

In brief, dPCR was performed using QuantStudio 3D Digital PCR System (Applied Biosystems). All dPCR reactions were performed in duplicates using QuantStudio 3D Master Mix v2 (Applied Biosystems). A TaqMan assay specific for the *NPM1* mutation W288fs\*12 (c.860\_863dup) was designed using the Custom TaqMan® Assay Design Tool (Thermo Fisher Scientific). 14,5µl of the Mastermix (Table 15) was loaded onto a QuantStudio 3D Digital PCR 20K Chip v2 (Applied Biosystems) using the QuantStudio 3D Digital PCR Chip Loader (Applied Biosystems). An average of 15ng (0.91-49.5ng) input DNA was used to perform dPCR. A control sample with a known *NPM1* W288fs\*12 mutation along with a no template control was added to each run. PCR was performed on a GeneAmp PCR System 9700 using Program C. Chips were imaged in the QuantStudio 3D Chip Reader. Raw data were analyzed using the relative quantification application in the QuantStudio 3D Analysis Suite Software (Applied Biosystems). The confidence level was set to 95% and the desired precision value was 10%.

### 2.4.3 Parallel sequencing

The FACS sorted fractions from patients with known mutations were analyzed by deep sequencing for the presence of any mutations at remission. NGS was performed in cooperation with the Institute of Pathology, Medical University of Graz, Austria using an Ion Torrent Sequencing platform and an amplicon panel MN (Myeloid Neoplasia) or AML V2 covering the frequently mutated genes.

For sequencing we used a protocol described previously by our group (Gaksch, Kashofer et al. 2018). In brief, NGS libraries were prepared using the AmpliSeq library kit 2.0 (Thermo Fisher Scientific) and the following Ion AmpliSeq Custom Next-Generation Sequencing DNA Panels: AMLv2-Panel covering the whole coding sequence of *CEBPA*, *DNMT3A*, *GATA2*, *TET2* and *TP53* as well as hotspot mutations in *ASXL1*, *BRAF*, *CBL*, *FLT3 (D835)*, *IDH1*, *IDH2*, *JAK2*, *KIT*, *KRAS*, *NPM1* (Tetrainsertion), *NRAS*, *PTPN11*, *RUNX1* and *WT1* and the MN Panel covering the whole coding sequence of *CEBPA*, *BCOR*, *DDX41*,

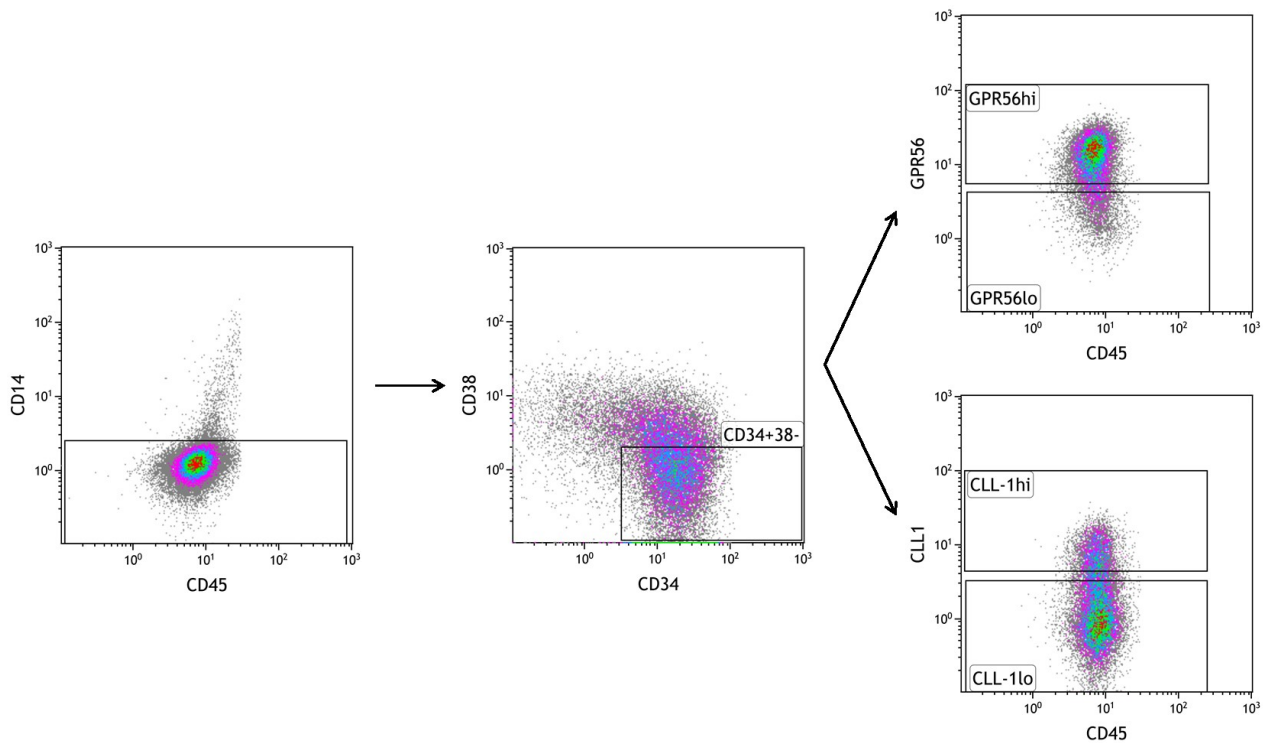
*DNMT3A, ETV6, GATA2, NF1, PHF6, SF3B2, SFRP1, SRP72, STAG2, TP53, ZRSR2* as well as hotspot mutations in *NPM1, ASXL1, BRAF, CALR, CBL, CSF3R, ETNK1, EZH2, FLT3, IDH1, IDH2, JAK2, KIT, KRAS, MPL, NRAS, PTPN11, RUNX1, SETBP1, SF3B1, SRSF2, STAT3, TET2, U2AF1* and *WT1*. *FLT3-ITD* mutations were not included in this panel due to reasons of patent law. Sequencing was performed on an Ion Proton benchtop sequencer (Thermo Fisher Scientific) to a length of 200 base pairs. On average, one million reads were obtained for each sample with more than 90% of bases above AQ20 and 87% to 93% reads on-target. Sequence information was obtained from tumor samples in duplicates. Initial data analysis was done using the Ion Torrent Suite Software Plug-ins (Thermo Fisher Scientific, open source, GPL, <https://github.com/iontorrent/>). Briefly, this included base calling, alignment to the reference genome (HG19) using the TMAP mapper and variant calling by a modified diBayes approach taking into account the flow space information. Called variants were annotated using open source software ANNOVAR (Wang, Li et al. 2010) and SnpEff (Cingolani, Platts et al. 2012). All coding, nonsynonymous mutations were further evaluated and visually inspected in IGV (<http://www.broadinstitute.org/igv/>) and variant calls resulting from technical read errors or sequence effects were excluded from the analysis.

## **2.5 Statistical analysis**

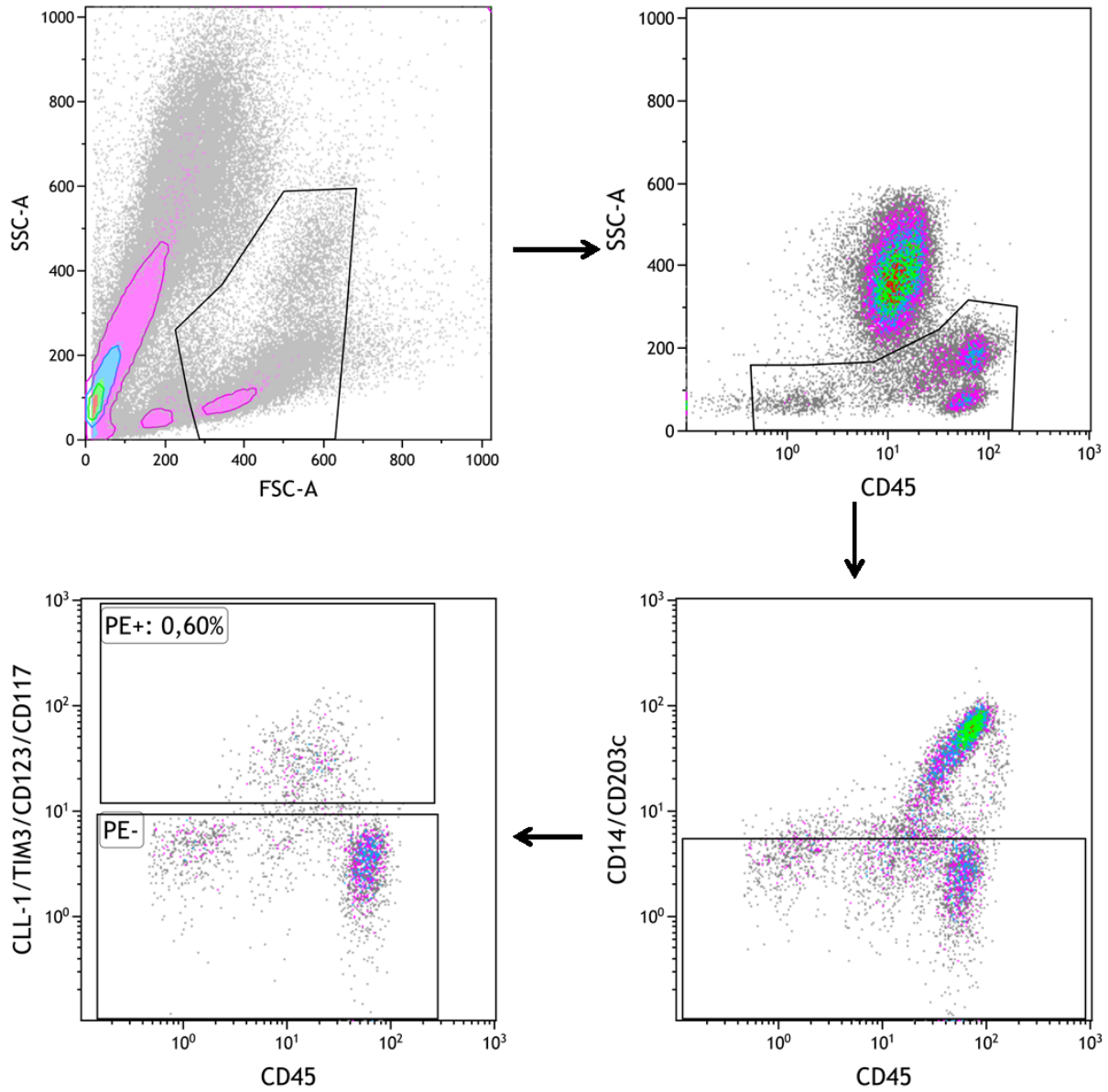
Differences in characteristics of patients were calculated using a two-sided Fisher's exact or Mann-Whitney test. Comparison between two groups concerning MFI values, percentages of marker-positive cells and gene expression values was done using the Mann-Whitney test in unpaired samples and using the Wilcoxon rank test in paired samples. Comparison between more than two groups in paired samples was done using the Kruskal-Wallis test. The Kaplan-Meier method was applied to generate the survival curves and differences were assessed by Log-Rank analysis. Cox regression was used for multivariate survival analysis. All statistical analyses were carried using GraphPad Prism software version 7.0 (GraphPad Software, La Jolla, CA, USA) and R 3.4.0 ([www.r-project.org](http://www.r-project.org)). All hypothesis testing was carried out for  $\alpha = 0.05$ .

**Table 10:** 5 color antibody panel used for sorting of leukemic cells based on expression of CLL-1 and GPR56 (Daga, Rosenberger et al. 2019).

| Tube #        | CD Marker     | Clone | Fluorophore | Company   | Volume per $5 \cdot 10^5$ cells |
|---------------|---------------|-------|-------------|-----------|---------------------------------|
| <b>Tube 1</b> | CD34          | 581   | APC         | BD        | 7.5                             |
|               | CD38          | HB7   | PE-Cy7      | BD        | 3                               |
|               | CD45          | 2D1   | APC-H7      | BD        | 3                               |
|               | CD371 (CLL-1) | 50C1  | PE          | BD        | 2.5                             |
|               | CD14          | MφP9  | BV421       | BD        | 5                               |
| <b>Tube 2</b> | CD34          | 581   | APC         | BD        | 7.5                             |
|               | CD38          | HB7   | PE-Cy7      | BD        | 3                               |
|               | CD45          | 2D1   | APC-H7      | BD        | 3                               |
|               | GPR56         | 4C3   | PE          | Biologend | 5                               |
|               | CD14          | MφP9  | BV421       | BD        | 5                               |



**Figure 6:** Sorting strategy for GPR56<sup>hi</sup> vs GPR56<sup>lo</sup> as well as CLL-1<sup>hi</sup> vs CLL-1<sup>lo</sup> CD34<sup>+</sup>38<sup>-</sup> AML cells, respectively. The CD14<sup>+</sup> monocytes were excluded and CD14<sup>-</sup> cells were further gated on CD34/CD38 to identify the CD34<sup>+</sup>38<sup>-</sup> cells.



**Figure 7:** Sorting strategy for leukemic cell enrichment. The MNCs were identified using SSC low and CD45 low. The CD14<sup>+</sup> monocytes and CD203c<sup>+</sup> basophils were excluded using CD14/CD203c. The CD14/CD203c<sup>-</sup> cells were gated on marker (PE) cocktail and the marker (PE) positive and marker (PE) negative fractions were sorted.

**Table 11:** 5 color antibody panels used for FACS based leukemic cell enrichment

|                             | <b>CD Marker</b> | <b>Clone</b> | <b>Fluorophore</b> | <b>Company</b> | <b>Volume per<br/>5*10<sup>5</sup> cells</b> |
|-----------------------------|------------------|--------------|--------------------|----------------|--|
| <b>Enrichment<br/>Panel</b> | CD34             | 581          | APC                | BD             | 7.5  |
|                             | CD38             | HB7          | PE-Cy7             | BD             | 3  |
|                             | CD45             | 2D1          | APC-H7             | BD             | 3  |
|                             | CD371 (CLL-1)    | 50C1         | PE                 | BD             | 2.5  |
|                             | CD366 (TIM3)     | 7D3          | PE                 | BD             | 2  |
|                             | CD123            | 7G3          | PE                 | BD             | 7.5  |
|                             | CD117            | 104D2        | PE                 | BD             | 7.5  |
|                             | CD14             | MφP9         | BV421              | BD             | 5  |
|                             | CD203c           | NP4D6        | BV421              | BD             | 3  |

**Table 12:** Reagents for cDNA synthesis

| <b>Mastermix for cDNA synthesis</b> | <b>Per sample (μl)</b> |
|-------------------------------------|------------------------|
| TaqMan RT Buffer 10x                | 10                     |
| MgCl                                | 22                     |
| dNTP Mix                            | 20                     |
| Random Hexamers                     | 5                      |
| RNase Inhibitor                     | 2                      |
| Multiscribe RT                      | 2.5                    |
| RNase free water                    | 36.5                   |
| RNA                                 | 2                      |

**Table 13:** Reagents for gene expression analysis by qPCR

| <b>Mastermix for qPCR</b>    | <b>Per sample (μl)</b> |
|------------------------------|------------------------|
| Mastermix Buffer             | 12.5                   |
| PDAR System target / control | 1.25                   |
| RNase free water             | 6.25                   |
| cDNA                         | 5                      |

**Table 14:** PCR programs used for cDNA synthesis and qPCR

|   | <b>Time/cycles</b>   | <b>Temperature</b> |
|---|----------------------|--------------------|
| <b>Program A</b><br><b>cDNA synthesis</b> | 10 min               | 25°C               |
|   | 30 min               | 48°C               |
|   | 5 min                | 95°C               |
|   | Hold                 | 4°C                |
| <b>Program B</b><br><b>qPCR</b>           | 2 min                | 50°C               |
|   | 10 min               | 95°C               |
|   | 15 seconds/40 cycles | 95°C               |
|   | 1 min/40 cycles      | 60°C               |
|   | Hold                 | 4°C                |

**Table 15:** Reagents for mutational analysis by dPCR

| <b>Mastermix for dPCR</b>    | <b>Per sample (µl)</b> |
|------------------------------|------------------------|
| QuantStudio 3D Master Mix v2 | 9                      |
| NPM1 TaqMan assay (20x)      | 0.9                    |
| Distilled water              | X                      |
| DNA                          | X                      |

**Table 16:** PCR program used for mutational analysis by dPCR

|                                 | <b>Time/cycles</b>   | <b>Temperature</b> |
|---------------------------------|----------------------|--------------------|
| <b>Program C</b><br><b>dPCR</b> | 10 min               | 96°C               |
|                                 | 2 min                | 56°C               |
|                                 | 30 seconds/44 cycles | 94°C               |
|                                 | 2 min/44 cycles      | 58°C               |

### 3 RESULTS

#### 3.1 High GPR56 expression correlates with a leukemic stem cell gene signature in CD34-positive AML (Daga, Rosenberger et al. 2019).

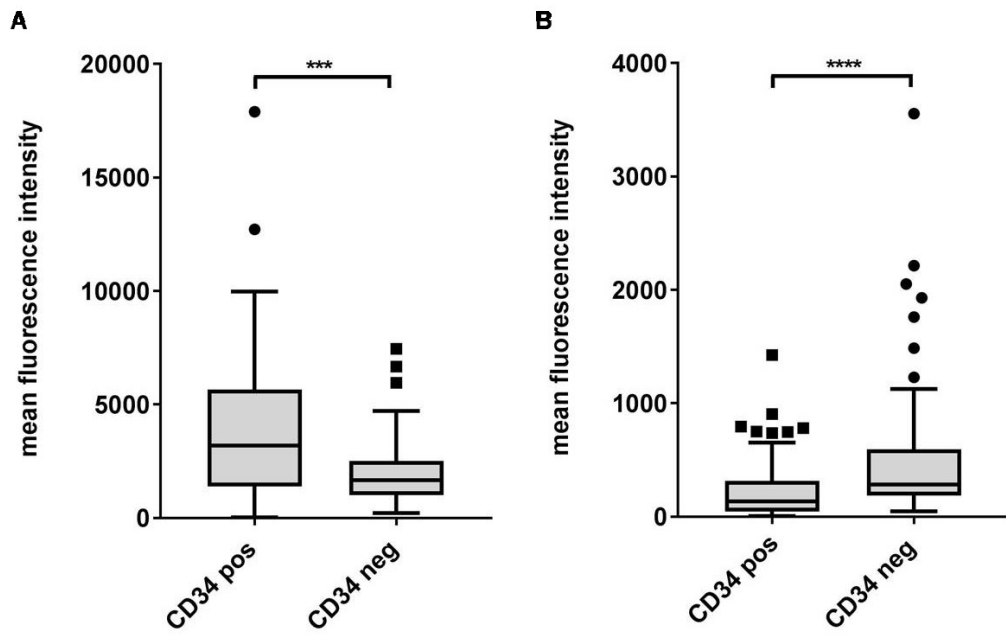
##### 3.1.1 Patient Characteristics

A total of 150 diagnostic AML patient samples were analysed using MFC panels encompassing 27 surface markers associated with AML (Table 9). Among this cohort, 88 patients had *de novo* AML, 41 patients had secondary and 21 patients had therapy-related AML. The patients' median age was 65 years (range 20–93). The sex ratio (M/F) was 1.2. Cytogenetic test results were available in 122 patients and there were 10 patients with a favorable karyotype, 76 with intermediate risk, and 36 with poor risk cytogenetics. Patient characteristics are listed in Table 17.

