Diploma thesis

Next generation deep sequencing for minimal residual disease detection in acute myeloid leukemia

submitted by

Lukas Gaksch

attaining the academic degree

Doktor(in) der gesamten Heilkunde

(Dr. med. univ.)

at the

Medical University Graz

conducted at the

Department of Internal Medicine
Division of Hematology

under supervision of

ao.Univ. Prof. Dr. Albert Wölfler
Mag. Dr. Karl Kashofer

Graz, 16. Februar 2017

Eidesstattliche Erklärung

Ich erkläre ehrenwörtlich, dass ich die vorliegende Arbeit selbstständig und ohne fremde Hilfe verfasst habe, andere als die angegebenen Quellen nicht verwendet habe und die den benutzten Quellen wörtlich oder inhaltlich entnommenen Stellen als solche kenntlich gemacht habe.

Graz, am 16. Februar 2017 *Lukas Gaksch eh*

I would like to dedicate my diploma thesis to my beloved parents
Ingrid and Helmut Gaksch.

Acknowledgements

First of all I would like to express my sincere gratitude to my supervisor ao.Univ.-Prof. Dr. Albert Wölfler for introducing me to scientific research, for his continuous support, encouragement, patience and motivation all through the time and for being a tremendous mentor for me.

I would like to show my gratitude to Assoz. Prof.in Priv.-Doz.in Mag.a Dr.in rer.nat. Ellen Heitzer, Institute of Human Genetics, and to Mag. Dr.phil. Karl Kashofer, Institute of Pathology, for their support, fruitful discussions and for sharing their knowledge during the course of this research.

I am also grateful to Univ.-Prof. Dr. Heinz Sill, who gave me access to the divisions laboratory and research facilities, and Assoz. Prof. PDDr. Armin Zebisch for their insightful comments, stimulating discussions and support.

I would like to thank Sybille Hofer, MSc for backing me in all the time and her continuous help. The door to her office used to be open whenever I had a question about my research or writing.

Moreover I am grateful to Angelika Rosenberger, BSc, Shruti Daga, MSC, Karin Lind and PDDr. Eduard Schulz for sharing their knowledge of various techniques and their help in research.

I would also like to thank Dr.in Bianca Huber-Kraßnitzer for arousing my interest in hematology.

Last but not the least, I would like to thank my family and my friends for supporting me spiritually throughout writing this diploma thesis and my life in general.

Zusammenfassung

Einführung: Die Akute Myeloische Leukämie (AML) ist die häufigste Form der akuten Leukämie bei Erwachsenen. Trotz des Erreichens einer vollständigen Remission nach intensiver Therapie, rezidivieren viele der AML Patienten mit normaler Zytologie aufgrund der persistierenden submikroskopischen Restkrankheit. In dieser Diplomarbeit haben wir uns die Frage gestellt, ob mit Parallelsequenzierung von DNA, die von Objektträgern mit Knochenmark isoliert wurde, AML-assoziierte Mutationen nachweisbar sind und ob diese Aberrationen als Nachweis für die persistierende molekulare Erkrankung mit einem erhöhten Risiko für ein Rezidiv und einem weniger günstigen klinischen Verlauf einhergehen.

Methodik: Wir inkludierten 34 de novo AML-PatientInnen, von denen uns Diagnosematerial und Objektträger nach mindestens einem Zyklus einer Konsolidierungstherapie zur Verfügung standen. Die extrahierte DNA wurde daraufhin auf 19 in der AML häufig vorkommende Mutationen mit einer Ion-Torrent-Target-Sequencing-Plattform parallel-sequenziert. Zusätzlich wurden die Allelefrequenzen bestimmter Mutationen durch eine digitale PCR und parallele Sequenzierung unter Verwendung eines Barcodierungsansatzes validiert.

Ergebnisse: Drei PatientInnen zeigten keine somatische Mutation im diagnostischen MaterialZusätzlich konnte aus vier Objektträgern nicht ausreichend DNA für die Sequenzierung des Remissionsmaterials isoliert werden. Daher ergab sich eine Gesamtgröße der Versuchsgruppe von 27 PatientInnen. Bei diesen PatientInnen wurden 68 somatische Mutationen zum Zeitpunkt der Diagnose nachgewiesen (im Median 3 Mutationen pro PatientIn, Bereich 1-5). 22 von diesen somatischen Aberrationen konnten noch bei 16 PatientInnen nach der Konsolidierungstherapie gefunden werden (median eine Mutation, Bereich 0-3). Verglichen mit der digitalen PCR oder dem Barcodierungsparallelsequenzierungsansatz korrelierten die mutierten Allelenfrequenzen von *NPM1* oder *DNMT3A* gut.

Die häufigsten persistierenden Mutationen wurden im *DNMT3A*-Gen (n=10) gefunden. Da diese aber bekanntlich in präleukämischen Stammzellen ohne jegliche Auswirkung auf das Rezidivrisiko zu finden sind, führten wir die Überlebens- und Rückfallrisikoanalyse ohne *DNMT3A*-Mutationen durch.

Unsere Studie ergab, dass das Persistieren von Nicht-*DNMT3A*-Mutationen nach Konsolidierungstherapie mit einem signifikant höheren Risiko eines AML-Rezidivs (7 von

8 PatientInnen gegenüber 6/19 PatientInnen, p=0,013) und mit einem kürzeren rezidivfreien Überleben (333 Tage vs. nicht erreicht; Log-rank p=0,0236) assoziiert war. Darüber hinaus zeigte sich ein Trend für ein schlechteres Gesamtüberleben (log-rank p=0,050).

Schlussfolgerungen: Die Persistenz von Nicht-*DNMT3A*-Mutationen nach Konsolidierungstherapie, wie sie durch Parallelsequenzierung nachgewiesen wurde, ist mit einem erhöhten Risiko eines Rückfalls bei zytogenetisch normaler AML assoziiert.

Abstract

Introduction: Acute myeloid leukemia (AML) is the most common form of acute leukemia in adults and despite achieving complete remission after intensive therapy. Most patients with cytogenetically normal AML relapse due to the persistence of submicroscopic residual disease. In this diploma thesis we asked whether broadly available targeted sequencing of DNA isolated from bone marrow slides displaying complete remission allows the detection of persisting AML-associated mutations and whether this persistent molecular disease is associated with increased risk for relapse and less-favorable clinical outcomes.

Methods: We included 34 de novo AML patients of whom diagnostic material and slides after at least one cycle of consolidation were available. Isolated DNA was screened for mutations in 19 genes recurrently mutated in AML using an Ion Torrent target sequencing platform. In addition, the variant allelic frequency of distinct mutations was validated by digital PCR and parallel sequencing using a barcoding approach.

Results: Three patients each revealed either no mutation in the diagnostic material or the somatic origin of the identified mutations could not be proven. In addition, DNA isolated from four slides was not sufficient for sequencing. Therefore, in total 27 patients could be analyzed for mutation clearance. In these patients we identified 68 somatic mutations (median of 3 mutations per patient, range 1-5) and 22 of these were still detected in 16 patients after consolidation therapy (median one mutation, range 0-3). The determined variant allelic frequencies of *NPM1* or *DNMT3A* correlated well compared with either digital PCR or a barcoding parallel sequencing approach.

The most frequent non-cleared mutations were found in *DNMT3A* (n=10 pts). Since these are known to persist in preleukemic stem cells without any impact on relapse risk, we performed survival and relapse risk analysis excluding *DNMT3A* mutations.

Importantly, persistence of non-*DNMT3A* mutations was significantly associated with a higher risk of AML relapse (7 out of 8 pts versus 6/19 pts; p=0,013) and with a shorter relapse-free survival (333 days vs. not reached; log-rank p=0,0236). In addition, there was a trend for worse overall survival (log-rank p=0,050).

Conclusions: Persistence of non-*DNMT3A* mutations after consolidation therapy as detected by broadly available targeted deep sequencing is associated with an increased risk of relapse in cytogenetically normal AML.

Table of contents

Abbreviations

ALL *Acute lymphoblastic leukemia*

AML *Acute myeloid leukemia*, *Acute myeloid leukemia*

AR *Adverse risk*

ASXL1 Additional Sex Combs Like 1

BM *Bone marrow*

BRAF B-Raf proto-oncogene

CBL CBL proto-oncogene

CEBPA CCAAT/enhancer-binding protein alpha

CN-AML C*ytogenetically normal AML*

CR *Complete remission*

DNMT3A DNA (cytosine-5)-methyltransferase 3A

dNTP *Deoxyribonucleotide triphosphate*

ELN *European Leukemia Net*work

FISH *Fluorescence in situ hybridization*

FLT3 Fms related tyrosine kinase 3

FR *Favorable risk*

GATA2 GATA binding protein 2

HSCs *Hematopoietic stem cells*

IDH1 Isocitrate dehydrogenase 1

IDH2 Isocitrate dehydrogenase 2

Ig *Immunoglobulin*

IGV *Integrative Genomic Viewer*

IR *Intermediate risk*

ISFET *Ion-sensitive field-effect transistor*

JAK2 Janus kinase 2

KIT KIT proto-oncogene receptor tyrosine kinase

KRAS KRAS proto-oncogene, GTPase, *Siehe*

LAIPs *Leukemia-associated immunophenotypes*

LSC *Leukemic stem cell*

MEDOCS *Medical Documentation and Communication System*

MFC *Multiparameter flow cytometry*

MRD *Measurable residual disease*

NGS *Next-generation sequencing*

NPM1 Nucleophosmin 1

NRAS Neuroblastoma RAS viral oncogene homolog

PB *Peripheral blood*

PCR P*olymerase chain reaction*

pre-LSC *Pre- Leukemic stem cell*s

PTPN11 Protein tyrosine phosphatase, non-receptor type 11

RT-PCR *Real-time PCR*

RUNX1 Runt related transcription factor 1

SB *Southern blotting*

SCT *Stem cell transplantation*

TCR *T-cell receptor*

TET2 Tet Methylcytosine Dioxygenase 2

TP53 Tumor protein p53

VAF *Variant allelic frequency*

WT1 Wilms tumor 1

Register of illustration

List of tables

1 Introduction

Acute myeloid leukemia (AML) is the most common form of acute leukemia in adults and, despite considerable progress in understanding the pathogenesis of the disease, its outcome is still adverse in most patients. (1–4) The main reason of this unfavorable prognosis is the high frequency of relapse due to persistence of trace amounts of chemoresistant leukemic cells after therapy. (4–6) Currently, the intensity of treatment, which may also include allogeneic hematopoietic stem cell transplantation, is based on risk stratification of patients at diagnosis. Specific chromosomal and molecular aberrations in three genes assign patients into four risk groups according to current European Leukemia Network (ELN) guidelines. While high-risk patients defined by complex chromosomal aberrations or monosomal karyotype should undergo allogeneic stem cell transplantation (SCT), high-dose cytarabine consolidation is the treatment of choice after induction therapy in good risk patients. However, more than 60% of AML patients cannot be assigned to one of these two groups and are therefore categorized into the so-called intermediate risk groups (IR). The majority of these patients has a normal karyotype and is therefore assigned to the intermediate IR group. However, clinical outcome and relapse risk vary considerably within these patients. (3,4,6–10)

Genomic approaches have identified recurrent somatic mutations in a limited number of genes in the vast majority of AML patients. (3,11) Besides increasing evidence that some of these mutations are associated with outcome, a recent studies has shown that genomic approaches detecting these mutations after induction chemotherapy can also be used to assess the clearance of leukemia cells after therapy. (5) In clinical practice this is still done by morphologic examination by microscope resulting in a low sensitivity of detecting persistent disease. Thus, broadly available, standardized higher-resolution targeted sequencing approaches may provide useful prognostic information by detecting residual leukemic cells after chemotherapy at higher sensitivity in clinical practice.

This diploma thesis is part of a pilot study, were we used a broadly available targeted sequencing approach encompassing nineteen recurrently mutated genes to determine the clearance of leukemia-associated mutations in patients with cytogenetically normal AML. We focused on remission samples obtained after at least one consolidation therapy and asked whether this approach is feasible throughout therapy using easily available remission

material, such as bone marrow slides, and whether persistent molecular disease is associated with an increased risk for relapse and a less-favorable clinical outcomes.

