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Pettersson, Louise

2021

Document Version:
Publisher's PDF, also known as Version of record

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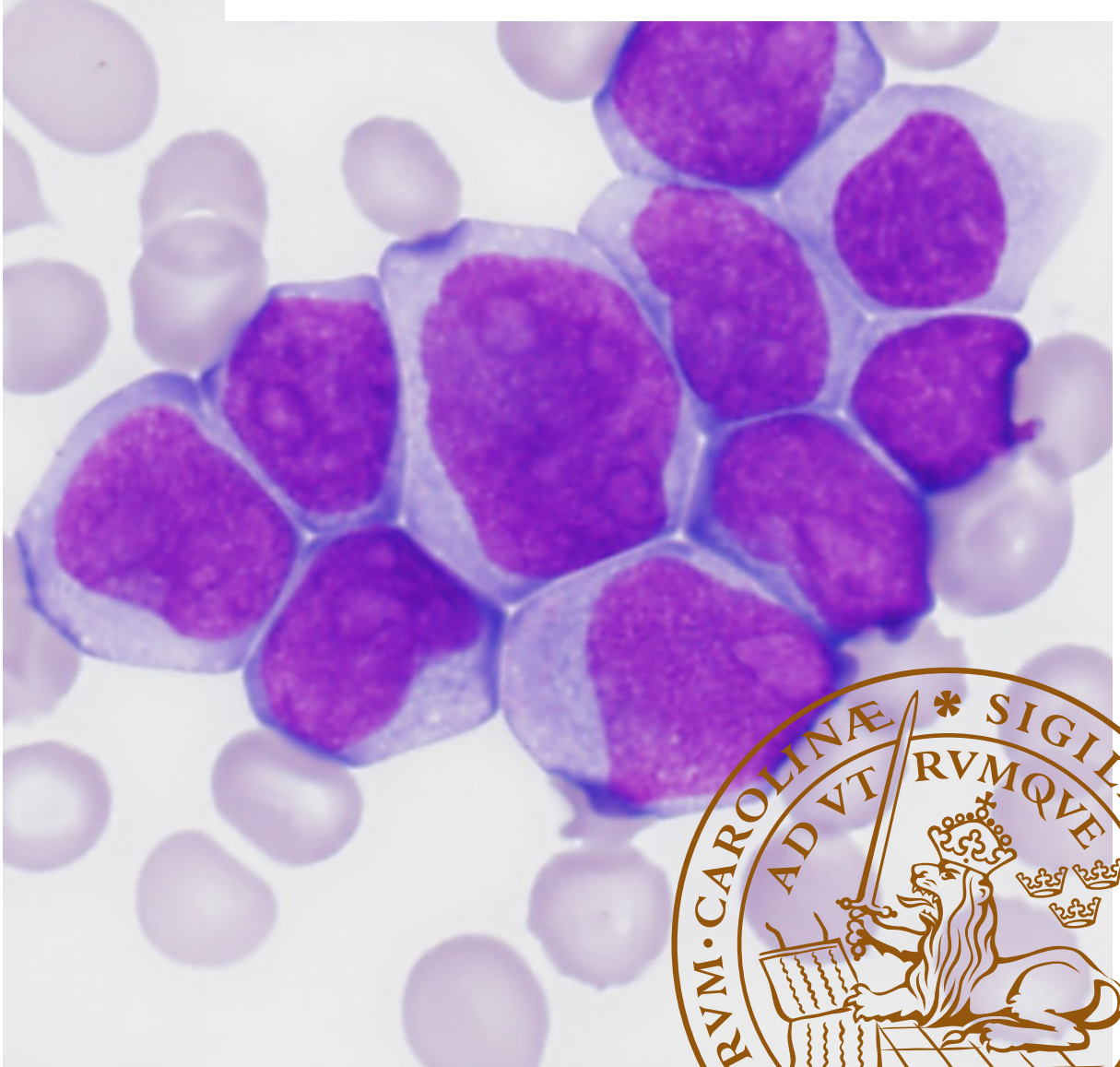

Measurable residual disease and clonal evolution in acute myeloid leukemia with focus on *NPM1*-mutations

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Louise Pettersson

LUND
UNIVERSITY

DOCTORAL DISSERTATION

by due permission of the Faculty of Medicine, Lund University, Sweden.
To be defended in Belfragesalen, BMC, Lund.
May 29, 2021, at 9 a.m.

Faculty opponent
Professor Wolfgang Kern
Munich Leukemia Laboratory (MLL), Munich, Germany

Department of Clinical Sciences, Lund, Division of Pathology, Faculty of Medicine, LUND UNIVERSITY	Doctoral dissertation
	Date of issue: May 29th, 2021
Author: Louise Pettersson	Sponsoring organization: -

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Abstract

Acute myeloid leukemia (AML) is the most common form of acute leukemia in adults, with mutations in the *NPM1* gene occurring in almost one third of all cases. The ability to detect residual leukemia below the resolution of conventional microscopy is crucial for evaluation of relapse risk after therapy. In principle, this can be achieved by measuring residual disease (MRD) with two different approaches, both used routinely in everyday hematological practice and in this thesis: multicolor flow cytometry (MFC) and molecular techniques. The latter include methods such as reverse transcription quantitative PCR (RT-qPCR), quantitative PCR (qPCR), droplet digital PCR (ddPCR) and next-generation sequencing (NGS). *NPM1* mutations are ideal targets for molecular MRD and the level of *NPM1*-MRD, as determined by quantification of RNA transcripts, is currently considered the most relevant prognostic factor after first-line treatment. Instead, to explore the clinical relevance of genomic DNA-based molecular MRD methods, this thesis has focused on targeting *NPM1* mutations, but also other AML-associated mutations, to decipher patterns of clonal evolution in AML before, during and after treatment.

First, a qPCR-based protocol for quantification of the *NPM1* type A mutation was refined, validated, and shown to be more sensitive than MFC for determination of MRD. This study was followed by an extensive comparison of DNA- and RNA-based methods for MRD assessment. The DNA-based methods proved highly accurate with respect to RNA thresholds of importance for treatment response. In addition, although RT-qPCR was more sensitive, it failed to detect leukemic transcripts in about 10% of samples with clear-cut *NPM1*-mutated DNA. Hence, DNA-based MRD techniques can add important information with respect to residual leukemia, of possible clinical relevance for MRD assessment. Next, several mutations in addition to *NPM1* were targeted with ddPCR and monitored in follow-up samples after treatment. This strategy revealed several patterns of clonal evolution in relapsing AML. In one pattern, all monitored mutations reappeared at relapse regardless of the number of subclones. In other relapses, a subclone different from the original leukemia was responsible for the recurrence. Finally, in some patients, the leukemia relapsed from persistent clonal hematopoiesis despite complete morphological and immunophenotypical remission. To explore the mutational landscape and clonal evolution in elderly patients, who are often treated outside clinical trials, a cohort of patients older than 75 years with *de novo* AML with mutated *NPM1* was analysed. The results indicate that the mutational pattern may differ between younger and older patients, with more *TET2* and *SRSF2* mutations but fewer *DNMT3A* mutations in the elderly.

In conclusion, this thesis shows that DNA-based methods are more sensitive than MFC for determination of MRD and that they may complement RT-qPCR, with possible consequences for risk assessment of patients treated for *NPM1*-mutated AML. Targeting several mutations with ddPCR or other DNA-based techniques may be relevant for accurate and complete MRD assessment in the personalised follow-up of most AML patients. Finally, the mutational landscape seems to differ between younger and elderly AML-patients, with possible implications for risk stratification, and ultimately, treatment.

Key words: AML, MRD, Measurable residual disease, *NPM1*, *NPM1*-mutation, *NPM1* type A mutation, clonal evolution, clonal patterns, subclones; genetic evolution; qPCR, RQ-PCR, ddPCR, deep seq, deep sequencing, NGS, RT-qPCR, old, elderly, MFC, flow cytometry

Classification system and/or index terms (if any)

Supplementary bibliographical information		Language: English
ISSN and key title 1652-8220		ISBN 978-91-8021-052-2
Recipient's notes	Number of pages 117	Price
	Security classification	

Signature:  Date: April 23rd, 2021

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Cover photo by the author.

The cover photo shows a group of myeloid blasts in a bone marrow smear from a patient with acute myeloid leukemia, stained with May-Grünwald-Giemsa.

Faculty of Medicine
Department of Clinical Sciences, Lund

ISBN 978-91-8021-052-2
ISSN 1652-8220

Printed in Sweden by Media-Tryck, Lund University
Lund 2021

To my beloved family

"What we know is a drop, what we don't know is an ocean."
Isaac Newton

Table of Contents

Abstract

Acute myeloid leukemia (AML) is the most common form of acute leukemia in adults, with mutations in the *NPM1* gene occurring in almost one third of all cases. The ability to detect residual leukemia below the resolution of conventional microscopy is crucial for evaluation of relapse risk after therapy. In principle, this can be achieved by measuring residual disease (MRD) with two different approaches, both used routinely in everyday hematological practice and in this thesis: multicolor flow cytometry (MFC) and molecular techniques. The latter include methods such as reverse transcription quantitative PCR (RT-qPCR), quantitative PCR (qPCR), droplet digital PCR (ddPCR) and next-generation sequencing (NGS). *NPM1* mutations are ideal targets for molecular MRD and the level of *NPM1*-MRD, as determined by quantification of RNA transcripts, is currently considered the most relevant prognostic factor after first-line treatment. Instead, to explore the clinical relevance of genomic DNA-based molecular MRD methods, this thesis has focused on targeting *NPM1* mutations, but also other AML-associated mutations, to decipher patterns of clonal evolution in AML before, during and after treatment.

First, a qPCR-based protocol for quantification of the *NPM1* type A mutation was refined, validated, and shown to be more sensitive than MFC for determination of MRD. This study was followed by an extensive comparison of DNA- and RNA-based methods for MRD assessment. The DNA-based methods proved highly accurate with respect to RNA thresholds of importance for treatment response. In addition, although RT-qPCR was more sensitive, it failed to detect leukemic transcripts in about 10% of samples with clear-cut *NPM1*-mutated DNA. Hence, DNA-based MRD techniques can add important information with respect to residual leukemia, of possible clinical relevance for MRD assessment. Next, several mutations in addition to *NPM1* were targeted with ddPCR and monitored in follow-up samples after treatment. This strategy revealed several patterns of clonal evolution in relapsing AML. In one pattern, all monitored mutations reappeared at relapse regardless of the number of subclones. In other relapses, a subclone different from the original leukemia was responsible for the recurrence. Finally, in some patients, the leukemia relapsed from persistent clonal hematopoiesis despite complete morphological and immunophenotypical remission. To explore the mutational landscape and clonal evolution in elderly patients, who are often treated outside clinical trials, a cohort of patients older than 75 years with *de novo* AML with mutated *NPM1* was analysed. The results indicate that the mutational pattern may differ between younger and older patients, with more *TET2* and *SRSF2* mutations but fewer *DNMT3A* mutations in the elderly.

In conclusion, this thesis shows that DNA-based methods are more sensitive than MFC for determination of MRD and that they may complement RT-qPCR, with possible consequences for risk assessment of patients treated for *NPM1*-mutated

AML. Targeting several mutations with ddPCR or other DNA-based techniques may be relevant for accurate and complete MRD assessment in the personalised follow-up of most AML patients. Finally, the mutational landscape seems to differ between younger and elderly AML-patients, with possible implications for risk stratification, and ultimately, treatment.

List of abbreviations

AI - artificial intelligence

AKD - activated kinase domain

allo-HSCT - allogeneic hematopoietic stem cell transplantation

AML - acute myeloid leukemia

APL - acute promyelocytic leukemia

ARCH - age-related clonal hematopoiesis

BM - bone marrow

Bp – base pair

BSA - bovine serum albumin

CA- California

CBF - core binding factor

CCA - conventional cytogenetic analysis

CD - cluster of differentiation

cDNA - complementary DNA

CH - clonal hematopoiesis

CHIP - clonal hematopoiesis of indeterminate potential

CHOP - clonal hematopoiesis with oncogenic potential

CR - complete remission

deep seq - deep sequencing

ddPCR - droplet digital PCR

DfN - different-from-normal

DLI - donor lymphocyte infusion

DNA- deoxyribonucleic acid

dPCR - digital PCR

Dx – diagnosis

EDTA - Ethylenediaminetetraacetic acid

ELN - European LeukemiaNet

FDA - Food and Drug Administration

FISH - fluorescence *in situ* hybridization

Flow - flow cytometry

FLT3 - Fms-like tyrosine kinase 3

FS - forward scatter

FU - follow-up

GEP - gene expression profile

HOX - homeobox genes

HSC - hematopoietic stem cell

HSCT - hematopoietic stem cell transplantation

IBSAFE® - in house developed droplet digital polymerase chain reaction; short for Incremental Before, Symmetric After, Fidelity Enhanced

Indels - insertions or deletions

inv - inversion

ITD - internal tandem duplication

LAIP - leukemia-associated immunophenotype

LoD - limit of detection

LU - Lund University

MFC - multicolor flow cytometry

MDS - myelodysplastic syndrome

MPN - myeloproliferative neoplasm

MPS - massively parallel sequencing

MRD - measurable residual disease

Mut – mutated

NES - nuclear export signal

NLS - nuclear localization signal

NGS - next generation sequencing

NK cells - natural killer cells

NPM1 - nucleophosmin 1

NPV - negative predictive value

OCI-AML3 – a *NPM1* type A mutated cell line used for qPCR-MRD

PB - peripheral blood

PCR - polymerase chain reaction

PPV - positive predictive value

PTD - partial tandem duplications

qPCR - real-time quantitative PCR

RNA - ribonucleic acid

RNA-seq - RNA sequencing

RQ-PCR - real-time quantitative PCR (qPCR)

RT-qPCR - reverse transcription quantitative PCR

SCT - stem cell transplantation

Seq - sequencing

SNP - single-nucleotide polymorphism

SNV - single nucleotide variations

SS - side scatter

t - translocation

tAML - therapy-related AML

TKD - tyrosine kinase domain

VAF - variant allele frequency

WES - whole exome sequencing

WGS - whole genome sequencing

WHO - World Health Organization

WTS - whole transcriptome sequencing

wt – wild-type

List of papers

This thesis is based on the original studies listed below, in the text referred to by their Roman numbers.

Included papers:

I. Pettersson L., Levéen P., Axler O., Dvorakova D., Juliusson G., Ehinger M. **Improved Minimal Residual Disease Detection by Targeted Quantitative Polymerase Chain Reaction in *Nucleophosmin 1* Type A Mutated Acute Myeloid Leukemia.** Genes, Chromosomes & Cancer 2016;55(10):750-66.

II. Pettersson L., Johansson Alm S., Almstedt A., Chen Y., Orrsjö G., Shah-Barkhordar G., Zhou L., Kotarsky H., Vidovic K., Asp J., Lazarevic V., Saal L. H., Fogelstrand L., Ehinger M. **Comparison of RNA- and DNA-based Methods for Measurable Residual Disease Analysis in *NPM1*-mutated Acute Myeloid Leukemia.** Accepted for publication in International Journal of Laboratory Hematology 2021.

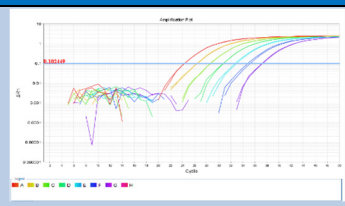
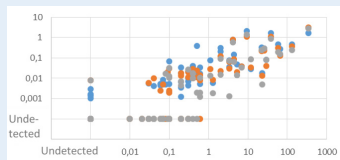
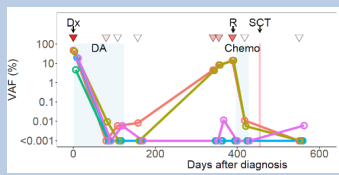
III. Pettersson L., Chen Y., George A.M., Rigo R., Lazarevic V., Juliusson G., Saal L. H., Ehinger M. **Subclonal patterns in follow-up of acute myeloid leukemia combining whole exome sequencing and ultrasensitive IBSAFE digital droplet analysis.** Leukemia & Lymphoma 2020;61:2168-2179.

IV. Pettersson L., Holmgren B., Juliusson G., Lazarevic V. and Ehinger M. **Mutational spectrum of *de novo NPM1*-mutated acute myeloid leukemia patients older than 75 years.** Leukemia and Lymphoma 2021. Published online: 12 March 2021.

Not included paper:

I. Ehinger M., Pettersson L. **Measurable residual disease testing for personalized treatment of acute myeloid leukemia.** APMIS 2019;127(5):337-351.