Defining CD34 positive AML by at least  $\geq 2\%$  CD34 expressing leukemic blasts within the bulk leukemia population we identified 108 CD34-positive samples (72%). Among these 57 samples displayed all three cellular compartments concerning differential CD34/38 expression namely CD34<sup>+</sup>38<sup>-</sup>, CD34<sup>+</sup>38<sup>+</sup>, and CD34<sup>-</sup>38<sup>+</sup> subcompartments.

##### 3.1.2 Flow cytometric analysis of putative leukemic stem cell markers

Among the 16 markers tested and reported to be associated with a LSC phenotype in the literature, CLL-1 and GPR56 were the most prominently differentially expressed surface markers among all CD34-positive versus CD34-negative AML samples as well as CD34/38 subcompartments in CD34-positive AML cases. Whilst expression of CLL-1 as calculated by mean fluorescence intensity (MFI) values was found to be higher in CD34-negative AML cases ( $215 \pm 246$  vs.  $578 \pm 700$ ;  $p < 0.0001$ , Figure 8A), expression of GPR56 was found to be higher in CD34-positive AML cases ( $3833 \pm 2991$  vs.  $2010 \pm 1625$ ;  $p < 0.001$ , Figure 8B). Other markers, which were differentially expressed between CD34-positive and -negative AML cases were CD33, CD44, CD45RA, CD47, CD49f, CD99, CD117, CD123, CD305 and IL1RAP. Details on these results are given in Table 18.



**Figure 8:** Expression GPR56 and CLL-1 in CD34-positive vs. CD34-negative AML samples at diagnosis. Surface expression levels of (A) CLL1 (B) GPR56 in CD34-positive (n=108) vs. CD34-negative (n=42) AML samples at diagnosis ( $p < 0.01$ ) as measured by mean fluorescence intensity (Daga, Rosenberger et al. 2019).

**Table 17:** Patient characteristics (Daga, Rosenberger et al. 2019)

|                                     |                  |
|-------------------------------------|------------------|
| <b>Gender</b> (female/male)         | 66/84            |
| <b>Age</b> (median, range in years) | 65 (20-93)       |
| age <60 years                       | 65               |
| age ≥60 years                       | 85               |
| <b>Type of AML</b>                  |                  |
| de novo AML                         | 88               |
| secondary AML                       | 41               |
| therapy-related AML                 | 21               |
| <b>WBC at diagnosis</b> (G/l)       | 40.8 (0.8-335.4) |
| WBC <30 G/l                         | 62               |
| WBC ≥30 G/l                         | 86               |
| not known                           | 2                |
| <b>Cytogenetic risk:</b>            |                  |
| favorable risk                      | 10               |
| intermediate risk                   | 76               |
| adverse risk                        | 36               |
| not known                           | 28               |

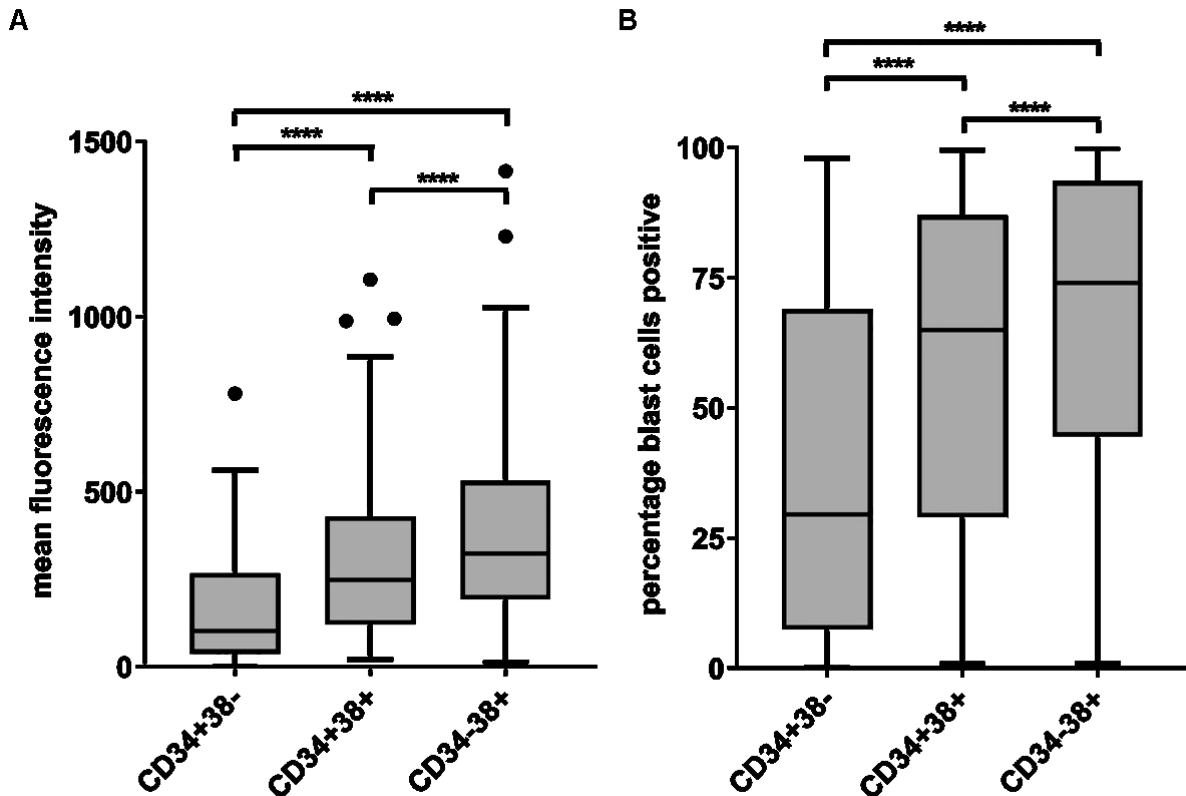
**Table 18:** Mean fluorescence intensity (MFI)  $\pm$  standard deviation of other surface markers tested in CD34-positive versus CD34-negative AML samples (n=150). (Daga, Rosenberger et al. 2019)

| Marker         | CD34-positive AMLs        | CD34-negative AMLs         | p-value               |
|----------------|---------------------------|----------------------------|-----------------------|
| <b>CD25</b>    | 119.6 $\pm$<br>269.16     | 120.37 $\pm$<br>260.95     | <b>0.5932</b>         |
| <b>CD33</b>    | 783.74 $\pm$<br>835.38    | 1496.0 $\pm$<br>1179.49    | <b>0.0001***</b>      |
| <b>CD44</b>    | 15256.47 $\pm$<br>7552.18 | 22588.91 $\pm$<br>14617.05 | <b>0.0146*</b>        |
| <b>CD45RA</b>  | 2607.0 $\pm$<br>2108.41   | 1709.69 $\pm$<br>1845.44   | <b>0.0097**</b>       |
| <b>CD47</b>    | 266.48 $\pm$<br>271.87    | 382.22 $\pm$<br>357.51     | <b>0.0330*</b>        |
| <b>CD49f</b>   | 884.53 $\pm$<br>921.34    | 502.10 $\pm$<br>559.77     | <b>0.0004***</b>      |
| <b>CD96</b>    | 160.93 $\pm$<br>113.21    | 152.20 $\pm$<br>97.70      | <b>0.9554</b>         |
| <b>CD99</b>    | 705.30 $\pm$<br>463.58    | 1011.39 $\pm$<br>672.33    | <b>0.0065**</b>       |
| <b>CD117</b>   | 3734.63 $\pm$<br>2723.56  | 1563.71 $\pm$<br>1183.72   | <b>&lt;0.0001****</b> |
| <b>CD123</b>   | 426.40 $\pm$<br>491.11    | 617.69 $\pm$<br>735.76     | <b>0.0381*</b>        |
| <b>CD305</b>   | 1636.58 $\pm$<br>1207.18  | 2797.77 $\pm$<br>2929.65   | <b>0.0095**</b>       |
| <b>IL-1RAP</b> | 189.56 $\pm$<br>214.99    | 294.45 $\pm$<br>286.16     | <b>0.0373*</b>        |
| <b>TIM-3</b>   | 895.57 $\pm$<br>796.19    | 876.31 $\pm$<br>1243.32    | <b>0.0822</b>         |
| <b>JAM-C</b>   | 747.33 $\pm$<br>610.81    | 998.69 $\pm$<br>1151.25    | <b>0.7356</b>         |

Differences in MFI values between groups were analysed using Mann-Whitney test.

### 3.1.2.1 CLL1 was highest expressed in CD34<sup>-</sup>38<sup>+</sup> leukemic cells

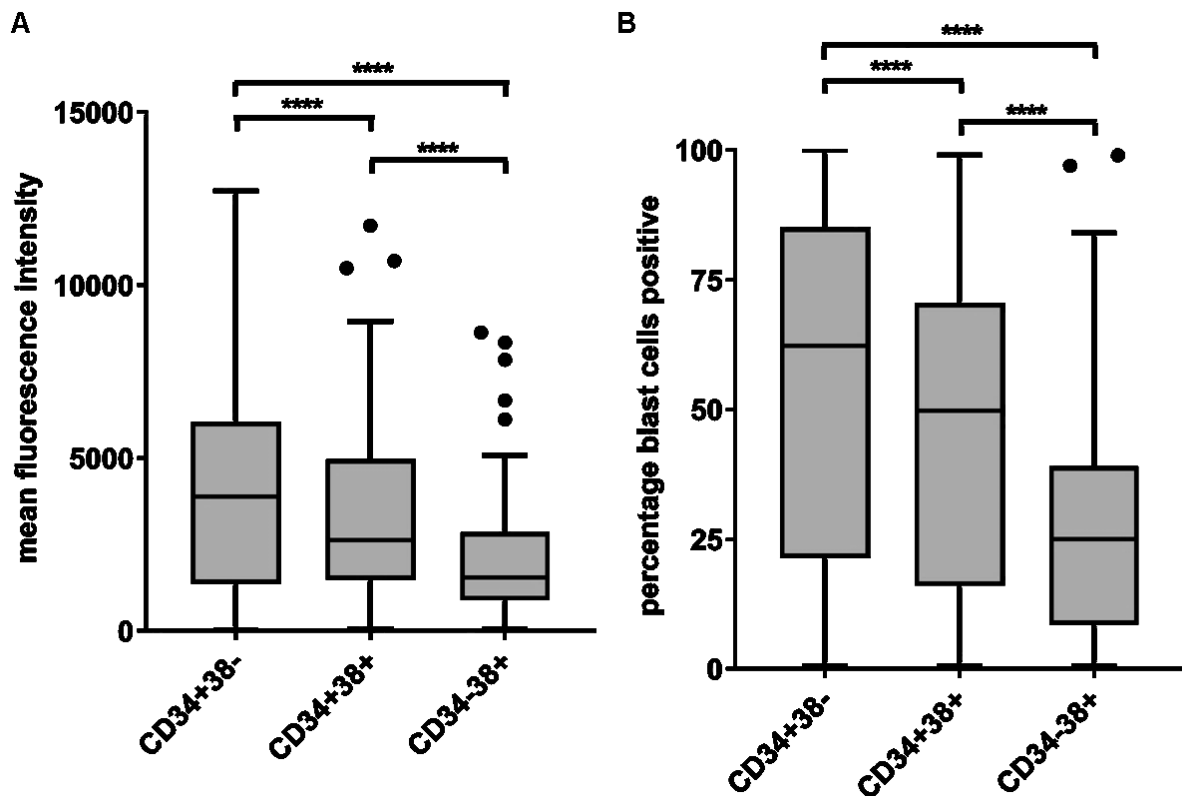
When we then analyzed CD34/38 subcompartments in CD34-positive AML samples, expression of CLL-1 was lowest in the LSC-enriched CD34<sup>+</sup>38<sup>-</sup> subpopulation, higher in the CD34<sup>+</sup>38<sup>+</sup> and highest in more mature CD34<sup>-</sup> cells as measured by both MFI values ( $173 \pm 178$  vs.  $320 \pm 258$  vs.  $432 \pm 349$ ;  $p < 0.0001$ , Figure 9A) as well as percentage of CLL-1<sup>+</sup> blasts ( $38.4 \pm 33.2$  vs.  $58.7 \pm 31.5$  vs.  $65.4 \pm 31.2\%$ ;  $p < 0.001$ , Figure 9B).



**Figure 9:** Expression of CLL-1 in CD34/38 cellular compartments of CD34-positive AML samples at diagnosis. Surface expression levels of CLL1 as measured by (A) mean fluorescence intensity (MFI) as well as (B) percentage of blasts positive for CLL-1 in different CD34/38 cellular compartments of AML samples at diagnosis (n=57) (\*\*  $p < 0.01$ ; \*\*\*\*  $p < 0.0001$ ) (Daga, Rosenberger et al. 2019).

### 3.1.2.2 GPR56 was highest expressed in CD34<sup>+</sup>38<sup>-</sup> leukemic cells

On the contrary, GPR56 expression as measured by MFI was highest in the LSC enriched CD34<sup>+</sup>38<sup>-</sup> population, lower in the CD34<sup>+</sup>38<sup>+</sup> cells and lowest in the CD34<sup>-</sup> compartment (4175±2949 vs. 3502±2701 vs. 2170±1992;  $p < 0.0001$ , Figure 10A). In accordance, the percentage of GPR56<sup>+</sup> blasts was also highest in the immature CD34<sup>+</sup>38<sup>-</sup> compartment and lowest in the more mature CD34<sup>-</sup> compartment (54.9±34.3% vs. 45.3±30.4% vs. 29.0±26.1%;  $p < 0.001$ , Figure 10B). Among other markers analyzed CD44 ( $p < 0.05$ ), CD117 ( $p < 0.01$ ), CD123 ( $p < 0.05$ ) and CD99 ( $p < 0.01$ ) were also differentially expressed between all three CD34/38 cellular compartments (Table 19).



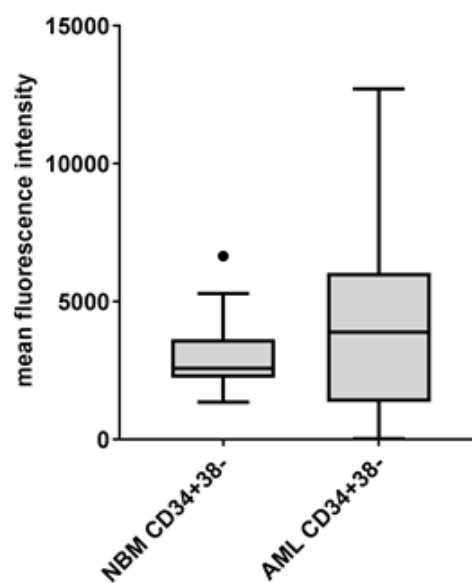
**Figure 10:** Expression of GPR56 in CD34/38 cellular compartments of CD34-positive AML samples at diagnosis. Surface expression levels of GPR56 as measured by (A) mean fluorescence intensity (MFI) as well as (B) percentage of blasts positive for GPR56 in different CD34/38 cellular compartments of AML samples at diagnosis (n=57) (\*\*  $p < 0.01$ ; \*\*\*\*  $p < 0.0001$ ) (Daga, Rosenberger et al. 2019).

**Table 19:** Mean fluorescence intensity (MFI)  $\pm$  standard deviation of other surface markers tested in CD34/38 compartments of CD34-positive AML samples (n=57). (Daga, Rosenberger et al. 2019).

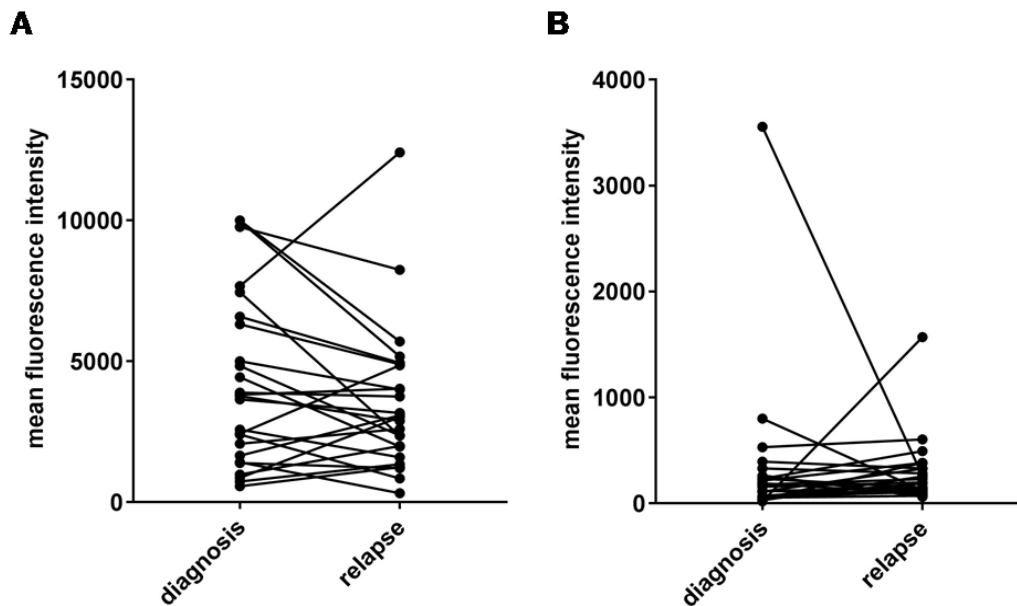
| Marker         | CD34 <sup>+</sup> 38 <sup>-</sup> | CD34 <sup>+</sup> 38 <sup>+</sup> | CD34 <sup>-</sup> 38 <sup>+</sup> | p-value         |
|----------------|-----------------------------------|-----------------------------------|-----------------------------------|-----------------|
| <b>CD25</b>    | 131.89 $\pm$<br>307.88            | 92.11 $\pm$<br>247.66             | 160.39 $\pm$<br>326.22            | <b>0.0822</b>   |
| <b>CD33</b>    | 752.86 $\pm$<br>662.31            | 1018.61 $\pm$<br>868.42           | 1108.49 $\pm$<br>907.74           | <b>0.0976</b>   |
| <b>CD44</b>    | 16849.36 $\pm$<br>8007.52         | 15422.48 $\pm$<br>7859.47         | 13084.93 $\pm$<br>8370.58         | <b>0.0348*</b>  |
| <b>CD45RA</b>  | 2700.16 $\pm$<br>2159.40          | 2515.54 $\pm$<br>1901.30          | 2028.51 $\pm$<br>1591.28          | <b>0.3047</b>   |
| <b>CD47</b>    | 244.23 $\pm$<br>250.91            | 340.39 $\pm$<br>392.03            | 295.46 $\pm$<br>314.29            | <b>0.6299</b>   |
| <b>CD49f</b>   | 937.74 $\pm$<br>1100.59           | 920.14 $\pm$<br>941.50            | 797.67 $\pm$<br>982.35            | <b>0.2528</b>   |
| <b>CD96</b>    | 189.91 $\pm$<br>167.94            | 200.48 $\pm$<br>277.40            | 150.52 $\pm$<br>129.29            | <b>0.5196</b>   |
| <b>CD99</b>    | 631.16 $\pm$<br>334.92            | 638.25 $\pm$<br>330.98            | 515.18 $\pm$<br>314.88            | <b>0.0099**</b> |
| <b>CD117</b>   | 3441.95 $\pm$<br>1985.58          | 4102.56 $\pm$<br>3469.46          | 3460.68 $\pm$<br>6635.33          | <b>0.0034**</b> |
| <b>CD123</b>   | 423.30 $\pm$<br>493.60            | 552.05 $\pm$<br>432.20            | 662.04 $\pm$<br>709.75            | <b>0.0186*</b>  |
| <b>CD305</b>   | 1605.98 $\pm$<br>1165.26          | 1782.81 $\pm$<br>1364.35          | 1764.11 $\pm$<br>1455.42          | <b>0.9039</b>   |
| <b>IL-1RAP</b> | 163.68 $\pm$<br>158.28            | 192.46 $\pm$<br>208.20            | 177.11 $\pm$<br>179.76            | <b>0.9390</b>   |
| <b>TIM-3</b>   | 878.05 $\pm$<br>724.80            | 966.37 $\pm$<br>738.04            | 712.0 $\pm$<br>655.80             | <b>0.0877</b>   |
| <b>JAM-C</b>   | 742.19 $\pm$<br>582.42            | 860.40 $\pm$<br>681.89            | 857.65 $\pm$<br>748.08            | <b>0.3301</b>   |

Differences in MFI values between groups were analysed using the Kruskal-Wallis test.