1.1 Scientific background and current status of research

1.1.1 Acute myeloid leukemia

AML is the most common form of acute leukemia in adults. It is characterized by a pathological rapid growth of malignant early myeloid cells in the bone marrow (BM) and by the resulting hematopoietic insufficiency causing variable degrees of peripheral blood cytopenia. The process of leukemic transformation of hematopoietic stem and progenitor cells is driven by a cascade of somatically acquired genetic and chromosomal aberrations, defining the biological characteristics of the disease. (1,12) Despite major advances in understanding the pathobiology and notable progress in treatment of the disease, its average long-term prognosis is still dismal with a 5-year survival rate of about 35% in patients younger than 60 years. Older patients have an even worse prognosis with a cure in 5 to 15%. (2–4) The main reason of this unfavorable prognosis is the high frequency of relapse due to persistence of trace amounts of chemoresistant leukemic cells after therapy. Various studies have shown that approximately 20% of AML patients cannot achieve complete remission (CR) after receiving initial induction chemotherapy and despite attaining CR approximately 50% develop a relapse. (4–6)

To assess the outcome, the intensity of treatment and the probability of relapse, risk stratification based on specific chromosomal and genetic aberrations is performed at diagnosis. (3,6–8) While the cytogenetic heterogeneity of AML has been noted for more than 30 years, knowledge of molecular heterogeneity has vastly increased over the last 15 years. (3) Nevertheless, the karyotype of the leukemic cells is still the most important factor for prognosis of response to induction therapy and for overall survival. Adult patients younger than 60 years can be divided into four risk groups according to the current ELN guidelines: favorable, intermediate I, intermediate II and adverse group. (Table 1) Mutations in distinct genes have allowed revealing the genetic diversity within defined cytogenetic groups, especially within the large group of AML patients with normal

karyotype (CN-AML). It has been shown that mutations in the *CEBPA*, *NPM1* and *FLT3* genes are among the most frequent molecular aberrations conferring prognostically relevant information. In addition, mutations such as the *RUNX1*, *ASXL1* and *TP53* genes have been associated with an adverse outcome and will be soon included as markers in clinical practice. (3,4,7,8,10) Patients with leukemia-specific translocations including core-binding factor genes or mutations affecting the *CEBPA* (biallelic mutations) or the *NPM1* gene without additional genetic aberrations are known to have a good risk profile and are classified within the favorable risk group (FR, Table 1). Conversely, complex chromosomal aberrations or a monosomal karyotype indicates an inferior outcome. These patients are allocated to the so called adverse risk group (AR). While high-risk patients should undergo allogeneic SCT, intermediate to high-dose cytarabine consolidation is the treatment of choice for good risk patients. (2,4,6,7) However, more than 60% of AML patients cannot be assigned to one of these two groups and therefore are categorized into the so-called intermediate risk (IR) groups. Patients with normal karyotype but less favorable mutations are classified as IR-I, while patients displaying cytogenetic abnormalities without clear adverse effect on prognosis are assigned as IR-II. (4,7–9) Unfortunately, overall survival of the heterogeneous IR-groups is still unfavorable with a 5-year survival rate of about 40 percent. (8) (See Figure 1)

Table 1: Current risk stratification of molecular genetic and cytogenetic alterations according to current ELN recommendations

Risk Profile	**Subsets**
Favorable	t(8;21)(q22;q22); RUNX1-RUNX1T1 inv(16)(p13.1q22) or t(16;16)(p13.1;q22); CBFB-MYH11 Mutated NPM1without FLT3-ITD (normal karyotype) Biallelic mutated CEBPA (normal karyotype)
Intermediate-I	Mutated NPM1 and FLT3-ITD (normal karyotype) Wild-type NPM1 and FLT3-ITD (normal karyotype) Wild-type NPM1 without FLT3-ITD (normal karyotype)
Intermediate-II	(9;11)(p22;q23); MLLT3-KMT2A Cytogenetic abnormalities not classified as favorable or adverse
Adverse	inv(3)(q21q26.2) or t(3;3)(q21;q26.2); GATA2–MECOM (EVI1) t(6;9)(p23;q34); DEK-NUP214 t(v;11)(v;q23); KMT2Arearranged −5 or del(5q); −7; abnl(17p); complex karyotype

Adapted from Döhner et al., The New England Journal of Medicine 2015 and Döhner et al., Blood 2010 (3,7)

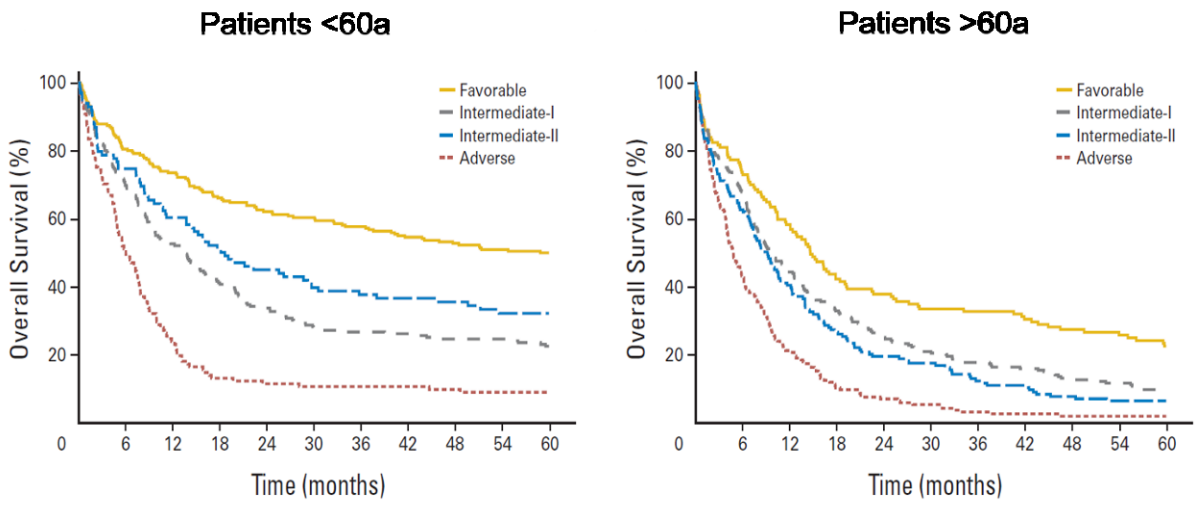


Figure 1: Probability of overall survival predicted by current risk stratification
Adapted from Rölling et al., Journal of Clinical Oncology (8)

1.1.2 Monitoring of measurable residual disease in leukemia

Despite the introduction of genetic and cytogenetic testing at diagnosis, estimation of treatment response and prognosis for a single patient is still challenging. (13) The aim of therapeutic interventions is to achieve and to maintain complete remission (CR). CR was defined in the 1970s based on the determination of peripheral blood counts and analysis of the bone marrow by light microscopy and required the levels of blood cells to be normal and the bone marrow to contain less than 5% blast cells. Nevertheless AML patients in CR can have a high diverse clinical outcome and many patients subsequently relapse due to the presence of residual leukemic cells below the microscopic detection limit. Due to the development of more sensitive tools, it is nowadays possible to measure residual disease burden below the microscopic detection limit. (See Figure 2) Detection of minimal or measurable residual disease (MRD) allows earlier recognitionof disease persistence and a better risk estimation for subsequent clinical relapse and possibly treatment decisions. (14–16)

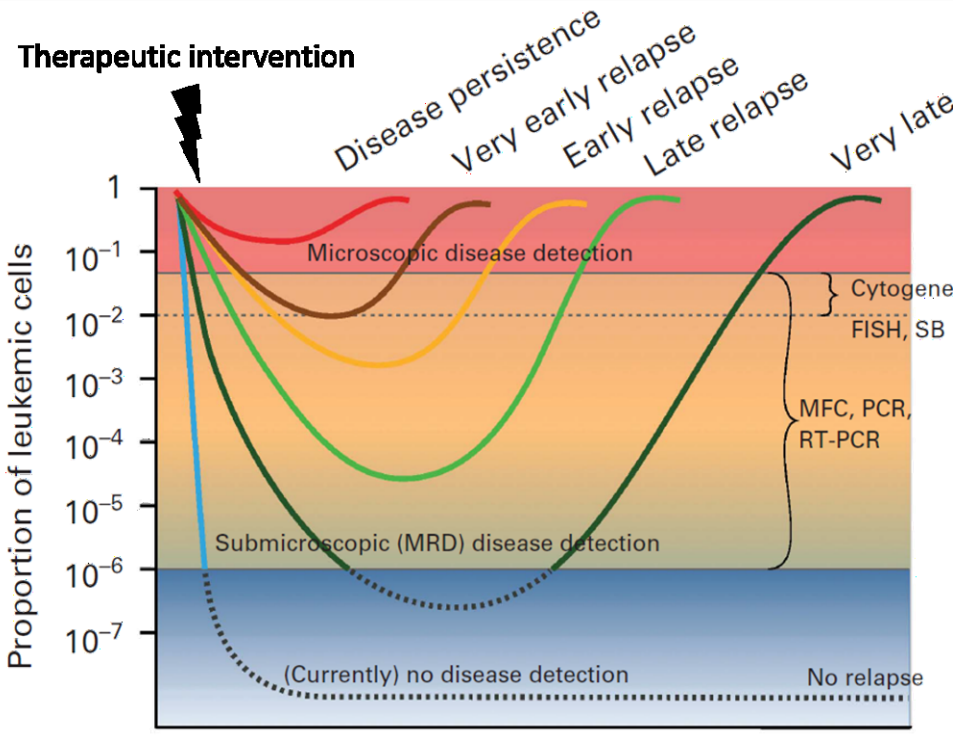


Figure 2: The concept of MRD and overview of MRD measurement methodologies

Patients with persistent disease (red line) can be assessed microscopically; Patients with complete remission butresidual disease can either have a rather high (brown and orange line) or low tumor burden (dark green line); patients with a higher residual disease burden are thought to have a highprobability of relapse and shorter relapse-free survival than those with low residual diseaseCure of patients can only be achieved when residual leukemia cells are either completely eradicated or controlled by the immune system (blue line); (FISH = Fluorescence in situ hybridization; MFC = Multiparameter flow cytometry; PCR = Polymerase chain reaction; RT–PCR = reverse transcriptase PCR; SB = Southern blotting)
Adapted from Buckley et al., Nature - Bone Marrow Transplantation 2013 (16)

It should be noted that the term “minimal” residual disease is debated controversially. It established in earlier times when techniques to measure residual leukemia burden after treatment were even less sensitive than today. Accurate MRD tests are limited to their sampling and threshold settings. This results in artificial estimates of sensitivity and may obscure the clinical reality. In fact, distinctions between high levels and low levels of “MRD” are arbitrary and without any biological meaning. Therefore J.M. Goldman et al. proposed to rename the acronym MRD to “measurable” residual disease. (14,15)

There have already been various attempts of measurable residual disease monitoring, most of them based on either multiparameter flow cytometry (MFC) – or on molecular polymerase chain reaction (PCR)-techniques. (4,7,14,16) Acute leukemia blasts express leukemia-associated immunophenotypes (LAIPs) defined as the presence of a combination of antigens and/or flow cytometric abnormalities, that can be used to differentiate these

cells to the remaining normal BM using MFC. (4,10,16,17) Despite the wide applicability of MFC (>90% of all AMLs have distinct LAIPs), its reproducibility and generalizability have been doubted, because MFC-based MRD assays are highly operator-dependent and erroneous interpretation can occur, if the interpreter has less extensive knowledge of normal hematopoietic immunophenotypic analysis. (16,18,19) In addition, standardized protocols for MRD measurement using MFC are still missing. By contrast, a reliable and well-standardized method for MRD monitoring with a higher detection sensitivity of down to 10^{-6} can be achieved with PCR-based approaches. (16) In acute lymphoblastic leukemia (ALL) PCR-based MRD detection is well established by targeting clonal rearrangements of immunoglobulin (Ig) and T-cell receptor (TCR) genes in approximately 90% of these patients. (16,20,21) Studies have demonstrated that MRD guided therapy can improve outcome in ALL patients. (21,22) Furthermore, latest therapeutic approaches to decrease the burden of MRD have been developed successfully in recent years resulting in prolonged survival in ALL. (23) Unfortunately, this type of MRD monitoring only applies to a subset of AML patients. (16) In these cases, quantitative real-time PCR (RT-PCR) enables monitoring of patients with known fusion transcripts, mutations and/or gene overexpression. (4,18,24,25) RT-PCR monitoring of mutations in the *NPM1* gene, which occur in about one third of AML patients, has been reported to allow quantification of MRD with a high prognostic impact on disease free and overall survival in a large data set of AML patients. (26)

Despite the obvious advantages, currently there are no clinically established MRD measurements available, which are suitable for clinical decision-making or drug development for the majority of AML patients. (14)

1.1.3 Impact of next-generation sequencing technologies on leukemia research

The availability of next-generation sequencing technologies (NGS, also termed as parallel or high-throughput sequencing) and data obtained by groups of the Cancer Genome Atlas Research Network have provided an unprecedented view of the genomic landscape of adult AML. This comprehensive analysis revealed that AML genomes display much fewer mutations than other adult cancers. An average of 13 gene mutations were found in each genome of 200 AML cases with an average of five found in genes recurrently mutated. (11) Using a selection of 18 genes therefore allowed detection of at least one potential driver mutation in >97% of adult AML patients, with most of them displaying two to five mutations. (27) In addition, using high-throughput sequencing technologies enables detection of low-abundance oncogenic aberrations at a resolution of one sequence per DNA clone, even with low tumor content, and offers the possibility to monitor the abundance of a mutated clone over time. (28–31) Recently a pilot study using parallel sequencing technologies successfully described tracking the clearance of leukemia associated mutations after induction therapy. These data suggests that this sequencing approach may also improve risk stratification of AML patients. (5)