Dissertation at a glance

	Question / Aim	Patients and Methods	Results		Conclusion
I	Compare MFC MRD to a new qPCR MRD method for assessing the *NPM1* type A mutation.	Samples from 15 *NPM1*-mutated AML patients were collected during a period of 43 months.	In 32/45 follow-up samples, MRD could be detected using qPCR compared to 2/45 by MFC.		qPCR for the *NPM1* type A mutation is more sensitive and reliable than is MFC for determination of MRD.
II	Compare MRD results measured using three different genomic DNA MRD methods to the gold standard RNA method RT-qPCR.	110 *NPM1* type A mutated AML samples in morpho-logical remission were analysed by qPCR, ddPCR, and deep seq.	Strong correlations were observed among the different methods and clinically relevant cut-offs for the DNA-based methods were proposed.		Excellent or substantial agreement among different MRD methods was revealed. Proposed cut-offs in BM for risk stratifi-cation are 0.1% for qPCR and 0.05% VAF for ddPCR and deep seq.
III	Can relapses in AML be identified using the new ddPCR MRD method IBSAFE that targets several mutations?	Ten relapsing and four non-relapsing AML patients were selected and retrospectively tested for molecular MRD using IBSAFE ddPCR in BM aspirates.	The IBSAFE ddPCR method appears to be applicable on virtually all newly diagnosed AML patients and is more sensitive than MFC.		IBSAFE ddPCR analyses detect leukemic clones missed by flow cytometry with possible clinical implications.
IV	Is there a difference in the mutational landscape between very old and younger *NPM1*-mutated AML patients?	22 diagnostic samples with *NPM1*-mutated AML in patients >75 years of age were sequenced and compared to younger patients.	76 mutations were identified. Compared with younger patients, a significant enrichment of *TET2* and *SRSF2* was observed.		The mutational pattern is different in the very old AML patients with possible implications for risk assessment.

Introduction

Author´s perspective

It is strange how things have turned out. When I was working at the Department of Surgery in Halmstad in the early 2000s and planning on starting a residency, I remember that it was really pleasant to not have to think about different kinds of hematological malignancies anymore. I only had to remember to keep them grouped at the back of my head for use in differential diagnosis. I found this area of disease to be quite challenging. A few years later, when I was completing my residency in pathology, molecular pathology and molecular biology were both somewhat difficult for me, and I was pleased that I did not have to explore the field. Close to 2010 when Mats and I began to discuss potential PhD programs, I made it quite clear that I was a morphology-person. Ten years later, I am a hematopathologist and have written a thesis on different techniques for molecular measurable residual disease (MRD). It is so typical of me to try to master something that I and people around me find difficult. It has been a journey, and sometimes a very lonely journey, being far from the university.

After working in hematopathology for a while, it was rather obvious to me that the prognosis for many acute myeloid leukemia (AML) patients was poor and that there was room for improvement in detecting potential AML relapses. A great deal of work has been performed recently in the field of AML, and the knowledge base is constantly increasing. It has been a privilege working in the field both as a scientist and as a pathologist seeing molecular MRD entering the Swedish AML guidelines. I observed first-hand the clinical problem, and I have been provided with the opportunity to choose to work in an attempt to increase the knowledge of the field for the benefit of the patients.

Acute Myeloid Leukemia

Acute myeloid leukemia (AML) is a heterogeneous neoplastic disease that is characterised by the proliferation of clonal immature bone marrow (BM)-derived cells (blasts), Figure 1, with impaired differentiation capacity that ultimately lead to BM failure. In addition to BM, AML often involves peripheral blood (PB) and

sometimes solid organs. If not clearly specified, AML in this thesis refers to all cases of AML with the exception of acute promyelocytic leukemia (non-APL-AML).

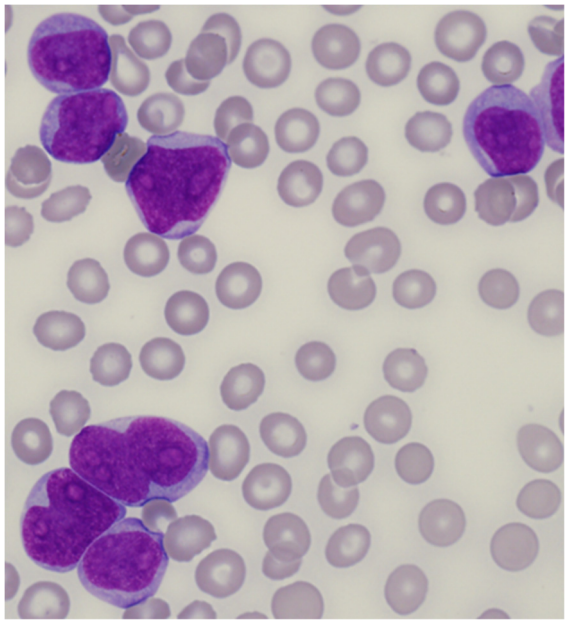


Figure 1. Leukemic cells (blasts) in a BM smear from a patient with AML, stained with May-Grünwald-Giemsa.

Historical perspective

Similar to most discoveries in medicine, there is no consensus regarding the discovery of leukemia. In what is by many considered to be the first published case of leukemia dating back to 1827, the French physician Velpeau described a 63-year-old patient who presented with fever, hepatosplenomegaly, and thick blood. However, it may also have been another French physician and pioneering microscopist, Donné, who first described the disease, reviewed in [1, 2]. He examined a specimen of blood in 1839 from a patient with an abdominal tumor and found that it looked like pus; however, his conclusion was that it was a new disease. He did not publish his findings until several years later. In 1845, the first two cases of leukemia were published within six weeks of each other. The Scottish pathologist Bennett described leukemia as both a clinical entity and a blood-related disease, and the German pathologist Virchow, a pioneer in the use of light microscopy in pathology, described only weeks later the presence of an abnormal number of white blood cells in patients exhibiting the clinical syndrome that was described by Velpeau. The term leukemia was first used by Virchow in 1847. ” Leukemia” was

merely a description that means “white blood” in Greek, as the cause of the disease was unknown. The distinction between acute and chronic leukemia was first proposed in 1857 to separate the more indolent (chronic) cases from the progressive and fatal ones (acute leukemia) [3]. A few years later in 1868, the link between the blood source and the BM was discovered [2], and the term myelogenous was coined after the Greek term “myelos” (bone in Greek), thus suggesting that leukemias arise from the BM [3]. In 1877, a technique for staining blood films was developed that made it possible to describe the leucocytes [2], and at approximately the same time, the first technique for BM examination to diagnose leukemia was presented. However, the mechanism by which cells can travel from the bone to the blood remained unclear [4]. In 1887, the stem cell concept was presented [2] and in 1900, the myeloblast was characterised by the Swiss hematologist Naegeli [2]. Several types of leukemia were known in the early twentieth century; however, none of these malignancies were treatable.

Classification of Acute Leukemias

In 1913, leukemia was classified into four types that include acute myeloid leukemia, chronic myeloid leukemia, acute lymphocytic leukemia and chronic lymphocytic leukemia [4].

The first standard criteria to classify AML were published in 1976 [5] and revised in 1985 by the French-American-British Working group, the FAB classification. They were based on morphology and cytochemistry and provided a 30% diagnostic cut-off for blasts, and when updated, this classification system divided AML into eight subtypes (M0-M7). However, the FAB classification did not consider the genetic or clinical diversity of the disease. In 2001, a new WHO classification was published that subdivided AML into four major categories [6]. The blast percentage required for acute leukemia diagnosis was lowered to 20%, primarily due to the observation of no significant prognostic differences between patients with 20-30% blasts and those with more than 30% blasts. To incorporate new knowledge, the WHO classification was updated in 2008 [7] and revised in 2016 [8, 9]. In the latest version, the major subtypes are:

- AML with recurrent genetic abnormalities
- AML with myelodysplasia-related changes
- Therapy-related myeloid neoplasms
- AML NOS
- Myeloid sarcoma (leukemic tumor outside the BM)
- Myeloid proliferations related to Down syndrome

The AML NOS category corresponds to the previously used FAB classification. AML with recurrent genetic abnormalities embraces eleven different balanced translocations or gene mutations, including AML with mutated *NPM1*. The latter is scrutinised in this thesis.

Incidence

AML is the most common form of acute leukemia in adults. The Swedish incidence is 3-4 / 100 000 inhabitants, and this corresponds to roughly 350 new cases each year. The incidence increases with age until 84 years of age. The median age at diagnosis is 71-72 years and the mean age is 68 years [10-13]. In the elderly, the incidence is higher in men than it is in women [11]. In children, acute lymphoblastic leukemia (ALL) is more common than AML that constitutes approximately 15-20% of childhood leukemias [14].

Etiology

The etiology of AML is not completely understood. It is established that approximately 20% of all AML cases were preceded by another type of hematological disease, and these are termed secondary AMLs (sAML). These include myelodysplastic syndromes (MDS), myeloproliferative neoplasms (MPN) and MDS/MPN [15, 16]. A number of cases occur after previous treatment with chemotherapy and/or radiation (therapy-related AML, tAML). Among children, the risk is increased for those with certain constitutional genetic aberrations such as Down syndrome and Fanconi anemia. Down syndrome increases the likelihood of developing AML by at least ten-fold [14]. Other risk factors include ionizing radiation and chemical exposures such as benzene and cigarette smoke. However, most cases of AML arise *de novo* without a known cause.

Clinical features

The clinical features of AML are dominated by BM failure that occurs due to the accumulation of malignant cells within the marrow. Anemia and thrombocytopenia are frequently observed, and there can be either an increase or decrease in white blood cell count. The most common cause is an increase due to the expansion of blasts within the blood. However, some patients present with leukopenia and low numbers of blood blasts. The severity of symptoms varies and often includes infections, bleeding, and fatigue, thus reflecting the insufficient production of

normal blood cells. Some patients exhibit alarming symptoms and require immediate treatment, while others can wait for a more definite sub-classification. Tumor cells can infiltrate a variety of tissues outside the BM, including gum tissue, and this is typical of myelomonocytic or monocytic AML subtypes.

Diagnosis of AML

Investigations

To achieve a complete diagnosis of AML, a BM aspirate is necessary and used for smears, flow cytometry, genetic analysis (next generation sequencing [NGS], karyotyping and often also fluorescence *in situ* hybridization [FISH] or PCR). A smear from PB is also often analysed. A trephine biopsy may occasionally be necessary, particularly if it is not possible to obtain an aspirate due to BM fibrosis ("dry tap").

The required mutation screening by NGS for proper diagnostic classification includes the *NPM1*, *CEBPA* and *RUNX1* genes and for prognostic information *FLT3*, *TP53* and *ASXL1*.

Diagnostic criteria

In the smears, a differential count is performed on at least 500 nucleated cells obtained from the BM and on 200 nucleated cells derived from PB. The diagnostic criteria for AML include at least one or several of the following features:

- $\geq$20% blasts in the BM or PB
- presence of a myeloid sarcoma (AML tumor outside of the BM)
- presence of certain chromosomal abnormalities in leukemic cells: t(8;21)(q22;q22.1), inv(16)(p13.1q22) / t(16;16)(p13.1;q22) or t(15;17)(q24.1;q21.2), regardless of the blast count [17].

Myeloblasts, monoblasts, and megakaryoblasts are included in the blast count, and in cases of AML with monocytic or myelomonocytic differentiation, promonocytes are considered as blast equivalents.

Prognosis and risk stratification

The prognosis for patients with AML is typically poor, although there have been improvements over the last few decades, at least for younger patients, with the advent of allo-SCT. Age at diagnosis is important, as a rapid decline in overall survival (OS) occurs after the age of 40-49 [18]. To assess the risk of relapse in individual patients, several factors are considered that include genetic alterations of the leukemia and response to therapy.

Genetic abnormalities at diagnosis are critical for risk stratification. Recent data have led to updated recommendations according to the European LeukemiaNet (ELN), Table 1 [17].

Favourable cytogenetics and mutations, Table 1, together with morphological remission and MRD-negativity after two courses of therapy will place the patient in a low-risk category. Failure to achieve complete remission and positive MRD after two courses of therapy or high-risk genetics confers a high risk of relapse. These patients are often offered SCT if allowed according to age and comorbidities. Combining baseline prognosticators and post-consolidation MRD status improves risk assessment [19]. Secondary disease, therapy-related disease, low performance status, severe comorbidity and high white cell count are all associated with adverse outcomes [20].

The importance of MRD for prognosis is discussed in more detail in the MRD section.

Table 1. Risk stratification by genetics for AML according to ELN 2017. Modified from the 2017 European LeukemiaNet [17].

Risk category	Genetic abnormality
Favorable	t(8;21)(q22;q22.1); *RUNX1-RUNX1T1*
	inv(16)(p13.1q22) or t(16;16)(p13.1;q22); *CBFB-MYH11*
	Mutated *NPM1* without *FLT3*-ITD or with *FLT3*-ITD$^{low(a)}$
	Biallelic mutated *CEBPA*
Intermediate	Mutated *NPM1* and *FLT3*-ITD$^{high(a)}$
	Wild-type *NPM1* without *FLT3*-ITD or with *FLT3*-ITD$^{low(a)}$ (without adverse-risk genetic lesions)
	t(9;11)(p21.3;q23.3); *KMT2A-MLLT3*[(b)]
	Cytogenetic abnormalities not classified as favorable or adverse
Adverse	t(6;9)(p23;q34.1); *DEK-NUP214*
	t(v;11q23.3); *KMT2A* rearranged
	t(9;22)(q34.1;q11.2); *BCR-ABL1*
	inv(3)(q21.3q26.2) or t(3;3)(q21.3;q26.2); *GATA2,MECOM(EVI1)*
	−5q or del(5q); −7; −17/abn(17p)
	Complex karyotype[(c)], monosomal karyotype[(d)]
	Wild-type *NPM1* and *FLT3*-ITD$^{high(a)}$
	Mutated *RUNX1*[(e)]
	Mutated *ASXL1*[(e)]
	Mutated *TP53*[(f)]

[a] low allelic ratio (<0.5); high allelic ratio (≥0.5).
[b] The presence of t(9;11)(p21.3;q23.3) takes precedence over rare, concurrent adverse-risk gene mutations.
[c] Three or more unrelated chromosome abnormalities in the absence of one of the WHO-designated recurring translocations or inversions, that is, t(8;21), inv(16) or t(16;16), t(9;11), t(v;11)(v;q23.3), t(6;9), inv(3) or t(3;3); AML with *BCR-ABL1*.
[d] Defined by the presence of one single monosomy (excluding loss of X or Y) in association with at least one additional monosomy or structural chromosome abnormality (excluding core-binding factor AML).
[e] These markers should not be used as an adverse prognostic marker if they co-occur with favorable-risk AML subtypes.
[f] *TP53* mutations are significantly associated with AML with complex and monosomal karyotype.

Normal hematopoiesis

After birth, hematopoiesis (blood cell formation) occurs in the BM during childhood and adult life. In adults, this process is confined to the central skeleton. With increasing age, the fraction of hematopoietic cells decreases, thus providing room for fat cells. Hematopoiesis gives rise to specialized cells in blood that include B- and T-lymphocytes, NK cells, erythrocytes, granulocytes (neutrophils, eosinophils and basophils), monocytes and platelets [21].

The hematopoietic stem cell (HSC)

Hematopoiesis can be considered to originate from pluripotent hematopoietic stem cells (HSCs). During HSC division, one of the new cells replaces the stem cell (self-renewal) and the other is committed to differentiation, ultimately evolving into

myeloid and lymphoid progenitors. These cells differentiate into all types of mature blood cells through many intermediate stages. The rate of proliferation and selection of cell lineage (commitment) is regulated by hematopoietic growth factors [21]. During hematopoiesis, BM cells become increasingly differentiated, gradually losing their ability for self-renewal and decreasing their multiplication rate. One single HSC can generate approximately 10^6 mature blood cells after 20 cell divisions. HSCs are rare and reside in special niches in the BM; however, they can also circulate in the PB. Specialized BM niches are formed by stromal cells (including adipocytes, fibroblasts, endothelial cells, osteoblasts, and macrophages) and their extracellular matrix. Different growth factors and cytokines are important regulators of the formation of these niches.

Leukemogenesis

In 1997, Bonnet *et al.* revealed that the development of AML (leukemogenesis) is organized as a hierarchy that originates from a primitive hematopoietic cell [22].

Leukemogenesis is a multistep process

A simple model of clonal expansion, such as what occurs during leukemogenesis, is that every newly acquired somatic mutation confers a selective advantage for growth. In 2002, Gilliland and Griffin presented a two-hit model of cooperativity for leukemogenesis with proposed class I and class II mutations [23]. Class I mutations, such as those in tyrosine kinases (e.g. *FLT3*), induce uncontrolled proliferation and confer a survival advantage (avoiding apoptosis), while class II mutations (myeloid transcription factors [e.g. *RARA*]) impair hematopoietic differentiation. Both classes of mutations are involved in AML development. However, during the last decade the existence of a more complex pathogenesis has been revealed. Hence, the two-hit hypothesis with class I and class II mutations [23] has been updated to include several new key classes [24, 25]:

- Class I signaling (e.g. *FLT3, KRAS, NRAS* and *KIT*)
- Class II transcription factors (e.g. *NPM1* and *RUNX1*)
- Class III epigenetic regulators (e.g. *ASXL1, DNMT3A, TET2* and *IDH1/2)*
- Class IV tumor suppressor genes (e.g. *TP53* and *WT1*)
- Class V RNA maturation (e.g. *U2AF1*, *SRSF2* and *SF3B1)*

All five classes are potential therapeutic targets. Up to 70% of *de novo* AML patients carry a single mutation in epigenetic modifiers (class III) such as *DNMT3A* and *TET2* [26]. Class IV mutations (mutations in tumor suppressor genes such as *TP53*

and *WT1*) regulate cells during cell division and replication. Class V mutations affect RNA regulation and include splicing factor genes such as *U2AF1*, *SRSF2* and *SF3B1*.

Leukemogenesis begins with the accumulation of genetic and epigenetic changes in HSCs, ultimately giving rise to preleukemic stem cells (pre-LSC). Further transformation events, including mutations, create fully transformed leukemia stem cells (LSCs) (illustrated in Figure 2) with the capacity to progress to overt leukemia by transformation into myeloblasts with or without additional mutations [27]. Over the last decade, it has become clear that the clonal architecture in AML, as in many tumor types, is complex [28]. Several subclones that are genetically diverse can coexist with the dominant clone [29], Figure 2. Leukemogenesis, similar to normal hematopoiesis, is regulated by specific transcription factors, epigenetic processes, and metabolic pathways.