Next, we compared CLL-1 and GPR56 surface expression levels between CD34<sup>+</sup>38<sup>-</sup> leukemic cells and their normal HSPC counterparts. As reported previously (van Rhenen, van Dongen et al. 2007, Pabst, Bergeron et al. 2016), we found GPR56 to be expressed on the majority of normal HSPCs with slightly, but not significantly lower MFI values in comparison to CD34<sup>+</sup>38<sup>-</sup> leukemic counterparts (p=0.42, Figure 11), while CLL-1 was hardly expressed in CD34<sup>+</sup>38<sup>-</sup> HSPCs. Analysis of paired diagnostic and relapse samples (n=25) indicated that both GPR56 and CLL-1 expression were conserved throughout the disease course in most cases (Figure 12).



**Figure 11:** Surface expression of GPR56 in normal CD34<sup>+</sup>38<sup>-</sup> HSPCs vs CD34<sup>+</sup>38<sup>-</sup> leukemic cells. Comparative surface expression levels of GPR56 as measured by mean fluorescence intensity (MFI) on normal CD34<sup>+</sup>38<sup>-</sup> HSPCs as well as their leukemic counterparts at diagnosis ( p = 0.42) (Daga, Rosenberger et al. 2019). .

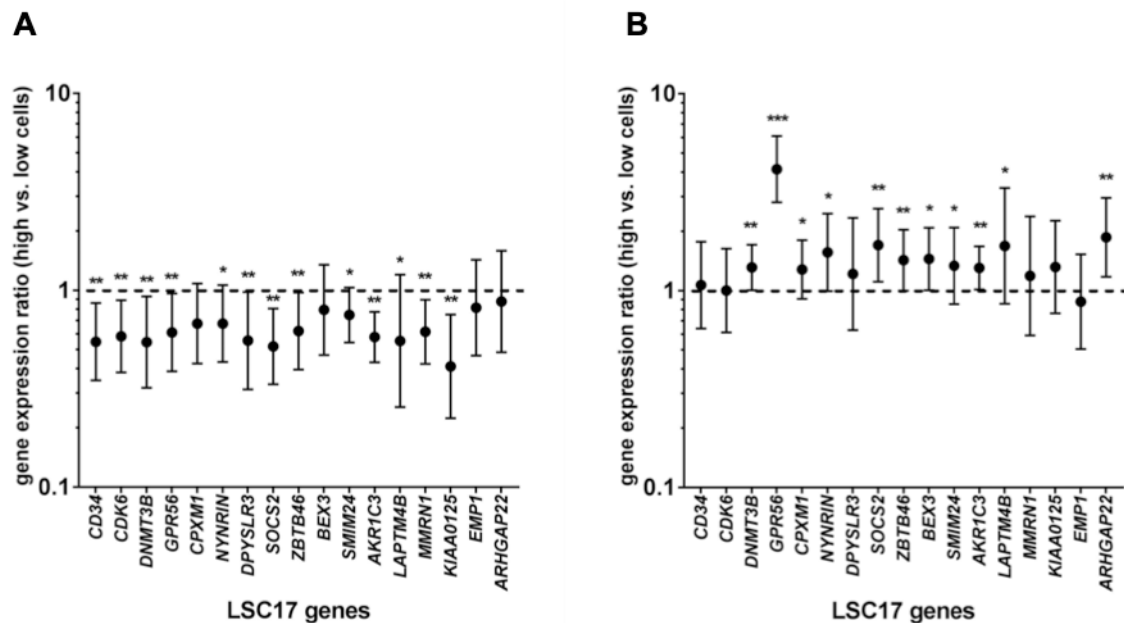


**Figure 12:** Expression GPR56 and CLL-1 in primary AML samples at diagnosis and relapse. Surface expression levels of (A) GPR56 (B) CLL1 in 25 paired diagnostic and relapse samples as measured by mean fluorescence intensity (MFI). MFI levels were not significantly different for both GPR56 ( $p=0.25$ ) and CLL-1 ( $p=0.29$ ) (Daga, Rosenberger et al. 2019).

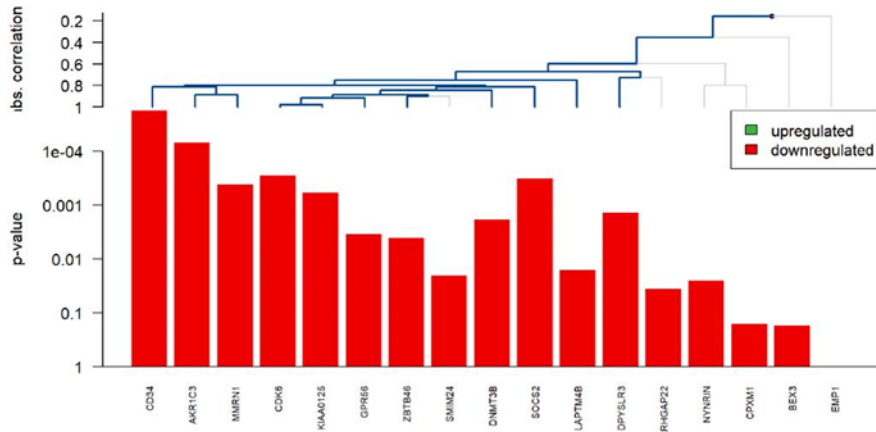
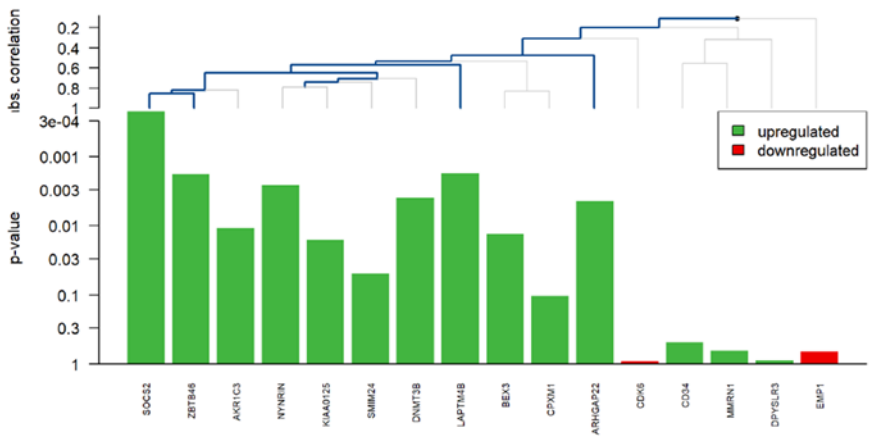
### 3.1.3 High GPR56 expression enriches for cells associated with LSC gene signature

In a comprehensive analysis involving 78 primary AML samples, Ng et al. identified a gene expression profile characteristic to LSCs based on differential expression of genes between the LSC-containing leukemic cell fractions and non-engrafting blast populations (Ng, Mitchell et al. 2016). They developed a so-called LSC17 score, which was based on the expression of the 17 most informative upregulated genes for LSC activity and found to be strongly associated with inferior outcomes in numerous AML cohorts. Interestingly, this LSC17 score also included the *GPR56* gene. To test the correlation of this LSC17 gene profile with surface expression of GPR56 or CLL-1, CD34<sup>+</sup>38<sup>-</sup> leukemic cells of 12 diagnostic AML samples were sorted based on their GPR56 and CLL-1 surface levels and the expression of the LSC17 genes was determined in GPR56 and CLL-1 high versus low expressing cells, respectively. Thirteen out of 17 genes were significantly lower expressed in CLL-1<sup>hi</sup> as compared to CLL-1<sup>lo</sup> CD34<sup>+</sup>38<sup>-</sup> leukemic cells (Figure 13A). While 11 out of 17 genes were significantly up-regulated in GPR56<sup>hi</sup> vs GPR56<sup>lo</sup> CD34<sup>+</sup>38<sup>-</sup> leukemic cells (Figure 13B), none of the genes was higher expressed in CLL-1<sup>hi</sup> leukemic cells. Using a

global statistical test described by Goelman et al. (2007), which evaluates the association of a group of genes with a distinct phenotype or factor, high CLL-1 surface expression in CD34<sup>+</sup>38<sup>-</sup> leukemic cells was associated with downregulation of the LSC17 gene panel ( $p < 0.001$ , Figure 14A). On the contrary, high GPR56 expression was significantly associated with upregulation of genes included in the LSC17 gene panel in CD34<sup>+</sup>38<sup>-</sup> AML cells ( $p < 0.0001$ ), even when the GPR56 qPCR data were omitted ( $p < 0.001$ , Figure 14B).



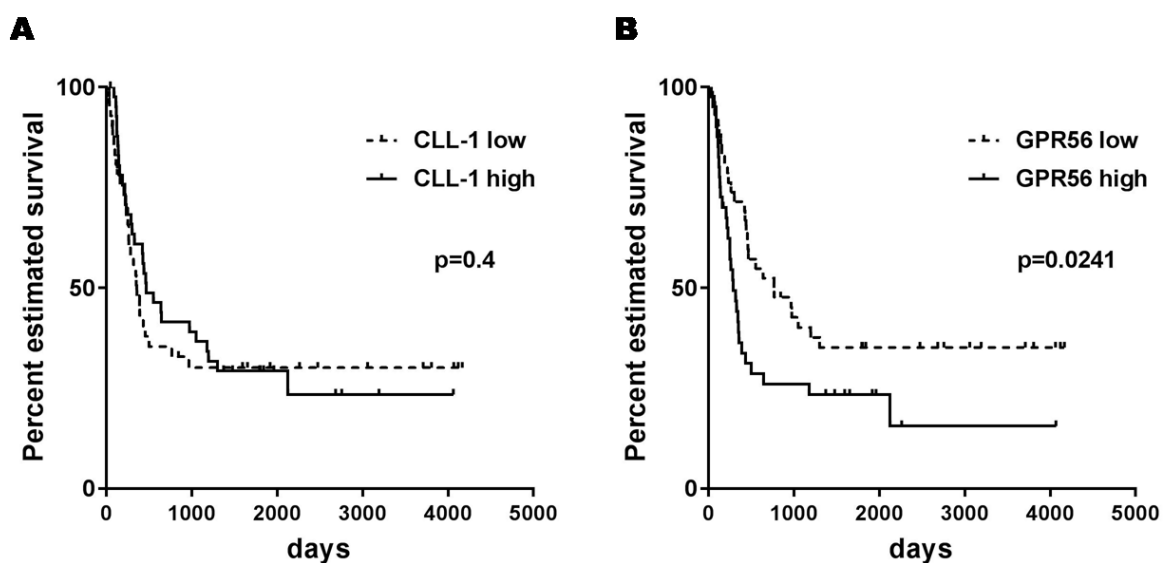
**Figure 13:** Mean geometric gene expression ratios of LSC17 genes in leukemic cells according to their CLL-1 as well as GPR56 surface levels. Mean geometric gene expression ratios ( $\pm$  geometric standard deviation) of LSC17 genes in sorted **(A)** CLL-1<sup>hi</sup> CD34<sup>+</sup>38<sup>-</sup> versus CLL-1<sup>lo</sup> CD34<sup>+</sup>38<sup>-</sup> cells (n=12); ratios <1 indicate lower expression of the respective gene in CLL-1<sup>hi</sup> CD34<sup>+</sup>38<sup>-</sup> cells. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ . **(B)** GPR56<sup>hi</sup> CD34<sup>+</sup>38<sup>-</sup> versus GPR56<sup>lo</sup> CD34<sup>+</sup>38<sup>-</sup> AML cells (n=12); ratios >1 indicate higher expression of the respective gene in GPR56<sup>hi</sup> CD34<sup>+</sup>38<sup>-</sup> cells (Daga, Rosenberger et al. 2019).

**A****B**

**Figure 14:** Association of LSC17 genes with high GPR56 and CLL1 surface expression using a global statistical analysis developed by Goelman et al., 2007. (A) High CLL-1 expression on CD34<sup>+</sup>38<sup>-</sup> leukemic cells was associated with downregulation (indicated in red) of genes included LSC17 panel ( $p < 0.001$ ). (B) High GPR56 expression on CD34<sup>+</sup>38<sup>-</sup> leukemic cells was significantly associated with upregulation (indicated in green) of genes included in the LSC17 panel ( $p < 0.0001$ ), even after exclusion of GPR56 ( $p < 0.001$ ). Bold lines indicate that the global test for association of the set of genes defined by cluster analysis with GPR56 and CLL-1 is significant even after correction for multiple testing (Daga, Rosenberger et al. 2019).

### 3.1.4 High GPR56 expression identifies patients with inferior outcomes

Since the LSC 17 gene expression signature has been associated with inferior outcome in AML patients, we next analyzed overall survival in 84 patients of our cohort receiving intensive chemotherapy according to the GPR56 and CLL-1 surface expression status. While no prognostic significance of CLL-1 expression was seen (median OS 463 vs. 352 days;  $p=0.4$ , Figure 15A), high GPR56 surface expression at diagnosis was associated with significantly lower OS (median OS 284 vs. 769 days;  $p=0.0241$ , Figure 15B). Interestingly, patients within the highest quartile of GPR56 expression had a lower complete remission rate after first induction chemotherapy as all other patients (38% versus 68%) suggesting that high GPR56 expression might be associated with resistance to chemotherapy. However, when tested in a multivariate analysis including cytogenetic risk, leukocyte counts, type of leukemia and receipt of an allogeneic stem cell transplantation GPR56 expression did not remain significant (Table 20) probably to the rather low number of patients in our cohort as well as to its known correlation with adverse cytogenetic markers (Pabst, Bergeron et al. 2016).



**Figure 15:** Overall survival in AML patients receiving intensive chemotherapy according to GPR56 and CLL-1 surface expression (n=84). While (A) CLL-1 expression levels were not informative, (B) high GPR56 expression was associated with inferior outcome (median overall survival 284 days vs. 769 days,  $p<0.05$ ) (Daga, Rosenberger et al. 2019).

**Table 20:** Multivariate analysis of factors affecting overall survival in AML patients having received intensive chemotherapy (Daga, Rosenberger et al. 2019).

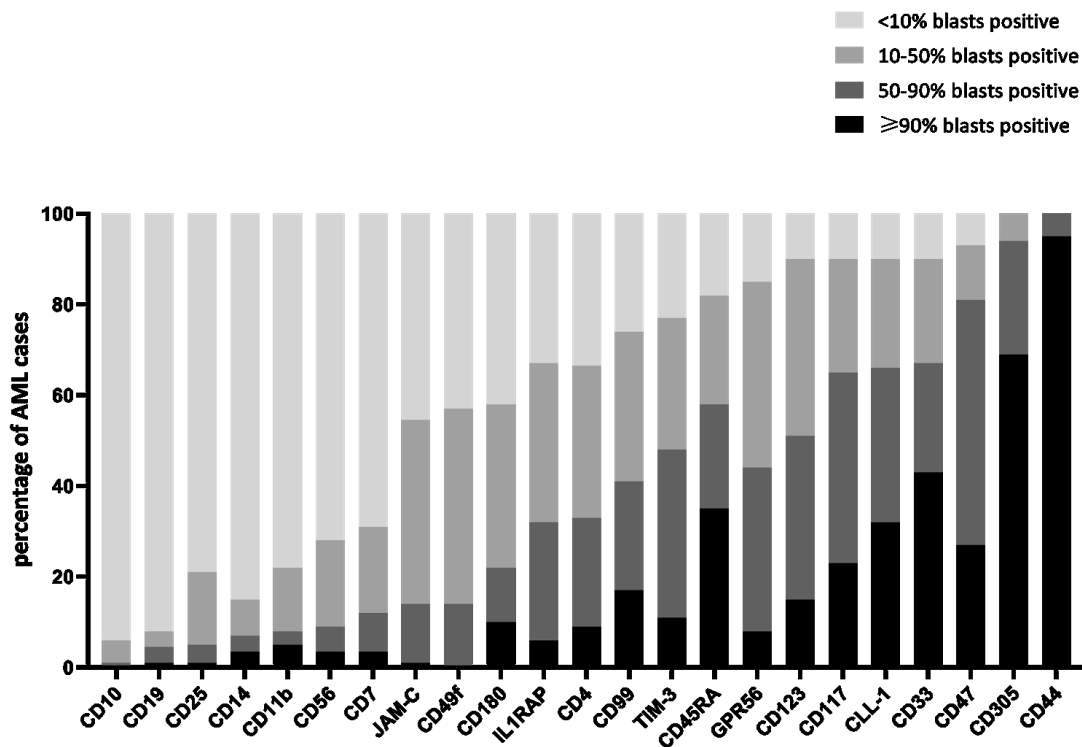
|  | <b>hazard ratio (95%CI)</b> | <b>p-value</b> |
|--|-----------------------------|----------------|
| <b>WBC</b><br>(high vs. low)               | 1.75 (1.0 - 3.215)          | <b>0.064</b>   |
| <b>type of AML</b><br>(de novo vs. others) | 0.57 (0.31 - 1.0)           | <b>0.066</b>   |
| <b>cytogenetic risk</b>                    | 1.75 (1.01 - 3.03)          | <b>0.044*</b>  |
| <b>allo-SCT</b>                            | 0.48 (0.27 - 0.83)          | <b>0.01*</b>   |
| <b>GPR56</b><br>(high vs. low)             | 1.49 (0.83 - 2.63)          | <b>0.17</b>    |
| <b>CLL-1</b><br>(high vs. low)             | 0.91 (0.5 - 1.56)           | <b>0.69</b>    |

GPR56 and CLL-1 expression levels were used as a dichotomous variable using the median value of the cohort as the cut-off level for high versus low expression

### 3.2 Novel method for detection of MRD by combining flow cytometry-based leukemic cell enrichment and mutational profiling of recurrently mutated genes in AML

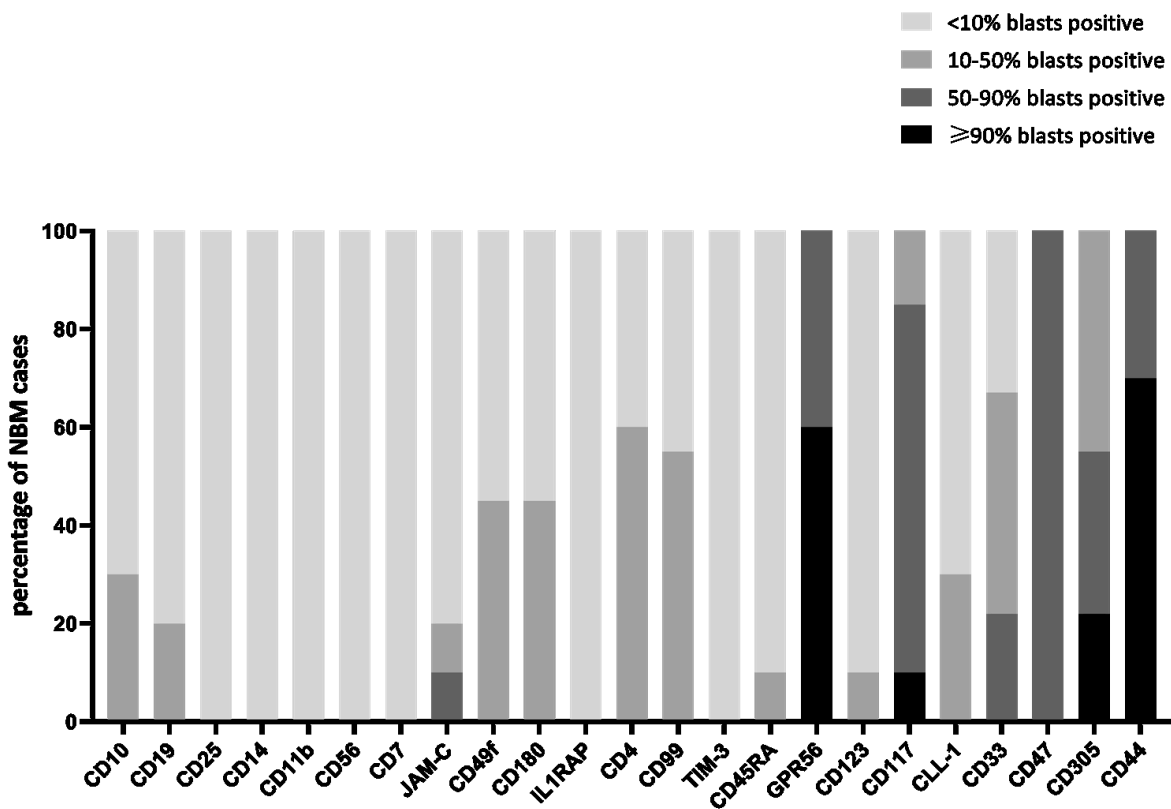
#### 3.2.1 Identification of cell surface markers suitable for flow cytometric based enrichment of leukemic cells

The expression of 23 potential cell surface markers for enrichment was analyzed on bulk leukemic cells in 150 diagnostic AML samples by MFC. For each of the 23 markers, the number of AML cases expressing them on more than 90%, 50-90%, 10-50% and less than 10% of blasts was determined (Figure 16). The highest numbers of samples with cells more than 90% positive for a marker were found for the markers CD44, CD305, CD33 and CD45RA. In contrast, CD4, CD7, CD10, CD11b, CD14, CD25, CD56, CD180 and JAM-C were hardly expressed on >90% of leukemic cells. When also including samples with a surface expression on 50-90% of cells, the markers CD47, CLL1, CD117, CD123, GPR56, TIM-3 and CD99 were prevalent in robust numbers of AML samples.