1.1.4 Ion semiconductor sequencing

In this study we used the Ion Torrent PGM System (life technologies) for high-throughput sequencing. This device is based on ion semiconductor sequencing, a method of DNA sequencing using a semiconductor chip. During a natural polymerization, binding a deoxyribonucleotide triphosphate (dNTP) into a growing DNA strand by DNA polymerase involves the release of pyrophosphate and a positively charged hydrogen ion. However, dNTP is only able to incorporate if there is a complementary leading unpaired nucleotide. This fact is used in ion semiconductor sequencing. A semiconductor chip consists of microwells containing copies of DNA templates to be sequenced and DNA polymerase. The system floods sequentially this microwells with unmodified A, C, G or T dNTP. Every

time an introduced dNTP is complementary to the unpaired nucleotide on the template strand by the DNA polymerase, a hydrogen ion is released. This reaction causes pH value change, which triggers the ISFET ion sensor. The semiconductor chip transmits the electrical impulses to a computer. If there is no complementary unpaired nucleotide for the introduced dNTP, there is not any incorporation nor biochemical reaction and the unpaired dNTP molecule is washed out before the next cycle of a different introduced dNTP starts. This allows to measure several templates at the same time. (32–36) (See Figure 3)

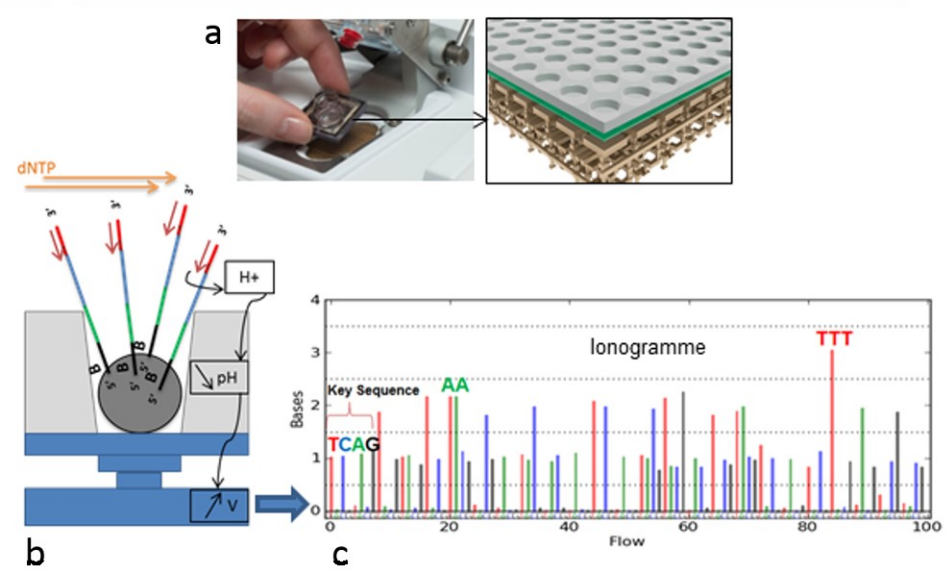


Figure 3: Ion semiconductor sequencing

a. semiconductor chip consisting of a layer of microwells and an ion sensitive layer, which is an ISFET ion sensor. **b.** The incorporation of dNTP into a growing DNA strand causes the release of hydrogen. This reaction causes pH value changes, which are detected by the ion sensor. **c.** The series of electrical pulses transmitted from the chip to a computer is translated into a DNA sequence. Key sequences allow the distinction between the different templates.
Adapted from Biogrami and life technologies™ (37,38)

1.2 Aim of the study

Based on the data and literature described above we hypothesized that broadly available targeted parallel sequencing of DNA allows the detection of persisting AML-associated mutations. We focused on remission samples obtained after at least one consolidation therapy and asked whether this approach is feasible throughout therapy using easily available remission material, such as bone marrow slides. In addition, we were interested whether persistent molecular disease is associated with an increased risk for relapse and less-favorable clinical outcomes.

2 Materials and methods

2.1 Patient selection

Patients included in this study were diagnosed with AML and treated for this disease at the Division of Hematology, Medical University of Graz. Recruitment criteria were defined as de novo AML, normal karyotype and a blast count of more than 20% in BM or peripheral blood (PB) at diagnosis. Diagnostic and remission material was either obtained directly from patients or available from the biobank at the Division of Hematology, Medical University of Graz. Clinical data were retrieved from the electronic documentation program MEDOCS (Medical Documentation and Communication System).

Thirty-four patients with CN-AML, who achieved a complete remission after induction chemotherapy and at least one consolidation therapy, were included in this study. Diagnosis and risk stratification of AML was made according to the ELN guidelines published in 2010. (8) Accordingly, in addition to cytogenetics, mutational analysis of the genes *NPM1*, *FLT3* and *CEBPA* was performed in all patients at diagnosis. All patients gave written informed consent and the study was approved by the institutional review board of the Medical University of Graz, Austria (26-050 ex 13/14).

2.2 DNA extraction

To prepare diagnostic samples for parallel sequencing, DNA was extracted using the QIAamp® Mini Kit (Qiagen®) as described in the protocol "DNA Purification from Blood or Body Fluids (Spin Protocol)" by Qiagen®. First of all, 20 µl QIAGEN Protease K was pipetted into the bottom of a 1.5 ml microcentrifuge tube. 200 µl sample and 200 µl Buffer AL were added into the tube and pulse-vortexed for 15 seconds. The samples were subsequently incubated at 56°C for 10 min. Then, 200 µl ethanol (96–100%) was added to the sample, and mixed again by pulse-vortexing for 15 s. This mixture was carefully applied to the QIAamp Mini spin column in a 2 ml collection tube and centrifuged (8000 rpm) for 1 min. Afterwards the QIAamp Mini spin column was placed in a clean 2 ml collection tube and the tube containing the filtrate discarded. Next, 500 µl Buffer AW1 was

carefully added to the QIAamp Mini spin column and centrifuged (8000 rpm) for 1 min. The QIAamp Mini spin column was placed again in a clean 2 ml collection tube and the tube containing the filtrate discarded. As a next step, 500 µl Buffer AW2 was pipetted into the QIAamp Mini spin column and centrifuged at full speed (14,000 rpm) for 3 minutes. Thereafter, the QIAamp Mini spin column was placed in a new 2 ml collection tube and centrifuged at full speed for 1 min. The old collection tube with the filtrate was discarded. As the last step, the QIAamp Mini spin column was placed in a clean 1.5 ml microcentrifuge tube and 200 µl Buffer AE was added. Then the mixture was incubated at room temperature (15–25°C) for 1 min and subsequently centrifuged at 8000 rpm for 1 min. After all, the eluted DNA was quantified and delivered to the laboratory for diagnostic genome analysis for parallel sequencing.

For sequencing of remission material we used BM slides obtained after at least one consolidation therapy. An example of a slide is shown in Figure 4. This allowed to estimate whether sufficient cellular material was available. DNA was extracted from BM slides using the QIAamp DNA Micro Kit (Qiagen®). Due to lower amounts of DNA obtained from the remission samples after consolidation therapy an average of 15,55 ng/µl DNA (1,03ng – 92,19 ng/µl) was used for these samples. All DNA concentrations were determined using Qubit dsDNA BR Assay Kits (Life Technologies).

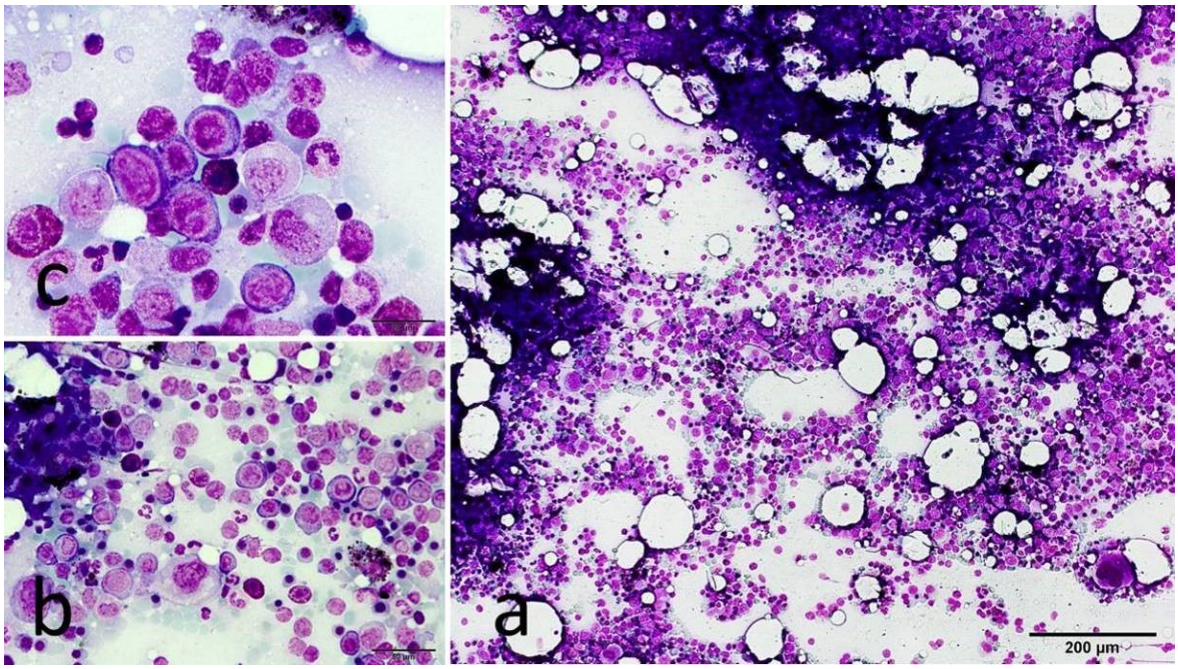


Figure 4: Example of a BM slide obtained after consolidation therapy

A digital image of all BM slides was taken with a microscope before DNA extraction. Microscopic magnification: a = 10x, b = 40x and c = 100x

2.3 Ion Torrent targeted deep sequencing

Targeted deep sequencing of diagnostic AML samples was done at the Laboratory for Diagnostic Genome Analysis, Institute of Pathology, Medical University of Graz, and was done as described by Schulz et al. (39) In brief, using 20 ng DNA isolated from diagnostic bone marrow or peripheral blood with more than 20% blasts, the entire coding sequences of *CEBPA, DNMT3A, GATA2, TET2, TP53* and mutational hotspots in *ASXL1, BRAF, CBL, FLT3 (D835), IDH1, IDH2, JAK2, KIT, KRAS, NPM1, NRAS, PTPN11, RUNX1,* and *WT1* were amplified by multiplexed PCR using the AMLv2 Panel (Life Technologies) encompassing 337 amplicons in total. Determination of *FLT3-ITD* was not included in this panel due to reasons of patent law. Library preparations were performed using the Ion AmpliSeq Library Kit 2.0 (Thermo Fisher Scientific). Emulsion PCR and sequencing were performed with the appropriate kits (Ion One Touch Template Kit v2 and Ion Proton 200 Sequencing Kit, [both from Thermo Fisher Scientific], respectively) on an Ion Torrent PGM sequencer using a single semiconductor chip. 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 performed using the Ion Torrent Suite Software[1]. Briefly, this included base calling, alignment to the reference genome (hg19) using the TMAP mapper and variant calling with a modified diBayes approach taking into account the flow space information. All called variants were annotated using open source software[2] and custom Perl scripts. (40,41) All identified potential disease-specific somatic mutations were verified using the Integrative Genomic Viewer[3]. (42,43) Screenshots for illustration are shown in Figure 5. To determine the analytic sensitivity of the assay, a dilution series using two samples with known mutations and normal bone marrow was performed. In both cases, a variant allelic frequency (VAF) of 0.5% was reliably detected.

[1] Thermo Fisher Scientific, open source, GPL, https://github.com/iontorrent

[2] ANNOVAR, http://www.openbioinformatics.org/annovar/; SnpEff, http://snpeff.sourceforge.net

[3] Integrative Genomic Viewer (IGV), http://software.broadinstitute.org/software/igv/