Hence, leukemogenesis is driven by multiple stepwise genetic and epigenetic changes in HSCs or committed progenitors, thus giving rise to pre-LSCs and ultimately fully transformed LSCs. LSCs are important for subclone formation and cellular hierarchies. They often persist in remission and can cause relapse [27, 30-32].

AML is often diagnosed beyond the pre-leukemic phase, and this has hampered the study of initiating lesions and the order of subsequent mutations. However, the advent of less expensive high-throughput genome sequencing platforms during the last few years has facilitated the discovery of genetic lesions responsible for the pathogenesis of many malignant tumors, including AML, and has thus increased our knowledge of leukemogenesis. In 2014, Slush *et al.* published the sequential order of mutations in AML. *DNMT3A*-mutations were determined to arise early in AML evolution, likely within HSCs, prior to the formation of *NPM1* and *FLT3*-mutations [33]. The *DNMT3A*-mutations in HSCs lead to a clonally expanded pool of HSCs from which AML evolves. These mutated pre-leukemic HSCs can survive chemotherapy and possess a repopulation advantage over non-mutated HSCs [33].

In addition to mutations, the BM microenvironment plays an important role during leukemia initiation and progression; however, little is known regarding the interactions between the non-cellular components of the BM niche and leukemic cells. The manner in which the leukemic niche differs from its non-leukemic counterpart has not yet been extensively studied. However, in 2019 Çelik *et al.* presented a proteomic profiling of the non-cellular compartment of the BM microenvironment in patients with AML and compared it to matched control individuals. They observed a significant difference for 168 proteins, with 91 upregulated and 77 downregulated proteins in leukemic BM [34].

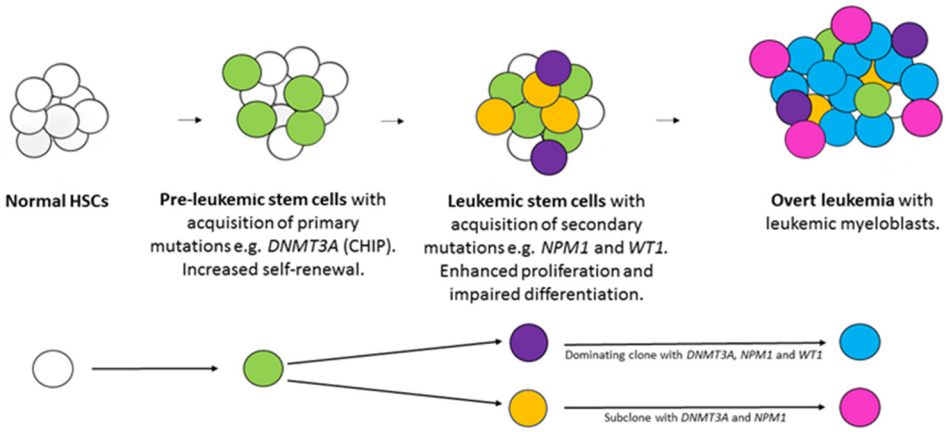


Figure 2. Leukemogenesis. A model of the multistep process and of the heterogeneity present in a leukemia with several emerging clones.
Acquisition of primary mutations such as *DNMT3A* in a hematopoietic stem cell (HSC; white) resulting in pre-leukemic stem cells (pre-LSCs; green) with increased self-renewal, giving rise to clonal hematopoiesis of indeterminate potential (CHIP). The process can stop or continue with the acquisition of secondary mutations such as mutations in *WT1* and/or *NPM1*, ultimately creating leukemic stem cells (LSCs) with *NPM1* and *WT1* mutations (purple) or *NPM1* mutation (orange). These secondary mutations enhance the proliferation capacity and impair differentiation. The LSCs expand to form myeloblasts of overt leukemia (*NPM1* and *WT1*; blue, *NPM1* alone; pink). These different cells create heterogeneity in leukemia. The myeloblasts may also have acquired additional mutations (not shown).

Clonal Hematopoiesis (CH)

Clonal hematopoiesis (CH) arises when a single HSC possessing an acquired (i.e. somatic) gene mutation contributes disproportionately to the population of mature blood cells due to an advantageous phenotype. The accumulation of somatic mutations in pre-leukemic HSCs occurs not only in pre-leukemic cells but also in HSCs during normal aging. This occurs in otherwise healthy individuals who do not develop AML or other hematological malignancies. In 2014, Jaiswal *et al.* and Xie *et al.* presented data on CH and the increased frequency of CH in the elderly [35, 36]. This phenomenon is termed age-related clonal hematopoiesis (ARCH) [37]. Detectable somatic mutations are rare in individuals less than 40 years of age but increase appreciably in frequency with age [35, 38], as CH is very common in the elderly. In 2017, Zink *et al.* demonstrated that CH is far more common among the elderly than previously thought. They used whole genome sequencing (WGS) rather than whole exome sequencing (WES) and candidate genes, and they identified CH in 0.5% of individuals younger than 35 years compared to >50% in individuals older than 85 years [38].

When the variant allele frequency (VAF) is ≥2%, ARCH should be described more specifically as clonal hematopoiesis of indeterminate potential (CHIP). The majority of patients with CHIP are older healthy individuals, and therefore, the

terms ARCH and CHIP are sometimes used synonymously. The following criteria for CHIP have been proposed and are adapted from Steensma *et al.* and Valent *et al.* [39, 40]:

- Absence of definitive morphological evidence of a hematological neoplasm and absence of persistent cytopenia (≥4 months).
- Presence of somatic mutations associated with hematological neoplasia at a VAF of ≥2% (e.g. *DNMT3A*, *TET2*, *ASXL1*, *SRSF2*, *SF3B1*, *JAK2* and *TP53*).
- Does not meet diagnostic criteria for paroxysmal nocturnal hemoglobinuria (PNH), monoclonal gammopathy of unknown significance (MGUS) or monoclonal B-cell lymphocytosis (MBL).

As long as the BM niche and the immune system can control the pre-LSCs possessing CHIP mutations that retain their full differentiation and maturation potential, the CHIP clone will remain indolent; however, as soon as it escapes these critical control mechanisms, it will expand [40].

The odds of progression to overt neoplasia from CHIP are approximately 0.5–1% / year [39]. The term CHIP should only be applied if the blood count is normal. In cases of cytopenia that lack the presence of a myeloid neoplasm, the term clonal cytopenia of undetermined significance (CCUS) is more appropriate [40].

The most commonly mutated genes in CHIP are *DNMT3A*, *ASXL1* and *TET2* (the "DAT"-mutations) [35]. Other CHIP mutations include *SRSF2*, *SF3B1*, *GNB1*, *GNAS*, *TP53*, *U2AF1* and *JAK2*. Unsurprisingly, CHIP mutations are also detected in various myeloid neoplasms (AML, MDS, MPN and MDS/MPN), in some more frequently than they are in others, and most of them are not disease-specific. Mutations in CHIP-genes are not reliable markers for MRD, as they can persist after treatment despite complete remission (CR) [41].

It is important to bear in mind that aged hematopoietic cells are often genetically and/or epigenetically altered, and this can result in differences in AML development in elderly individuals compared to that in younger individuals [42].

Different mutations in the same gene do not convey the same risk of transformation from CHIP to AML. For example, Young *et al.* observed an increased risk of transformation to AML in individuals with variants at the *DNMT3A* R882 locus, and this is unsurprising, as *DNMT3A* R882-mutations are common in AML [43].

In 2018, Abelson *et al.* were able to stratify healthy individuals with CH by demonstrating notable differences in the mutational landscape of ARCH and pre-AML [44]. *TP53* and *U2AF1*-mutations were associated with a relatively high risk of subsequent AML transformation, while mutations in other genes such as *DNMT3A* and *TET2* confer a lower risk of malignant transformation. The term clonal hematopoiesis with oncogenic potential (CHOP) has been coined for

oncogenic mutations associated with a substantial risk of AML transformation in an effort to separate them from the more indolent CHIP mutations that can be found in healthy individuals who never develop leukemia [40]. Examples of CHOP mutations include *FLT3*, *RUNX1*, *WT1*, *NPM1*, *NRAS*, and *TP53*. CHOP mutations correspond to the secondary mutations presented in Figure 2 that can transform a pre-LSC to a LSC, ultimately giving rise to overt leukemia that often possesses additional mutations.

Finally, it should be mentioned that CHIP mutations may affect the normal function of systems beyond hematopoiesis. In a groundbreaking paper from 2014, Jaiswal *et al.* demonstrated that persons with CHIP mutations have an increased overall mortality and increased risk of cardiometabolic disease [35]. Individuals with CHIP have a risk of coronary heart disease that is almost twice as high as that of non-carriers [45].

Clonal Evolution

Many of the mutations in AML are random events that occur in the HSCs prior to the initiating mutation, and the history of these random events is "captured" in the funding clone [46]. This clone may later acquire additional mutations, thus creating subclones, Figure 2. Subclones emerge when such mutations confer a competitive advantage over the ancestral clone(s). Multiple divergent subclones may have been produced prior to overt leukemia [47]. Proteomics is a method used to identify genetically distinct AML subclones with different phenotypes, drug sensitivity, growth, and engraftment behaviors [48].

Intra-tumor heterogeneity caused by clonal evolution is a major problem in cancer treatment. A subclone can be resistant to chemotherapy and occurs as the dominant clone at relapse. This adaptation, by changing the mutational profile, is a key factor for the survival and regrowth of leukemic cells [49]. In relapsing leukemia, two major paths of clonal evolution can be discerned. Either the dominant clone in the primary tumor acquires mutations and evolves into the relapsing clone or a minor subclone that survives therapy gains new mutations and expands, thus causing relapse [50]. These two major patterns of relapse can also be explained as leukemia relapsing from either rare LSCs or from larger subclones of immunophenotypically committed leukemia cells that retain stem cell-like properties [51]. The presence of genetically diverse LSCs is a limitation of therapies that target the dominant clone and not the subclones.

Clonal evolution and CH are intertwined processes with different possible scenarios in treated AML patients, summarized below according to Hasserjian *et al.* [52]:

1. Driver mutations at low molecular levels despite CR.
2. Persistence of the CH detected at diagnosis.

3. A new mutation implying CH, detected at low levels.
4. Persistence of one or several mutations despite CR with morphological and clinical evidence of another myeloid neoplasm.
5. Persistent germline mutation.
6. Relapse after CR with the disappearance of the mutations and the presence of the same mutations that were detected at diagnosis.
7. Relapse with the presence of some but not all mutations present at diagnosis and also with the acquisition of new mutations in the relapsed clone.
8. Diagnosis of a new AML with a genetic profile completely unrelated to the original AML.
9. Being in CR, emergence of CH of donor origin after allo-SCT.

Immunphenotypic shift

Leukemic blasts exhibit an immunophenotype that typically but not always differ from normal/benign blasts. Different subclones may gain a specific immunophenotype due to clonal evolution. Additionally, the immunophenotype is sometimes unstable following chemotherapy, thus resulting in a new phenotype (i.e. immunophenotypic shift) [53].

Using multicolor flow cytometry (MFC), different immunophenotypes of various leukemic subpopulations can be readily detected. This technique is commonly used in MRD analysis [54]. An advantage of MFC as an MRD method is that it can be used regardless of the cytogenetic and molecular genetic abnormalities present [55]. A drawback is the presence of immunophenotypic shifts that may hamper the identification of leukemic cells [53, 54].

Genetic alterations

Genetic alternations include chromosomal abnormalities and gene mutations.

Chromosomal abnormalities

Chromosome abnormalities, as the name reveals, affect chromosomes, most often during cell division. They can be either numeric (affecting the number of chromosomes) or structural. Structural abnormalities include translocations (one part of a chromosome moving to another chromosome), duplications (gain of additional sequence), deletions (a portion of a chromosome is lost), or inversions (rotated portion of a chromosome). Copy number variation (CNV) is defined as "a DNA segment of one kilobase (kb) or larger that is present at a variable copy number in comparison with a reference genome" [56, 57].

Chromosomal abnormalities can be either hereditary or acquired. Acquired or somatic clonal chromosomal abnormalities are observed in 50% of AML cases [26], and the frequency is higher in the elderly and in patients with secondary leukemia. Classic examples of chromosomal abnormalities in AML include the gene rearrangements *PML-RARA*, *CBFB-MYH11*, *RUNX1-RUNX1T1* and *BCR-ABL1*.

Gene mutations

A gene mutation is a shorter (compared to chromosomal abnormality) change in the DNA sequence. Mutations can be caused by environmental factors such as irradiation or can occur randomly during DNA replication. They can exist as point mutations (single base substitutions, i.e. single nucleotide variations [SNVs]), deletions or insertions. Similar to chromosome abnormalities, gene mutations can be either hereditary (germline) or acquired (somatic). Somatic mutations can occur at any time-point throughout life.

There are also variations in the DNA sequence, and these are known as single nucleotide polymorphisms (SNPs). These variations in the human genome are observed in at least 1% of the population. SNPs are responsible for traits such as eye color and account for the normal differences among individuals.

Mutational pattern in AML

AML possesses fewer mutations compared to those present in other extensively sequenced adult cancer types. In 2008, Ley *et al*. sequenced the first whole cancer genome that was derived from a cytogenetically normal AML [58]. The rapid development of high-throughput sequencing techniques during the last decade has provided new insights into the molecular landscape of myeloid neoplasms [59]. When we began to discuss this PhD program in 2013, little was known regarding the mutational basis of AML. This year, The Cancer Genome Atlas Research Network (TCGA) published the ground-breaking paper "Genomic and Epigenomic Landscapes of Adult *De Novo* Acute Myeloid Leukemia" that provided important insights into the mutational pattern of AML [26]. They generated a comprehensive catalogue of leukemia genes by sequencing 200 samples from patients with *de novo* AML. A total of 237 genes were mutated in two or more cases, and 23 were recurrently mutated at an average of five recurrently mutated genes/sample. The mutated genes were classified into one of nine functional categories, Table 2. Almost all samples possessed a mutated gene in one of the categories, and many samples had mutated genes in several categories. They also identified biological relationships among several of the genes and categories. For example, the significant co-occurrence among mutations in *FLT3*, *DNMT3*A and *NPM1* [26].

In 2016, Papaemmanuil *et al.* presented a new genomic AML classification [60]. They sequenced samples from 1,540 patients, and by adding cytogenetic profiling,

they identified patterns of co-mutations in the driver landscape of AML. These patterns separated the samples into 14 different genomic classes, thus reflecting the evolution of AML. The different subgroups were also relevant for prognostication and disease classification. Eleven of the classes were non-overlapping and expressed a distinct clinical phenotype and outcome, and three classes were heterogeneous (e.g., "AML with mutated chromatin, RNA-splicing genes, or both"). Some classes were similar to the TCGA categories (e.g., "AML with *NPM1* mutation") or combined several of the TCGA categories into broader groups. For example, DNA methylation, chromatin modifiers, spliceosome, and cohesin complex groups of TCGA categories fit into the group "AML with mutated chromatin, RNA-splicing genes, or both". Others were new, for example, "AML with no detected driver mutations", "AML with driver mutations but no detected class-defining lesions", and "AML with biallelic *CEBPA* mutations".

The different cohorts in this classification included patients aged ≤65 years. Therefore, the new knowledge is not necessarily relevant for the elderly. In 96% of the samples, at least one driver mutation could be identified, and in 86% of samples, two or more were observed [60]. The most frequent driver mutations were *FLT3* (33%), *NPM1* (27%), *DNMT3A* (23%) and *NRAS* (18%). Papaemmanuil *et al.* also

Table 2: Organization of mutations into categories of related genes with exemples of genes or fusions in each category. Adapted from Ley *et al.*, 2013 (TCGA) [26]. Samples from 200 adults with *de novo* AML. One sample can have several mutations (i.e. belong to several categories), and thus, the percentage exceeds 100%.

Mutational Cathegories [26]	% of cases	Example of genes / fusions
Transcription factor fusions	18%	*CBFB-MYH11* (inv16 / t16;16)
		RUNX1-RUNX1T1 (t8;21)
		PML-RARA (t15;17)
NPM1	27%	*NPM1*
Tumor suppressors	16%	*TP53*
		WT1
DNA Methylation	44%	*DNMT3A/B*
		IDH1/2
		TET2
Activated signaling	59%	*FLT3*
		KIT
		KRAS
		NRAS
Myeloid transcription factors	22%	*CEBPA*
		RUNX1
Chromatin-modifiers	30%	*ASXL1*
		EZH2
		KMT2A-fusions
Cohesin complex	13%	*STAG2*
		RAD21
Spliceosome	14%	*U2AF1*
		SRSF2

identified many new pairwise gene–gene correlations and found different patterns of co-mutations for certain genes. For example, *NPM1*-mutations are associated with *NRAS* G12/13 but not with *NRAS* Q61.

The proposed genomic classification of AML by Papaemmanuil *et al.* [60] was modified by the European LeukemiaNet (ELN) in their updated recommendations for AML in 2017 [17]. The slightly revised classification by ELN contains 16 molecular classes of AML compared to the 14 described by Papaemmanuil *et al.* New classes include rare balanced rearrangements such as t(9;22)(q34.1;q11.2), *BCR-ABL1*.