**Figure 16:** Antigen expression of the 23 potential markers on bulk leukemic cells in primary AML samples at initial diagnosis (n= 150). Data represent the general prevalence of the specific marker (percentage of samples) within the number of AML samples evaluated.

Next we analyzed the expression of these markers in normal CD34<sup>+</sup>38<sup>-</sup> HSPCs, since - to facilitate enrichment of residual leukemic cells - it is advantageous that a marker was low/negative and infrequently expressed on normal HSPCs. In 16 samples of normal bone marrow analyzed, CD44 and GPR56 were expressed on >90% of CD34<sup>+</sup>38<sup>-</sup> cells in the majority of cells. When including samples with a surface expression on 50-90% of cells, the markers CD117, CD305, CD47, CD33 and CD99 were prevalent in substantial numbers of normal HSPCs. In contrast, the remaining markers were hardly expressed on the CD34<sup>+</sup>38<sup>-</sup> HSPCs (Figure 17).



**Figure 17:** Antigen expression in normal CD34<sup>+</sup>38<sup>-</sup> HSPCs (n= 16). Data represent the general prevalence of the specific marker (percentage of samples) within the number of NBMC samples evaluated.

### 3.2.2 Possible markers for enrichment

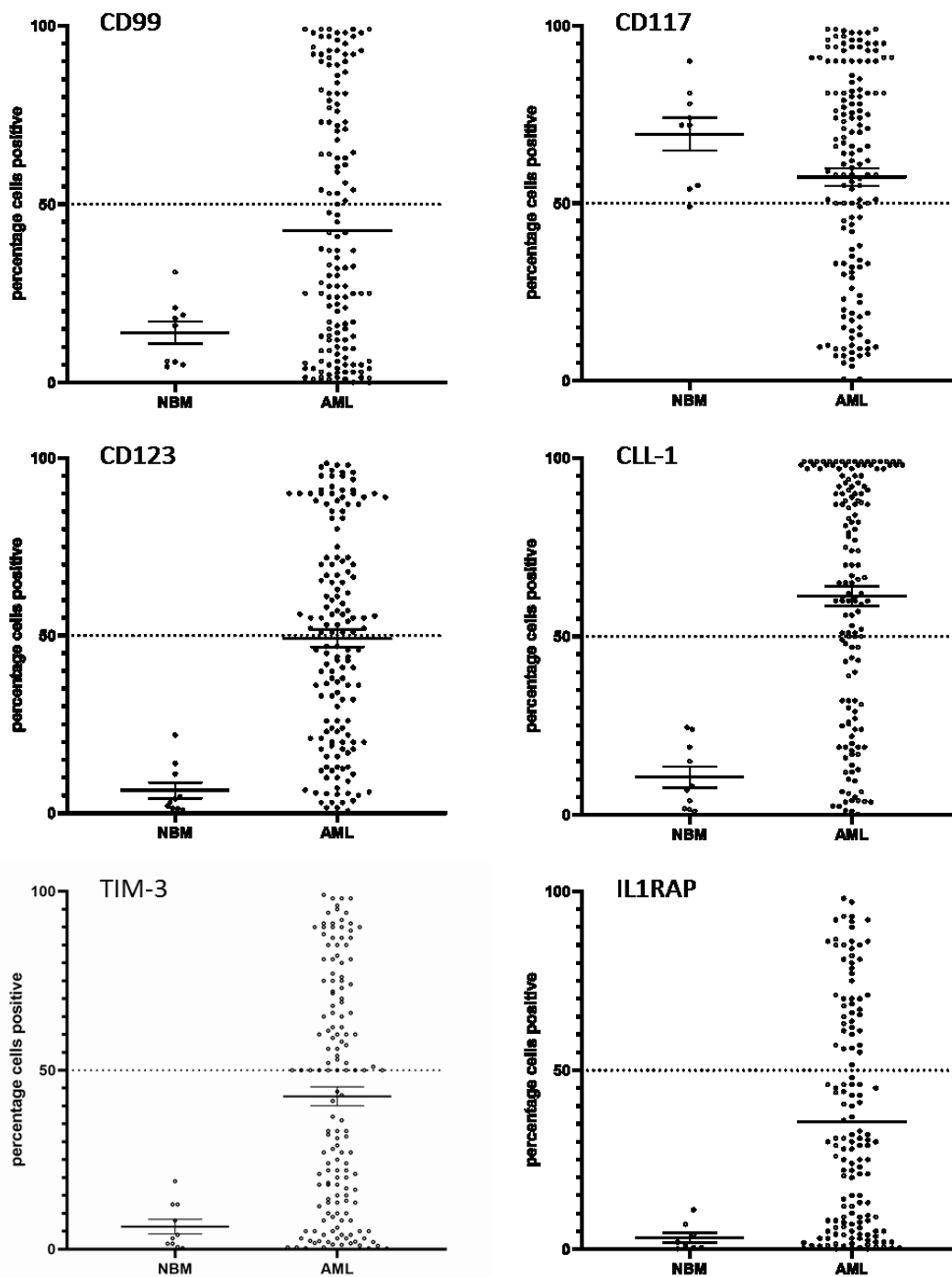
Since no single marker with an ideal expression profile could be identified, we aimed for the best marker combination that could enable enrichment of residual leukemic cells in a

maximum number of AML cases. Although CD47, CD33, CD305 and CD45RA were expressed on >90% blasts of the majority of the AML cases, these markers were not considered further for enrichment, because they were also widely expressed on normal hematopoietic cell subtypes including CD34<sup>+</sup>38<sup>-</sup> HSPCs. From that perspective CD123, CLL-1, CD99, TIM-3, IL1RAP and CD117 were the most promising markers, since they are only expressed on distinct progenitor cells, such as CD123 and CD117, or on distinct small subtypes of mature leukocytes, such as CLL-1, TIM-3 or IL1RAP (van Rhenen, van Dongen et al. 2007, Kikushige, Shima et al. 2010, Ågerstam, Karlsson et al. 2015).

In order to identify the most optimal combination of these six markers that could enable enrichment of residual leukemic cells in the vast majority of AML samples we determined the number of AML cases where the markers were positive meaning that the markers were expressed on more than 90% of leukemic blasts. Among the 150 AML cases analyzed, CLL-1 was positive in 32% cases, CD117 in 23%, CD99 in 17%, CD123 in 15%, TIM-3 in 12% and IL1RAP in 5% of the samples. In order to be able to target the vast majority of the AML cases we also determined the number of AML cases where the above markers were dim-positive meaning that the markers were expressed on 50-90% of leukemic blasts. When also including samples with a dim-positive surface expression on leukemic cells, CLL-1 was expressed in 66% cases, CD117 in 65%, CD123 in 51%, TIM-3 in 49%, CD99 in 40%, and IL1RAP in 31% of all samples. Of note is that more than one marker may be positive in the same AML sample.

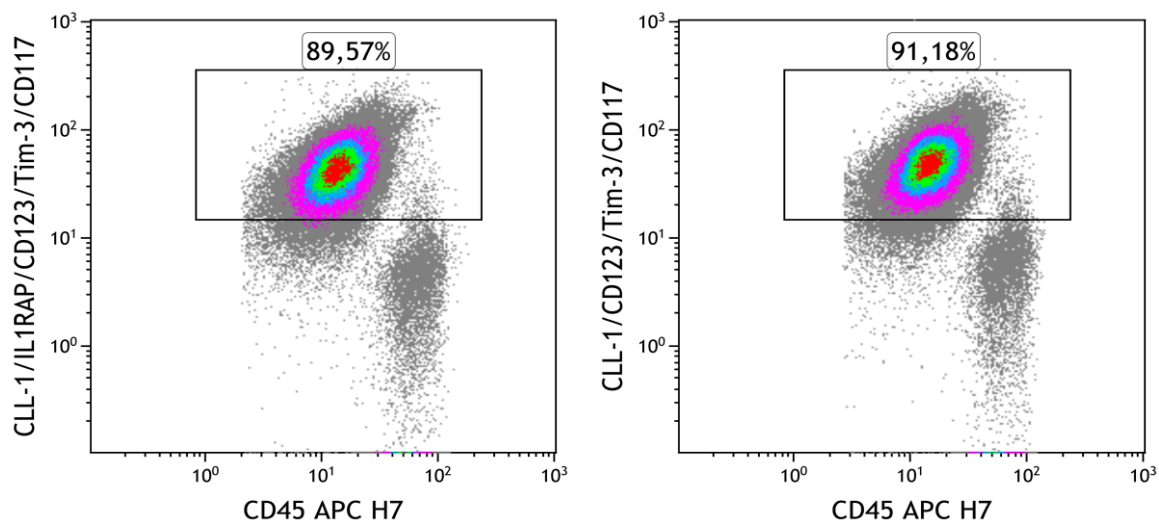
### **3.2.3 Selection of markers for enrichment**

Among these remaining 6 markers, difference in expression between leukemic cells and normal HSPCs was significant ( $p < 0.0001$ ) for TIM-3, IL1RAP, CD123 and CLL-1. In contrast, expression of CD117 in AML was not statistically significant as compared to normal HSPCs ( $p = 0.46$ ), and CD99 was only of borderline significance ( $p = 0.04$ ) (Figure 18). Moreover, detailed analysis revealed that the expression of CD99 positively correlated with CD123 ( $r = 0.25$ ,  $p = 0.0044$ ) and CLL-1 ( $r = 0.44$ ,  $p < 0.0001$ ) indicating that CD99 probably could not contribute considerably to enrichment. In addition, in the majority of AML cases (60%) CD99 was expressed on <50% of leukemic cells. Thus, CD99 was not considered further as a marker for enrichment.



**Figure 18:** Comparative expression of the enrichment markers on bulk leukemic cells in primary AML samples at diagnosis ( $n = 150$ ) and on  $CD34^{+}38^{-}$  HSPCs in NBM ( $n = 16$ ). Each dot represents one patient sample. While expression of CD117 was not different ( $p = 0.46$ ), expression of CD123, IL1RAP, CLL-1 and TIM-3 ( $p < 0.001$ ) was highly significantly different. Expression analysis of CD99 yielded a borderline significance ( $p = 0.04$ ).

Based on above results concerning performance, prevalence, redundancy and stability on HSPCs and leukemic cells of the different markers, we designed one tube that could enable enrichment of residual leukemic cells in remission BM samples. To begin with, we evaluated the enrichment potential of a single tube using antibodies against CD123, CD117, CLL-1, TIM-3 and IL1RAP in the PE-fluorescence channel along with a backbone marker, CD45. However, further analysis revealed that expression of IL1RAP correlated highly positively with CD123 ( $r=0.57$ ,  $p<0.0001$ ) and TIM-3 ( $r=0.40$ ,  $p<0.0001$ ) respectively in AML patients. In addition to the rather low incidence of IL1RAP (31% cases with IL1RAP expressed on  $\geq 90\%$  leukemic cells) we therefore determined, whether inclusion of IL1RAP did indeed further enhance enrichment. Enrichment potential was compared in 16 primary diagnostic AML samples using panels with or without an antibody against IL1RAP. Notably, the majority of the blasts ( $\geq 90\%$ ) were identified within the PE (marker) positive gate even after exclusion of the antibody against IL1RAP indicating that IL1RAP did not further improve enrichment (Figure 19). Therefore, IL1RAP could also be excluded as an enrichment marker.



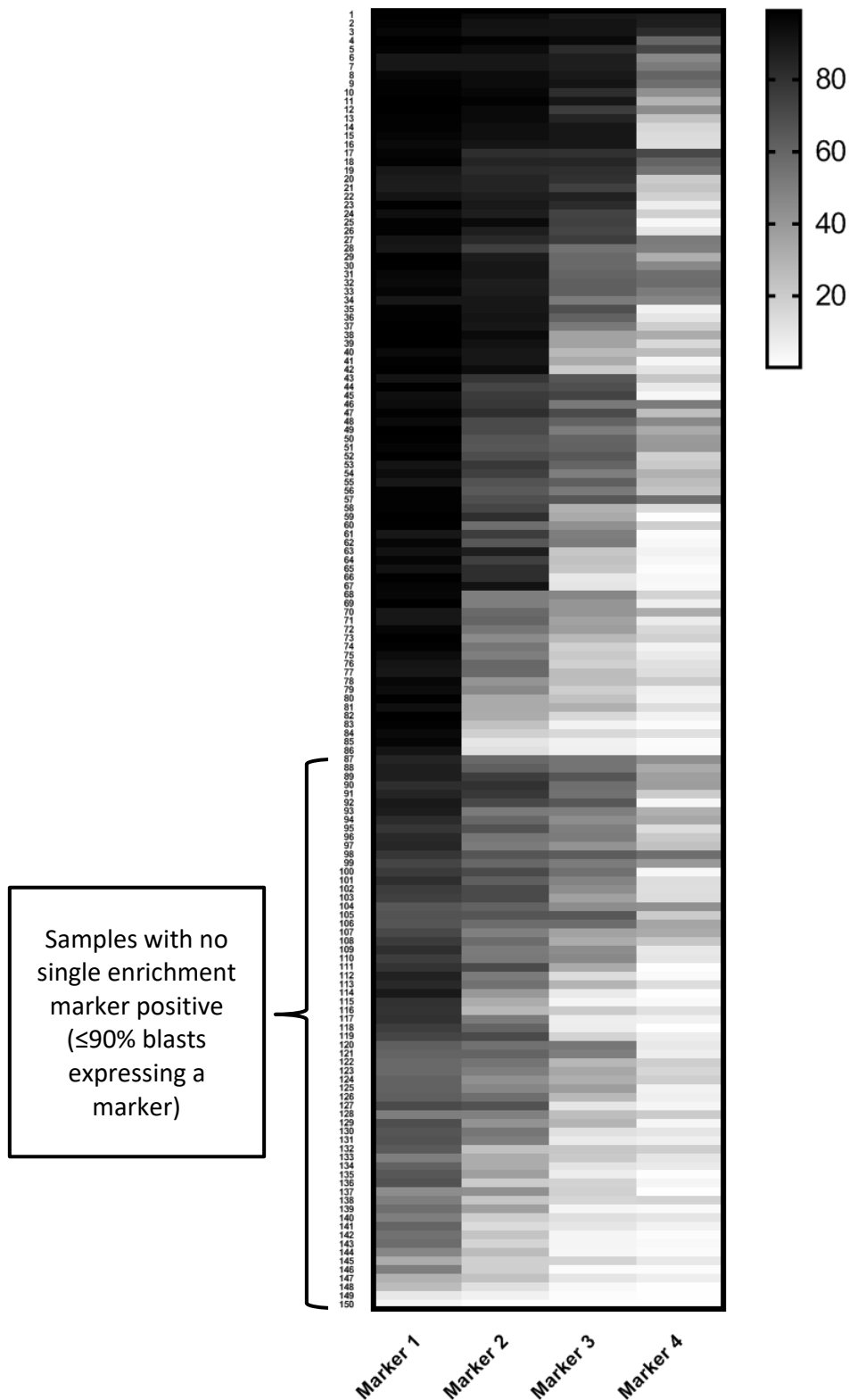
**Figure 19:** Comparison of enrichment potential after exclusion of IL1RAP for leukemic cells in primary AML samples. A representative example of the analysed primary AML samples at diagnosis ( $n = 16$ ) using enrichment panel (A) including IL1RAP as well as a panel (B) excluding IL1RAP is shown. Majority of blasts were identified within the PE (marker) positive gate indicating that IL1RAP did not further enhance enrichment.

Finally, we wondered whether CD117 could also be omitted as a marker for enrichment, since CD117 is expressed on a significant fraction of normal HSPCs (see also Figure 17). However, a combination of antibodies against CD123, TIM-3 and CLL-1 enabled coverage of the majority of cells in less than 75% of AML cases. Thus, in order to be able to cover the majority of AML samples CD117 was needed along with the other selected markers. Moreover, CD117 has also been used as a backbone marker for detection of MRD in previous studies (Roug, Larsen et al. 2014, Coustan-Smith, Song et al. 2018). Based on these data we eventually assigned the markers CD117, CD123, TIM-3 and CLL-1 to be used in our enrichment panel.