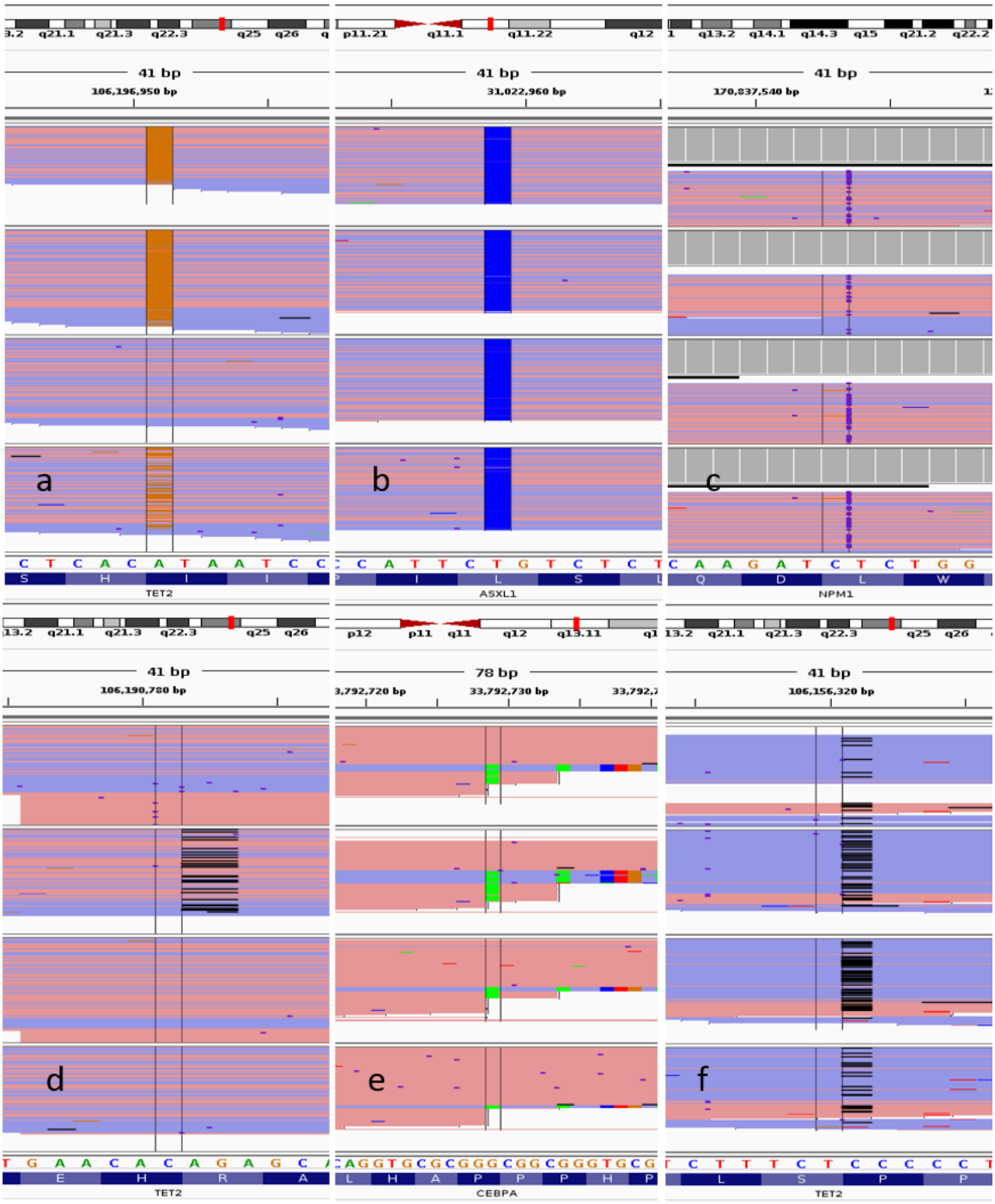


Figure 5: Screenshots of the Integrative Genomic Viewer (IGV)

The IGV was used to verify identified potential disease-specific somatic mutations and to exclude error artefacts, which can arise during sequencing. **a.** Example of a real mutation in *TET2*, which could be found at diagnosis and was cleared after consolidation therapy; **b.** Example of a known common SNP mutation in *ASXL1*, as verified by reference genome; **c.** Example of a real insertion mutation in *NPM1*; **d.** Example of a real deletion mutation in *TET2* with following extinguishing artifact; **e.** Example of an artifact in *CEBPA*; **f.** Example of an artifact in *TET2*. Caused by a limitation of the ion semiconductor sequencing system. If a template DNA strand to be sequenced constains homopolymer repeats of the same nucleotide (e.g. CCCC), multiple nucleotides are incorporated during a sequencing cycle and far more hydrogen ions are released. This results in an increasing pH value and a proportionally greater electronic signal resulting in an artifact (32);

2.4 High resolution mutation analysis of DNMT3A and NPM1 hotspot mutations

In order to validate the results in sensitivity obtained by the Ion Torrent sequencing we also performed digital PCR on the most frequent *NPM1* mutation and the Safe-SeqS method for other mutations in *NPM1* as well as the hotspot mutation in *DNMT3A* in collaboration with Dr. Ellen Heitzer at the Institute of Human Genetics, Medical University of Graz. These two genes are most commonly mutated in AML.

The *NPM1* hotspot mutation c.860_863dup, p.W288fs*12 was analyzed using digital PCR (dPCR) performed on the QuantStudio 3D platform (Life Technologies), which enables accurate quantitation of a target DNA molecule through partitioning a sample into 20k reactions on a single chip. A TaqMan assay specific for the detection of the mutation was designed using the Custom TaqMan® Assay Design Tool (Thermo Fisher). (See Table 2) Specificity of the assays was tested on a StepOne Plus instrument (Life Technologies) using the TaqMan Genotyping Master Mix (Life Technologies) according to the manufacturer's recommendations. The analytical sensitivity of 0.8% for the assay was determined using a serial dilution of a sample with known mutated fraction in the background of a normal control DNA (Promega). (See Figure 6) For dPCR 18 µl of Digital PCR Master Mix (2X) were mixed with 1,8µl of the TaqMan assay (20X) and a total amount of input DNA of 120 ng to a final volume of 36 µl for diagnostic samples. Due to limited availability of DNA from the remission samples after consolidation therapy an average of 18ng DNA (0.75ng - 60ng) was used for these samples. All samples were run in duplicates, therefore 14.5 µl each were loaded on two Digital PCR 20k Chip using the QuantStudio 3D Digital PCR Chip Loader (Life Technologies). The chips were then thermally cycled in a two-step PCR using the GeneAmp PCR System 9700, starting with the activation of the polymerase for 10 min at 96°C, followed by 44 cycles of 56°C for 2 min and 94°C for 30 s with a final extension of 2 min at 58°C. Chips were imaged in the QuantStudio 3D instrument. Raw data were analyzed using the relative quantification module of the QuantStudio 3D Analysis Suite Software. The confidence level was set to 95% and the desired precision value was 10%.

Mutant allele frequencies of other mutations in *NPM1* as well as the hotspot mutation c.2645G>A, p.Arg882His in *DNMT3A* were assessed using the Safe-SeqS method. Safe-SeqS is a targeted resequencing approach with substantially improved accuracy compared to conventional deep sequencing. By decreasing the presumptive sequencing errors by at least 70-fold, it is an extremely useful approach for rare variant detection. (44) Primers were designed using primer 3 software[4]. Sites with known common SNPs were excluded from the design in order to ensure equal amplification of both alleles. The amplicon-specific sense primers contained a 12-base UID (unique identifier) for encoding each template molecule with a unique molecular barcode. In addition both primers contained Illumina-specific sequences at their 5´ ends for a later amplification step and the addition of sample-specific indices. For primer sequences please see Table 2. For the *DNMT3A* mutation the same dilution series as prepared for the dPCR *NPM1* assay was used to assess an analytical sensitivity of 0.4% for the Safe-SeqS assay. (See Figure 6)

Briefly, 20ng of DNA for diagnostic samples and an average of 10.5ng (range 3ng - 20ng) for consolidation samples were amplified using 1U Phusion polymerase (Thermo Fisher), 0.25mM dNTPs and 02.µM amplicon-specific primers in 10 cycles of amplicon-specific PCR (30 s at 98°C, followed by of 98°C for 10 s and 58°C for 2 min with a final extension of 10 s at 72°C). To remove first-round primers, the PCR products were purified using Ampure XP beads (Beckman Coulter) and eluted in 16µl nuclease-free H2O. A total of 15µl were used in a second round of PCR to attache Illumina specific adapters and Indices to the 5' ends for 35 cycles (1 min at 98°C, followed by of 98°C for 10 s and 65°C for 15 s with a final extension of 15 s at 72°C). After the second round of amplification, PCR fragments were purified again using Ampure XP beads (Beckman Coulter) and eluted 12µl of nuclease-free H_2O. For quality control and quantification samples were run on an Agilent Bioanalyzer DNA 7500 chip (Agilent Technologies). All samples from one patient were pooled equimolarily and sequenced on an Illumina MiSeq in a 2x150 bp paired-end run.

Generated reads were grouped to read families according to the UID which was derived from the same PCR template. Reads containing an "N" in the UID were discarded. After grouping, a consensus sequence of each read family was generated by picking a base that

[4] Primer 3 software, http://bioinfo.ut.ee/primer3-0.4.0

occurs in at least 80% of all the reads assigned to a family at that position. If no consensus was found "N" was used as the consensus output base at that position. Based on the grouping output a new fastq-file was generated form the consensus sequences of each read family comprising at least 5 reads and containing a maximum of 2 N positions at the forward and reverse consensus sequence, respectively. Forward and reverse sequences were merged using FLASH (45) and the resulting FastQ file was aligned to the human reference genome (hg19) using BWA (46) and samtools. (47) Alignments were visualized in IGV (42) in order to detect variations.

Table 2: Primer sequences

Primer name	**Sequence[1, 2]**	**Assay**
UID NPM1_F	*5´ACACTCTTTCCCTACACGACGCTCTTCCGATCTNNNN NNNNNNNNNT*GTCTATGAAGTGTTGTGGTTCC-*3´*	Safe-SeqS (1st PCR)
UID NPM1_R	*5´GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTC*G GTAGGGAAAGTTCTCACTC-3´	Safe-SeqS (1st PCR)
UID_DNMT3A_F	*5´ACACTCTTTCCCTACACGACGCTCTTCCGATCTNNNN NNNNNNNNNN*GGTATTTGGTTTCCCAGTCC***-3´***	Safe-SeqS (1st PCR)
UID_DNMT3A_R	*5´GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT*CA CGCAAAATACTCCTTCAGC-3´	Safe-SeqS (1st PCR)
Illumina_index_F	*AATGATACGGCGACCACCGAGATCTACACTCTTTCCCT ACACGACGCTCTTCCGATCTATCGGGAAGCTGAAG*	Safe-SeqS (2nd PCR)
Illumina_index_R	*CAAGCAGAAGACGGCATACGAGAT**NNNNNN**GTGACT GGAGTTCAGACGTGTGCTCTTCCGATCTATCCGACGGT AGTGT*[3]	Safe-SeqS (2nd PCR)
NPM1_W288fs12_F	5´TCTATGAAGTGTTGTGGTTCCTTAAC***-3´***	dPCR
NPM1_W288fs12_R	5´AGCCAGATATCAACTGTTACAGAAATGAA***-3´***	dPCR
NPM1_W288fs12_VIC	5´CACTGCCAGACAGAGAT***-3´***	dPCR (probe)
NPM1_W288fs12_FAM	5´CTCCACTGCCAGAGAT***-3´***	dPCR (probe)

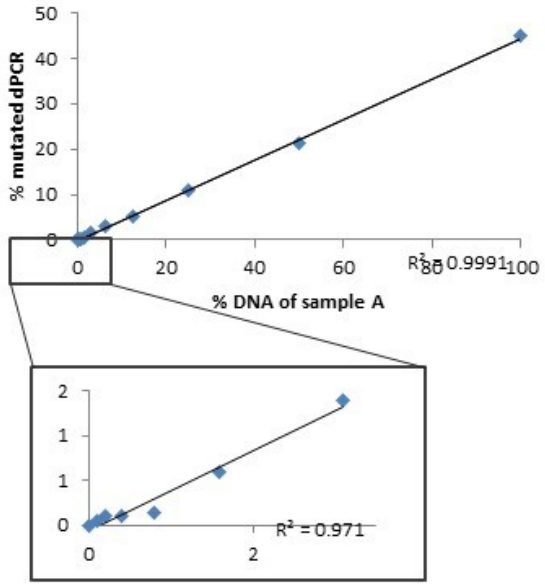
[1] Sequences in italics refer to the 12bp unique identifiers and the Illumina-specific adaptor sequences

[2] Primer were designed based on reference sequences (RefSeq) NM_002520.6 (*NPM1)* and NM_175629.2 (*DNMT3A*)

[3] *NNNNNN* denotes a 6bp Illumina specific index-sequence

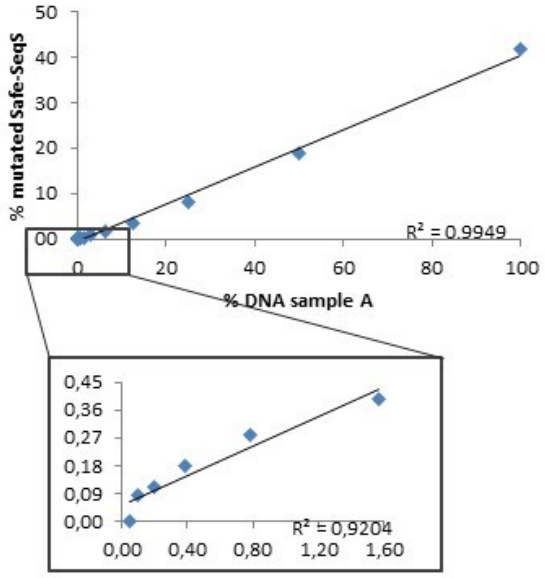
(By Ellen Heitzer)

Assessment of analytical sensitivity of NPM1 dPCR assay
Serial dilution of sample A



% sample DNA	% mutated dPCR
100	45.0
50	21.1
25	10.9
12.5	5.1
6.3	3.2
3.1	1.7
1.6	0.7
0.8	0.2
0.4	0.1
0.2	0.1
0.1	0.1
0	0.0

Assessment of analytical sensitivity of DNMT3A Safe-SeqS
Serial dilution of sample A



% sample DNA	WT reads	Mutated reads	% mutated
100	37275	15532	41.7
50	3910	732	18.7
25	17175	1396	8.1
12.5	15561	540	3.5
6.3	5431	85	1.6
3.1	16569	155	0.9
1.6	6053	24	0.4
0.8	11434	32	0.3
0.4	6043	11	0.2
0.2	27683	31	0.1
0.1	11772	10	0.1
0.0	19183	0	0.0

Figure 6: Assessment of analytical sensitivity of *NPM1* dPCR and of *DNMT3A* Safe-SeqS assay
(By Ellen Heitzer)

2.5 Statistical analysis.

Differences in characteristics of patients were calculated using a two-sided Fisher´s exact or Mann-Whitney tests. Comparison between groups concerning VAF or sequencing depth of distinct amplicons was also done using the Mann-Whitney test. The Kaplan-Meier method was applied to generate the survival curves and differences were assessed by Log-Rank analysis. All statistical analyses were carried using GraphPad Prism software version 7.0 (GraphPad Software, La Jolla, CA, USA). All hypothesis testing were carried out for an alpha = 0.05 with no adjustment for multiplicity.