In 2016, a revised WHO classification was presented based on the numerous advances in the identification of acute leukemic and myeloid biomarkers with possible improvements in diagnosis and prognosis [8]. In the revised classification, new entities are introduced. For example, AML with mutated *NPM1* is now recognized as a distinct entity. The balanced cytogenetic abnormalities (e.g. AML with *BCR-ABL1*), that are not included as new entities, but as provisional entities, in the revised classification, are rare. Other changes include updated gene names e.g. the change from *MLL* to *KMT2A*. In this classification, mutations in *NPM1* triumphs the presence of multilineage dysplasia in patients without MDS-associated cytogenetic findings. Hence, these patients should be classified as possessing AML with mutated *NPM1* and not MDS. Similarly, concomitant *NPM1*-mutation and del(9q) should be classified as AML with mutated *NPM1* [8]. This is in contrast to most AMLs, where del(9q) is considered a myelodysplasia-associated abnormality.

Mutations in epigenetic regulators occur early in leukemogenesis as pre-leukemic mutations (CH), and they typically persist in remission [46, 61].

Mutations in chromatin altering genes such as DNA methylation genes and in genes involved in histone modification and chromatin looping (the so called "landscaping" genes), occur early in the evolution of AML as pre-leukemic mutations, while mutations in genes involved in proliferation tend to occur at later stages [61].

NPM1-mutations, the most frequent AML-defining molecular lesions, are associated with several other driver mutations [62] such as those in genes involved in DNA methylation (“CHIP-mutations”) (*DNMT3A*, *TET2*) and RNA splicing (*SRSF2*, *SF3B1*) or in tyrosine-kinase (*FLT3*) and RAS pathway genes or cohesin complex genes (*STAG2*). In as many as 75% of *NPM1*-mutated cases, a CHIP mutation can be identified [59]. Both the blast phenotype and the presence of co-mutations can impact disease biology and outcome in AML with mutated *NPM1* [63, 64].

The sequence by which mutations are acquired in *de novo* AML can be illustrated as a three-step genetic hierarchy that include CHIP-mutations, followed by mutations in *NPM1* or in a transcription factor gene (*RUNX1*, *CEBPA*, *GATA2*) and then a mutation in a signaling pathway gene (*FLT3*, *RAS*, *KIT*) [65]. Modern

technologies such as single-cell RNA-sequencing (RNA-seq) are very helpful for revealing AML hierarchies [66].

Some mutations in AML appear to be mutually exclusive, including *IDH1*/2-mutations and *TET2*-mutations, due to their similar roles in leukemogenesis [67]. Another example is *IDH* R172 mutations, an *IDH2*-variant that is mutually exclusive to *NPM1* [60].

Several review articles have summarized the knowledge regarding the clinical outcomes of different gene mutation combinations [64, 68, 69], including the adverse prognosis of patients with mutations in *NPM1*/*DNMT3A*/*FLT3*-ITD [60].

Some mutations are associated with clonal disorders in the elderly, and these include spliceosome gene mutations *SRSF2* and *SF3B1* [70]. They are rarely observed before the age of 70. Even though AML is predominantly a disease of the elderly, they are underrepresented or excluded in many clinical trials. Both the mutational pattern and the relevance of driver gene mutations may differ between older and younger patients [71, 72].

Recurrent genetic aberrations

NPM1 mutations

Epidemiology, discovery and classification

Among the most common recurrent mutations in AML are driver mutations in the Nucleophosmin1 (*NPM1*) gene. They are observed in approximately 30% of adult AML cases [73], and they are even more common (up to 45–64%) in the cytogenetically normal group [9, 73-75] and relatively uncommon in childhood AML [76]. *NPM1*-mutations are slightly more common among females, and all patients tend to be younger at diagnosis (median 65 years) compared to the age of patients diagnosed with other types of AML [63, 77]. Characteristic clinical features at diagnosis include higher numbers of BM blasts, white blood cells, and platelet counts (even though still thrombocytopenia) compared to those observed with other AML subtypes [9, 74, 75, 77-79].

The *NPM1* gene was first discovered in rats in 1973 and was initially named B23 [80]. *NPM1*-mutations were first described in a ground-breaking work by Falini *et al*. in 2005 [73], and their findings were soon confirmed by others [75, 77, 81, 82]. Prior to 2005, which was well before the NGS era, it was known that the *NPM* gene (or the *NPM1* gene) was involved in translocations of leukemias and lymphomas and that the NPM1 protein shuttled between the nucleus and the cytoplasm with a predominant localization to the nucleus. One possible explanation was that *NPM1*

had a tumor suppressor function. The Falini group was searching for novel *NPM1* rearrangements using immunohistochemistry. Surprisingly, of their 591 patients, 35% exhibited cytoplasmic staining. Reverse transcription quantitative PCR (RT-qPCR) and direct sequencing confirmed the presence of mutations primarily affecting exon 12 of the *NPM1* gene in these patients. Thus, *NPM1* mutations were discovered.

In the 2008 WHO classification of myeloid neoplasms, AML with mutated *NPM1* was included as a provisional entity; however, since 2016, it has been recognized as a distinct entity in the revised classification [7-9]. It is one of only two WHO leukemia entities defined by a single gene mutation, together with AML with a biallelic mutation in *CEBPA* [9].

NPM1 gene and protein structure and the different mutations

NPM1 encodes the nuclear protein nucleophosmin1 (NPM1). It shuttles between the nucleus and cytoplasm [83] but is preferably located in the nucleolus under normal conditions. Mutated NPM1, in contrast, is observed in the cytoplasm. The gene contains 12 exons [84] located on chromosome 5q35.1 encoding three major transcript variants/isoforms: NPM1.1 (NM_002520.6), NPM1.2 (NM_199185.3) and NPM1.3 (NM_1037738.2) [85, 86]. NPM1.1 contains 11 exons and 294 amino acids and is the longest and most prevalent variant [87-89] i.e. NPM1. The literature states that a little more than 50 different *NPM1* mutations have been described [78, 89]; however, many more are known. In paper II we used an in-house (Sahlgrenska/ Fogelstrand group) developed tool for deep sequencing 'NPM1 Deep Seq' https://github.com/ClinicalGenomicsGBG/NPM1_DeepSeq that includes a list of almost 90 variants, with new variants continuously being added.

NPM1 mutations almost always occur (>98%) [78] as a tetranucleotide (4 bp) insertion in the final exon, exon 12 [85, 90]. This exon is sometimes referred to as exon 11, as exon 10 of the *NPM1* gene is absent in the most biologically relevant transcript NPM1.1 [91]. The most common mutation is type A (c.863_864insTCTG), (technically a duplication c.860_863dup). This mutation constitutes 70-80% of all *NPM1* mutations. It is a duplication of the bases TCTG (thymine, cytosine, thymine and guanine) that creates an insertion at the W288 codon (tryptophan=Trp=W) [9, 73, 75, 77, 81, 82, 89]. Type A mutations are followed in frequency by type B (c.863_864insCATG) and type D (c.863_864insCCTG), where each constitutes approximately 5% of the mutations [9, 73, 75, 77, 81, 82, 90]. All other reported *NPM1* mutations are rare. The most common *NPM1*-mutations are typically referred to as type A, B and D and the remainder are referred to by their Human Genome Variation Society (HGVS) nomenclature.

The NPM1 protein (both wild-type [wt] and mutant) possesses several functional domains, including export and import signaling. NPM1 wt possesses three major

functional domains, including: 1) two nuclear export signals (NESs) at the N-terminus, 2) a nuclear localization signal (NLS) at the C-terminus with W288 and W290 and 3) other NLSs located between these domains. In the wt state, nuclear import predominates over nuclear export [92, 93]. Wt NPM1 and mutant NPM1 differ only in the very last portion of the C-terminus of the protein [73, 81, 94]. *NPM1* mutations introduce a new NES at the C-terminal and simultaneously replace one or both tryptophan residues (W288 and W290 or rarely W290 alone) that are critical for nucleolar localization [89, 95], thus resulting in delocalization to the cytoplasm. The complex nuclear-cytoplasmic shuttling of wt NPM1 is critical for most of its functions [83, 92] and distinct functional domains regulate this shuttling activity.

NPM1 - a multifunctional protein

The major functions of wt NPM1 are DNA replication, ribosome biogenesis, and cell cycle regulation. More specifically, these functions include the following:

- inhibition of centrosome duplication during the cell cycle, thus maintaining genomic stability
- cooperation in ribosome biogenesis, thus moderating cell growth and proliferation
- action as a histone chaperone and participating in nucleosome formation (histones are essential for chromatin function)
- involvement in genomic stability and DNA repair (DNA damage response)
- regulation of apoptosis
- binding to TP53 enhancing its stability and transcriptional activity
- modulation of growth suppressive pathways through ARF (alternate reading frame protein), (regulation of ARF-p53 tumor suppressor pathway) and
- participation in ribosomal ribonucleic acid (rRNA) 2'-O-methylation to maintain an optimal translational program [78, 85, 88-90, 96-98].

Despite the growing body of knowledge, more studies are needed to fully understand this multifunctional protein.

The role of NPM1 mutants in leukemogenesis

The role of mutated *NPM1* in leukemogenesis can partly be explained by the altered function of several nuclear proteins due to cytoplasmic delocalization. It is not only mutated NPM1 that is delocalized to the cytoplasm. Intriguingly, mutant NPM1 induces cytoplasmic delocalization of several other nuclear proteins, including ARF and APE1. These proteins are involved in apoptosis, DNA repair, and differentiation

[90]. The full extent of their interactions with NPM1 is uncertain; however, mutations in *NPM1* with subsequent cytoplasmic delocalization result in persistent expression of homeobox (*HOX*) genes, with an expression as high as that of HSCs. This expression does not decrease as observed in the differentiation of normal progenitors. The persistent expression of *HOX* is likely at least partly responsible for maintaining the undifferentiated state of leukemic cells [99-101].

The nuclear exporter Exportin-1 (XPO1) (also named CRM1) is responsible for cytoplasmic shuttling of proteins that contain a nuclear export signal, and as *NPM1* mutants contain this signal [85], XPO1 can shuttle mutated NPM1 to the cytoplasm. The XPO1 / *NPM1*mut interaction is essential for the maintenance of AML [100].

NPM1 and karyotype

NPM1 mutations are not detected in individuals with clonal hematopoiesis [70] and they are associated with a normal karyotype (in 85% of cases) [102], likely due to the ability of both wt *NPM1* and *NPM1* mutants to regulate centrosome duplication [103]. Approximately 15% of cases carry chromosomal aberrations, particularly +8 [102]. *NPM1* mutations are always heterozygous [73], and complete loss of *NPM1*wt is embryonically lethal [104].

NPM1 and immunophenotype

NPM1-mutations can occur in all types of AML, with the exception of APL with *PML-RARA*, thus implying variations in the immunophenotype of *NPM1*-mutated AML. An early study revealed a higher expression of monocytic differentiation–associated antigens and a lower expression of CD34 compared to those present in *NPM1* wt AML [81]. However, other studies have shown that *NPM1*-mutated AML can be subdivided both by morphology and immunophenotype into two major subgroups, myeloid and monocytic, both lacking expression of CD34 [105, 106]. The myeloid subgroup can be further subdivided; one lacking monocytic differentiation and one with neither CD34 nor HLA-DR expression [63]. The monocytic subgroup expresses monocytic markers such as CD64, CD14, CD11b and HLA-DR, while myeloid markers are not expressed in most cases. Lack of CD34 and HLA-DR in the myeloid subgroup may potentially mimic APL with *PML-RARA*. [107]. Different methods by which to discriminate between these two entities according to MFCs have been published [108]. The presence of some expression of CD15 or HLA-DR or the presence of a few monocytes would argue in favor of AML with mutated *NPM1*.

NPM1 mutated cases may contain CD34 positive cells in MFC. These cells have also been demonstrated to display *NPM1*-mutations [109].

Co-mutations and gene expression profile

NPM1-mutations frequently co-occur with mutations in *DNMT3A* (43%), *FLT3* (41%), *IDH1/2* (39%) and *TET2* (23%) [63]. However, *NPM1* mutations alone appear to be insufficient for leukemogenesis, thus implying molecular synergisms promoting AML development [33, 60, 110]. It is not fully understood if *NPM1*-mutations drive leukemia through loss of function, gain of function, or both [111]. They are most often secondary events, reflected by a higher VAF% of at least one co-mutation [62]. There is no strong correlation between *NPM1* VAF% and the percentage of blasts [112], which could have been assumed, as *NPM1* is a heterozygous mutation and AML with *NPM1*-mutations often lack copy-number aberrations. However, a high *NPM1* VAF% at diagnosis (≥ 44%) is associated with a poor clinical outcome independent of other prognostic markers such as clinical variables and co-mutations [113].

AML with mutated *NPM1* exhibits a unique gene expression profile compared to that of *NPM1*wt AML, with overexpressed *HOX* genes that are independent of other co-occurring mutations [82, 88]. *HOX*-genes are HSC-related genes that encode transcription factors.

Prognosis

Early, it was discovered that *NPM1*-mutations predicted a relatively favourable prognosis [77, 81]; however, the prognostic impact differs depending on the associated mutations. Mutant *NPM1* is only a favourable factor if *FLT3*-ITD is negative or if its allelic ratio is below 0.5, as shown in Table 1 [17]. However, different studies have provided contradictory results in older *NPM1*mut / *FLT3*-ITDwt patients. In a study from 2015, Ostronoff *et al*. revealed that patients with *NPM1* mutations only exhibited a relatively favourable prognosis if they were between 55 and 65 years of age at diagnosis and not if they were older than 65 years [114]. In contrast, Juliusson *et al*. presented data in 2020 where the favourable effect of *NPM1*mut / *FLT3*-ITDwt was more pronounced in older (60-74 years) patients than it was in younger patients [115]. A possible explanation for these discrepancies could be the use of different inclusion criteria.

Co-mutated *NPM1* and *NRAS* confer favourable prognosis [116]. The prognosis is much better than that of the *NPM1*mut / *FLT3*-ITDmut combination [60, 117]. The adverse effect of *FLT3*-ITD appears to be most clinically relevant in patients with concomitant *DNMT3A* and *NPM1*-mutations [60]. If an *NRAS*G12/13 mutation is identified instead of *FLT3*-ITD (*NPM1*mut / *DNMT3A*mut / *NRAS*G12/13mut), the prognosis is relatively good [60]. In 2017, Dovey *et al*. presented an explanation for the latter observation by comparing the effects of the two combinations on hematopoiesis and leukemogenesis in knock-in mice [117]. They found many shared features between the two groups, including *HOX* gene overexpression; however, they also observed differences such as an altered gene expression profile

in the *NPM1*mut / *FLT3*-ITDmut group compared to that of the *NPM1*mut / *NRAS*mut group and the wt progenitors.

CD34-/HLA-DR- cases often carry *TET2* or *IDH1/2*-mutations, and these patients appear to exhibit a significantly better survival than do those expressing CD34 and/or HLA-DR [63, 107].

With increasing knowledge, it is fair to conclude that AML with mutated *NPM1* is a diverse rather than a uniform group of diseases.

NPM1 and MRD

In the early 2000s, molecular MRD analysis of AML was largely restricted to those with fusion transcripts such as t(8;21), inv(16) / t(16;16) or t(15;17) [118]. Soon after the discovery of mutant *NPM1* in AML, it became clear that molecular MRD had a prognostic impact in this type of AML and that certain thresholds of *NPM1*-MRD could subdivide patients into different prognostic groups [119-122]. The level of *NPM1*-MRD was determined to be the most relevant prognostic factor after first-line treatment [122]. *NPM1* mutations are ideal targets for MRD, as they are relatively frequent and stable over the course of the disease and most often present at relapse. Additionally, they typically disappear with the achievement of remission and are not associated with clonal hematopoiesis. In the ELN guidelines, MRD monitoring in AML is recommended as part of the standard of care [41]. For AML with mutated *NPM1*, molecular MRD is recommended over the use of MFC, and reverse transcription quantitative PCR (RT-qPCR) is currently considered the gold standard.

Further aspects of MRD in AML, including *NPM1*-MRD, will be discussed in the MRD-paragraph below.

New therapeutic strategies

With increasing knowledge of the role for mutant *NPM1* in leukemogenesis, new therapeutic strategies have been uncovered that may improve treatment [88]. *NPM1* mutations are believed to be suitable candidates for targeted therapy, as they are often present in the entire leukemic population. XPO1 inhibitors exist but with low efficacy and with cytotoxic side effects [123]. Newer nuclear export inhibitors are currently under development.

By better understanding of the *NPM1-HOX* interactions, new therapeutic possibilities may evolve in the future [88]. Other new tools that can be used to manipulate mutated *NPM1* are yet to be discovered. The emergence of CRISPR technologies may also help to develop targeted therapies for AML with mutated *NPM1* [100].