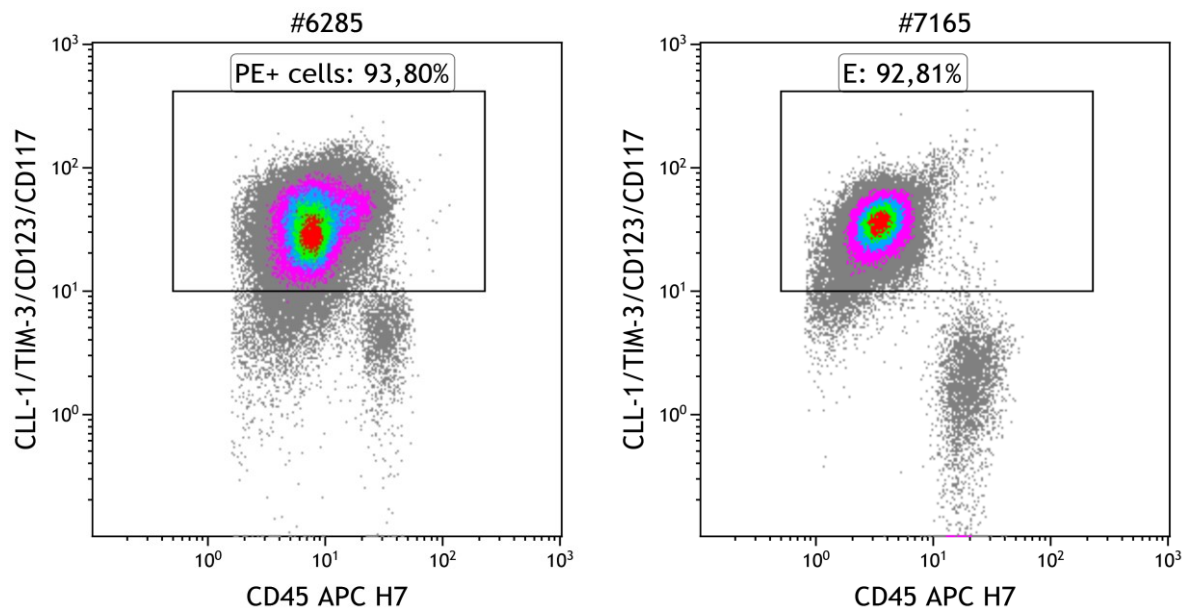
### **3.2.4 Establishment of the enrichment assay**

To establish the numbers of AML samples covered sufficiently by our enrichment panel, we reanalysed our total AML cohort. First, we identified all AML samples, where >90% of cells were positive with at least one of the four markers indicating sufficient staining with our enrichment panel. In total, 58% of the AML cases (86/150) showed expression of at least one marker on  $\geq 90\%$  blasts in our initial analysis, which was done for each marker using a separate fluorochrome for each antibody (Figure 20).

Of the remaining 64 AML samples, which did not have any single marker expressed on >90% of cells, 60 were then analyzed using an antibody cocktail targeting all four enrichment markers (CLL-1, TIM-3, CD117 and CD123) labelled with the same fluorochrome (PE). Four samples could not be determined due to the lack of sufficient material. In 48 samples the vast majority of blasts ( $\geq 90\%$ ) were identified within the PE (marker) positive gate (Figure 21) indicating that the cocktail including antibodies against CD117, CD123, TIM-3 and CLL-1 sufficiently labelled >90% of AML cells. Thus, in total 134 out of 146 AML samples (91.7%) showed adequate enrichment using the enrichment panel which included antibodies against CLL-1, TIM-3, CD117 and CD123. In summary, through our selection process we were able to identify four markers with high expression in AML cells covering about 90% of AML samples of our clinical cohort.

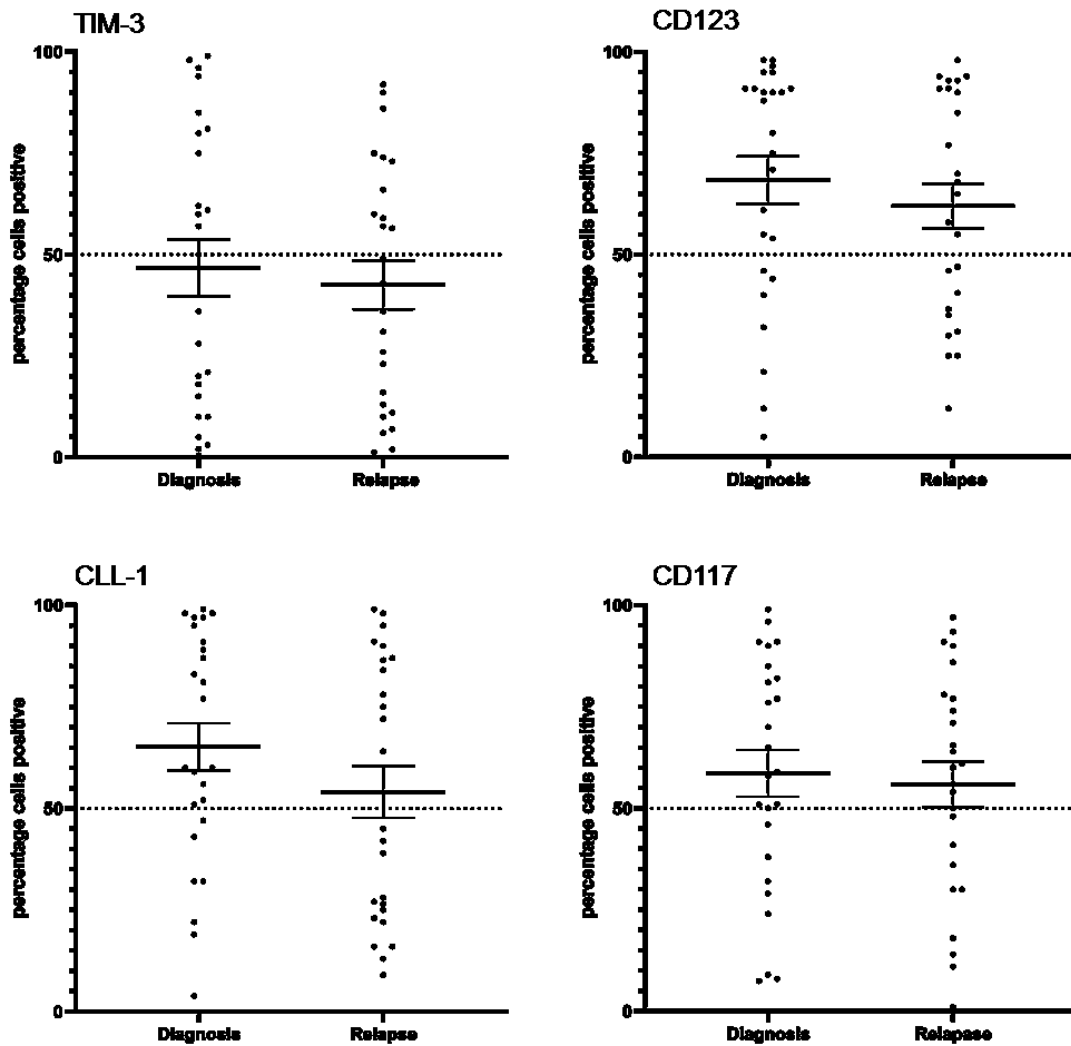


**Figure 20:** Expression of selected enrichment markers in 150 primary AML samples. The makers are arranged in descending order with the marker displaying the highest percentage of cells at the left while the marker displaying the lowest percentage of cells positive is on the right. Each row represents one patient sample.



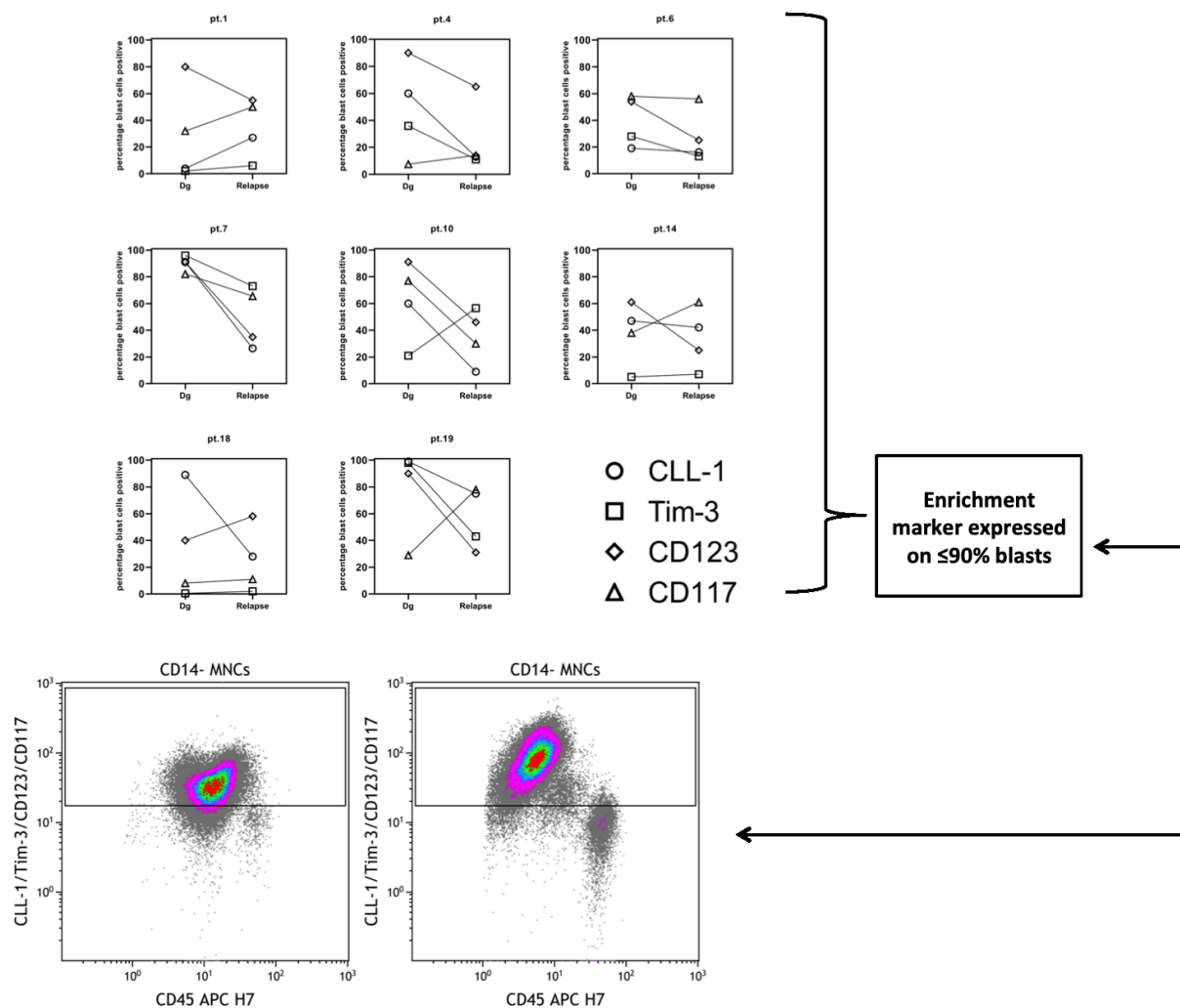
**Figure 21:** Performance of enrichment markers stained with antibodies using the same fluorochrome (PE) in samples where no single enrichment marker was expressed on >90% of blasts. Two representative examples of primary AML samples (n = 60) are shown. The majority of blasts ( $\geq 90\%$ ) were identified within the PE (marker) positive gate.

Next we compared the expression of markers in paired diagnostic and relapse samples (n=25) to ensure that marker persisted on leukemic cells at relapse and remained stable during course of disease and/or treatment. Expression of all the four enrichment markers was conserved throughout the disease course with either an increase or no significant change between diagnosis and relapse (Figure 22).



**Figure 22:** Comparative expression of the enrichment markers on leukemic cells in paired AML samples at diagnosis and at relapse (n= 25). Each dot represents one patient sample. While expression of CD123 was increased at relapse ( $p = 0.043$ ), expression of CD117 ( $p = 0.67$ ); CLL-1 ( $p = 0.26$ ) and TIM 3 ( $p = 0.95$ ) was not significantly different.

Most importantly, among the 25 patients studied, 17 samples had at least one of the selected enrichment markers positive and expressed on >90% of cells. The remaining 8 samples, which did not have any enrichment marker expressed on >90% of cells were then analyzed using an antibody cocktail targeting all four enrichment markers (CLL-1, TIM-3, CD117 and CD123) labelled with the same fluorochrome (PE). In all the 8 samples the vast majority of blasts ( $\geq 90\%$ ) were identified within the PE (marker) positive gate (Figure 23) indicating that the cocktail including CD117, CD123, TIM-3 and CLL-1 sufficiently labelled >90% of AML cells both at diagnosis and at relapse in all 25 patients studied ensuring that enrichment was not hampered by immunophenotypic shifts.

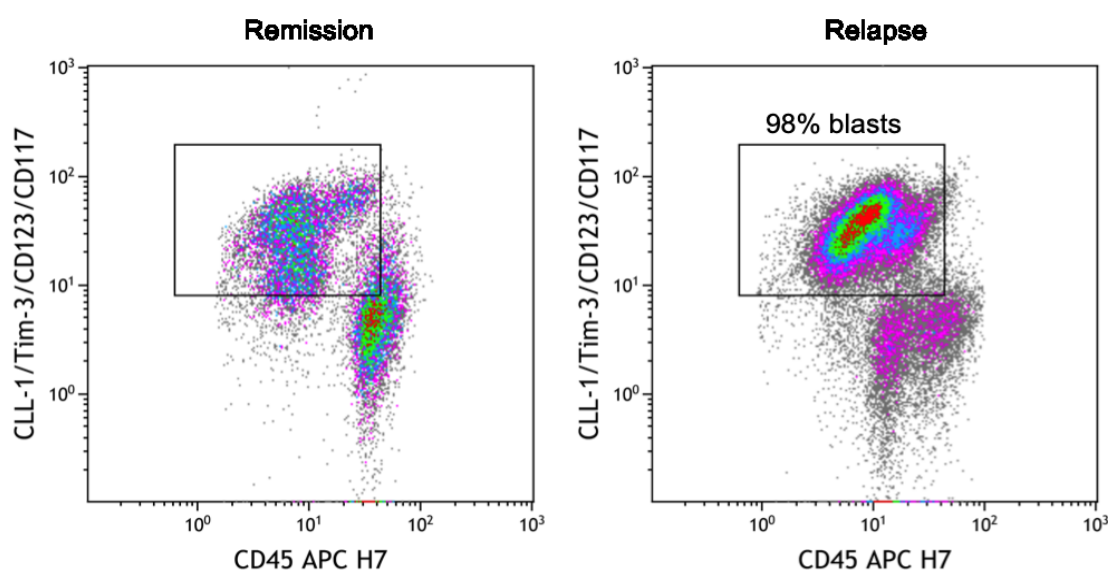


**Figure 23:** Representative examples of where AML cases where none of enrichment markers were positive at relapse. Each marker is indicated by a symbol. These 8 AML samples were verified for enrichment of leukemic cells using a cocktail of the four enrichment markers in PE fluorochrome. The majority of blasts ( $\geq 90\%$ ) were identified within the PE (marker) positive gate.

### 3.2.5 Composition and validation of enrichment panel

For flow cytometry based enrichment of residual leukemic cells in remission BM samples of AML patients, a single tube was then designed by combining antibodies targeting the chosen enrichment markers which included CLL-1, TIM-3, CD117 and CD123 in the PE-fluorescence channel along with the backbone markers CD45, CD14, CD34 and CD38. Furthermore, an antibody against CD203c, a basophil marker, was included to improve enrichment for leukemic cells by exclusion of CD123<sup>+</sup> basophils (Table 11). The cells were sorted into PE (marker) positive and, in some samples, PE (marker) negative fractions according to the gating strategy described in methods section (Figure 7).

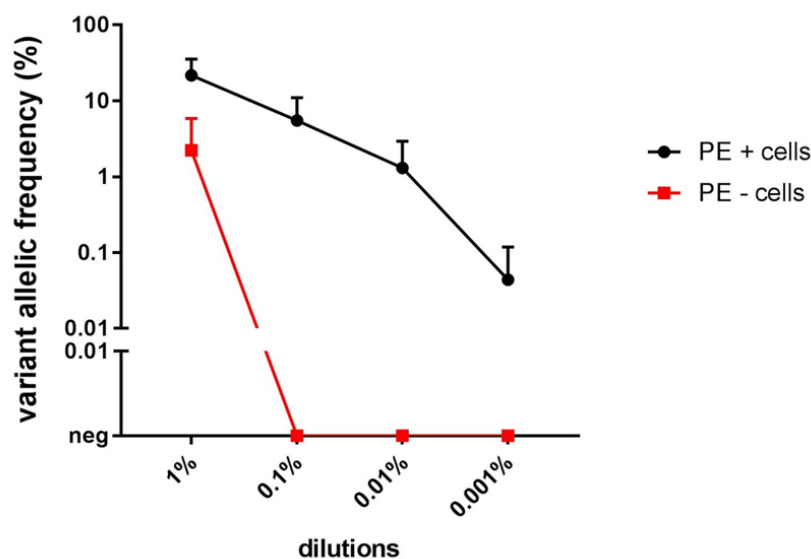
Accuracy of this enrichment panel was then verified by comparing matched remission and relapse samples. If the enrichment panel was accurate, the majority of blasts (>90%) at relapse should be found within the same PE (marker) positive gate as in remission sample. Analysis of matched remission and relapse samples (n = 3) revealed that the majority of blasts from the relapse sample was indeed within the same the same PE (marker) positive gate as in remission sample (Figure 23).



**Figure 24:** Evaluation of matched remission and relapse samples using the enrichment panel. A representative example is shown. The mononuclear cells were identified using SSC and CD45 expression. After excluding CD14 positive monocytes, the CD14 negative cells were gated onto the PE (marker) positive cocktail.

### 3.2.6 Dilution experiment for determining the sensitivity of a combined (“two-step”) approach of flow cytometry based leukemic cell enrichment and mutational analysis

In order to determine the sensitivity of this newly developed two-step MRD assay to detect residual leukemic cells, serial dilutions experiments were performed. BM from three different patients harboring *NPM1* mutations were mixed with normal BM cells. Frozen *NPM1*-mutated leukemic cells were carefully thawed and diluted with normal BM cells at concentrations 1%, 0.1%, 0.01% and 0.001% (1:10<sup>2</sup> to 1:10<sup>5</sup>). We then performed cell sorting of the mixed BM samples using our established enrichment panel. The average numbers of PE-positive and PE-negative cells sorted were 75000 and 273582, respectively. Using dPCR, cells with an *NPM1* mutation were clearly enriched in the PE (marker)-positive samples (Figure 24). The *NPM1* mutation was detected in all sorted PE (marker)-positive samples up to the dilution of 1:10000. In 2 out of 3 samples tested, we even were able to trace the *NPM1* mutation in PE positive cells in dilutions 1:100000. In contrast, *NPM1*-mutations were not detected in PE (marker) negative cells in dilutions higher than 1:100. Thus, the sensitivity to detect a *NPM1*-mutated AML cell of this approach was 10<sup>-4</sup> to 10<sup>-5</sup>.



**Figure 25:** Sensitivity level of two-step MRD assay based on dilution experiments of *NPM1* mutated leukemic cells mixed with normal BM cells. Sorted PE (marker) positive fractions are represented in black while sorted PE (marker) negative fractions are represented in red.

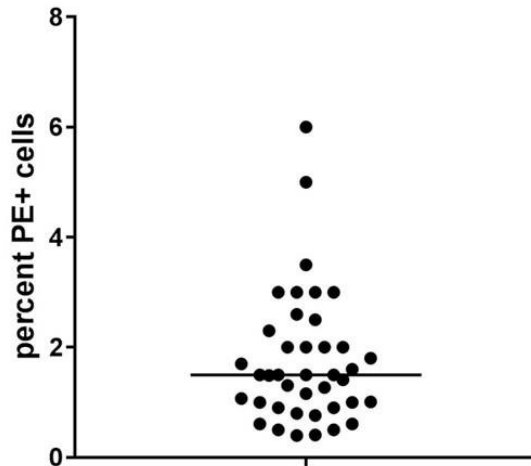
## **4 Validation of the two-step MRD assay as a prognostic biomarker for relapsing disease in a prospective cohort**

### **4.1 Patient Characteristics**

The two-step MRD assay was validated as a biomarker for relapsing disease in a cohort comprising a total of 41 patients who received intensive chemotherapy. Among the 41 patients, 36 cases had de novo AML, 4 had secondary AML and 1 case had therapy related AML. The patients' median age was 60 (range 34–75) years. The sex ratio (M/F) was 1.2. ELN risk classification was available for all patients and there were 13 patients with a favorable risk, 16 with intermediate risk, and 12 with poor risk. Remission samples were obtained after the first induction therapy in 37 patients, while four patients achieved CR after the second induction cycle. Nineteen patients received allogeneic stem cell transplantation as consolidation therapy. Complete patient characteristics are listed in Table 21.