3 Results

Thirty-four patients with AML and normal karyotype were included in this study and referred to parallel sequencing of diagnostic material. In three cases no informative mutation could be identified, either because no mutation was found (two cases) or the somatic nature of the mutation could not be unequivocally determined (one case; the experimental flow-chart is given in Figure 7). Of the remaining 31 patients bone marrow slides were available after consolidation, but one was excluded due to low cell counts rather reflecting blood than bone marrow. In three cases DNA quality of cells isolated from bone marrow slides was not adequate for parallel sequencing using the Ion Torrent platform. Therefore, twenty-seven patients were available for full analysis and subsequent results rely on this cohort. Fourteen of these patients relapsed, while thirteen stayed in complete remission during follow-up. The clinical characteristics of these patients are given in Table 3.

Table 3: Patient characteristics

	relapse (n=13)	**relapse-free** (n=14)	**p-value**
gender (female/male)	8/5	9/5	1.0
age (median, range in years)	48 (22-69)	47 (26-79)	0.24
WBC at diagnosis (10^9/l)	71.6 (11.5-178.5)	35.5 (0.9-170)	0.07
percent BM blasts (median, range)	89 (55-100)	76 (25-95)	0.64
ELN risk score (fav/int-1)	5/8	4/10	0.69
molecular genetics:			
FLT3-ITD	4	7	0.44
NPM1 mutated (without FLT3-ITD)	5	2	0.21
biallelic CEBPA mutation	0	2	0.48
remission status (CR/CRi)	10/3	11/3	1.0
therapy:			
induction (number of cycles)	1 (1-2)	1 (1-2)	1.0
consolidation (cycles)*	2 (1-3)	2 (1-4)	0.59

* five patients received an allogeneic stem cell transplantation as part of their consolidation therapy before residual disease testing (one in the relapse and four in the relapse-free group, respectively)

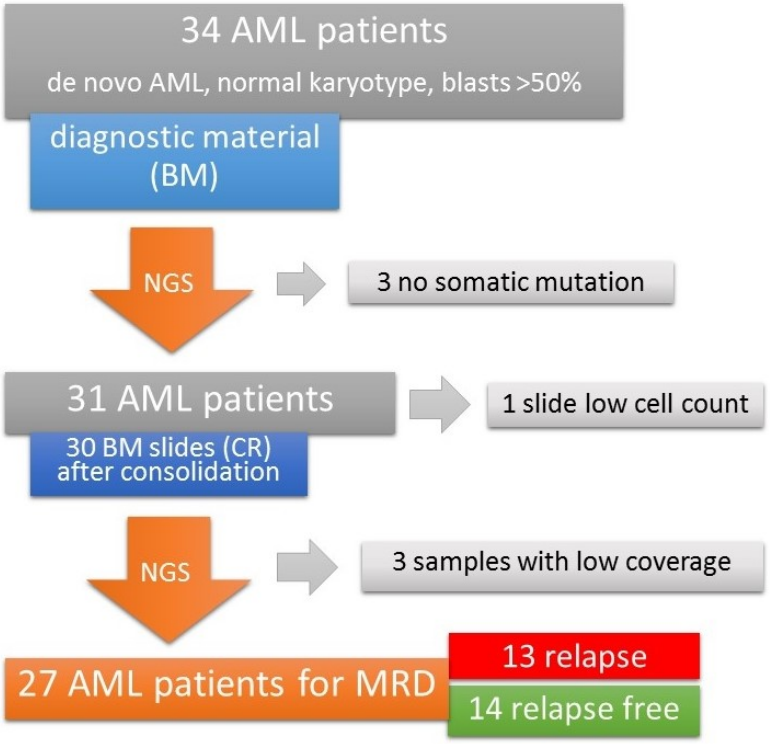


Figure 7: Experimental flow chart of the project

With an average reading depth of 6680x per amplicon indicating adequate sequencing of samples, 68 somatic mutations were identified in total at diagnosis in these 27 patients (median number of mutations per patient: 3, range 1-5, Figure 8). In accordance to the published literature the most commonly affected genes were *DNMT3A* and *NPM1*, followed by *TET2* and *CEBPA*. (see Figure 9)

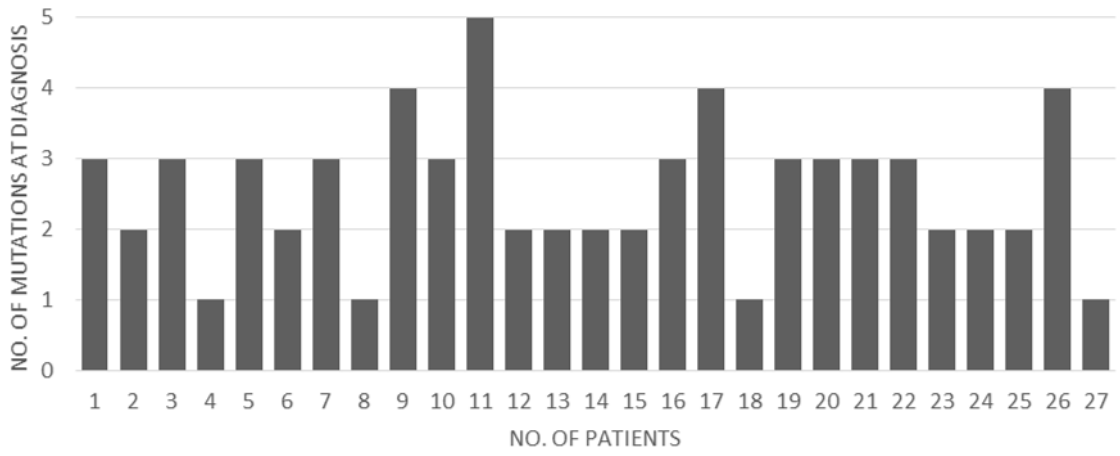


Figure 8: Number of all identified somatic gene mutations per patient at diagnosis

Overall 69 somatic mutations could be detected at diagnosis in all 27 patients (median of 3 mutations per patient, range 1-5)

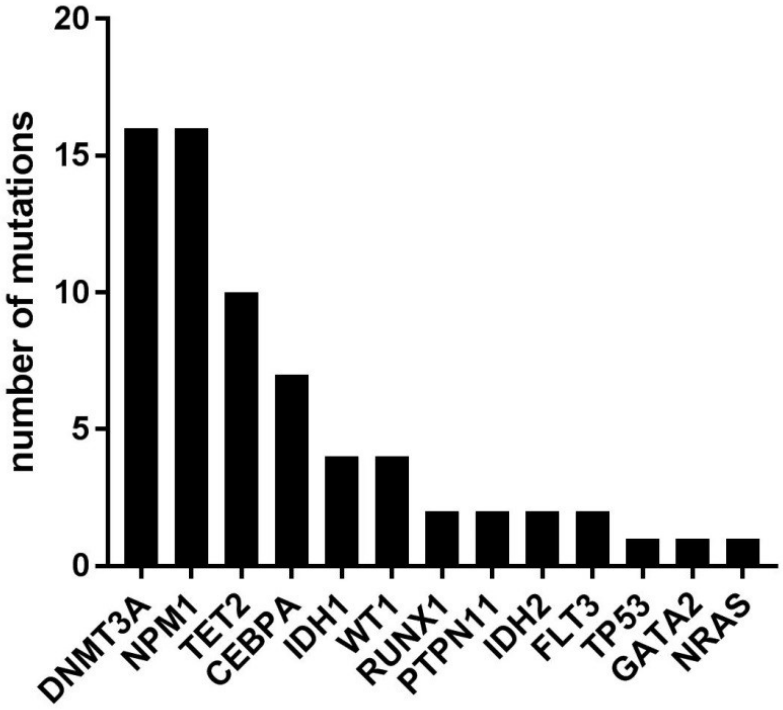


Figure 9: Number of mutations identified per gene at diagnosis in 27 patients with cytogenetically normal AML

Before we analyzed DNA from material obtained after consolidation, we determined the sensitivity and specificity of detecting the mutated variant of the involved genes by this sequencing approach. First, we performed a series of dilutions of two patient samples and observed a sensitivity to detect 1 mutated cell among 99 normal bone marrow cells. By

analyzing the 1:99 dilutions the VAF for the heterozygous mutation was 0.62% and 0.69% in DNMT3A and ASXL1, respectively (see Table 4). However, when using higher dilutions (1:999, 1:9999), we were not able to discriminate the VAF from false positive background values.

Table 4: Dilutions series of two patient samples containing *DNMT3A* and *ASXL1*

DNMT3A	**cGc/cAc**	**R659H**	**chr2**	**25457242**	**C**	**T**	**46,3549522**
		bp	**A**	**G**	**C**	**T**	**MAF**
K151-39_100_BC56	**100**	25457242	1	39	16140	13950	**46,36%**
K151-35_10_BC57	**10**	25457242	2	57	23250	1310	**5,33%**
K151-30_10_BC51	**10**	25457242	1	61	25488	1537	**5,69%**
K151-36_1_BC58	**1**	25457242	1	40	24994	155	**0,62%**
K151-31_1_BC52	**1**	25457242	2	67	24871	156	**0,62%**
K151-37_01_BC59	**0,1**	25457242	0	80	30305	16	**0,05%**
K151-32_01_BC53	**0,1**	25457242	2	51	19665	20	**0,10%**
K151-38_001_BC60	**0,01**	25457242	2	59	27699	36	**0,13%**
K151-33_001_BC54	**0,01**	25457242	1	53	28534	21	**0,07%**
K151-41_0_BC61	**0**	25457242	2	47	24971	11	**0,04%**
K151-40_0_BC55	**0**	25457242	0	53	30190	20	**0,07%**
ASXL1	**gaG/gaT**	**E1023D**	**chr20**	**31023821**	**G**	**T**	**46,3291139**
		bp	**A**	**G**	**C**	**T**	**MAF**
K151-39_100_BC56	**100**	25457242	18	4090	79	3810	**48,23%**
K151-35_10_BC57	**10**	25457242	0	107	0	7	**6,14%**
K151-30_10_BC51	**10**	25457242	10	4628	16	349	**7,01%**
K151-36_1_BC58	**1**	25457242	5	2081	1	13	**0,62%**
K151-31_1_BC52	**1**	25457242	26	8658	36	67	**0,77%**
K151-37_01_BC59	**0,1**	25457242	14	6390	19	4	**0,06%**
K151-32_01_BC53	**0,1**	25457242	24	4423	8	4	**0,09%**
K151-38_001_BC60	**0,01**	25457242	17	5884	6	3	**0,05%**
K151-33_001_BC54	**0,01**	25457242	17	5756	29	7	**0,12%**
K151-41_0_BC61	**0**	25457242	6	3145	9	2	**0,06%**
K151-40_0_BC55	**0**	25457242	8	3348	8	2	**0,06%**

Next, we determined the percentages of false positive reads for selected mutations reflecting base substitutions in patient samples not carrying the mutation of interest and calculated the numbers reflecting a z-score of 3 for each of these mutations. This approach allowed us to define a value for each identified mutation, above which the detected VAF is truly positive with a specificity of 99.87%. (48,49) The percentages of false positive reads

per mutation and the according z-score of 3 as well as the sequencing depths of the respective amplicons are given in Table 5. The mean z-scores of 3 of all point mutations tested was 0.28%. With the exception of *IDH2* R140Q (z-score 3: 0.63%) and *CEBPA* T310A (z-score 3: 0.8.%), all mutations from diagnostic material tested displayed a z-score of 3 of less than 0.5%. From these data and calculations we concluded that with this approach we were able to detect one cell carrying a heterozygous mutation among 99 normal bone marrow cells and therefore defined persistent residual disease as a detected VAF of >0.5% with sufficient specificity.

Table 5: Z-score of 3 and sequencing depths (Coverage) of selected mutations

	Coverage		**z-Score 3**	
Mutation	*diagnosis*	*remission*	*diagnosis*	*remission*
DNMT3A - p.R882C	7908 x	16496 x	0,23 %	0,30 %
DNMT3A - p.R882H	8051 x	16804 x	0,19 %	1,17 %
IDH1 - p.R132G	4318 x	9817 x	0,24 %	0,30 %
IDH2 - p.R140Q	6315 x	8528 x	0,63 %	0,21 %
RUNX1 - p.R162K	7029 x	1110 x	0,22 %	0,45 %
GATA2 – p.A318T	4605 x	8222 x	0,36 %	0,46 %
FLT3 - p.D835Y	3303 x	3345 x	0,40 %	0,27 %
WT1 - p.R462Q	5901 x	9175 x	0,27 %	0,79 %
TP53 - p.P89R	7885 x	18176 x	0,10 %	0,08 %
NRAS - p.G12C	3974 x	7444 x	0,02 %	0,13 %
PTPN11 - p.E69K	7092 x	8867 x	0,29 %	0,56 %
CEBPA - p.T310A	7000 x	10326 x	0,80 %	0,55 %
TET2 - p.R544*	12316 x	30540 x	0,17 %	0,26 %
TET2 - p.G1282D	9821 x	8146 x	0,20 %	0,30 %
TET2 - p.F1300I	4676 x	18995 x	0,08 %	0,01 %

By applying this definition 22 of the initial 68 mutations were still detected in 16 patients after at least one consolidation therapy (median one mutation per patient, range 0-3; Figure 10). Changes of the variant allelic frequencies in the most commonly affected genes are shown in Figure 11.