FLT3 mutations

Fms-like tyrosine kinase 3, also known as the *fetal liver tyrosine kinase 2* (*FLT3*) gene, is located on chromosome 13 and is commonly mutated in AML [69]. Activating *FLT3*-mutations in AML consist of two types, either internal tandem duplications (ITD) or point mutations. ITDs are repeated sequences of variable length that range from three to several hundred nucleotides and often result in constitutive tyrosine kinase activation. Point mutations (single amino acid substitutions) occur in the tyrosine kinase domain (TKD) [23]. *FLT3* signaling promotes proliferation and inhibits apoptosis [23]. *FLT3*-ITDs are present in nearly one-third of AML patients [124] while *FLT3*-TKDs are less common (~10%) [125, 126]. ITDs are associated with adverse clinical outcomes; however, their prognostic impact depends upon the absence or presence of associated mutations such as *NPM1*, Table 1 [17, 124]. TKDs exhibit a different mechanism of receptor activation compared to that of ITDs and a different biology, and they are not associated with the same inferior prognosis as ITDs [125, 126]. In *FLT3*-ITD mutated cases, the allelic ratio (the ratio of ITD-mutated alleles to wt alleles) is also important. A high ratio (≥0.5) is associated with adverse outcomes [17, 127]. More recently, *FLT3*-mutations have been shown to be therapeutic targets through the use of tyrosine kinase inhibitors (TKIs) [128, 129]. TKIs can impair the constitutive proliferative signals induced by mutations that affect leukemia. However, resistance due to various cellular mechanisms, including *FLT3*-TKD mutations, is a major problem [130].

FLT3-ITD mutations are potential MRD markers, as although *FLT3*-ITD-mutated blasts are chemosensitive at diagnosis, they tend to return and cause relapse in a substantial number of patients, particularly in those who have not received allo-SCT [131]. However, in a considerable proportion of relapses, approximately 25% of patients, the mutation has been lost [131]. Hence, ELN recommends against the use of *FLT3* mutations for MRD analysis [41]. Other problems with the use of *FLT3*-mutations for MRD assessment are low sensitivity and the necessity to design patient-specific assays due to the different lengths of nucleotide insertions [131]. With the rapid development of new NGS techniques for MRD assessment, these issues may be resolved in the future.

DNMT3A mutations

Mutations in DNA methyltransferase 3α (*DNMT3A*) are observed in myeloid, lymphoid, and mixed hematological malignancies [132]. Approximately 20% of AML patients carry a mutation in the *DNMT3A* gene [133] and the mutation is even more common in cytogenetically normal AML cases. These mutations are the most frequent mutations in ARCH [35]. *DNMT3A*-mutations are also the most common co-mutations in *NPM1*-mutated AML [134]. The gene, located on chromosome 2,

generates an enzyme involved in DNA methylation (the addition of methyl groups to the DNA). DNA methylation is vital for gene expression regulation by chromatin modification and impinges on fundamental biological processes such as developmental patterning during embryogenesis [24, 133]. In HSCs, the methylation patterns generated by *DNMT3A* promote maturation (differentiation) into different blood cell types. Mutations in *DNMT3A* may alter DNA methylation, and this can play an important role in leukemogenesis. It has long been assumed that aberrant DNA methylation may be involved in cancer pathogenesis [133]. This is now well established and DNA methyltransferase inhibitors (e.g. azacitidine) are widely used to treat patients with AML and MDS. More recent data have increased the knowledge of gene regulation, and unsurprisingly, it is far more complex than first thought. Rather than working in isolation, DNA methylation is likely intertwined with other gene-silencing mechanisms [24].

In 2010, Ley *et al*. published the discovery of highly recurrent *DNMT3A* mutations in AML [133]. One of the most frequently mutated sites in *DNMT3A* is the arginine 882 (R882) hotspot, harboring 60% of the mutations [133] with the most common subtype presenting as R882H. When analysing *DNMT3A* mutations alone, there is no impact on prognosis, even when restricting the analysis to mutations at the R882 locus [135]. Neither in combination with *NPM1* mutations, do the *DNMT3A* mutations appear to affect prognosis, irrespective of the *DNMT3A* mutational subtype [135]. However, in combination with *FLT3*-ITD, *DNMT3A* mutations (especially the R882 mutation) appear to have a worse prognosis [60, 135]. In *NPM1*/*DNMT3A/FLT3*-ITD co-mutated cases, the presence of the R882 subtype mutation is associated with an inferior prognosis compared to that of the non-R882 mutated cases [135].

Interestingly, *NPM1*/*DNMT3A* mutated cases display a co-mutational pattern that is different from that of *NPM1*-mutated AML in the absence of *DNMT3A* mutation, thus suggesting that the former group may represent a distinct biological entity [135].

DNMT3A mutations (particularly the R882 subtype) are significantly more common in younger *NPM1*-mutated AML patients than they are in elderly patients [135]. This is interesting and contrasts to the age distribution of other CHIP-mutations such as *TET2* that are more often found in elderly with *NPM1*-mutated AML and indeed in the elderly population as a whole [135]. By comparing mutated allele ratios (VAF%), it has become clear that *DNMT3A*-mutations are often the first hit in leukemogenesis [62, 135]. However, most patients with CHIP, including those with *DNMT3A*-mutations, will likely never develop AML. Nevertheless, the risk is increased, and the loss of *DNMT3A* function in a murine model predisposed these mice to AML development [136].

As mutations in *DNMT3A* frequently persist in remission, they are not suitable targets for MRD [137].

Treatment

Early attempts to treat leukemia include arsenic, blood transfusions and X-rays. During the Second World War chemotherapy was introduced [2]. For the past five decades (since 1973), intensive combination chemotherapy with DA (i.e. daunorubicin and cytarabin) referred to as "the DA protocol", with some modifications of the variation in dose intensities, has been the mainstay of therapy [138, 139]. However, during the last five years, the AML treatment landscape has begun to change with the emergence of new targeted drugs [139, 140].

The main treatment options include intensive chemotherapy, low-intensity regimens and palliative treatment. The initial assessment evaluates if a patient can be considered for intensive induction chemotherapy or not. Age at diagnosis, poor performance status and significant comorbidities are all important factors for this decision and age alone should not be the decisive determinant to guide therapy [17].

Ideally, all patients should be included in a clinical trial if feasible.

Hyperleukocytosis (white blood count > 100×10^9/L) is a severe risk factor during induction therapy. In such cases, lowering the white blood count prior to the initiation of treatment should be considered [20].

Intensive chemotherapy

In Sweden, all patients up to the age of approximately 70 years and those between 70-80 years of age without severe comorbidity should be considered for intensive combination chemotherapy with DA with an aim of inducing complete remission (CR). The national Swedish guidelines recommend a maximum of four courses of DA [20]. The course(s) given to induce CR is called induction therapy and the subsequent courses are referred to as consolidation therapy.

For *FLT3*-mutated and CBF- (core binding factor) AML patients, adding a drug to the intensive chemotherapy should be considered [17]. In the case of an *FLT3*-mutation (both ITD and TKD mutations), therapy with protein kinase inhibitors such as midostaurin, is currently recommended in combination with intensive chemotherapy [20]. Gemtuzumab ozogamicin (GO), a CD33-directed antibody-drug conjugate, should be considered for CBF-AML, i.e. *de novo* AML with inv(16)(p13.1q22) / t(16;16)(p13.1;q22) or t(8;21)(q22;q22.1), in combination with intensive chemotherapy [20].

Low-intensity regimens including hypomethylating agents and venetoclax

DA may be poorly tolerated, with a risk of induction mortality in older patients and in those with severe comorbidities. Besides, the duration of the remission can be short, particularly for those with high-risk genetics. Consequently, these patients and those who reject intensive therapy are frequently offered low-intensity regimens

that primarily include hypomethylating drugs such as azacitidine. Azacitidine may also be considered as consolidation after induction with intensive chemotherapy, if the patient is unlikely to tolerate further intensive treatment [141].

More recently, the bcl2 inhibitor venetoclax (a mitochondrial inhibitor) has been shown in different combinations to increase the remission rate in this group that is not suited for high-dose regimens. Overall survival was longer and the remission rate was higher in patients who received azacitidine plus venetoclax than were these values in those who received azacitidine alone [142].

Palliative treatment

If the performance status, age and comorbidities make a patient unfit for intensive or alternative treatment, the choice is best supportive care. The treatment of unfit and elderly patients with AML is currently unsatisfactory. The ELN recommends these patients to be enrolled in clinical trials [17]. Several risk scoring systems exist and should be used to determine the choice of intensive or alternative treatment or best supportive care.

Allogenic stem cell transplantation (allo-SCT) and donor lymphocyte infusion (DLI)

Allogenic stem cell transplantation (allo-SCT) is used as consolidation therapy in remission [143]. Transplantation is the most effective consolidation regimen, and AML is the most frequent indication for allo-SCT [17]. Patients with high-risk genetics, and many with intermediate risk without *NPM1* mutation and without severe comorbidity, below the age of approximately 70 years, or with positive MRD are considered for allo-SCT as part of the consolidation. Additionally, there must be an available and suitable donor and the patient must accept a hard and long treatment with risk for treatment-related complications. That is why it is of utmost importance to discuss SCT at an early stage. With reduced-intensity conditioning (RIC) regimens, elderly (up to 75 years) patients can be considered for allo-SCT [17]. Allo-SCT is estimated to reduce the risk of leukemia relapse by approximately half; however, there is a significant risk for treatment-related complications, including death [20]. Therefore, the risk-benefit ratio must always be assessed. Allo-SCT is often recommended when the relapse incidence without transplantation is expected to be >35-40%. The higher the expected relapse risk, the higher is the acceptable risk of non-relapse mortality [17]. The donor may be an HLA-identical sibling, a matched unrelated donor (MUD) or a haploidentical donor.

Donor lymphocyte infusion (DLI) refers to the administration of lymphocytes from the SCT donor. This treatment is given to enhance a graft-versus-leukemia (GvL) immune response. For example, if a pending relapse is suspected or after cytoreductive treatment of a relapse, this treatment can be used. In the case of molecular relapse (MRD-detected without morphological relapse), DLI may prevent overt relapse.

Salvage treatment including SCT

Most patients achieve complete remission with current chemotherapeutic regimens, but unfortunately, many of them eventually relapse. Some patients have refractory disease [55, 119, 144-148]. Salvage chemotherapy regimens such as FLAG-Ida (fludarabine and idarubicin) are sometimes offered to patients with refractory or relapsing disease. Allo-SCT can be considered for patients in CR2 (i.e. a second CR after relapse) who have not been previously transplanted, particularly for those with low-risk cytogenetics [149] and occasionally for those with refractory disease. Re-transplantation may be considered for patients with good health status if a new CR has been achieved.

Novel therapies

As mentioned above, several new targeted drugs, including new combination regimens, have emerged in recent years. These targeted drugs can be used either as monotherapy or in new combination therapies. Novel therapies are typically first evaluated in older patients, in those not fit for standard intensive chemotherapy, or in those with relapsed or refractory disease [17].

Venetoclax plus low-dose cytarabine has been shown to be effective for AML patients who are unfit for intensive chemotherapy [150]. The latter combination can be provided for a considerable amount of time [151]. A recent study demonstrated promising results for venetoclax in combination with low-dose cytarabine or azacitidine to reduce the relapse risk in patients (age 25–81 years) with persistent or rising *NPM1*-mutated MRD [152]. The authors concluded that these combinations have the potential to replace salvage chemotherapy/SCT for this molecularly defined subgroup of patients.

In a recent publication, a study is presented showing promising results by combining venetoclax and intensive chemotherapy in patients above 60-65 years [153].

Patients with high-risk genetics may benefit from the combination of azacitidine and venetoclax (mentioned above as a low-intensity regimen alternative for those not fit for intensive chemotherapy) due to the lower remission rates with DA in this group compared to those with low- and intermediate-risk genetics [154].

For patients with AML and *IDH1/2* mutations, epigenetic modulators are available and approved by the FDA under certain circumstances. They act as inhibitors of IDH1 (ivosidenib) or IDH2 (enasidenib) [20].

Other new options, particularly for secondary acute myeloid leukemia (sAML), include an improved liposomal delivery of standard therapies such as a liposomal formulation of daunorubicin and cytarabine (CPX-351) [140, 155].

Both FLT3 and IDH1/2 inhibitors have been tried together with venetoclax, but also triplet combination regimens adding a hypomethylating backbone [139].

Post-consolidation immunotherapy with interleukin-2 and histamine dihydrochloride was thought to improve leukemia-free survival by activating the anti-leukemic functions of T-cells and natural killer (NK) cells, particularly in acute myelomonocytic leukemia and acute monoblastic and monocytic leukemia [156-159]. However, this regimen has little place in current treatment strategies.

Criteria for remission

The criteria for complete remission (CR) are (modified from the 2017 European LeukemiaNet (ELN) recommendations).

- bone marrow blasts <5%
- absence of blasts with Auer rods
- signs of regenerating hematopoiesis
- absence of extramedullary disease
- absence of circulating blasts
- absolute neutrophil count $\geq 1.0 \times 10^9$/L
- platelet count $\geq 100 \times 10^9$/L
- independence of red blood cell transfusions [17, 20, 118].

Relapse

Unfortunately, AML carries a poor prognosis and is associated with a high risk of relapse (>35%) [160, 161]. For relapsed AML, the prognosis is even worse, with an overall 1-year survival rate after the first relapse of 29% and 11% after 5 years. [162]. These numbers are affected by several factors such as age, treatment response, cytogenetics at diagnosis, and white blood count. Relapse after allo-SCT remains a major problem, with a 1-year post-relapse overall survival of ~20% [160]. The time from allo-SCT to relapse is important. Patients who relapse ≥6 months after allo-SCT exhibit better survival and may benefit from a second allo-SCT [160]. Additionally, age >40 years, an unrelated donor, adverse cytogenetics, and graft-versus-host disease (GvHD) at the time of relapse are all associated with worse survival [160, 163].

The causes of relapse can include insufficient eradication of neoplastic cells; however, subclones that are resistant to chemotherapy, clonal evolution including

immunophenotypic shift, or a new AML induced by therapy (tAML) may all also cause recurrence of the leukemia. This, in turn, may generate drawbacks for MRD assessment, particularly if only one method is used, as the re-emerging leukemic cells may escape detection.

Measurable Residual Disease (MRD)

The ability to detect residual disease below the resolution of conventional microscopy is an important tool for the risk classification of acute leukemia. Historically, remission or residual disease was simply determined by a 5% blast threshold after counting 500 nucleated cells in the microscope. Everyone familiar with BM microscopy knows that it can be difficult just by looking at a blast to determine if it is a malignant, leukemic blast, or a benign, regenerating blast. Additionally, the number of remaining blasts can be overwhelming even after the successful reduction of the leukemic cell burden by 95%. Considering the normal number of cells in the BM of an adult 70 kg individual (approximately 10^{12} cells) [164], the number of cells in a leukemic BM with increased cellularity is even higher. The number of remaining blasts at a 5% level after treatment will still be in the order of 10^{10} to 10^{11}. Thus, more sensitive methods are required to determine residual disease. Measurable residual disease (MRD), previously termed minimal residual disease, is defined as the number of leukemic cells remaining during or after treatment when the patient has reached complete morphological remission (CR) where the levels are too low to be detected by conventional microscopy. Several techniques have been evaluated; however, some were discarded due to their low sensitivity. For example, using FISH, it is difficult to increase the resolution below 0.3-5% [165]. In general, techniques possessing sensitivities at or below 10^{-3} are suitable for MRD determination [41]. Even with high-resolution techniques such as reverse transcription quantitative PCR (RT-qPCR) (down to 10^{-6}), it is important to note that MRD negativity is not equivalent to the absence of leukemic cells after therapy. Most patients achieve CR and molecular remission; however, numerous patients eventually relapse because of residual undetected disease [166].

The major MRD-related questions include what MRD targets to test for, what time-points to use, which thresholds are appropriate, what technique to use, and which tissue to examine [167].

Methods to determine MRD

Two different approaches are used for the detection of MRD in routine clinical diagnostic workups. The first and most common is multicolor flow cytometry (MFC), and it depends upon the immunophenotype of the leukemic cells. The other

approach for MRD assessment is examining target genetic markers of leukemia and it includes several molecular techniques such as RT-qPCR, quantitative PCR (qPCR), digital PCR and next-generation sequencing (NGS)-based technologies.

For accurate MRD assessment of RT-qPCR and MFC, the diagnostic sample should ideally be analysed at the same laboratory with the same method, since the diagnostic information in most cases is used as reference. For the DNA-based methods it is sufficient to have an NGS-analysis performed at diagnosis to be able to perform an MRD-analysis.

The first consensus guidelines for MRD analyses in AML were published in 2018 by the European LeukemiaNet (ELN) [41]. Based on these guidelines, it is recommended that patients with mutant *NPM1*, *RUNX1-RUNX1T1*, *CBFB-MYH11*, or *PML-RARA* should be monitored using molecular techniques i.e. RT-qPCR and the remainder of the patients should be monitored with MFC [41]. However, as molecular markers can be identified in almost all cases [26] and due to the rapid development of ddPCR and high-throughput sequencing techniques, it is likely that we will have new recommendations for the use of genomic DNA in the future.

Multicolor flow cytometry (MFC)

Normal differentiation of BM cells, including blasts, results in reproducible patterns of antigen expression [121]. MFC can be used for MRD assessment in ~90% of AML patients, as most leukemic blasts display an aberrant phenotype that differs from that of normal blasts [17, 168]. Two alternative approaches have been used in MFC-MRD diagnostics: the different-from-normal (DfN) and the leukemia-associated immunophenotype (LAIP) [41, 169].

Different types of aberrancies include antigen overexpression, lack of antigen expression and asynchronous expression. An LAIP may for example consist of CD45 (a pan-leukocyte marker), a blast marker (CD34, CD117, CD133), a myeloid antigen (CD33, CD13) and aberrantly expressed markers [170]. The LAIP(s) are defined at diagnosis and tracked in the follow-up samples. The sensitivity depends upon the aberrant phenotype of the leukemic blasts, but also on the presence of normal BM blasts with the specific leukemic phenotype (the “background noise”) [55]. Another approach to analyse MRD by MFC is the different-from-normal approach (DfN) that is independent of the diagnostic phenotype. With this approach, aberrant populations are sought in follow-up samples without specific reference to the LAIP.