### **4.2 Cell sorting for flow cytometry based enrichment of residual cells**

The enrichment panel was then assessed for its enrichment potential in the prospective cohort comprising of 41 patients. Remission bone marrow was sorted using a panel of antibodies against CD117, CD123, CLL-1 and TIM-3 all labelled with the fluorochrome PE into PE (marker) positive fractions according to the gating strategy described in methods section (Figure 7). We observed between 0.4% and 6% PE (marker) positive cells with an average of 1.5% cells in the tested remission samples. Interestingly, the two outliers with 5% and 6% PE (marker) positive cells, respectively, were samples from patients with secondary AML. This indicated an enrichment of  $\approx 15$ -250-fold with a median enrichment of 67-fold using our marker combination (Figure 26).



**Figure 26:** Percentage of sorted marker positive (PE positive) calculated as percentage of total nucleated BM cells obtained after FACS based cell sorting in 41 remission samples.

### 4.3 Mutational Profiling

#### 4.3.1 Detection of mutations at diagnosis

Mutational analysis with NGS using the myeloid panel was available at diagnosis in 31 patients. The remaining 10 patients displayed either a mutation in *NPM1*, *FLT3* or *CEBPA* as detected by routine Sanger sequencing at diagnosis and could therefore also be included in the study. In accordance with the published literature the following genes were found to be commonly affected at diagnosis: *NPM1* (n = 14), *FLT3* (n = 11), *DNMT3A* (n = 11), *TET2* (n = 9), *RAS* (n = 8), *IDH1/2* (n = 7), *PTPN11* (n = 5), *RUNX1* (n = 5), *CEBPA* (n = 4), *WT1* (n = 3), *ASXL1* (n = 3), *GATA2* (n = 3), *NF1* (n = 3), *TP53* (n = 2) and *CBL* (n = 2). On average, we detected 2.4 mutations with at least one informative mutation per patient, which could serve as a marker for residual disease.

**Table 21:** Patient characteristics for prospective cohort

|                                     |                    |
|-------------------------------------|--------------------|
| <b>Gender</b> (female/male)         | 19/22              |
| <b>Age</b> (median, range in years) | 60 (34-75)         |
| age <60 years                       | 27                 |
| age ≥60 years                       | 14                 |
| <b>Type of AML</b>                  |                    |
| de novo AML                         | 36                 |
| secondary AML                       | 4                  |
| therapy-related AML                 | 1                  |
| <b>Mean WBC</b> at diagnosis (G/l)  | 34.5 (0.64-197.13) |
| WBC <30 G/l                         | 25                 |
| WBC ≥30 G/l                         | 14                 |
| <b>ELN Risk Classification</b>      |                    |
| favorable risk                      | 13                 |
| intermediate risk                   | 16                 |
| adverse risk                        | 12                 |

### 4.3.2 Detection of mutations at CR

Next, we performed targeted NGS to detect persistent mutations at remission after intensive chemotherapy in the FACS sorted PE (marker)-positive bone marrow cells. Numbers of sorted cells in PE (marker)-positive population ranged from 1300 to 7200 with an average of 6500 cells. In 39 samples, sufficient DNA was available to perform mutational analysis. In the remaining two sample DNA concentrations was too low. Therefore, data from these 39 patients were included for further analysis.

We previously reported that the sensitivity of detecting a mutated variant using our sequencing approach was 0.5%. However, insertions as found for example with *NPM1* mutations were reliably detected with a sensitivity of 0.1% (Gaksch, Kashofer et al. 2018) as also reported by others (Jongen-Lavrencic, Grob et al. 2018; Onecha, Linares et al. 2019). Therefore, we defined persisting residual disease as a detected VAF of >0.5%. However, in the case of insertions we used a cutoff of 0.1%.

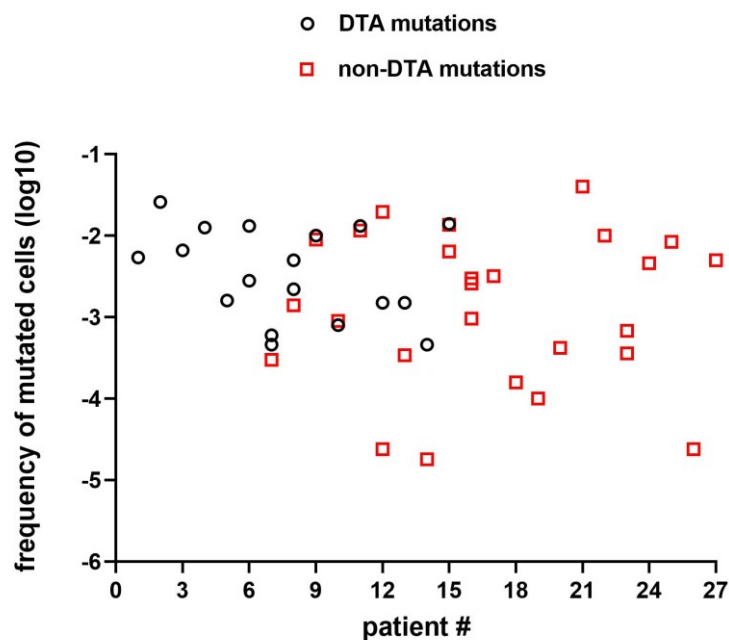
By applying the above defined cut-off values, 44 of the 93 mutations at diagnosis (47.3%) still persisted in 27 patients (69%). Through mutational analysis we detected 1 to 4 mutations per patient indicating average of at least one recurrently mutated genes still persisting at remission. The VAF ranged from 0.1 to 47% (Figure 27). The following genes were found to persist in CR: *DNMT3A* (n = 11), *TET2* (n = 7), *NPM1* (n = 6), *RUNX1* (n = 4), *IDH1/IDH2* (n = 4), *TP53* (n = 2), *NRAS* (n = 2), *KRAS* (n = 1), *NFI* (n = 1), *WT1* (n = 1), *PTPN11* (n = 1), *CBL* (n = 1), *FLT3* (n=1) and *ASXL1* (n = 1). The majority of mutations in genes *RAS*, *FLT3*, *PTPN11*, *GATA2* and *CEBPA* were cleared after induction therapy.

The most commonly non-cleared mutations at CR were *DNMT3A* (n = 11) and *TET2* (n= 7). We and others have previously shown that *DNMT3A*, *TET2* and *ASXL1* (DTA) mutations can persist in regenerating BM despite clearance of AML blasts (Thol, Damm et al. 2011, Gaksch, Kashofer et al. 2018, Jongen-Lavrencic, Grob et al. 2018). In 6 of 27 (22%) patients who had both DTA mutations and non-DTA mutations at diagnosis, we observed that while DTA mutations still persisted after induction therapy the non-DTA mutations were usually cleared. These observations were consistent with the idea that residual cells bearing DTA mutations often represent preleukemic clones, which repopulate the BM after induction therapy rather than persisting true leukemic cells (Jan, Snyder et al. 2012, Corces-Zimmerman, Hong et al. 2014). Moreover, no prognostic impact of persisting DTA mutations

has recently been shown (Jongen-Lavrencic, Grob et al. 2018). Therefore, we performed further analysis after excluding the DTA mutations.

Importantly, persistence of non-DTA mutations within the PE (marker)-positive fraction was found in twenty-one samples with a median variant allelic frequency (VAF) of 5% (range: 0.1%-45%). The calculated frequencies of mutated leukemic cells in PE (marker)-positive fractions ranged between  $1.4 \times 10^{-2}$  –  $2.4 \times 10^{-5}$  (Figure 27, Table 22). Eighteen samples tested negative for leukemia specific mutations in PE (marker) positive populations. Assuming a sensitivity of 0.5% for mutational analysis (Gaksch, Kashofer et al. 2018), the calculated sensitivities for MRD negative samples ranged between  $2.3 \times 10^{-5}$  –  $7 \times 10^{-5}$  (Table 23).

In 19 of the 39 samples we also did mutational analysis of the PE (marker)-negative sorted cell fraction. Notably, leukemia specific mutations were absent in the PE (marker)-negative cells of all these samples tested according to our detection algorithm. Since 9 out of these 19 samples were MRD positive in the PE (marker)-positive cells, these results clearly suggested a leukemic cell enrichment using our enrichment panel. However, in one MRD-positive sample, a mutation in *NPM1* was also detected in the PE (marker)-negative cell population albeit at a lower VAF (0.02%) as our defined detection limit (0.1%).



**Figure 27:** Calculated frequencies of mutated cells detected during complete remission using our two-step MRD detection method. Black circles denote mutated cell frequencies as detected by DTA mutations and red squares display mutated cell frequencies as detected by non-DTA mutations.

**Table 22:** Cell numbers and frequencies of mutated cells in sorted remission bone marrow samples tested MRD positive in which both, PE (marker) positive as well as negative, cell fractions were analysed.

| Pat.ID |     | No: of sorted cells | % total cells | % mutated                             | frequency of mutated cells   |
|--------|-----|---------------------|---------------|---------------------------------------|--|
| 7558   | PE+ | 38700               | 2.2           | 7.3% NPM1                             | $3.2 \times 10^{-3}$   |
|        | PE- | 82300               | 8.8           | Neg                                   | Neg  |
| 7608   | PE+ | 5718                | 1.5           | 1.4% NPM1                             | $4.2 \times 10^{-4}$   |
|        | PE- | 8603                | 4.2           | Neg                                   | Neg  |
| 8758   | PE+ | 3680                | 0.7           | 1.0% NPM1                             | $1.4 \times 10^{-4}$   |
|        | PE- | 18106               | 2.3           | Neg                                   | Neg  |
| 7704   | PE+ | 4391                | 1.3           | 0.79% NPM1                            | $2 \times 10^{-4}$   |
|        | PE- | 36129               | 13.9          | Neg                                   | Neg  |
| 8319   | PE+ | 3077                | 1.5           | 35%TET2<br>30% RUNX1                  | $1 \times 10^{-2}$<br>$9 \times 10^{-3}$                           |
|        | PE- | 125563              | 37.2          | Neg                                   | Neg  |
| 8408   | PE+ | 2796                | 1.2           | 3% IDH2                               | $7 \times 10^{-4}$   |
|        | PE- | 21952               | 9.4           | Neg                                   | Neg  |
| 8415   | PE+ | 6500                | 1.4           | 37% WT1                               | $1 \times 10^{-2}$   |
|        | PE- | 26120               | 4.5           | Neg                                   | Neg  |
| 8733   | PE+ | 7326                | 1.2           | 0.1% NPM1                             | $2.4 \times 10^{-5}$   |
|        | PE- | 95000               | 12.4          | 0.02% NPM1                            | $5 \times 10^{-5}$   |
| 8446   | PE+ | 4391                | 1.3           | 0.9% NPM1<br>1.8% DNMT3A<br>2.3% TET2 | $2.4 \times 10^{-4}$<br>$4.6 \times 10^{-4}$<br>$6 \times 10^{-4}$ |
|        | PE- | 36129               | 13.9          | Neg                                   | Neg  |

**Table 23:** Cell numbers and frequencies of mutated cells in sorted remission bone marrow samples tested MRD negative in which both, PE (marker) positive as well as negative, cell fractions were analysed.

| Pat.ID |     | No: of sorted cells | % total cells | % mutated | frequency of mutated cells<br>(calculated hypothetical sensitivity) |
|--------|-----|---------------------|---------------|-----------|---|
| 7359   | PE+ | 3585                | 1.16          | 0.00      | Neg ( $6 \times 10^{-5}$ )  |
|        | PE- | 30900               | 20            | 0.00      |   |
| 7874   | PE+ | 4756                | 0.61          | 0.00      | Neg ( $3 \times 10^{-5}$ )  |
|        | PE- | 15700               | 2,55          | 0.00      |   |
| 8732   | PE+ | 1840                | 0.64          | 0.00      | Neg ( $3.2 \times 10^{-5}$ )  |
|        | PE- | 5456                | 3.28          | 0.00      |   |
| 7400   | PE+ | 5374                | 0.63          | 0.00      | Neg ( $3.1 \times 10^{-5}$ )  |
|        | PE- | 16358               | 3.39          | 0.00      |   |
| 7473   | PE+ | 6974                | 0.86          | 0.00      | Neg ( $4.3 \times 10^{-5}$ )  |
|        | PE- | 35000               | 7.56          | 0.00      |   |
| 8671   | PE+ | 2519                | 0.47          | 0.00      | Neg ( $2.3 \times 10^{-5}$ )  |
|        | PE- | 29000               | 3.54          | 0.00      |   |
| 7934   | PE+ | 20976               | 1.4           | 0.00      | Neg ( $7 \times 10^{-5}$ )  |
|        | PE- | 26376               | 11.09         | 0.00      |   |
| 8315   | PE+ | 6499                | 1.41          | 0.00      | Neg ( $7 \times 10^{-5}$ )  |
|        | PE- | 26000               | 4.5           | 0.00      |   |
| 7766   | PE+ | 1267                | 0.76          | 0.00      | Neg ( $3.8 \times 10^{-5}$ )  |
|        | PE- | 3745                | 2.55          | 0.00      |   |
| 8550   | PE+ | 8054                | 1.27          | 0.00      | Neg ( $6.3 \times 10^{-5}$ )  |
|        | PE- | 33200               | 7.6           | 0.00      |   |

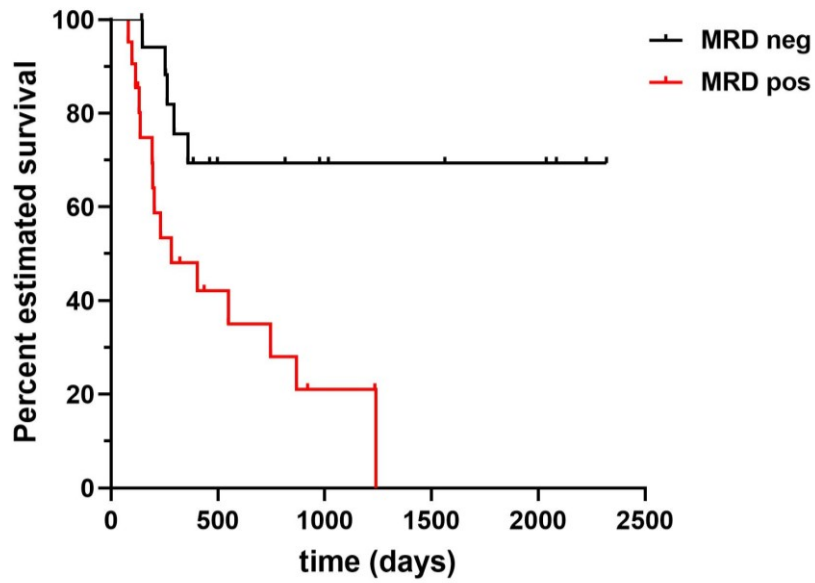
#### 4.4 The two-step MRD assay as a biomarker for relapsing disease

Next we analyzed relapse free survival (RFS) in relation to the MRD status of patients in our cohort. Median follow-up time was 559 days. Patients harboring only *DNMT3A*, *TET2* or *ASXL1* (DTA) mutations (n=6) were termed MRD negative since persistence of these mutations are known to be not predictive for relapse (Thol, Damm et al. 2011, Gaksch, Kashofer et al. 2018, Jongen-Lavrencic, Grob et al. 2018). Of the 39 patients tested in our cohort, twenty-one patients tested MRD positive, while eighteen patients tested MRD negative. Among the 21 MRD positive patients, 15 patients relapsed (71.4%), while among the 18 MRD negative patients, five patients relapsed (27.8%; p=0.0065). Therefore, the calculated sensitivity and specificity of our two-step method of MRD detection to predict relapse was 0.75 and 0.684, respectively.

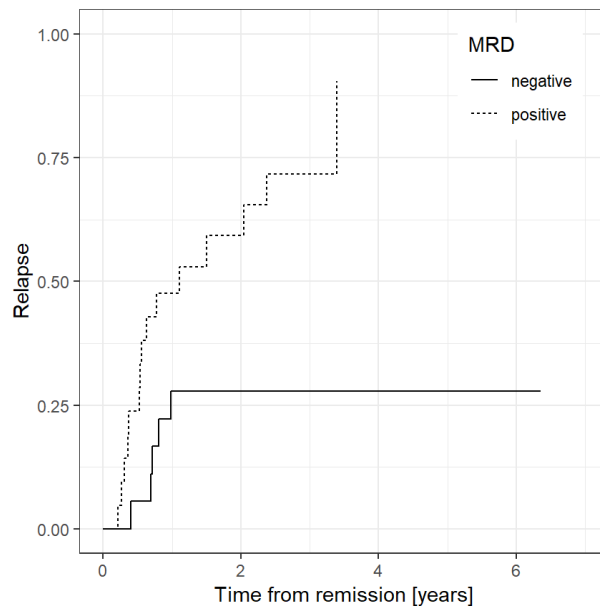
Patients with a positive MRD status had a shorter RFS than MRD negative patients (median RFS 283 vs. not reached, p=0.0031) (Figure 28). Accordingly, the cumulative incidence of relapse (CIR) was significantly higher for MRD positive patients than for MRD negative patients (5-year CIR: 90.5% vs 28%, p<0.001, Figure 29). However, when we analyzed overall survival according to MRD status, no prognostic significance of MRD status on overall survival was seen in our cohort (p=0.0849, Figure 30).

#### 4.5 Prognostic significance of the two-step MRD assay

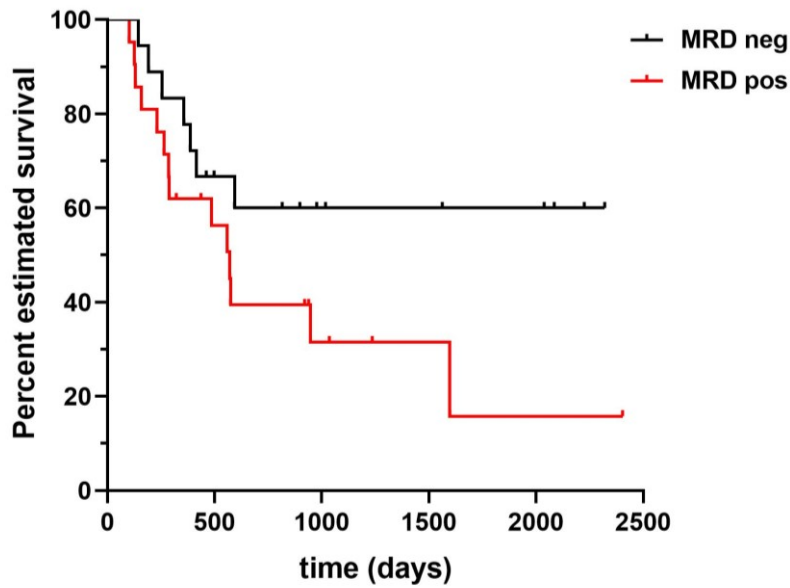
Four variables including age, MRD status, ELN cytogenetic risk and leukocyte counts were considered in univariate and multivariate analysis for cumulative incidence of relapse (CIR). In the univariate analysis, MRD positivity was the only risk factor for CIR (HR, 4.2; 95% [CI], 1.5-11.7; p = 0.0062) (Figure 31A; Table 24). When tested in a multivariate analysis, MRD positivity (HR, 7.07; 95% CI, 2.26-22; p = 0.000756), as well as intermediate/adverse ELN risk groups (HR, 4.1; 95% CI, 1.24-13.6; p = 0.0205), age  $\geq 60$  (HR, 3.24; 95% CI, 1.1-9.5; p = 0.0324) and leukocyte counts above 30 G/l (HR, 2.84; 95% CI, 1.05-7.64; p = 0.0373) were statistically significant risk factors for CIR (Figure 31B; Table 25).