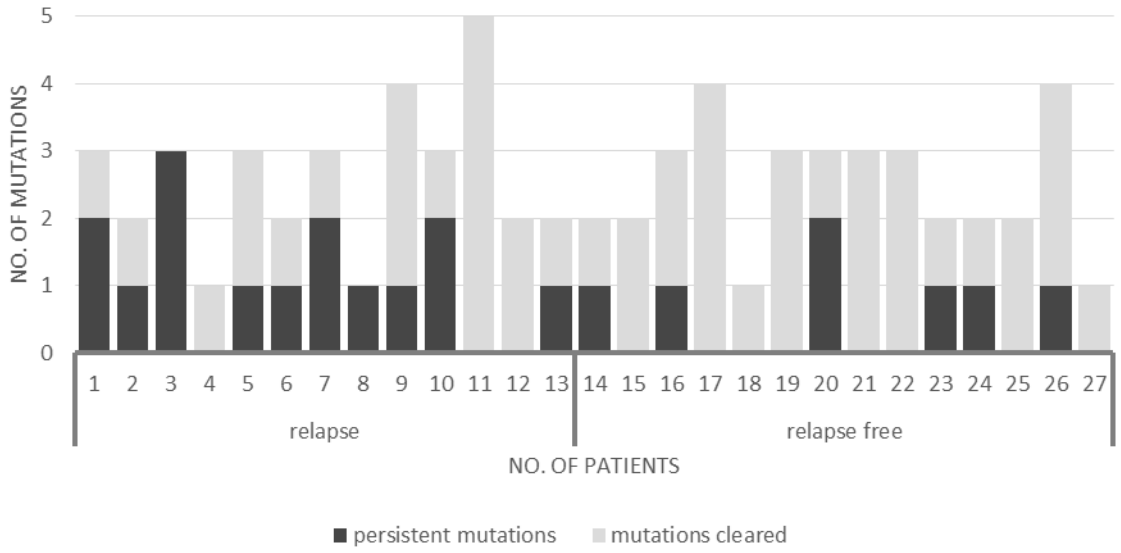


Figure 10: Mutation clearance - number of all identified somatic gene mutations per patient after at least one consolidation therapy

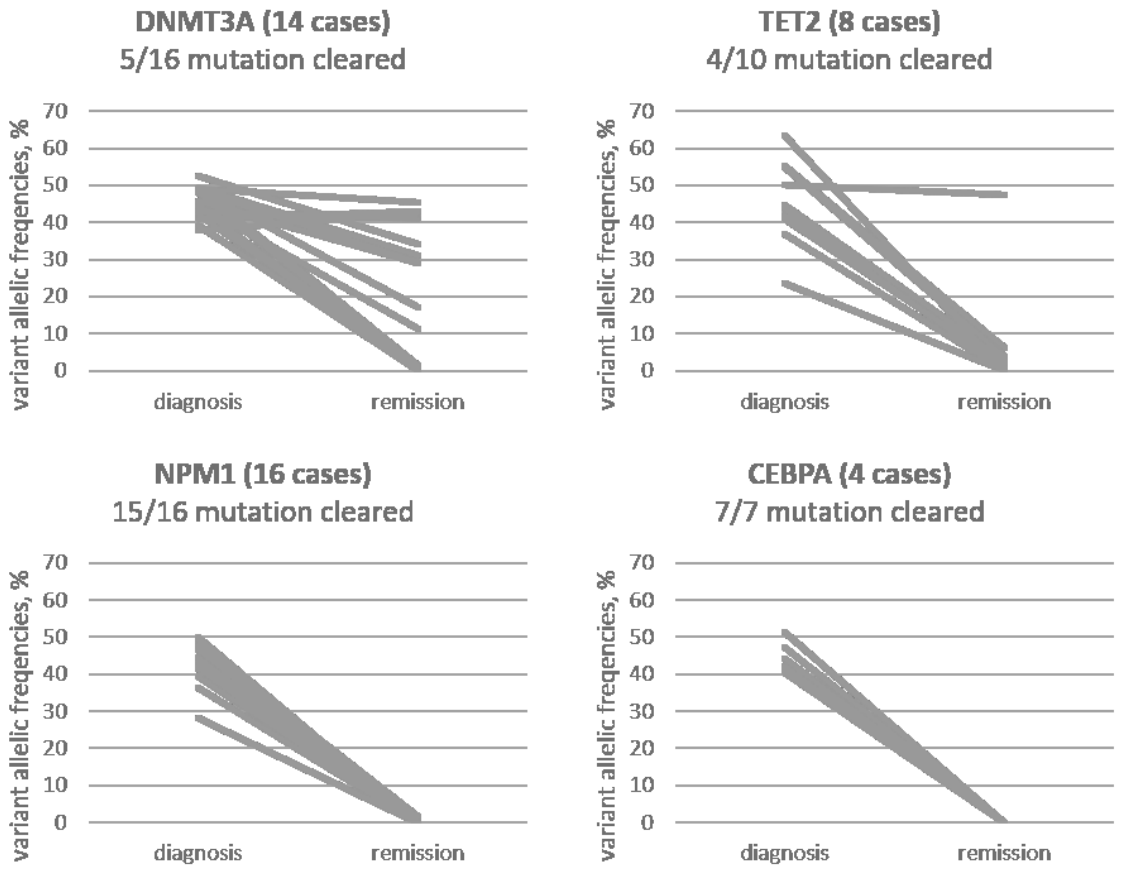


Figure 11: Variant allelic frequencies in the most commonly affected genes at diagnosis and remission

Of the remaining 46 initial mutations coverage was sufficient in 45 cases in the remission material. Only in one initially identified mutation (*PTPN11* E76G) coverage was not sufficient in the remission material. Interestingly, despite lower input DNA obtained from the remission material when compared to diagnostic material, the average sequencing depth per amplicon per patient was comparable (6680x vs. 11733x; see also for selected amplicons Table 5).

In order to validate the results obtained by the Ion Torrent sequencing for the two most commonly mutated genes (*DNMT3A* and *NPM1*) we also performed digital PCR on the most frequent *NPM1* mutation and the Safe-SeqS method for other mutations in *NPM1* as well as the hotspot mutation c.2645G>A, p.Arg882His in *DNMT3A* (for the respective dilution series see Figure 6). For values above 0.5%, the determined VAF of NPM1 or *DNMT3A* correlated well when compared with either digital PCR or the Safe-SeqS approach, respectively (r=0.996 for *NPM1* and r=0.969 for *DNMT3A*; Figure 12).

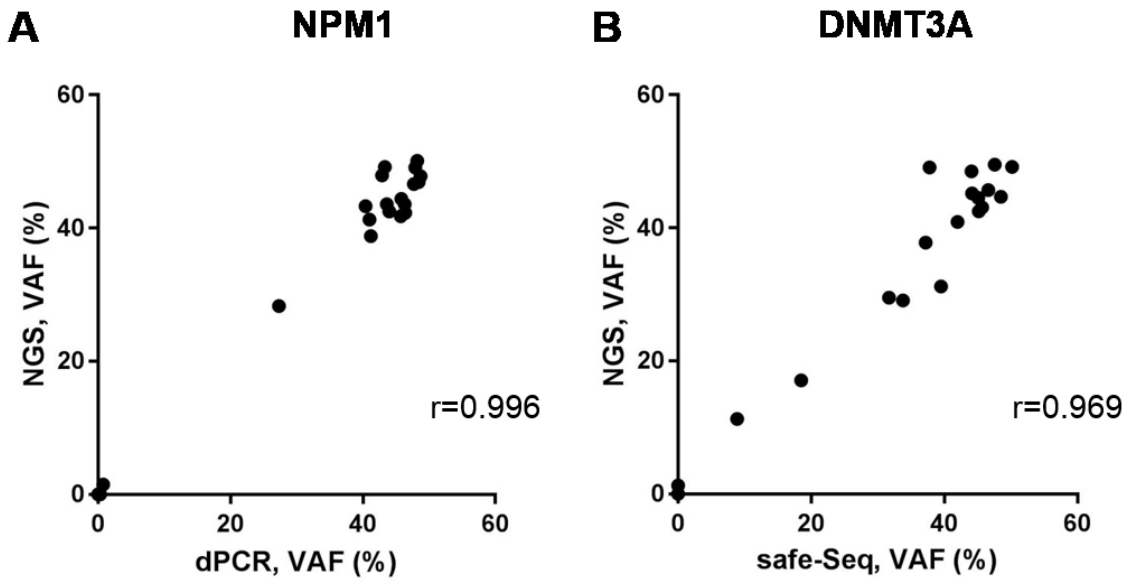


Figure 12: A. Correlation of variant allelic frequencies (VAF) of NPM1 detected by targeted parallel sequencing (NGS) or digital PCR (dPCR) and B. VAF of DNMT3A detected by NGS or safe-Seq

The most frequent non-cleared mutations were found in *DNMT3A* (n=10 pts) and, interestingly, while most mutations displayed a marked reduction of the VAF in the DNA obtained after consolidation, VAF of *DNMT3A* mutations persisted on an almost heterozygous level in several samples despite complete remission (comparison of VAF of *DNMT3A* vs. non-*DNMT3A* mutations at remission, $p<0.001$). These data indicate that *DNMT3A* mutations can persist in regenerating normal bone marrow despite clearance of AML blasts. Indeed, *DNMT3A* has been reported to occur in a high allelic frequency at highly purified hematopoietic stem cells (HSCs), progenitor cells and mature blood cell fractions in AML patients in CR. It has been shown that these mutations promote self-renewal and lead to their clonal expansion with and repopulation advantage over non-mutated HSC resulting in a pre-leukemic stem cell (pre-LSC) population. Pre-LSCs themselves do not induce disease and maintain the aptitude to differentiate into mature lymphoid and myeloid daughter cells. Only when a pre-LSC acquires a cooperating mutation (e.g. *FLT3-ITD*), it leads to a transformation into a leukemic stem cell and subsequently to AML blasts. (See Figure 13) Pre-HSCs have been reported to be found in remission samples and are thus suspected to survive chemotherapy. (50,51) Thus, since *DNMT3A* mutations have been shown to persist in pre-LSCs after therapy without any impact on relapse risk (52,53), we performed survival and relapse risk analysis excluding *DNMT3A* mutations (as seen in Figure 14).

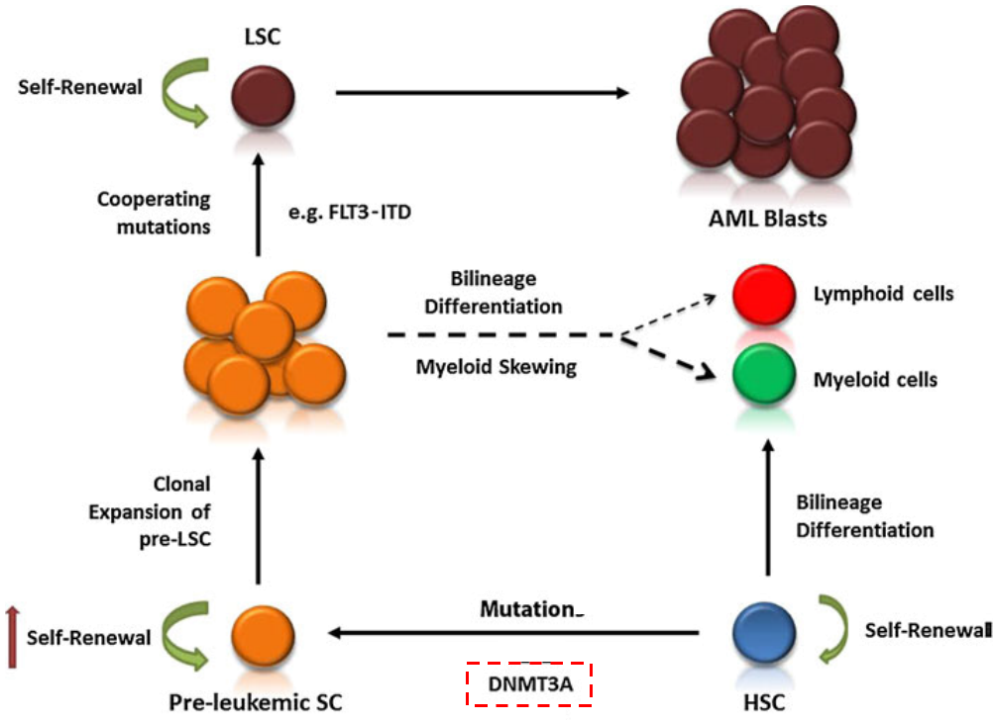


Figure 13: Model of development a hematopoietic stem cell into a fully leukemic stem cell