The major drawback of using MFC for the determination of MRD in AML is the limited sensitivity of this method, with approximately 0.1% leukemic cells in many cases [55]. A potential way to improve sensitivity, and hence the prognostic value, could be to add monitoring of leukemic stem cells (LSCs). This would offer an opportunity to monitor patients who lack aberrant phenotypes suitable for standard MFC-MRD investigation [171].

The specific immunophenotypic aberrancies of leukemic blasts can change during treatment. At least one antigen is altered in up to 90% of AML patients [55, 172], a phenomenon known as immunophenotypic shift, and this may lead to the escape from detection by MFC-MRD. This is a potential drawback. Another limitation is that there are some elements of subjectivity in the assessment that require experienced and skilled staff for accurate analysis. ELN recommends that MFC-MRD should be performed in experienced laboratories until further standardization is performed [17].

Molecular techniques

Molecular MRD assessments are either PCR- or sequencing-based approaches using DNA or complementary DNA (cDNA) from RNA. In general, molecular techniques are more sensitive than are MFC [54], and RT-qPCR is considered the gold standard among molecular techniques [41]. However, a major limitation is that the use of RT-qPCR is restricted to a subset of patients displaying a specific genetic marker (fusion genes or *NPM1* mutation). In younger AML patients (0-60 years), ∼60% possess a trackable molecular marker that is suitable for RT-qPCR assays compared to ∼30% in patients that are older than 60 [17, 54]. Any MRD platform should be able to detect leukemic cells down to a level of at least 10^{-3} (0.1%), which is equivalent to one mutated cell among 1000 normal cells [41].

RT-qPCR

Molecular MRD for AML patients was first used for fusion transcript quantification by RT-qPCR, including *RUNX1-RUNX1T1*, *CBFB-MYH11* and *PML-RARA* transcripts [64, 173, 174]. The sensitivity of RT-qPCR is often very high; however, it depends upon the level of expression of the molecular target [54]. For example, *KMT2A-MLLT3* assays exhibit low sensitivity (10^{-3}) in contrast to the high sensitivity of *NPM1* assays (10^{-6} or below) [17]. In addition to analysing fusion gene transcripts, RT-qPCR can also be used to monitor the expression of somatic mutations, for example those in *NPM1* or in aberrantly expressed genes such as *WT1* [41]. However, the latter is not recommended by ELN due to its low sensitivity [41]. Another advantage of RT-qPCR in addition to its high sensitivity is the inherent false negative control that is performed through the independent amplification of a housekeeping gene such as *ABL1* [54]. In addition to the sensitivity, the kinetics of MRD response to therapy differ among markers, where for example a slower reduction of *RUNX1-RUNX1T1* compared to that of the *NPM1* transcript levels has been observed [17, 122, 134, 175-182].

Due to its high sensitivity compared to that of MFC, RT-qPCR is currently considered the gold standard for MRD of AML with mutated *NPM1* [41]. Other reasons for *NPM1* mutations being particularly suitable as leukemia-specific MRD targets are that they are common (∼30%), typically occur *de novo*, are present in the whole leukemic cell population, and are often stable over the course of the disease [183].

DNA-based techniques

Molecular MRD can also be determined by monitoring mutated DNA using various techniques, including qPCR, droplet digital PCR (ddPCR) and deep sequencing (deep seq), offering several advantages. This is particularly true for *NPM1*-mutations, scrutinized in this thesis. The false-positive error rate generated from sequencing is lower for indel mutations such as *NPM1* than it is for substitutions (SNVs), thus making them suitable targets for sequencing-based MRD [184]. In general, with DNA-based methods MRD analysis is not restricted to genes targetable by RT-qPCR, such as highly expressed genes or fusion transcripts, and the results are not affected by variations in gene expression. qPCR based on genomic DNA is highly sensitive [119, 185, 186] and neither ddPCR nor deep seq require standard curves or reference genes [187, 188]. Additionally, DNA protocols are more flexible than are RNA protocols due to the inherent instability of RNA.

With WGS, WES or current standard myeloid panels of recurrently mutated genes, sequencing can identify at least one molecular mutation in more than 90% of adult AML patients at diagnosis [26, 60, 64]. In addition, most AML patients harbor several mutations that can be employed as putative MRD targets (CHIP-mutations excluded, as they often persist in remission) [41, 64]. Both NGS and ddPCR can simultaneously detect several mutations. Multi-target MRD approaches, either using ddPCR or NGS, have been tested and seem to be able to predict prognosis [64]. NGS possesses limited sensitivity using standard platforms; however, modern technology has found several ways to overcome this issue through the use of for example computational error-correction, utilization of unique molecular indexes (UMIs) and targeted deep sequencing [189, 190]. With these new techniques, it is possible for NGS to achieve a sensitivity comparable to that of qPCR [191]. Thus, NGS-based MRD is emerging and appears to be widely applicable to patients with AML [192].

Novel techniques

Targeted RNA-seq possesses a high multiplexing capacity and the ability to identify novel fusion breakpoints or insertions and is also a potential technique for MRD determination [193]. High-throughput single-cell sequencing (SCS) is another novel technique that can evaluate the clonal dynamics of AML with implications for the risk of relapse. Decreased clonal diversity at CR is associated with longer relapse-free survival [194]. Although NGS is not a standard method for AML-MRD today, simultaneous DNA and RNA sequencing can be used together for full coverage of both fusion and non-fusion mutations in future MRD assessments [189].

The prognostic and predictive role of MRD

MRD is used both to assess treatment response and to monitor remission status after therapy to detect early signs of relapse [17]. Different MRD thresholds at different time-points may have variable meanings in different patient risk groups [41, 195].

A recent meta-analysis including 11,151 patients revealed that the achievement of MRD negativity is associated with superior disease-free survival and overall survival in patients with AML regardless of age, AML subtypes, time of MRD assessment, specimen source and MRD detection methods [196].

Time-points for MRD assessment with RT-qPCR

The optimal time-point for MRD assessment depends upon the type of leukemia, thus reflecting its inherent biology and disease kinetics. For example, for *RUNX1-RUNX1T1* leukemias, a later time-point than that for *NPM1*-mutated leukemias may be more prognostically relevant to assess treatment response [134, 182, 197]. In addition, the molecular relapse kinetics may differ among specific types of leukemia, where for example a significantly longer median doubling time of the *CBFB-MYH11* leukemic clone may be observed compared to that of clones harboring *RUNX1-RUNX1T1*, *PML-RARA* or *NPM1* [198]. A reasonable strategy in everyday practice and in clinical trials could be to use the same checkpoints for all AML patients. Ideally, the timing and number of these checkpoints should aim to cover the kinetics of all AML subtypes.

For molecular MRD assessment, ELN recommends the following time-points as a minimum [41]:

- After the second standard course of chemotherapy (induction/ consolidation) (BM and PB*)
- After consolidation therapy (end of treatment) (BM and PB*)
- Before allo-HSCT, after the last consolidation, and not earlier than four weeks before conditioning treatment** (BM and PB*)
- After the end of treatment, including post-allo-SCT, every third month during the first two years*** (BM and PB*)

*Both BM and PB samples, as PB may provide better prognostic stratification [41]. **The treatment given before SCT. ***The relapse risk is highest during the first two years after treatment, hence the recommendation of follow-up for two years after the end of treatment [199].

Time-points for MRD assessment with MFC

For MFC-MRD, the same time-points are recommended to assess the treatment response. BM-samples are recommended over PB due to their higher sensitivity. To avoid hemodilution, ELN strongly recommends that the first BM pull should be submitted for MRD analysis [41]. The optimal time-points for MFC-MRD during follow-up are unknown. Therefore, a pragmatic approach could be to use the same MRD time-points for all types of AML [197].

MRD thresholds with RT-qPCR

For molecular MRD, different expression thresholds have been suggested to define prognostic groups during follow-up and after treatment [122, 134, 175-177, 200]. In general, BM-MRD cut-off levels are 1 $\log_{10}$ higher than those in PB, thus reflecting the higher sensitivity in BM samples [176, 200]. It is not necessarily measurable leukemic disease (truly MRD-positive) versus unmeasurable leukemic disease (truly MRD-negative) that provides the best prognostic cut-off. In other words, a low level of residual disease may be more favourable than are higher levels. To account for this possibility, the MRD positive group is sometimes subdivided into "MRD high" and "MRD low" both in BM and PB samples. The prognostic impact of detectable and undetectable thresholds has been studied in both PB and BM [134, 175, 176].

Different cut-offs or thresholds have been studied for different molecular targets. For example, for *NPM1*-mutations in BM samples, a less than 3 $\log_{10}$ reduction of *NPM1* transcripts as compared to the diagnostic level and/or a failure to reduce *NPM1* mutated transcript levels to $<200/10^4$ *ABL* copies after two cycles of chemotherapy identify patients at high risk of relapse [175, 177]. In PB, the persistence of *NPM1* mutated transcripts after two courses of cytoreductive therapy has long been known to be an independent prognostic factor for death [134]. Balsat *et al*. have showed that achieving a 4 $\log_{10}$ reduction in *NPM1*-mutant transcripts in PB compared to the value at the baseline (diagnostic) level was the best cut-off to predict relapse and overall survival after induction therapy [200].

For AML with *RUNX1-RUNX1T1*, a 3 $\log_{10}$ reduction at remission (after induction or after first consolidation) of the *RUNX1-RUNX1T1* transcript level in the BM is a strong predictor of relapse [179, 181, 201]. The achievement of <0.001% *RUNX1-RUNX1T1* / *ABL1* transcripts in PB after the end of consolidation therapy has been demonstrated to be predictive of a lower likelihood of subsequent relapse [202]. A more recent study revealed that achievement of MRD negativity at the end of treatment in both BM and PB was an independent and favourable prognostic factor for both relapse and overall survival [203]. This investigation confirmed the reduced risk of relapse after achievement of >3 $\log_{10}$ reduction after the second treatment cycle. Yin *et al*. have shown that >500 *RUNX1-RUNX1T1* transcripts in BM or >100 transcripts in PB during follow-up is associated with a 100% relapse rate [181].

For AML with *CBFB-MYH11,* >10 *CBFB-MYH11* copies / 10^5 *ABL* copies in PB at remission/after the first course of induction chemotherapy was the most useful prognostic variable for relapse risk according to a multivariate analysis [181]. The same threshold was the most significant prognostic factor after the third course. For BM samples, no log reduction threshold for relapse risk could be established. After consolidation chemotherapy and during follow-up, MRD thresholds associated with a 100% relapse risk were >50 *CBFB-MYH11* copies / 10^5 *ABL* copies for BM samples and >10 copies for PB samples [181].

After treatment, a patient with complete morphological remission can be either MRD-positive (molecular persistence at low copy numbers) or MRD-negative (complete molecular remission), Figure 3. The MRD-negative group may develop molecular relapse and the MRD-positive group may develop molecular progression. To detect early relapse, the MRD should be monitored continuously. A persistent high-level positivity or a rising level of transcripts is a strong sign of approaching relapse [17, 41, 54, 197]. The definitions for molecular MRD according to ELN are [41]:

- Complete molecular remission (CR_{MRD^-}). A patient in complete morphological remission (CR) with two successive MRD-negative samples obtained within an interval of ≥ four weeks.
- Molecular persistence at low copy numbers. A patient in CR with positive MRD at low copy numbers <1% to 2% of the target to reference gene [175, 176] or increase <1 log, between any two positive samples.
- Molecular progression. A patient with molecular persistence at low copy numbers with an increase in MRD copy numbers ≥1 $\log_{10}$ between any two positive samples.
- Molecular relapse. A previously MRD-negative patient with an increase in MRD level of ≥1 $\log_{10}$ between two positive samples.

ELN recommends that clinicians intervene if transcript levels rise by >1$\log_{10}$ [41], thus defining molecular relapse or molecular progression (even if the patient can still be in CR), Figure 3.

MRD thresholds with MFC

For MFC-MRD, ELN recommends a cut-off of 0.1% to distinguish MRD-positive from MRD-"negative" BM samples [41]. This threshold often coincides with the sensitivity level. However, with an informative LAIP, the limit of detection may be below 0.1%. In such cases, MRD levels should be reported. MRD-positive patients below 0.1% may have a lower risk of relapse than those with MRD >0.1% [204]. PB samples are not recommended for MFC-MRD.

Material for MRD

Both PB and BM samples are used at different thresholds, for different time-points and for different types of leukemia. For certain time-points and for some subtypes, either PB or BM may be the most useful prognostic variable for relapse risk.

BM aspiration may not be representative of the whole body due to the heterogeneous distribution of AML cells. A variation in skill levels in BM aspiration is also a problem that might contribute to false-negative results. Using PB instead of BM for quantifying MRD may provide a strategy to address these limitations [205, 206]. However, further studies are needed to confirm this hypothesis.

The main criticism of PB MRD is that it possesses a lower sensitivity than that of BM MRD [176]. However, the specificity of PB can be higher due to the lower frequency of background noise caused by normal myeloid progenitors [207]. Another advantage is that the sampling procedure with PB results in less discomfort to the patient.

The fact that some studies have demonstrated a good correlation between PB and BM MRD and others not, was discussed in a recent paper [167]. As a consequence, particularly for MFC-MRD, the use of PB is not currently recommended [41].

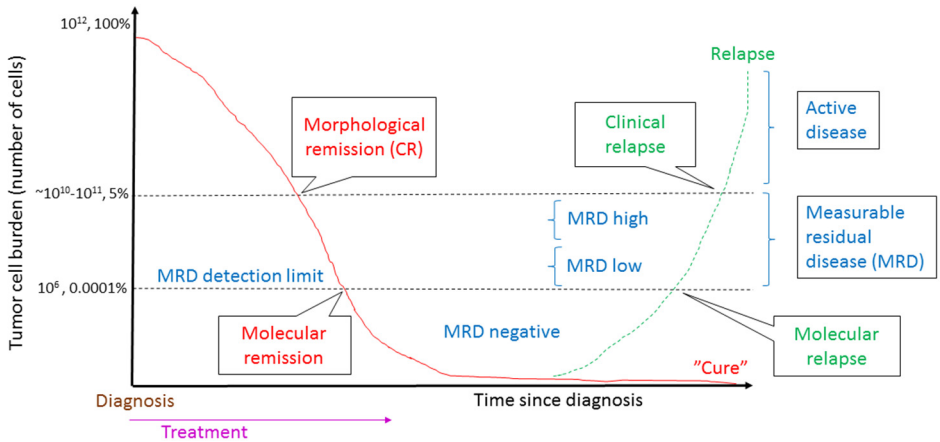


Figure 3. A schematic diagram of tumor cell burden (number of leukemic cells) compared to the values for different molecular Measurable Residual Disease (MRD) thresholds. The figure illustrates the number of leukemic cells at the different remission thresholds (y-axis). A patient in complete morphological remission (CR) may still have as many as ~10^{10}-10^{11} leukemic cells, thus highlighting the need for submicroscopic MRD analyses. A patient in molecular remission and regarded as truly MRD-negative can still have as many as 10^{6} leukemic cells left in the BM. Note the huge difference in leukemic burden between the different remission thresholds.

MRD and NPM1

The importance of MRD for prognostication of AML with mutated *NPM1* has been demonstrated in numerous studies, most of which employ RT-qPCR as the MRD method [119, 122, 176, 177, 200, 208, 209]. In 2011, Kronke *et al*. showed that the level of *NPM1*-mutated transcripts after the end of treatment (intensive chemotherapy or SCT) is an independent prognostic factor with a four-year overall survival of 80% for the MRD-negative group compared to 44% for the MRD-positive group using the cut-off >2% *NPM1*-mutated/*ABL1* for MRD positivity [175]. Although cDNA has been most commonly used for *NPM1*-MRD, several studies (qPCR, NGS and ddPCR) have been performed using DNA [119, 187, 210-216], including the study by Gorello *et al*. as early as in 2006, where assays for both cDNA and DNA were presented [120].

In an exhaustive review from 2018, Forghieri *et al*. summarized the results of published studies investigating the clinical relevance of MRD in patients with AML with mutated *NPM1* [217]. Transcripts should be monitored in both the PB and BM to improve risk assessment. If MRD in PB remains negative but is positive in BM after the end of treatment, transcripts should be monitored every 4 weeks for at least 3 months to detect any increase in MRD [41].

Although *NPM1* is considered a stable marker for MRD, it is important to remember that approximately 10% of AML with mutated *NPM1* relapse as *NPM1* wt leukemia [79, 175]. The relapse of AML with mutated *NPM1* occurs earlier with *NPM1* mutated relapse than it does with *NPM1* wt relapse (14 vs. 43 months) [79].

Precision medicine

In 2015, US President Barack Obama launched a precision medicine initiative that aimed to accelerate progress toward a new era of individualized therapy [218]. Precision medicine is personalised treatment based on individual patient-specific characteristics [219], i.e. precision medicine is care for patients based on their genetic aberrations applicable to groups of patients, while personalised medicine is an individualized treatment for the unique patient.