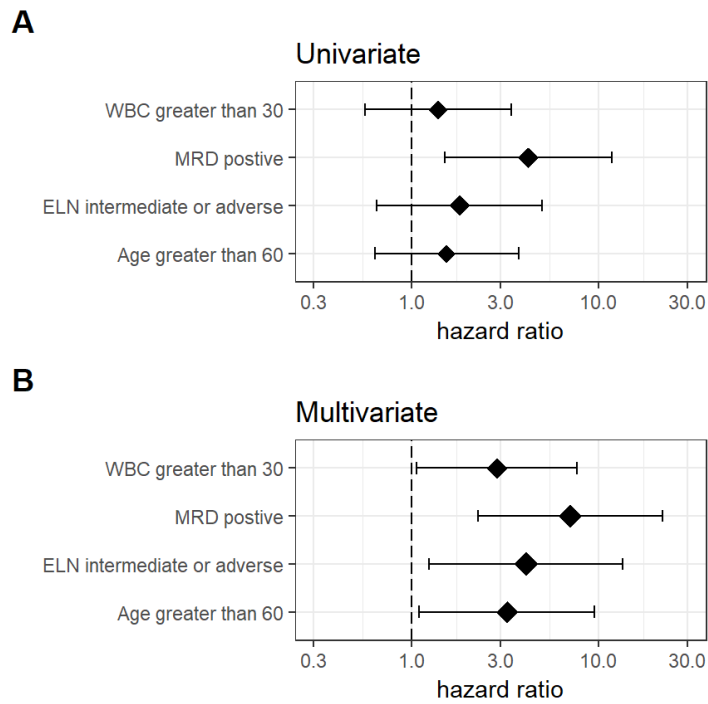
**Figure 28:** Relapse free survival of AML patients according to MRD status (n =39). Patients with a positive MRD status as measured by the two-step MRD assay had a significantly shorter duration of RFS (p=0.0031).



**Figure 29:** Competing risk analysis for cumulative incidence of relapse in AML patients according to their MRD status.



**Figure 30:** Overall survival of AML patients of prospective cohort according to their MRD status (n =39). No significant impact of a positive MRD status as measured by two-step MRD assay was seen on overall survival (p=0.0849).



**Figure 31:** Uni- and multivariate analysis of factors affecting CIR in AML patients having received intensive chemotherapy

**Table 24:** Univariate analysis of factors affecting CIR in AML patients having received intensive chemotherapy

|  | <b>hazard ratio (95%CI)</b> | <b>p-value</b> |
|--|-----------------------------|----------------|
| <b>WBC <math>\geq</math> 30 G/l</b>    | 1.38 (0.562-3.4)            | 0.48           |
| <b>Age <math>\geq</math> 60</b>        | 1.55 (0.637-3.75)           | 0.336          |
| <b>MRD</b>                             | 4.2 (1.5-11.7)              | <b>0.0062*</b> |
| <b>ELN Risk (intermediate/adverse)</b> | 1.8 (0.651-5)               | 0.256          |

**Table 25:** Multivariate analysis of factors affecting CIR in AML patients having received intensive chemotherapy

|  | <b>hazard ratio (95%CI)</b> | <b>p-value</b>   |
|--|-----------------------------|------------------|
| <b>WBC <math>\geq</math> 30 G/l</b>    | 2.84 (1.05-7.64)            | <b>0.0373*</b>   |
| <b>Age <math>\geq</math> 60</b>        | 3.24 (1.1-9.5)              | <b>0.0324</b>    |
| <b>MRD</b>                             | 7.07 (2.26-22)              | <b>0.000756*</b> |
| <b>ELN Risk (intermediate/adverse)</b> | 4.1(1.24-13.6)              | <b>0.0205*</b>   |

## 5. Discussion

### 5.1 High GPR56 expression correlates with a leukemic stem cell gene signature in CD34-positive AML (Daga, Rosenberger et al. 2019).

In AML leukemic stem cells represent an attractive cellular target for therapy, since these are the key source of residual as well as relapsing disease. Numerous cell surface markers have been described as potential LSC targets. In the first part of the present study we aimed to characterize the expression of potential LSC markers within the immature LSC compartment as compared to more mature leukemic cell subsets along with their prognostic impact on the outcome of AML patients.

Although the expression of some of the LSC markers used in this study has been shown on CD34<sup>+</sup>CD38<sup>-</sup> LSCs by others, our study is probably the first of kind to investigate the expression of 16 potential LSC markers simultaneously on the different CD34/38 cellular compartments of CD34-positive AML samples. In this comprehensive analysis of potential LSC markers GPR56 and CLL-1 were the most prominently differentially expressed surface markers in AML in CD34/CD38 sub-compartments at diagnosis. While expression of GPR56 was highest within the LSC containing CD34<sup>+</sup>38<sup>-</sup> subpopulation as compared to CD34<sup>+</sup>38<sup>+</sup> and CD34<sup>-</sup>, CLL-1 was lowest expressed in CD34<sup>+</sup>38<sup>-</sup> leukemic cells and increased in the more mature CD34<sup>+</sup>38<sup>+</sup> and CD34<sup>-</sup> cells. Moreover, we showed that high surface expression of GPR56 on CD34<sup>+</sup>38<sup>-</sup> leukemic cells correlated with a recently published LSC gene expression signature (Ng, Mitchell et al. 2016). Notably, these results indicate that high expression of GPR56 allows identification of leukemic cells with a gene expression profile representative of LSCs and therefore confirm and extend data from Pabst et al. (Pabst, Bergeron et al. 2016), who demonstrated that GPR56 identifies leukemic subpopulations with high repopulating ability in immunocompromised mice irrespective of their CD34/38 status, an important characteristic attributed to LSCs. Interestingly, expression of GPR56 was found to be related to LSC function in several gene expression studies aimed at identifying a “stemness signature” characteristic to leukemic stem cells (Eppert, Takenaka et al. 2011, Ng, Mitchell et al. 2016). For example, GPR56 expression was found to be higher in the LSC-enriched fraction as compared to the non-engrafting bulk leukemic cells (Ng, Mitchell et al. 2016). A related study found that expression of GPR56 was higher on the CD34<sup>+</sup> LSC progeny with LMPP and GMP phenotype in comparison to CD34<sup>-</sup> cells without LSC activity (Daria, Kirsten et al. 2016). Therefore, flow-cytometric analysis of GPR56 surface expression may allow to identify the pool of AML cells with LSC activity at diagnosis. This is

particularly of clinical significance, since the number of LSC (Terwijn, Zeijlemaker et al. 2014, Hanekamp, Denys et al. 2018) as well as LSC gene signatures in AML cells (Eppert, Takenaka et al. 2011, Ng, Mitchell et al. 2016) have been shown to be associated with an inferior outcome. Interestingly, RNA expression levels of GPR56 were also correlated with treatment outcome in two independent prospective clinical trials of the Austrian–German Study group consisting of 423 patients (Daria, Kirsten et al. 2016). Using the median expression level of GPR56 as cutoff, high GPR56 expression was associated with lower event-free and overall survival in this study. Furthermore, RNA-seq data from Pabst et al. revealed that the GPR56<sup>high</sup> group was enriched for patients with high-risk genetics, poor survival and those who did not achieve CR (Pabst, Bergeron et al. 2016). In the present study, we found that overall survival in patients receiving intensive chemotherapy was affected by their GPR56 protein expression status at diagnosis, since patients with a high GPR56 expression as assessed by flow cytometry showed an inferior overall survival in univariate analysis. However, due to our rather small cohort more comprehensive studies are needed to establish a definite role of GPR56 surface expression as an independent adverse risk factor in AML outcome.

GPR56 expression has already been linked to tumorigenesis before. GPR56 has been shown to be upregulated in several cancers, such as pancreatic (Huang, Fan et al. 2008) and lung cancer (Kausar, Sharma et al. 2011) as well as glioblastoma (Shashidhar, Lorente et al. 2005). In leukemia GPR56 expression was first described in EVI1<sup>high</sup> AML wherein EVI1 was shown to bind directly to the promoter region of *GPR56* (Saito, Kaneda et al. 2013). GPR56 knockdown in EVI1<sup>high</sup> AML cell lines reduced viability and the cells displayed increased susceptibility to chemotherapy drugs (Saito, Kaneda et al. 2013). In an AML cohort encompassing 179 patients GPR56 mRNA expression levels correlated with the expression of drug efflux transporters ABCG1, ABCC1 and ABCA2 indicating an association of GPR56 expression and drug resistance (Pabst, Bergeron et al. 2016). In other tissues surface GPR56 was shown to exert its cellular functions by interacting with protein ligands present in the extracellular matrix, such as collagen III and tissue transglutaminase 2 (Luo, Jin et al. 2012). It was therefore speculated that GPR56 is involved in adhesion and repopulating activity of HSPCs as well as LSCs indicating a probable role for GPR56 in the crosstalk between LSCs and their microenvironmental niche mediating chemoresistance (Daria, Kirsten et al. 2016). Consistent with these mechanisms we found lower CR rates after first induction chemotherapy in patients within the highest quartile of GPR56 expression.

Up till now, there is only very limited data correlating surface expression of distinct LSC markers with genes associated with LSC activity. We found that expression of eleven LSC-associated genes (LSC17 profile) was higher in CD34<sup>+</sup> GPR56<sup>hi</sup> cells as compared to CD34<sup>+</sup> GPR56<sup>lo</sup> cells. This observation is further complemented by RNA-seq data from Pabst et al. wherein eight of these 11 genes were also significantly upregulated in CD34<sup>+</sup> GPR56<sup>hi</sup> cells (Pabst, Bergeron et al. 2016). Given these data and its surface expression in AML cells with LSC activity, GPR56 might represent an interesting target for antibody-directed therapy. However, as reported in this study as well as others, GPR56 is also expressed on normal HSPCs (Daria, Kirsten et al. 2016, Pabst, Bergeron et al. 2016) and other tissues (Luo, Jin et al. 2012), which might hamper its therapeutic targeting. GPR56 has been previously associated with stem cell biology in the neural system, wherein neural stem and progenitor cells express high levels of GPR56 but decreased in the more differentiated cells (Bai, Du et al. 2009). Concerning the hematopoietic system it was shown that long-term (LT) as well as short-term (ST) HSCs express high levels of GPR56, but it was downregulated in the more differentiated B cells and cells of the myeloid lineage (Saito, Kaneda et al. 2013). Moreover, GPR56 knockout mice were reported to have lower numbers of HSPCs in comparison to wild type mice. However, another group did not detect a significant effect of GPR56 deficiency on function and maintenance of HSPCs in mice (Rao, Marks-Bluth et al. 2015). In a recent paper, Daria et al. indeed demonstrated that the human AML cell line MV4-11 as well as primary AML patient samples were efficiently targeted by a blocking anti-GPR56 antibody resulting in a major reduction of engraftment potential as well as improved survival in immunocompromised mice (Daria, Kirsten et al. 2016). These observations are encouraging, although future investigations will have to show whether normal human HSPC engraftment depends on GPR56 to the same extent as human LSCs. Further experiments investigating a therapeutic role of anti-GPR56-antibodies in mouse xenograft models of human AML will be required to better elucidate the potential of such antibodies and their effect on normal hematopoiesis.

Unlike GPR56, CLL-1 is hardly expressed on normal HSPCs with the exception of committed myeloid progenitor cells (Cate, De Bruyn et al. 2010, Bill, B. van Kooten Niekerk et al. 2018). Accordingly, CLL-1 has not been implicated in stem cell biology and CLL-1<sup>+</sup> HSPCs seeded in long-term colony formation assays (LTC) did not generate colony-forming cells suggesting that CLL-1<sup>+</sup> normal hematopoietic cells do not possess long term stem cell properties (Bill, Aggerholm et al. 2018). However, a study encompassing 89 AML samples identified CLL-1 to be expressed on CD34<sup>+</sup>38<sup>-</sup> AML-LSCs. In three samples tested sorted

CD34<sup>+</sup>CLL-1<sup>+</sup> AML cells were able to engraft and generate CLL-1<sup>+</sup> blasts after transplantation in immunodeficient mice (van Rhenen, van Dongen et al. 2007). Thus, CLL-1 has been considered as a LSC marker and therefore to be an attractive target for CAR T-cell or antibody based therapeutics. In contrast to these considerations, however, we found that CLL-1 expression was lowest in the LSC-enriched CD34<sup>+</sup>38<sup>-</sup> subpopulation and inversely correlated with the LSC17 gene signature in CD34-positive AML samples. Our data therefore confirm and extend recent findings by Perner et al. and Haubner et al., who found that expression of CLL-1 was significantly less on CD34<sup>+</sup>38<sup>-</sup> LSC as compared to bulk leukemic blasts. In accordance, low CLL-1 expression both at the protein and gene expression level was associated with increased LSC frequency (Perna, Berman et al. 2017; Haubner, Perna et al. 2018). To the best of our knowledge, ours is the first report to inversely correlate surface expression of CLL-1 with a distinct LSC gene signature. Altogether these results support the notion that high CLL-1 expression is not a suitable marker for identification of leukemic cells with LSC activity among AML bulk cells. These findings may also explain the fact, why CLL-1-targeting CAR T cells only displayed modest activity against primary human AML blasts xenografted into immunocompromised mice (Kenderian, Ruella et al. 2016), although a CLL-1 targeting approach has been proven to be very effective in AML cell line xenografts (Tashiro, Sauer et al. 2017; Wang, Chen et al. 2018). Interestingly, primary human AML cell killing could be enhanced by CAR T cells targeting a combination of CLL-1 with other surface markers such as CCR1 or LILRB2 (Perna, Berman et al. 2017). First feasibility results of an ongoing clinical trial with a combinatorial approach using CAR T cells targeting CLL-1 as well as CD33 were recently published (Liu, Pinz et al. 2018).

Concerning a potential prognostic impact of CLL-1, its expression was recently evaluated in a cohort of 123 patients with de novo CD34<sup>+</sup> Non-M3 AML (Wang, Chen et al. 2017). Interestingly, the CLL-1<sup>lo</sup> group was enriched for patients with poor survival and those who did not achieve CR. This is in contrast to the results of our study, where CLL-1 expression did not show any significant impact on overall survival. Thus, further studies with higher number of patients are needed to clearly establish a prognostic impact of CLL-1 surface expression in AML.

In conclusion, we found that surface expression of GPR56 was high in LSC-enriched CD34<sup>+</sup>38<sup>-</sup> leukemic cells and correlated with a LSC gene signature in CD34-positive AML as well as an adverse clinical outcome. Our data therefore further strengthen the use of GPR56 not only as a marker for LSC activity among bulk leukemia cells in CD34-positive AML at

diagnosis but also as a promising prognostic marker. In contrast, CLL-1 expression was lower in CD34<sup>+</sup>38<sup>-</sup> AML cells enriched for LSCs and correlated inversely with an LSC gene signature and may therefore have limited potential for identification of LSC among AML cells. However, due to its aberrant expression on AML cells as compared to normal HSPCs, it may still represent a powerful antigen for combinatorial targeted therapy approaches and may prove useful for residual disease detection by flow cytometry.

## **5.2 Development of a highly sensitive method for detection of MRD by combining flow cytometry-based leukemic cell enrichment and mutational profiling of recurrently mutated genes in AML.**

Although high CR rates are achieved after intensive chemotherapy, the majority of AML patients eventually relapse (Estey and Döhner 2006). It is the persisting residual leukemic cells during remission that cause relapse, thereby making AML cure challenging. These persisting cells representing MRD cannot be detected with conventional methods. With the development of more sensitive assays there has been growing interest in incorporating MRD detection into clinical routine. The current routine methods to measure MRD in AML include either leukemia-specific immunophenotype analysis using multiparameter flow cytometry (MFC) and the detection of molecular aberrations using PCR based techniques (Ravandi, Walter et al. 2018, Schuurhuis, Heuser et al. 2018). Although MFC and molecular methods have proven their values in predicting relapse-free and overall survival, there is no current standard to detect MRD in AML patients. Challenges to adopt MRD detection in routine clinical practice for AML patients include lack of consensus concerning important parameters such as type of specimen, MRD markers, methodology (for example: qPCR or MFC) and cut-off values (Schuurhuis, Heuser et al. 2018). In the second part of this thesis, we therefore aimed for establishing a novel method for monitoring MRD in AML by combining flow cytometry-based leukemic cell enrichment applying antibodies against CD117, CD123, CLL1 as well as TIM-3 and mutational analysis using NGS or dPCR. Using this method in a pilot study MRD detection has been possible with an improved and clinically relevant sensitivity of 1 leukemic cell in up to 10,000 normal bone marrow cells or even more. This two-step MRD method may also help to address and overcome some of the limiting issues of current MRD detection making its use attractive for further clinical development: First, this approach of MRD detection can be used successfully in the vast majority of AML patients. Using a combination of four markers we were able to enrich leukemic cells in 91.7% percent

of diagnostic AML samples and in our prospectively collected samples DNA quality of sorted cells was sufficient for mutational analysis in 39 out of 41 samples (95%). In all samples tested an informative leukemia-specific mutation was present. Second, there is no need for specific reagents for distinct samples involving multiple antibody combinations as used by others (Kern, Haferlach et al. 2010, Béné, Nebe et al. 2011) allowing standardization and even automation of this method more easily. Third, by using a combination of four enrichment markers in one fluorescence channel with our approach, an immunophenotypic shift in one marker might affect MRD measurement only minimally. This is in contrast to MRD detection by MFC alone, where an immunophenotypic shift of one surface marker during relapse may result in false negative reports due to the loss of the LAIP (Kern, Bacher et al. 2010).

The enrichment markers used in this study were previously proposed as robust markers either for MRD detection or for the identification of leukemic stem cells (LSCs). Recently, C type-like Lectin like molecule (CLL-1) was identified as a stable and reliable AML antigen expressed in 90% of diagnostic AML samples (Larsen, Roug et al. 2012). CD123 was found to be highly expressed in AML patients (Jordan, Upchurch et al. 2000). Moreover CD34<sup>+</sup>CD123<sup>+</sup> cells isolated from AML patients were able to establish leukemia in immunodeficient mice (Testa, Riccioni et al. 2002). TIM-3 was found to be expressed on CD34<sup>+</sup>CD38<sup>-</sup> LSCs from most AML types except for M3 (Kikushige, Shima et al. 2010). Separation of normal HSC from residual LSCs in AML samples based on the differential expression of TIM-3 was also described (Jan, Chao et al. 2011). CD117 was shown to be positive in almost two-thirds of AMLs and was expressed at a much higher level in myeloblasts than in normal myeloid precursors (Wells, Bray et al. 1996, Scolnik, Morilla et al. 2002). A marker combination similar to ours, involving CLL-1 and CD123 along with backbone markers CD34 and CD117 was evaluated for its potential to detect MRD in a cohort of 69 AML patients (Roug, Larsen et al. 2014). The sensitivity of this assay ranged from 10<sup>-2</sup> to 10<sup>-4</sup>. Detection of high levels of CD123/CLL-1 LAIPs post-induction was found to be a strong prognostic marker for relapse for patients in CR (p<0.001). A comprehensive study involving analysis of six LSC markers in a cohort of 302 primary AML samples, found co-expression of CLL1/TIM3 in a majority of AML samples while being absent on HSPCs to be a promising antigen combination for targeted therapy (Haubner, Perna et al. 2018). An alternative approach to detect MRD using MFC, which was proposed recently, is the direct monitoring of LSCs. LSCs are hypothesized to selectively survive chemotherapy and to subsequently grow out causing relapse (van Rhenen, Feller et al. 2005). A recent study

combined MRD and LSC assessments after CR to show that patients with MRD<sup>high</sup>/LSC<sup>high</sup> were at highest risk of relapse compared to MRD<sup>low</sup>/LSC<sup>high</sup>, MRD<sup>high</sup>/LSC<sup>low</sup>, and particularly MRD<sup>low</sup>/LSC<sup>low</sup> patients (Zeijlemaker, Grob et al. 2018). In this studies LSCs were defined using markers CLL-1, CD22, CD44, TIM-3, CD133, CD123 and CD96, which were shown to be aberrantly expressed on LSCs in comparison to normal HSPCs. Routine flow cytometry panels which are designed based on expression of LAIPs only, may fail to detect persisting LSCs post therapy, which explains treatment failure in otherwise flow cytometry MRD-negative patients (Bachas, Schuurhuis et al. 2012, Buccisano, Maurillo et al. 2012). Since three of the proposed LSC markers (CLL-1, TIM-3, and CD123) were included in our enrichment panel, our two-step approach could offer an additional advantage by probably labelling therapy-resistant LSCs.