If a hematopoietic stem cell acquires a mutation in genes involved in regulation of DNA methylation like *DNMT3A*, this leads to development of pre-leukemic stem cell population, which is not disease causing. Only the acquisition of cooperating mutations (e.g. *FLT3-ITD*) leads to a conversion into a fully leukemic stem cell and subsequently to AML blast.
Adapted from Majeti et al., IJH 2013 (50)

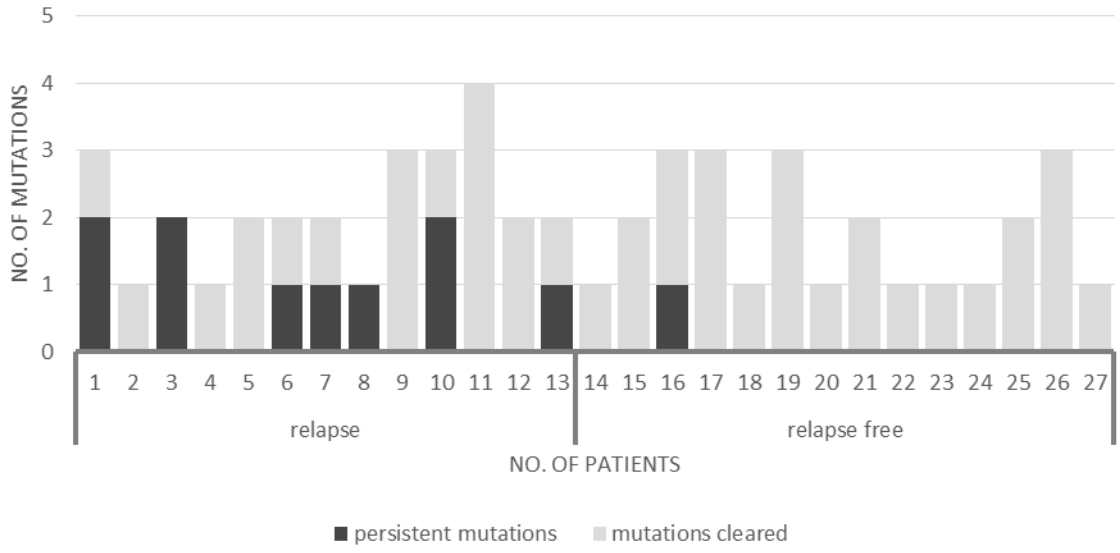


Figure 14: Number of all identified somatic gene mutations excluding *DNMT3A* per patient after at least one consolidation therapy

Importantly, persistence of non-*DNMT3A* mutations was significantly associated with a higher risk of AML relapse (7 out of 8 pts versus 6/19 pts; p=0,013, see Figure 14) and with a shorter relapse-free survival (333 days vs. not reached; log-rank p=0,0236; see Figure 15A). In addition, there was a trend for worse overall survival (log-rank p=0,050; see Figure 15B).

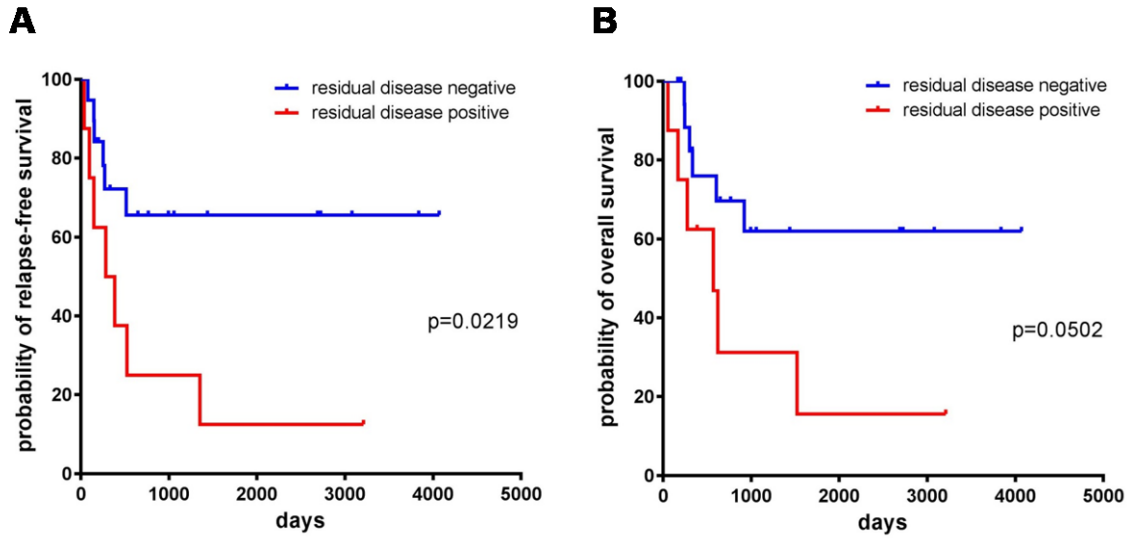


Figure 15: A. Relapse-free and B. overall survival in patients according to residual disease detected after at least one consolidation

4 Discussion

This diploma thesis was part of a pilot study where we used parallel sequencing with an Ion Torrent platform and a commercially available pre-designed targeted gene panel to determine the clearance of leukemia-associated mutations on remission samples obtained after at least one consolidation therapy in de novo AML patients with normal karyotype. Monitoring by this method proved to be feasible and effective as persistence of non-*DNMT3A* mutations were prognostic for relapse risk.

The mutation frequency found with our targeted approach encompassing 19 genes corresponded to what has been previously reported (11) and allowed identification of a somatic mutation suitable for residual disease detection in 31 out of 34 patients (91.2%). Additional sequencing of other recurrently mutated genes, such as *SRSF2*, *STAG2*, *SF3B1*, *BCOR*, and *EZH2*, might not only increase the number of patients with a trackable mutation but also the number of mutations per patient suitable for residual disease detection. The latter is of increasing importance, since several recent studies have reported the existence of recurrent mutations in AML not only in leukemic cells but also in hematopoietic stem cells and in differentiated hematopoietic cells of several lineages, thus constituting preleukemic mutations. (50,51) As a result, mutations in genes implicated in AML leukemogenesis are not always leukemia-specific and thus probably not suitable for residual disease analysis. In addition, mutations in these genes, such as *DNMT3A*, *TET2*, *TP53* and *ASXL1*, can also be found in healthy adults with clonal hematopoiesis. (54) In our study, we found that *DNMT3A* mutations recurrently persisted at high VAF as compared to all other mutations indicating that *DNMT3* mutations indeed had occurred in preleukemic HSC in these patients. In contrast, all but one *TET2* mutations identified in our patients showed markedly decreased VAF levels in remission samples rather excluding the possibility that these mutations had occurred in preleukemic HSC. Based on our own and other findings (5,53) we thus excluded *DNMT3A* mutations as marker for residual disease in the analysis. However, further prospective and more comprehensive studies are needed to clearly define which mutational markers are best suitable for detection of residual leukemic cells to identify patients with an increased risk of relapse. A combination of mutations may probably be best informative in this situation.

In contrast to methods based on flow cytometry, residual disease detection using parallel sequencing usually does not allow a quality control about the material used for analysis. For example, it was shown that in some aspirates depending on the volume the amount of detected residual disease was significantly reduced due to dilution of the bone marrow with blood. (55) With our approach using DNA isolated from slides we could ensure that the analyzed DNA was indeed derived from bone marrow cells and therefore could circumvent this potential pitfall. Interestingly, although the amount of input DNA was lower in the remission material, the reading depth per amplicon was comparable between diagnostic and remission material suggesting that in the majority of cases DNA isolated from BM slides is sufficient for targeted parallel sequencing.

In AML patients with cytogenetic aberrations (about 50% of all patients), persistence of clonal cytogenetic markers in remission samples has shown an increased risk of relapse. (56,57) Interestingly, Klco et al. reported that the clearance of AML-associated mutations as detected by a NGS approach was more sensitive as cytogenetic analysis: while all five patients with residual cytogenetic findings also had non-clearance of mutations, five out of eight patients without cytogenetic findings had persistent mutations, suggesting that both methods are correlating well but that parallel sequencing is more sensitive. (5) Our data extend these findings to patients with cytogenetically normal AML. Using our approach a marker for residual disease detection could be successfully identified and applied in almost 80% of such patients (27 out of 34). In our study we used digital PCR to validate the results of parallel sequencing of the most frequent *NPM1* mutation (p.W282fs) and observed a good correlation between the results. Although studies involving larger numbers of patients are not yet available, reports on small case series indicate a fairly good correlation of residual disease detection with parallel sequencing and other techniques such as detection of *NPM1* mutation by qPCR or multiparameter flow cytometry. (58) However, in some cases different methods of residual detection may give discrepant results either due to changes in leukemia-associated immunophenotypes or changes in mutational patterns both indicative of clonal evolution. (58) Thus, also in that perspective prospective studies are needed to validate the various methods of residual disease detection for risk stratification in AML patients.

Another important issue for detection of residual disease is the definition of mutation clearance. Klco et al. could show that after induction therapy mutation clearance either defined by a VAF <2.5% or VAF<1% was comparably prognostic concerning event-free

and overall survival. However, as expected, with the lower VAF median survival was longer in both groups when compared to the survival times of separated by a VAF of 2.5%. A comparable definition of mutation clearance was used by Kohlmann and colleagues by analyzing the role of persistent *RUNX1* mutations in AML patients. (59) Good responders were classified by a VAF <3.61% at first follow-up and had a significantly better event-free and overall survival as poor responders displaying a VAF >3.61%. With our approach of detecting a VAF of 0.5% after at least one consolidation therapy we were able to identify 7 out of 13 (53.8%) patients who relapsed and only one patients with a VAF>0.5% stayed in complete remission. In a comprehensive analysis of patients with mutated *NPM1* and serial measurements of minimal residual disease using RT-PCR for detection of mutant *NPM1*. Ivey and colleagues showed that results obtained from peripheral blood after the second cycle of chemotherapy were most informative concerning overall survival. (60) All these data indicate that different thresholds defining mutation clearance at different time points during therapy may be needed for early and reliable identification of patients at increased risk for relapse. One could envision that in the future distinct thresholds of mutation clearances will define persisting residual disease during the various phases of therapy and therefore establish the requirement for an optimal response to therapy in AML. Patients failing to reach such a response may undergo early allogeneic stem cell transplantation. In a recent study of the Acute Leukemia French Association group, Balsat et al. demonstrated an overall survival benefit in patients with persistent mutated *NPM1* after induction undergoing allogeneic stem cell transplantation. (61)

5 Conclusion

To sum up, our pilot study clearly shows that using a commercially available pre-designed targeted gene panel to determine the clearance of leukemia-associated mutations was feasible for residual disease detection and effective as persistence of non-*DNMT3A* mutations were prognostic for relapse risk in de novo AML patients with normal karyotype. The results from this diploma thesis suggest that this sequencing approach can improve risk stratification for patients with AML. However, further prospective and more comprehensive studies involving larger numbers of patients are needed, to clearly validate the various methods of residual disease detection and to identify patients with an increased risk of relapse.

6 References

1. Estey E, Döhner H. Acute myeloid leukaemia. The Lancet. 2006;368(9550):1894–1907.

2. Rowe JM, Tallman MS. How I treat acute myeloid leukemia. Blood. 2010 Oct 28;116(17):3147–56.

3. Longo DL, Döhner H, Weisdorf DJ, Bloomfield CD. Acute Myeloid Leukemia. N Engl J Med. 2015 Sep 17;373(12):1136–52.

4. Buccisano F, Maurillo L, Del Principe MI, Del Poeta G, Sconocchia G, Lo-Coco F, et al. Prognostic and therapeutic implications of minimal residual disease detection in acute myeloid leukemia. Blood. 2012 Jan 12;119(2):332–41.

5. Klco JM, Miller CA, Griffith M, Petti A, Spencer DH, Ketkar-Kulkarni S, et al. Association Between Mutation Clearance After Induction Therapy and Outcomes in Acute Myeloid Leukemia. JAMA. 2015 Aug 25;314(8):811.

6. Burnett A, Wetzler M, Lowenberg B. Therapeutic Advances in Acute Myeloid Leukemia. J Clin Oncol. 2011 Feb 10;29(5):487–94.

7. Döhner H, Estey EH, Amadori S, Appelbaum FR, Büchner T, Burnett AK, et al. Diagnosis and management of acute myeloid leukemia in adults: recommendations from an international expert panel, on behalf of the European LeukemiaNet. Blood. 2010;115(3):453–474.

8. Rollig C, Bornhauser M, Thiede C, Taube F, Kramer M, Mohr B, et al. Long-Term Prognosis of Acute Myeloid Leukemia According to the New Genetic Risk Classification of the European LeukemiaNet Recommendations: Evaluation of the Proposed Reporting System. J Clin Oncol. 2011 Jul 10;29(20):2758–65.

9. Rockova V, Abbas S, Wouters BJ, Erpelinck CAJ, Beverloo HB, Delwel R, et al. Risk stratification of intermediate-risk acute myeloid leukemia: integrative analysis of a multitude of gene mutation and gene expression markers. Blood. 2011 Jul 28;118(4):1069–76.

10. Buccisano F, Maurillo L, Spagnoli A, Del Principe MI, Fraboni D, Panetta P, et al. Cytogenetic and molecular diagnostic characterization combined to postconsolidation minimal residual disease assessment by flow cytometry improves risk stratification in adult acute myeloid leukemia. Blood. 2010 Sep 30;116(13):2295–303.