Morphology will, at least for many years to come, be the forefront of hematological diagnostics, as it is both rapid and cost-effective. When complemented with immunophenotyping, the minimal turnaround time for a preliminary diagnosis is only a few hours. Molecular characterization often requires a longer time depending on the technique used; however, it is likely to become more rapid. When new specific treatments are entering the therapeutic arsenal as alternatives to classical induction with cytotoxic drugs, early molecular characterization and information on gene mutations for targetable disease mechanisms are becoming increasingly important for individualized treatment decisions [17]. Conventional cytogenetic analysis (CCA) (performed by chromosome banding of metaphases) is necessary for genetic characterization, is quite time-consuming [220] and is often complemented by FISH or PCR for specific translocations. During the last ten years, DNA sequencing (myeloid gene panels or WES) for recurrent mutations has become mandatory as a complement to CCA and FISH/PCR for correct disease classification [17]. However, CCA is already challenged by NGS-based approaches, including whole genome sequencing (WGS) and whole transcriptome sequencing (WTS), i.e. RNA-seq, for accurate prognostication [221, 222]. These NGS-based techniques possess the potential to display the full spectrum of molecular aberrations in AML. The combination of WGS and WTS may identify new aberrations with diagnostic, prognostic, and therapeutic relevance. These advanced sequencing approaches will likely be used in the clinical routine in many countries

in the not so distant future as a source of information for individualized treatment of AML patients. Current challenges include high costs and long turnaround times.

Additionally, further research examining the epigenome could potentially lead to improved classification of AML [223]. Gene expression profiles (GEPs) have been shown to serve as potential prognostic markers [224] and can predict drug response [225]. Both GEP and DNA methylation analyses may provide complementary diagnostic and prognostic information in the future [223].

The present investigation

Aims

The overall aim of this thesis was to implement and compare new methods for MRD detection and to explore patterns of clonal evolution in AML with a special focus on *NPM1* mutations.

The more specific aims for each paper were to:

I. Establish a new DNA-based MRD method for *NPM1* type A mutation detection in AML.

II. Compare the gold standard RT-qPCR method for *NPM1*-MRD detection in AML to three different DNA-based methods to determine how the DNA-based methods can be used for quantification of clinically relevant molecular MRD.

III. Investigate if AML relapses can be identified and predicted by targeting several mutations using droplet digital PCR, thereby producing information on multiple putative subclones.

IV. Explore the mutational spectrum of *NPM1*-mutated AML in patients older than 75 years and compare it to that of younger patients.

Methods

Ethics approval statement

The studies were approved by the Regional Ethical Review Board at Lund University (diary number 2014/505 and diary number 2017/850) and for study II also by the Regional Ethical Review Board at the University of Gothenburg (diary number 2017/1138).

Patients and cell samples

Samples from adult AML patients from the southern healthcare region were included in all papers I-IV, starting from July 2012 to June 2019. Paper I also included 12 *NPM1* type A positive BM samples from the University Hospital Brno, Brno, Czech Republic. In paper II, the majority of patients came from the western healthcare region from 2013 to 2018.

In paper I, *NPM1* type A positive patients were selected. Paper II included patients with type A, B and DD5 mutations and paper IV patients with different types of *NPM1* mutations. In paper III, two groups of non-relapsing and relapsing AML patients were selected regardless of *NPM1* mutational status.

In paper III, only BM samples were analysed, while all other papers included both PB and BM samples. Papers I-III contained both diagnostic and follow-up samples, while paper IV only contained diagnostic samples.

Multicolor flow cytometry (MFC)

Immunophenotypic data were acquired using a Navios flow cytometer (Beckman Coulter, Brea, CA) on erythrocyte-lysed whole blood or BM samples in heparinized tubes according to routine clinical protocols at the Department of Pathology in Lund. A broad panel of antibody combinations for AML was used. Analyses were performed using the Kaluza software (Beckman Coulter). For MRD monitoring, leukemia-associated immunophenotypes (LAIPs) were identified in the diagnostic

samples by identifying and gating leukemic blasts with aberrant phenotypes. An example of the gating strategy is shown in Figure 4.

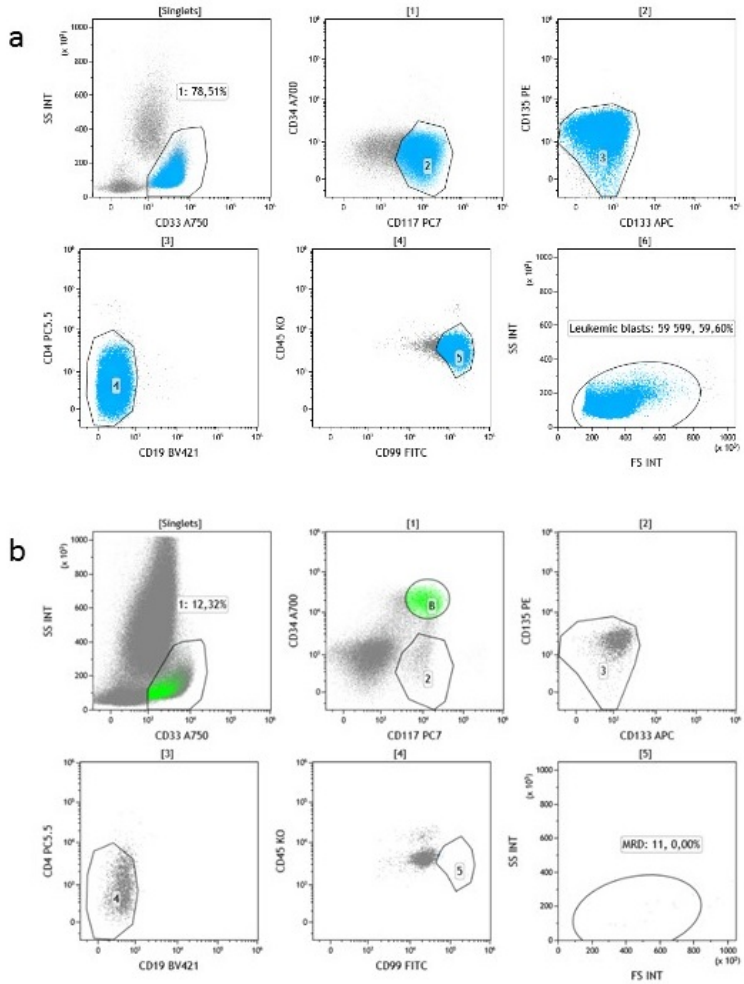


Figure 4. MRD-analysis by MFC on a bone marrow (BM) aspirate from a patient suffering from AML with mutated *NPM1*.
a) Diagnostic BM with sequential gating on the leukemic blasts revealing 60% myeloid blasts with a characteristic LAIP i.e. CD34-/CD117+/CD33+/CD135dim/CD99++. 100.000 events (cells) were collected.
b) Follow-up sample after the first course of treatment with the same gating strategy revealing virtual disappearance of the leukemic blasts (<0.1%). There are 11 events (blue) in the MRD gate, bottom right, disqualifying for measurable disease (possible random events). Note the normal regenerating blasts CD34+/CD117+/CD33+ (green). 500.000 events (cells) were collected.
SS, side scatter; FS, forward scatter.

At least 500,000 cells were analysed for each MRD sample. A minimum of 20 cells were required to qualify as a leukemic cluster, i.e. LAIP [226]. The sensitivity level (0.01% - 1%) varied depending on the phenotypic aberrancies of leukemic blasts compared to the phenotype of normal or regenerating BM cells.

Quantitative PCR (qPCR)

In paper I, real-time quantitative PCR is abbreviated as RQ-PCR. In the following papers and in this thesis, the abbreviation qPCR is used instead to distinguish it from RT-qPCR. qPCR is also a more commonly used term.

To detect *NPM1* mutations, the method described by Thiede *et al.* was used [227]. Patient DNA from the diagnostic sample was analysed using *NPM1*-specific capillary electrophoresis to distinguish the wt allele from the mutated allele according to size, Figure 5. *NPM1*-mutation positive samples were selected, and in the following PCR a clamping strategy was used to block amplification of the wt allele [227]. The PCR product was purified by agarose gel electrophoresis and gel extraction prior to Sanger sequencing.

This first screening and classification step is now replaced by NGS. Currently, panel sequencing using NGS is performed on most diagnostic AML samples in Sweden. The myeloid panel is nationally designed (Genomic Medicine Sweden) and includes the *NPM1* gene.

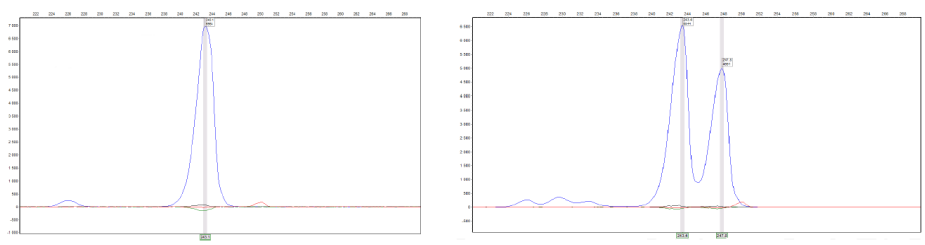


Figure 5. Capillary electrophoresis. The *NPM1* wild-type (wt) allele is distinguished from the mutated allele according to size. **A,** Amplicons of the wt allele and **B,** amplicons with both the wt allele (leftmost) and the mutated allele (rightmost) showing the typical four base pair (bp) insertion.

For MRD assessment in follow-up samples, a specific *NPM1* type A qPCR was designed (paper I) partially based on the methods described by Chou *et al.* [119] and Gorello *et al.* [120]. A type A mutation-specific reverse primer was used, resulting in amplification of the type A mutant allele alone with no amplification of the wt allele or other types of *NPM1* mutations. All reactions (25µl) were performed

in duplicates. A volume of 5µl of template with a concentration of (50-)100ng/µL was used, thus providing a DNA input of (250-)500ng. Prior to MRD analysis, an exact quantification of the sample was performed by qPCR amplification of the *albumin* gene.

For quantification of *NPM1* type A gene mutations, a standard curve was generated from a dilution series of DNA extracted from the *NPM1* type A mutated cell line OCI-AML3 (Leibniz Institute DSMZ, no.: ACC 582, Braunschweig, Germany) (4 × dilutions except for the first step [2,5 ×]), Figure 6A-C. A total concentration of 100 ng/µL of DNA for each dilution step was achieved by dilution in DNA (100 ng/µL) from a wt *NPM1* leukemic cell line (NB4, Leibniz Institute DSMZ, no.: ACC 207). To monitor non-specific amplification, a negative control (*NPM1* type A wt-cell line NB4) and a non-template control (water) were included.

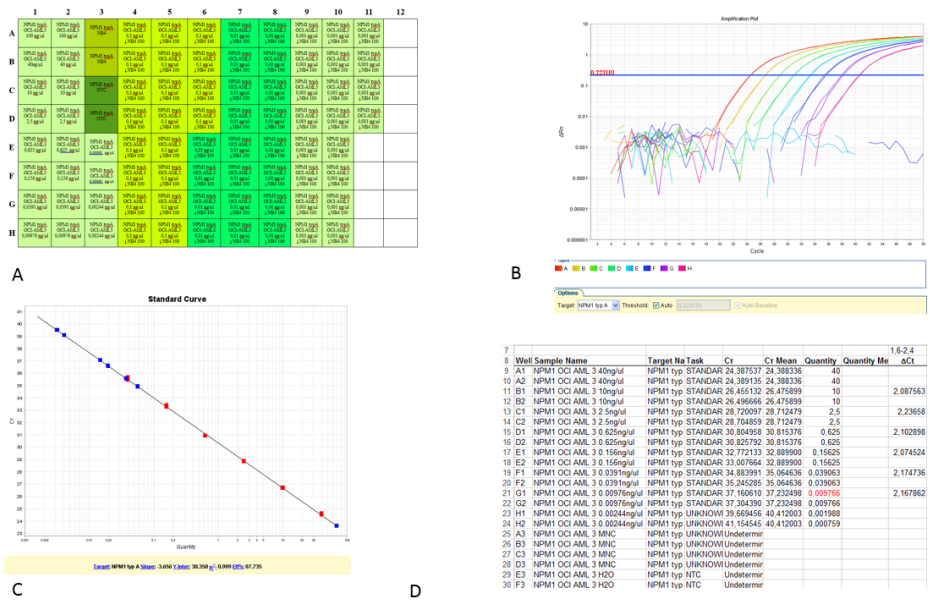


Figure 6. A, Sample plate from a limit of detection (LoD) experiment. **B,** Amplification plot. Representative amplification curves of serial dilutions of the *NPM1* type A mutation positive cell line OCI-AML3 in duplicates, 40ng/µL; leftmost - 0,0098ng/µL; rightmost. **C,** Standard curve with serial dilution of OCI-AML3 (red dots) and patient samples (blue dots). **D,** Example of data sheet underlying the determination of the reproducible sensitivity.

To standardize the quantification of AMLs with *NPM1* type A mutation and to determine the reproducible sensitivity of each qPCR, we adhered to the criteria previously defined for MRD analysis of acute lymphatic leukemia (ALL) by van der Velden [228] and for *NPM1*-mutated AML by Gorello [120]. These criteria use the amplification curves, Figure 6B, by identifying the first ΔCt between two consecutive dilutions that possess a value outside the interval of 1.6-2.4. The

expected theoretical value is 2 with a deviation of ± 20%. The highest concentration of these two consecutive dilutions is defined as the reproducible sensitivity, given that this and previous dilutions showed a ΔCt for duplicates ≤ 1.5. Other criteria include the standard curve, where the slope must be in the interval -3.0 to -3.9, the $R^2 \geq 0.98$ and both samples in a duplicate must be positive for the sample to be considered as positive, Figure 6C.

For clinical use, reproducible sensitivity is used as a cut-off to define quantifiable samples. Samples above the cut-off are reported with a numeric value, while samples below the cut-off are regarded as positive but not quantifiable and are not assigned an exact numeric value, Figure 6D. The cut-off for reproducible sensitivity depends upon both the sample curves and the standard curve, and may thus vary between runs. In paper I, an inter-experimental variation in reproducible sensitivity between 0.00061 and 0.16% (ng/µL), i.e. 10^{-3}-10^{-5}, leukemic DNA between the different runs was observed. For the limit of detection (LoD), LoD_{50} was used, i.e. the DNA concentration at which 50% of the samples were positive. LoD_{50} was 0.001% leukemic DNA. No false positives were observed neither in the experiment to determine the specificity of the assay (20 wt AML samples in duplicates), nor in the negative control *NPM1* wt samples included in each PCR run.

Samples were reported as the percentage of residual mutated leukemic DNA (MRD%). The MRD% is derived from the *NPM1* and *albumin* PCR standard curves, respectively, using the formula: MRD% = 100 × (concentration of *NPM1* type A mutation-positive DNA / total concentration of DNA).

In Lund, heparinized tubes were up to recently used for sample collection for MFC and DNA/RNA isolation. As observed by us and by others, heparin in the tubes inhibits the PCR reaction, thus leading to potential underestimation of the MRD levels [229]. To investigate possible inhibitory factors, we performed several experiments (unpublished) comparing: 1) samples collected in EDTA versus heparin, 2) DNA purification methods (column-based versus non-column-based), 3) different polymerases such as Immolase, a more specific enzyme than the usual TaqMan polymerase and 4) a higher concentration of magnesium in the reaction than the usual concentration. The inhibition observed by heparin was virtually completely alleviated by the addition of bovine serum albumin (BSA), regardless of the DNA purification method, magnesium concentration or type of polymerase. The degree of inhibition without BSA was highly sample dependent. The BSA concentration (0.32 mg/mL) was chosen according to van der Velden [230].

Reverse transcription quantitative PCR (RT-qPCR)

Reverse transcription quantitative polymerase chain reaction (RT-qPCR) for the *NPM1* type A mutation in paper II was performed according to Kronke *et al.* [175] at Sahlgrenska University Hospital, Gothenburg, as part of the clinical routine.

RT-qPCR is used when the starting material is RNA, and this method measures gene expression rather than the fraction of mutated cells or the variant allele frequency (VAF) as obtained by DNA methods.

After lysing the erythrocytes, total RNA is isolated and transcribed into complementary DNA (cDNA) using reverse transcriptase. Thereafter, qPCR for the *NPM1* type A mutation and the housekeeping gene *ABL1* are performed. The result obtained is the number of transcripts of mutated *NPM1* divided by the number of *ABL1* transcripts and is expressed as %. For follow-up samples, if possible, the result is also calculated as MRD in relation to the diagnostic level. Therefore, the diagnostic sample must be analysed. The formula for MRD is the follow-up sample *NPM1/ABL1* (%) divided by the diagnostic *NPM1/ABL1* (%).

For optimal analysis of all types of *NPM1* transcripts, a specific assay is required for each type of mutation. Currently, analysis of type A transcripts is available at Sahlgrenska University Hospital, using a type A mutation-specific reverse primer. The LoD is 15 *NPM1* type A transcripts in at least two of the three replicates based on the mean+3SD of 25 *NPM1* wt-samples.

RT-qPCR is the gold standard method for *NPM1* MRD based on its high sensitivity and its common use in large clinical trials [41, 120, 122, 134, 175-177].