More recent studies have established the utility of NGS to monitor AML patients with frequent mutations during treatment. With the help of now commercially available multigene panels, patient-specific assays are no longer required, since recurrent mutations can be identified in AML patients for the purpose of MRD monitoring. We and others previously reported that a MRD positive status as detected by NGS (NGS-MRD) was associated with higher risk of relapse in pilot studies (Klco, Miller et al. 2015, Gaksch, Kashofer et al. 2018, Patkar, Kodgule et al. 2018). Klco et al. demonstrated in a cohort of 50 patients that leukemia specific mutations still persist at CR in 48% patients. These patients with a persisting mutation were at a higher risk of relapse (Klco, Miller et al. 2015). In a pilot study comprising of 34 AML patients, we analyzed the clearance of leukemia-specific mutations in remission material obtained after at least one consolidation therapy in cytogenetically normal AML patients using parallel sequencing on an Ion torrent platform (Gaksch, Kashofer et al. 2018). We found that persisting non-*DNMT3A* mutations were associated with an increased risk of relapse ( $p=0.013$ ) and a significantly lower relapse-free survival (333 days vs. not reached,  $p=0.0219$ ). However, in both these studies the sensitivity for detection of leukemia-specific mutations was rather low, namely 2.5 and 0.5% VAF, respectively. Another issue limiting NGS for determination of MRD is the fact that only some of the detected gene mutations are true leukemia-specific mutation, which act as driver that are essential in leukemic transformation. Some mutations which include *DNMT3A*, *TET2* and *ASXL1* (DTA) are acquired early and usually occur in preleukemic clones implying that these mutations alone are insufficient to initiate AML (Papaemmanuil, Gerstung et al. 2016). Moreover, these mutations can also be found in elder persons with clonal hematopoiesis but without any evidence of leukemic disease (Genovese et al, 2014, Steensma et al, 2015, Jaiswal et al,

2012). Therefore, no adverse effect of persisting DTA mutations in the absence of other genetic alterations was described (Jongen-Lavrencic, Grob et al. 2018), when NGS was used for MRD detection in AML. The detection of persisting DTA mutations alone during CR did not correlate with an increased risk of relapse. This makes the discrimination of true leukemia specific mutations a challenging issue when using NGS. Our combined approach may help to address this problem. Parallel to analysis of cells, which are enriched for residual leukemic cells and therefore stained with our antibody panel (PE (marker)-positive cell fractions), total bone marrow which comprises predominantly granulocytes, which may have arisen at least in part from preleukemic stem cells (Corces-Zimmerman et al, 2014, Jan et al, 2012, Slush et al, 2014) could also be analysed by NGS. At remission, true leukemia-specific mutations must be present with a frequency  $\leq 5\%$  in total BM, since the number of blasts in a remission sample is  $< 5\%$ , but their frequency might be increased in PE (marker)-positive sorted cell fractions. Such a discrepancy in the frequency of a mutation may doubtlessly allow the identification of true leukemic mutations.

Only a few studies have explored using more than one MRD detection method simultaneously, such as MFC and molecular MRD as a prognostic biomarker for relapsing disease. A study by Pera et al. (Perea, Lasa et al. 2006) found that the simultaneous use of MFC and qPCR improved MRD detection. MFC in addition to *WT1*-based molecular assessment of pre-transplant MRD could predict the risk of post-transplant relapse in AML patients (Marani, Clavio et al. 2013, Zhao, Yan et al. 2013). In a study comparing 10-color MFC with multigene NGS to measure MRD in AML patients before HSCT found that the presence of MRD as detected by either method prior to HSCT was predictive for relapse risk (MFC: hazard ratio, 4.62; and NGS: hazard ratio, 4.35) (Getta, Devlin et al. 2017). Residual disease detected in 340 AML patients using a NGS panel targeting 54 genes as well as MFC concomitantly conferred the highest relapse risk in patients, who were positive by both assays. Patients with a discordant status had an intermediate risk, while patients who were negative in both assays had the lowest relapse risk (Jongen-Lavrencic, Grob et al. 2018). These data show that a simultaneous use of more than one MRD detection technique results in a clear refinement of prognostication. Our combined approach takes advantage of the MFC technique followed by molecular analysis, applicable to the majority of AML patients. From that perspective it is interesting that previous studies of FACS sorted cells positive for one or more of our enrichment markers have shown to be enriched for AML-related molecular aberrations. Overexpression of CD123 has been shown in 62% of AMLs harboring *NPM1* mutations and in 83% of AMLs harboring *FLT3-ITD* mutations (Rollins-Raval, Pillai et al.

2013, Al-Mawali, Gillis et al. 2016). Abnormal expression of CLL-1 and TIM-3 has been observed in AMLs with *RUNX1-RUNX1T1* gene fusions (Coustan-Smith, Song et al. 2018). Overexpression of TIM-3 has been shown in AMLs harboring *CEBPA* mutations (Jan, Chao et al. 2011). Consistent with these studies mutational profiling of our FACS sorted sample indeed supported the flow cytometry based enrichment of residual cells, since leukemia specific mutations were present within sorted marker positive (PE positive) cells and absent within marker negative (PE negative) cells (Table 23).

Our pilot study also has some limitations. Despite the fact that in up to 90% of AML patients MRD can be detected using the selected enrichment markers, a significant proportion of patient samples may lack expression of the enrichment markers. Interestingly, of the 5 patients who tested MRD negative but relapsed, three patients fell in the latter category and lacked expression of the enrichment markers. For the remaining two patients this observation could not be confirmed due to unavailability of diagnostic material. The latter patient group also included patients with CD14<sup>+</sup> monocytic AMLs. The monoblasts display significant overlap with normal/regenerating monocytes, which makes MRD detection using our combined approach in such patients challenging because our sorting strategy excludes CD14<sup>+</sup> monocytes. However, despite these limitations of the enrichment process, sensitivity of the two-step MRD assay was high with 0.75 and only 28% of MRD-negative patients relapsed. Previous studies exploring the prognostic significance of either mutation-based or MFC-based MRD detection reported even higher relapse rates in MRD negative patients. In a study measuring MRD using mutant NPM1 transcripts found that 35% of the MRD negative patients relapsed (Ivey, Hills et al. 2016) while another study measuring MFC-MRD found that nearly 40% of MRD negative patients relapsed (Terwijn, van Putten et al. 2013).

Another observation was that although most of the patients positive for their leukemia specific mutation relapsed, some (7 out of 21) patients tested positive with our MRD approach and they did not relapse and thus, specificity of our assay was 0.648. Among the 7 patients, 3 patients died due to non-transplant related mortality after receiving allogeneic transplantation and hence could not be followed up for a longer period. Thus, it cannot be ruled out that those patients with a positive MRD status may have relapsed at a later time point. The low level positivity possibly represents a low level of residual disease held in check by the immune system. The observation that the threshold for relapse is higher in transplanted patients (Ommen et al. 2014) might support this concept; however additional studies are necessary to draw any firm conclusions. In a comprehensive analysis of patients

harbouring *NPM1* mutations using qPCR for detection of mutant *NPM1* several groups showed that residual disease measured after two cycles added most important information to the already known pre-treatment risk factors concerning relapse risk and survival (Krönke et al., 2011; Lambert et al., 2014). In contrast to this, we assessed MRD after completion of one cycle of induction therapy with a sensitivity to detect residual leukemic cells reaching  $10^{-5}$ , similar to that achieved by quantification of mutated *NPM1* transcripts. After the first induction, some persisting leukemic cells may have led to a positive MRD signal, but these cells might get cleared after receiving additional therapy. Therefore, further studies are needed to establish distinct thresholds of residual leukemic cells after distinct cycles of therapy that will reliably discriminate clinically relevant MRD-positive from MRD-negative disease with increased specificity allowing optimal prediction of impending relapse. Given the high accuracy to detect low levels of leukemic cells (reaching  $10^{-5}$ ) with our two-step approach, it seems appropriate to test this method also after completion of one cycle of consolidation therapy. The test may then be even more informative for relapse risk as well as better comparable to the current gold standard of highly sensitive MRD detection by quantification of *NPM1* mutations.

Another issue is that expression of distinct markers, such as CD117, on normal HSPC hampers sufficient enrichment of residual leukemic cells for subsequent sequencing. On average, a 67-fold enrichment could be achieved with our marker combination in the present study. In order to improve the enrichment use of other newly identified markers, which might be more specific for leukemic cells, may be useful. A recent study revealed CD93 as a unique LSC marker in *MLL*-rearranged leukemias (Iwasaki, Liedtke et al. 2015). Furthermore, CD70, CD191 and *LILRB2* (also known as CD85d) were found to be expressed on more than 75% of bulk as well as LSCs in almost all AML patient samples (Perna, Berman et al. 2017). These markers were found to be expressed at very low levels in normal BM making them ideal candidates for enrichment of residual leukemic cells. Additional studies to assess the potential of these markers to further improve enrichment of residual leukemic cells are therefore warranted.

We and others have previously demonstrated that already known mutations are detectable reliably at a sensitivity of 0.5% to 1% by NGS (Schmitt, Kennedy et al. 2012, Gaksch, Kashofer et al. 2018). Thus, the sensitivity of MRD detection by NGS using a multigene panel is limited in comparison to qPCR which offers sensitivities reaching up to  $10^{-6}$ . In NGS, artifacts can arise from library preparation and the sequencing procedure itself causing

errors in DNA base calling. This challenges the identification of true leukemic mutations at lower allelic frequencies thereby preventing sensitive detection of MRD in AML. In order to overcome this hurdle, molecular bar coding has recently been introduced. Molecular bar codes are random DNA-barcodes that are tagged to label each DNA molecule before the first PCR amplification. When the same mutation is found in DNA molecules labelled with different barcodes, it can be reliably termed as a true positive mutation. The use of molecular identifiers thus increases the sensitivity of the assay by being able to distinguish between sequencing artefacts from true positive mutations. Young et al. first reported the use of this error corrected sequencing approach using samples from AML patients. Using 16 bp DNA-barcodes, they were able to reliably detect tumor DNA with 1:10,000 sensitivity. Error corrected NGS (EC-NGS) thereby allowed identification of leukemic subclones below the threshold of regular NGS (Young, Wong et al. 2015). Thol et al. recently established an EC-NGS approach that can be applied to any somatic gene mutation. This approach was evaluated in 116 AML patients in CR undergoing transplantation. To decrease the sequencing error rate, they used a proofreading polymerase for PCR; introduced molecular barcodes tagged to target DNA and performed the first PCR amplification with only 5 cycles. The 5 year relapse risk for patients with a positive MRD status as assessed by EC-NGS was found to be 66% in comparison to 17% for patients with a negative MRD status (hazard ratio 5.58) (Thol, Gabdoulline et al. 2018). Another approach to increase sensitivity is to use statistical methods to reliably differentiate background noise from true leukemic mutations (Jongen-Lavrencic, Grob et al. 2018). In a recent study Patkar et al. analyzed the clinical significance of single gene NGS-MRD in a cohort of 83 AML patients harboring *NPM1* mutations at the end of either induction or consolidation therapy. With a high sensitivity of 0.001% to detect an *NPM1* mutation due to ultradeep sequencing, NGS-MRD was predictive of inferior outcomes. When tested in a multivariate analysis NGS-MRD was the only independent prognostic factor for lower OS (hazard ratio, 3.64) as well as lower RFS (hazard ratio, 4.8) (Patkar, Kodgule et al. 2018). Thus, recent studies show that ultrasensitive or error-corrected sequencing approaches increased sensitivity of NGS to detect leukemic cells to about  $10^{-5}$  and was highly predictive for relapse risk. Application of molecular bar coding to our two-step MRD method in future studies might help to increase the sensitivity as well as specificity of this method.

Finally, inadequate sampling might be another limitation of this study. Although the material was collected prospectively, the cells were cryopreserved until use and thawed at the time of analysis. Despite the fact that 95% of samples had sufficient cells, cryopreserved material

does not represent an ideal source for MRD detection (Mulé et al, 2016). In addition to the fact that remission material itself contains lesser numbers of cells, cryopreservation and subsequent thawing results in significant loss of cells. Aliquoting a single aspirate into several vials might lead to dilution of the residual leukemic cells thereby resulting in unsatisfactory sorting of residual leukemic cells. Furthermore, a single aliquot of the remission material may not completely represent the total AML burden of the patient. Immediate analysis using freshly aspirated BM for sorting might help overcome this issue in a future study.

In the present study, we were able to show that patients with a positive MRD status as assessed by our two-step MRD assay had a higher risk of relapse, therefore confirming and extending data from previous studies who have established that a positive MRD test identifies patients with higher risk of relapse. Irrespective of the method used, a strong association between MRD status and relapse risk has been shown in many studies. For example, two large, prospective, multicenter studies identified MFC-based MRD as an independent prognostic indicator of relapse in adults with AML. In a study by HOVON/SAKK group assessing MFC-MRD after one and two cycles of induction therapy on young AML patients aged 18-60 years found that a MRD-negative status was associated with a decreased relapse risk when tested in a multivariate analysis (42% vs 72%) (Terwijn, van Putten et al. 2013). In a study by UK MRC group assessing MFC-MRD after one or two cycles of therapy in a cohort of 483 patients aged  $\geq 60$  years found that the 3-year relapse risk for patients in CR after one cycle of therapy with positive MRD status was 83% in comparison to 71% for patients with negative MRD status while for patients in CR after two cycles of therapy with positive MRD status was 91% in comparison to 79% for patients with negative MRD (Freeman, Virgo et al. 2013). MRD detection by molecular techniques has proven to be equally informative for relapse risk. In a cohort of 346 patients, Ivey et al. showed that a MRD-positive status as detected by presence of *NPM1* mutated transcripts post intensive chemotherapy was found to be an independent predictor of relapse-free survival (Ivey, Hills et al. 2016). The 3-year relapse risk for patients with persisting *NPM1* mutations after the second induction cycle was higher (82% vs. 30%; hazard ratio, 4.80) and associated with an inferior survival (24% vs. 75%; hazard ratio for death, 4.38). When tested in a multivariate analysis a positive MRD status was the only independent prognostic factor for death. A study by German-Austrian group comprising of 245 young adults harboring *NPM1* mutations, showed that MRD positive status after induction therapy was associated with a lower rate of survival in comparison to patients with MRD negative status (51 vs. 90%) (Krönke, Schlenk

et al. 2011). In contrast to these large studies, we could not detect a significant impact of the MRD status as assessed with our two-step method on overall survival. This may be attributed to the rather low number of patients in our pilot study. Moreover, our cohort comprised rather old patients making age one of the major factors contributing to poor prognosis in addition to death from non-leukemia related reasons such as GVHD and other co-morbidities. Nevertheless, results from our prospective cohort clearly indicate that MRD detection using our combined approach display a high sensitivity and allows identification of patients at high risk of relapse.

Although a high sensitivity to detect residual leukemic cells and clinical applicability of the assay could be established with this pilot study, a proper prospective evaluation in larger multicenter cohorts including uniformly treated patients is required. Moreover, comparisons of our two-step MRD assay with the current gold standard methods of MFC-MRD as well as qPCR (including patients harboring *NPM1*-mutations) for their applicability; reproducibility and specificity are warranted to further solidify its performance. In conclusion, our two-step MRD assay is predictive of impending relapse and allows for faster simpler MRD detection in more than 90% of the AML patients with high sensitivity making this novel test highly attractive for further clinical development.

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### 7.3 List of Abbreviations

|         |   |
|---------|---|
| MRD     | Minimal residual disease                |
| AML     | Acute myeloid leukemia                  |
| LSCs    | Leukemic stem cells                     |
| MFC     | Multiparameter flow cytometry           |
| BM      | Bone Marrow                             |
| PB      | Peripheral blood                        |
| NBM     | Normal bone marrow                      |
| MNCs    | Mononuclear cells                       |
| HSPCs   | Hematopoietic stem and precursor cells  |
| CR      | Complete remission                      |
| sAML    | Secondary AML                           |
| tAML    | Therapy related AML                     |
| ELN     | European Leukemia Net                   |
| WHO     | World Health Organization               |
| FAB     | French-American-British system          |
| MDS     | Myelodysplastic syndrome                |
| MPN     | Myeloproliferative neoplasms            |
| CN-AML  | Cytogenetically normal AML              |
| CBF-AML | Core factor binding AML                 |
| NGS     | Next generation sequencing              |
| TCGA    | The Cancer Genome Atlas                 |
| NPM1    | Nucleoplasmin 1                         |
| CEPBA   | CCAAT Enhancer Binding Protein $\alpha$ |
| FLT3    | FMS like tyrosine kinase 3              |
| IDH1/2  | Isocitrate dehydrogenase 1 and 2        |
| TET2    | Ten eleven translocation 2              |
| MLL     | Mixed Lineage Leukemia                  |
| ASXL1   | Additional sex comb like genes          |
| DNMT3A  | DNA (cytosine) methyltransferase 3A     |
| FISH    | Fluorescence in-situ hybridisation      |

|              |  |
|--------------|--|
| DNA          | Deoxyribonucleic acid  |
| RNA          | Ribonucleic acid   |
| WBC          | White blood count  |
| RFS          | Relapse free survival  |
| EFS          | Event free survival  |
| CR           | Complete Remission   |
| HSCT         | Hematopoietic stem cell transplantation                      |
| OS           | Overall survival   |
| PCR          | Polymerase chain reaction                                    |
| qPCR/RT-qPCR | Quantitative reverse transcription Polymerase chain reaction |
| LAIP         | Leukemia associated aberrant immunophenotypes                |
| dPCR         | Digital Polymerase chain reaction                            |
| VAF          | Variant allele frequency                                     |
| HSCs         | Hematopoietic stem cells                                     |
| LMPP         | Lymphoid primed multipotent progenitors                      |
| GMP          | Granulocyte-macrophage progenitors                           |
| CLL-1        | C-type lectin like molecule 1                                |
| GPR56        | G protein coupled receptor 56                                |
| EVI-1        | Ecotropic viral integration site-1 high                      |
| MFI          | Mean fluorescence intensity                                  |
| HR           | Hazard ratio   |
| CI           | Confidence interval  |
| EC-NGS       | Error corrected Next generation sequencing                   |
| ALL          | Acute lymphoblastic leukemia                                 |
| MEDOCS       | Medical Documentation and Communication System               |