11. The Cancer Genome Atlas Research Network. Genomic and Epigenomic Landscapes of Adult De Novo Acute Myeloid Leukemia. N Engl J Med. 2013 May 30;368(22):2059–74.

12. Lowenberg B, Downing JR, Burnett A. Acute myeloid leukemia. N Engl J Med. 1999;341(14):1051–1062.

13. Walter RB, Othus M, Paietta EM, Racevskis J, Fernandez HF, Lee J-W, et al. Effect of genetic profiling on prediction of therapeutic resistance and survival in adult acute myeloid leukemia. Leukemia. 2015 Oct;29(10):2104–7.

14. Hokland P, Ommen HB, Mulé MP, Hourigan CS. Advancing the Minimal Residual Disease Concept in Acute Myeloid Leukemia. Semin Hematol. 2015 Jul;52(3):184–92.

15. Goldman JM, Gale RP. What does MRD in leukemia really mean|[quest]|. Leukemia. 2014 May 1;28(5):1131–1131.

16. Buckley SA, Appelbaum FR, Walter RB. Prognostic and therapeutic implications of minimal residual disease at the time of transplantation in acute leukemia. Bone Marrow Transplant. 2013;48(5):630–641.

17. Walter RB, Buckley SA, Pagel JM, Wood BL, Storer BE, Sandmaier BM, et al. Significance of minimal residual disease before myeloablative allogeneic hematopoietic cell transplantation for AML in first and second complete remission. Blood. 2013 Sep 5;122(10):1813–21.

18. DiNardo CD, Luger SM. Beyond morphology: minimal residual disease detection in acute myeloid leukemia. Curr Opin Hematol. 2012 Feb;1.

19. Kröger N, Bacher U, Bader P, Böttcher S, Borowitz MJ, Dreger P, et al. NCI First International Workshop on the Biology, Prevention, and Treatment of Relapse after Allogeneic Hematopoietic Stem Cell Transplantation: Report from the Committee on Disease-Specific Methods and Strategies for Monitoring Relapse following Allogeneic Stem Cell Transplantation. Part I: Methods, Acute Leukemias, and Myelodysplastic Syndromes. Biol Blood Marrow Transplant. 2010 Sep;16(9):1187–211.

20. Campana D. Minimal residual disease monitoring in childhood acute lymphoblastic leukemia: Curr Opin Hematol. 2012 Jul;19(4):313–8.

21. Gaipa G, Basso G, Biondi A, Campana D. Detection of minimal residual disease in pediatric acute lymphoblastic leukemia: MRD in Pediatric ALL. Cytometry B Clin Cytom. 2013 Nov;84(6):359–69.

22. Eckert C, Henze G, Seeger K, Hagedorn N, Mann G, Panzer-Grumayer R, et al. Use of Allogeneic Hematopoietic Stem-Cell Transplantation Based on Minimal Residual Disease Response Improves Outcomes for Children With Relapsed Acute Lymphoblastic Leukemia in the Intermediate-Risk Group. J Clin Oncol. 2013 Jul 20;31(21):2736–42.

23. Topp MS, Gökbuget N, Zugmaier G, Degenhard E, Goebeler M-E, Klinger M, et al. Long-term follow-up of hematologic relapse-free survival in a phase 2 study of blinatumomab in patients with MRD in B-lineage ALL. Blood. 2012;120(26):5185–5187.

24. Østergaard M, Nyvold CG, Jovanovic JV, Andersen MT, Kairisto V, Morgan YG, et al. Development of standardized approaches to reporting of minimal residual disease data using a reporting software package designed within the European LeukemiaNet. Leukemia. 2011;25(7):1168–1173.

25. Yin JAL, O'Brien MA, Hills RK, Daly SB, Wheatley K, Burnett AK. Minimal residual disease monitoring by quantitative RT-PCR in core binding factor AML allows risk stratification and predicts relapse: results of the United Kingdom MRC AML-15 trial. Blood. 2012 Oct 4;120(14):2826–35.

26. Shayegi N, Kramer M, Bornhauser M, Schaich M, Schetelig J, Platzbecker U, et al. The level of residual disease based on mutant NPM1 is an independent prognostic factor for relapse and survival in AML. Blood. 2013 Jul 4;122(1):83–92.

27. Patel JP, Gönen M, Figueroa ME, Fernandez H, Sun Z, Racevskis J, et al. Prognostic relevance of integrated genetic profiling in acute myeloid leukemia. N Engl J Med. 2012;366(12):1079–1089.

28. Thomas RK, Nickerson E, Simons JF, Jänne PA, Tengs T, Yuza Y, et al. Sensitive mutation detection in heterogeneous cancer specimens by massively parallel picoliter reactor sequencing. Nat Med. 2006 Jul;12(7):852–5.

29. Kohlmann A, Grossmann V, Klein H-U, Schindela S, Weiss T, Kazak B, et al. Next-Generation Sequencing Technology Reveals a Characteristic Pattern of Molecular Mutations in 72.8% of Chronic Myelomonocytic Leukemia by Detecting Frequent Alterations in TET2, CBL, RAS, and RUNX1. J Clin Oncol. 2010 Aug 20;28(24):3858–65.

30. Ding L, Ley TJ, Larson DE, Miller CA, Koboldt DC, Welch JS, et al. Clonal evolution in relapsed acute myeloid leukaemia revealed by whole-genome sequencing. Nature. 2012 Jan 11;481(7382):506–10.

31. Walter MJ, Shen D, Ding L, Shao J, Koboldt DC, Chen K, et al. Clonal Architecture of Secondary Acute Myeloid Leukemia. N Engl J Med. 2012 Mar 22;366(12):1090–8.

32. Davies K. Powering Preventative Medicine [Internet]. Bio-ITWorld. 2011 [cited 2017 Jan 11]. Available from: http://www.bio-itworld.com

33. Rusk N. Torrents of sequence. Nat Meth. 2011 Jan;8(1):44–44.

34. Churchill JD, King JL, Chakraborty R, Budowle B. Effects of the Ion PGM™ Hi-Q™ sequencing chemistry on sequence data quality. Int J Legal Med. 2016 Sep;130(5):1169–80.

35. Pennisi E. Semiconductors inspire new sequencing technologies. Science. 2010;327(5970):1190–1190.

36. Rothberg JM, Hinz W, Rearick TM, Schultz J, Mileski W, Davey M, et al. An integrated semiconductor device enabling non-optical genome sequencing. Nature. 2011 Jul 20;475(7356):348–52.

37. Blervaque R. Principe Ion torrent PGM | biorigami [Internet]. Biorigami. 2013 [cited 2017 Jan 12]. Available from: http://www.biorigami.com/?tag=principe-ion-torrent-pgm

38. Ion PGM™ System for Next-Generation Sequencing [Internet]. Thermo Fisher Scientific. [cited 2017 Jan 12]. Available from: https://www.thermofisher.com/uk/en/home/life-science/sequencing/next-generation-sequencing/ion-torrent-next-generation-sequencing-workflow/ion-torrent-next-generation-sequencing-run-sequence/ion-pgm-system-for-next-generation-sequencing.html

39. Schulz E, Kashofer K, Heitzer E, Mhatre KN, Speicher MR, Hoefler G, et al. Preexisting TP53 mutation in therapy-related acute myeloid leukemia. Ann Hematol. 2015 Mar 1;94(3):527–9.

40. Wang K, Li M, Hakonarson H. ANNOVAR: functional annotation of genetic variants from high-throughput sequencing data. Nucleic Acids Res. 2010 Sep 1;38(16):e164–e164.

41. Cingolani P, Platts A, Wang LL, Coon M, Nguyen T, Wang L, et al. A program for annotating and predicting the effects of single nucleotide polymorphisms, SnpEff: SNPs in the genome of Drosophila melanogaster strain w ^{1118}; iso-2; iso-3. Fly (Austin). 2012 Apr;6(2):80–92.

42. Robinson JT, Thorvaldsdóttir H, Winckler W, Guttman M, Lander ES, Getz G, et al. Integrative genomics viewer. Nat Biotechnol. 2011 Jan;29(1):24–6.

43. Thorvaldsdottir H, Robinson JT, Mesirov JP. Integrative Genomics Viewer (IGV): high-performance genomics data visualization and exploration. Brief Bioinform. 2013 Mar 1;14(2):178–92.

44. Kinde I, Wu J, Papadopoulos N, Kinzler KW, Vogelstein B. Detection and quantification of rare mutations with massively parallel sequencing. Proc Natl Acad Sci. 2011 Jun 7;108(23):9530–5.

45. Magoc T, Salzberg SL. FLASH: fast length adjustment of short reads to improve genome assemblies. Bioinformatics. 2011 Nov 1;27(21):2957–63.

46. Li H, Durbin R. Fast and accurate short read alignment with Burrows-Wheeler transform. Bioinformatics. 2009 Jul 15;25(14):1754–60.

47. Li H. A statistical framework for SNP calling, mutation discovery, association mapping and population genetical parameter estimation from sequencing data. Bioinformatics. 2011 Nov 1;27(21):2987–93.

48. Minarik G, Repiska G, Hyblova M, Nagyova E, Soltys K, Budis J, et al. Utilization of Benchtop Next Generation Sequencing Platforms Ion Torrent PGM and MiSeq in Noninvasive Prenatal Testing for Chromosome 21 Trisomy and Testing of Impact of In Silico and Physical Size Selection on Its Analytical Performance. Shomron N, editor. PLOS ONE. 2015 Dec 15;10(12):e0144811.

49. Jeon YJ, Zhou Y, Li Y, Guo Q, Chen J, Quan S, et al. The Feasibility Study of Non-Invasive Fetal Trisomy 18 and 21 Detection with Semiconductor Sequencing Platform. Hahn S, editor. PLoS ONE. 2014 Oct 20;9(10):e110240.

50. Chan SM, Majeti R. Role of DNMT3A, TET2, and IDH1/2 mutations in pre-leukemic stem cells in acute myeloid leukemia. Int J Hematol. 2013 Dec;98(6):648–57.

51. Shlush LI, Zandi S, Mitchell A, Chen WC, Brandwein JM, Gupta V, et al. Identification of pre-leukaemic haematopoietic stem cells in acute leukaemia. Nature. 2014 Feb 12;506(7488):328–33.

52. Bhatnagar B, Eisfeld A-K, Nicolet D, Mrózek K, Blachly JS, Orwick S, et al. Persistence of *DNMT3A* R882 mutations during remission does not adversely affect outcomes of patients with acute myeloid leukaemia. Br J Haematol. 2016 Oct;175(2):226–36.

53. Debarri H, Lebon D, Roumier C, Cheok M, Marceau-Renaut A, Nibourel O, et al. IDH1/2 but not DNMT3A mutations are suitable targets for minimal residual disease monitoring in acute myeloid leukemia patients: a study by the Acute Leukemia French Association. Oncotarget. 2015 Oct 12;6(39):42345–53.

54. Clonal Hematopoiesis and Blood-Cancer Risk. N Engl J Med. 2015 Mar 12;372(11):1071–2.

55. Helgestad J, Rosthøj S, Johansen P, Varming K, Østergaard E. Bone marrow aspiration technique may have an impact on therapy stratification in children with acute lymphoblastic leukaemia. Pediatr Blood Cancer. 2011 Aug;57(2):224–6.

56. Chen Y, Cortes J, Estrov Z, Faderl S, Qiao W, Abruzzo L, et al. Persistence of Cytogenetic Abnormalities at Complete Remission After Induction in Patients With Acute Myeloid Leukemia: Prognostic Significance and the Potential Role of Allogeneic Stem-Cell Transplantation. J Clin Oncol. 2011 Jun 20;29(18):2507–13.

57. Marcucci G. Abnormal Cytogenetics at Date of Morphologic Complete Remission Predicts Short Overall and Disease-Free Survival, and Higher Relapse Rate in Adult Acute Myeloid Leukemia: Results From Cancer and Leukemia Group B Study 8461. J Clin Oncol. 2004 Jun 15;22(12):2410–8.

58. Malmberg EB, Ståhlman S, Rehammar A, Samuelsson T, Alm SJ, Kristiansson E, et al. Patient-tailored analysis of minimal residual disease in acute myeloid leukemia using next generation sequencing. Eur J Haematol [Internet]. 2016 May [cited 2016 Sep 26]; Available from: http://doi.wiley.com/10.1111/ejh.12780

59. Kohlmann A, Nadarajah N, Alpermann T, Grossmann V, Schindela S, Dicker F, et al. Monitoring of residual disease by next-generation deep-sequencing of RUNX1 mutations can identify acute myeloid leukemia patients with resistant disease. Leukemia. 2014 Jan;28(1):129–37.

60. Ivey A, Hills RK, Simpson MA, Jovanovic JV, Gilkes A, Grech A, et al. Assessment of Minimal Residual Disease in Standard-Risk AML. N Engl J Med. 2016 Feb 4;374(5):422–33.

61. Balsat M, Renneville A, Thomas X, de Botton S, Caillot D, Marceau A, et al. Postinduction minimal residual disease predicts outcome and benefit from allogeneic stem cell transplantation in acute myeloid leukemia with NPM1 mutation: a study by the acute leukemia French Association Group. J Clin Oncol. 2016;JCO–2016.