Droplet digital PCR (ddPCR)

Droplet digital PCR (ddPCR) combines conventional quantitative PCR, compartmentalization into miniature droplets and combinatorial mathematics and statistics. After mixing the reagents for the PCR reaction, a “droplet generator” is used to generate droplets using oil to divide the sample into many thousands of nanoliter-sized droplets (up to 20 000 droplets/tube). The generation of droplets randomly divides the reaction components, including primers, probes, and DNA (both mutant and wt), into spheres, Figure 7. A PCR or similar thermocycling reaction is performed, which generates fluorescent signals inside each droplet depending on the presence or absence of the target(s) of interest. The droplets are then “read” by a droplet reader that measures the fluorescent intensities of each droplet in the fluorescent channels being used (all studies herein were performed using a 2-color system). The method is called “digital”, as each droplet is scored as either 0 or 1 (negative or positive) for each color channel depending on the intensity

meeting a threshold as shown in Figure 8. As the method is based on mathematical calculation assuming a Poisson distribution of mutant and wt DNA, one can utilize the counts of negative and positive droplets to mathematically calculate the number of target molecules that were present in the initial input sample. For the mathematical calculation to work, one requirement is that for each color channel, at least one droplet must be empty (no target DNA inside). If this is met, then across a rather wide range of input concentrations, highly accurate molecular counts can be achieved. The 95% confidence intervals for quantification are quite narrow until there are very few negative droplets or if there are too many droplets containing both mutant and wt DNA, Figure 7. Together, this ddPCR approach allows for accurate calculation, using Poisson statistics, of the copy number concentration of mutant and wt alleles for the selected locus, and the VAF% can thus be obtained.

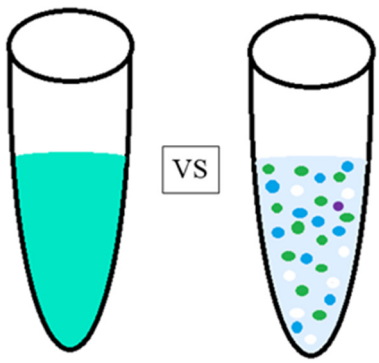


Figure 7. Illustration of the difference between conventional PCR (left), where there is one PCR reaction per tube (a single compartment) and ddPCR (right), where the PCR has been partitioned into thousands of compartmentalized reactions (one per droplet) in one tube. In the ddPCR tube, the four different colors illustrate droplets containing wild-type (wt) DNA (blue), mutated DNA (white), both mutant and wt DNA (purple) or empty droplets (green).

Calculation of copy number concentration of the mutant and the wt alleles:

$$C_{V_i} = \frac{-\ln(1-\frac{P}{T})}{V_d} \times \frac{V_r}{V_i}$$

where C_{V_i} is the copy number concentration of the target (mutant or wt allele) in the input BM DNA sample (copy/ng), P is the number of positive droplets for the target, T is the number of total droplets analysed, V_d is the volume of a droplet (0.85×10^3 µL), V_r is the total volume of a ddPCR reaction (20 µL) and V_i is the input volume per ddPCR reaction of the BM DNA sample.

Calculation of the variant allele frequency (VAF):

$$VAF = \frac{{C_{V_i}}^{Variant}}{{C_{V_i}}^{Variant} + {C_{V_i}}^{Wild-type}} \times 100\%$$

One advantage of using ddPCR for MRD is that ddPCR does not require standard curves or reference genes. ddPCR has been tested for *NPM1*-MRD by us and by others [188, 231, 232].

In papers II and III, an optimized and improved version of ddPCR was used (IBSAFE®) developed by the Saal group at Medicon Village, Lund, and is now commercialized by SAGA Diagnostics AB. An issue with conventional ddPCR is that there can be false-positive droplets caused by polymerase error and other sources of error that hinder sensitivity. IBSAFE® is an improved assay design, chemistry, and thermocycling, which improves the effective lower LoD down to approximately 0.001-0.003% VAF based on the amount of DNA analysed per sample [188, 233-235], and this is approximately 100-fold lower than that of conventional ddPCR. Each IBSAFE® assay was tested with zero false-positive droplets using 60 ng of *NPM1* wt-control DNA run in triplicate. IBSAFE® was performed using the Bio-Rad QX Droplet Digital PCR System (droplet generator and droplet reader) (Hercules, CA).

Briefly, IBSAFE® minimizes the polymerase base misincorporation error, which is a main source of false-positive signals that affects all PCR-based technologies, by applying an alternative two-stage thermocycling chemistry including an enriching first linear stage where one of the primers is reversibly suppressed. With this first linear amplification stage, even if polymerase misincorporation events occur, the ratio of true to false positive signals within each droplet will significantly increase, and this in turn enables a higher sensitivity. In the following exponential amplification stage, the previously temporarily inhibited primer is activated. After the two-stage PCR the droplets are read in the droplet reader. The mutant and wt probes possess different fluorchromes to allow for interpretation as shown in Figure 8.

In paper III, mutations were selected (SNVs and small indels) from WES data with priority for: 1) mutations in genes known to be recurrently mutated in AML according to The Cancer Genome Atlas Research Network (TCGA) [26] and 2) mutations present at both diagnosis and relapse. For a small number of patients, certain mutations present only at relapse were chosen to backtrack possible emerging clones. The probe and primer designs and the validation were performed using the diagnostic sample as a positive control, or in a small number of cases, the relapse sample. All accepted assays were confirmed to possess zero false positive droplets and were analysed in triplicate using wt control DNA as shown in Figure 8B. In paper II, assays for *NPM1* mutations type A, B and DD5 were used.

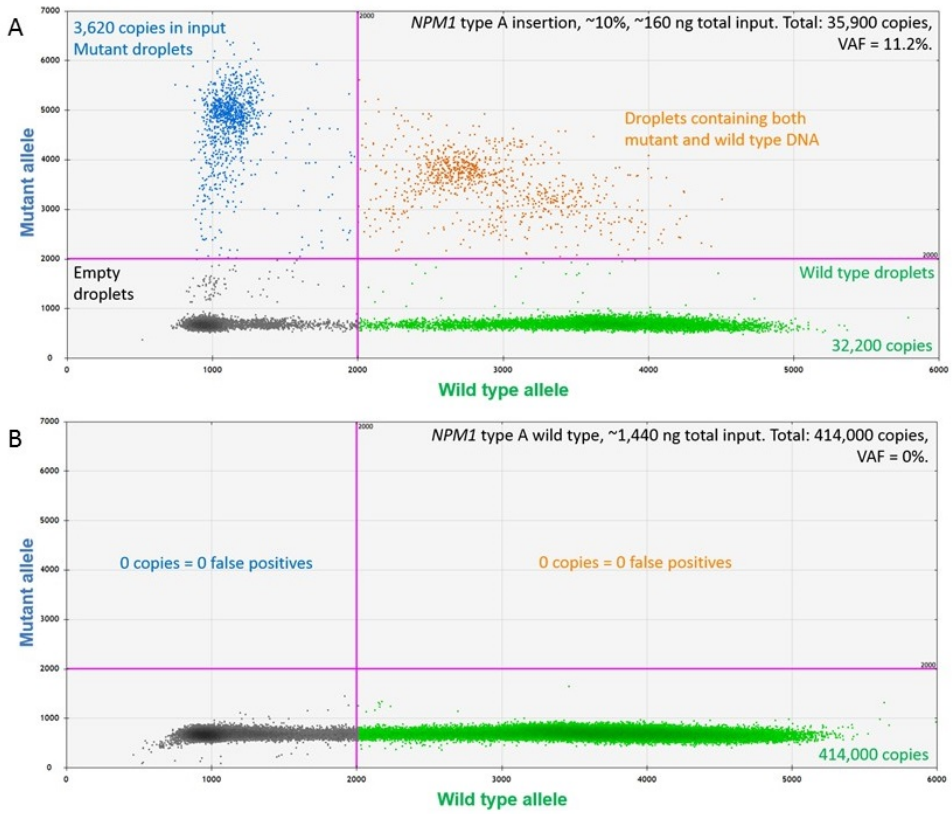


Figure 8. Two examples of IBSAFE® results. Droplets containing mutant DNA are presented in the upper left (blue dots), droplets containing wild-type (wt) DNA are at the bottom right (green dots), droplets containing both mutant and wt DNA are at the top right (orange dots) and empty droplets (black dots) are at the bottom left. **A,** A sample from a dilution series of the *NPM1* type A mutation. The VAF by ddPCR was 11.2%. **B,** A negative control sample (wt) analysed with the same *NPM1* type A mutation assay generating zero false positive droplets. Adapted with permission from originals by Yilun Chen and Lao H Saal.

Next generation sequencing (NGS)

Next generation sequencing (NGS), also known as massive parallel sequencing (MPS), is a sequencing technology that has revolutionized the field in recent years and has replaced Sanger sequencing. Using this technique, either genes or part(s) of genes in differently sized panels, whole exomes (whole exome sequencing [WES]), the whole genome (whole genome sequencing [WGS]) or different sized portions of the transcriptome (transcriptome sequencing), i.e. RNA-sequencing (RNA-seq) can be sequenced. The exome is a part of our DNA (genome) that remains within the mature RNA after RNA splicing and is also known as the transcribed portion. The exome harbors most of the mutations that cause disease and constitutes

approximately 1% of the genome. The transcriptome is the transcribed RNA within a cell type, and it changes from cell-type to cell-type based on the function of the cells. Not the whole exome is transcribed and translated into proteins in every cell. This makes the transcriptome different from the exome, as the latter is constant in diverse cells. The depth (or coverage) is the number of reads in a sequence. Various platforms using different techniques from different producers are available.

For MRD purposes, the sensitivity must be much higher than that of standard NGS. This can be solved in several ways, such as using error-correcting bioinformatics (algorithms) and/or unique molecular indexes (UMIs) [236, 237]. Sequencing only a small portion of the genome (parts of a gene or genes), and thus generating a large number of reads for this/these gene(s) (deep sequencing [deep seq]), is another solution to achieve high sensitivity [184, 189, 190, 213, 238-241]. One of the advantages of deep seq as an MRD-method for *NPM1* mutations is that it covers all known *NPM1* mutations without the need for mutation-specific primers or probes. Insertions such as *NPM1* are particularly suitable for NGS MRD as the false-positive error rate from sequencing is lower than that for substitutions (single nucleotide variations [SNVs]) [184]. Another advantage is that NGS does not require standard curves or reference genes for quantification.

The sequencing (paired-end sequencing) described in this thesis was performed using Illumina platforms (Illumina, San Diego, CA) both in Lund and Gothenburg. It was performed according to sequencing by synthesis. Briefly, the DNA of interest was flanked by adaptor oligonucleotides that bind to the flow cell. DNA was replicated to form clusters of identical strands. During sequencing, each cluster emits a specific fluorescent signal for each nucleotide incorporated, and the signal is then read. Sequencing was repeated for the reverse strand. The acquired data were aligned to the human reference genome prior to analysis.

The deep seq described in paper II was performed at the Sahlgrenska Academy, Gothenburg. All known insertions in exon 12 of *NPM1* were included [215]. The in-house developed script included approximately 90 *NPM1* variants and was updated in December 2020. The mean coverage for MRD analyses with this assay was approximately 1×10^6, and all samples yielded at least $>7 \times 10^5$ reads [187]. For a sample to be defined as positive, eight mutated reads were required (based on simultaneous sequencing of >20 wt samples,) and this corresponded to a LoD of approximately 0.001% VAF. The virus genome PhiX was used to increase library diversity to facilitate sequencing and to avoid misreading. Additionally, low diversity was used with a cluster density of approximately 600 to facilitate the deep sequencing.

In paper III, the mutational profile of each leukemia was determined at diagnosis and at the first relapse by WES at the Department of Clinical Genetics, Lund, by the group led by Prof. Fioretos. Cultured skin fibroblasts were used as germline controls

to filter out individual variations to identify somatic variants only [242]. The cut-off for somatic variants was (3-)5% VAF.

In paper IV, the diagnostic samples were sequenced using the Illumina Trusight Myeloid Sequencing Panel (Illumina, San Diego, CA) that included 54 genes either at the Center for Translational Genomics (CTG) at Lund University (LU) or the Center for Molecular Diagnostics (CMD) at the Skåne Regional Laboratories. All samples were bioinformatically analysed by our research group without germline control. The cut-off for somatic variants was 5% VAF.

Results

Paper I

Improved Minimal Residual Disease Detection by Targeted Quantitative Polymerase Chain Reaction in Nucleophosmin 1 Type a Mutated Acute Myeloid Leukemia

Multicolor flow Cytometry (MFC) is an established and rather sensitive method for MRD detection applicable on the majority of AML patients. When this paper was published in 2016, MFC was the recommended MRD technique for most AML patients including the *NPM1* mutated. However, there are certain disadvantages with MFC-MRD, including a limited sensitivity. Therefore, we decided to validate and refine a qPCR-based protocol for quantification of the *NPM1* type A mutation and simultaneously compare the two methods. Earlier qPCR-protocols for *NPM1*-mutations suffer from clear definitions of quantifiability, why we sought to define it in a reproducible and standardised manner. For accurate comparison, the experiments were performed on the same 45 BM follow-up specimens from 15 patients with both MFC and qPCR to determine the relative strength of each method.

All patients displayed at least one leukemia-associated immunophenotype (LAIP). For each LAIP the fraction of cells in normal and regenerating marrows carrying that LAIP was determined and was seen to reside between 0.001% and 1.0%. The reproducible sensitivity of MFC varied depending on the LAIP ranging from 10^{-4} - 10^{-2} (0.01% to 1%). The sensitivity level was at least 10^{-3} for 86% of the patients. Experiments for precision, repeatability and linearity proved them to be precise and repeatable with a coefficient of variation (CV) ranging from 3% at the 1% dilution to approximately 20% at the 0.001% level. Good linearity was shown down to the reproducible sensitivity level.

For reproducible sensitivity of our qPCR assay, indicating the DNA-concentration down to which the sample can be precisely quantified, we adhered to criteria defined for ALL [228]. An inter-experimental variation for the reproducible sensitivity between 10^{-3}-10^{-5} was seen. To determine limit of detection (LoD), serial dilutions (20 samples of each dilution) of the *NPM1* type A mutation positive cell-line OCI-AML3 were analysed. Our results showed that LoD was close to 10^{-5}. To test specificity, we analysed 20 *NPM1* type A wt patients samples and in addition all negative control samples from each performed experiment. Specific amplification

was never seen. The precision was determined from the LoD experiment and defined as two standard deviations from the mean. The precision depended on the concentration, with better precision at higher concentrations. To investigate repeatability, we analysed samples at two different occasions and looked at the differences between the two measurements. The agreement between these measurements was good. For reproducibility of our qPCR assay an exchange of samples with an external laboratory was organized. Despite different conditions, assays and experimental approaches, good agreement between the independent measurements was seen with a wider range in the follow-up samples as compared to the diagnostic samples.

In 32 of the 45 follow-up samples (71%), MRD was detectable by qPCR compared to 2 samples by MFC (4%), a statistically significant difference ($p<0.0001$). Fifteen of the 32 detectable samples by qPCR (33%) were also quantifiable, meaning that they were above the reproducible sensitivity level in their respective PCR runs. Thus, qPCR was more sensitive than MFC and detected residual leukemia in a significantly higher number of patient samples.

Paper II

Comparison of RNA- and DNA-based Methods for Measurable Residual Disease Analysis in NPM1-mutated Acute Myeloid Leukemia

In the consensus document written by the ELN working party from 2018 [41], molecular MRD is recommended over the use of MFC for *NPM1*-mutated AML. RT-qPCR, based on analysis of cDNA, is the method of choice due to its high sensitivity and frequent use in large clinical studies. However, DNA-based methods do have some advantages, and since we were aware of the power of both qPCR (paper I), ddPCR (paper III) and deep seq [187], we wanted to compare all these DNA-based methods with RT-qPCR.

First, we compared the detectability of the *NPM1* type A mutation with the DNA-based methods versus RT-qPCR in both BM (n=67) and PB (n=43) MRD samples. A statistically significant correlation was seen between RT-qPCR and the different DNA methods with the highest correlation for qPCR, followed by ddPCR and deep seq, Table 3. In BM, ddPCR and deep seq detected leukemic signals with very high specificity and PPV (100%), while qPCR showed superior diagnostic accuracy in PB, Table 3. The DNA-based methods failed to detect residual disease in some samples where it was found by RT-qPCR. The false negative rate depended on the method and the type of specimen (PB or BM) and was in the range 8-16% for qPCR, 29-31% for ddPCR and 39-40% for deep seq, Table 3. However, also RT-qPCR failed to detect transcripts in as many as 10% of the samples detected by any of the DNA-based methods. This finding is useful and novel information. All three DNA-

based methods were validated in the present and previous studies with no false positives. The RNA-quality was double checked in these samples with a high level of *ABL* transcripts, reflecting adequate RNA quality.

Next, the performance of the different DNA-based methods was compared with excellent agreement with respect to detectability of *NPM1*-mutated DNA.

Last, since there is no single threshold that is the most predictive of relapsing leukemia, we set out to identify thresholds for the DNA-based methods in BM samples using already established cut-offs for RT-qPCR as gold standard. Both the cut-off less than 3 $\log_{10}$ reduction of *NPM1* mutated transcripts compared to the diagnostic level [177], and the cut-off >200 mutant *NPM1* copies/10^4 *ABL* copies [175] were applied, because they classified our BM samples in exactly the same way. ROC analyses were performed to find optimal thresholds for the DNA methods. The cut-off 0.1% proved optimal for qPCR, aiming at a high specificity to avoid possible risks of overtreatment if risk stratifying with the DNA-based methods. Since ddPCR and deep seq measure not only the mutant allele but also the wt allele, and the *NPM1*-mutation is heterozygous, 0.05% VAF was chosen as cut-off for these methods. With these thresholds, the positive predictive value (PPV) was very high, but also the negative predictive value (NPV), well above 90%, Table 3. At the expense of the chosen high specificities, the sensitivities were lower, especially for qPCR. Here, the lower sensitivity was influenced by a few detectable samples just below the chosen cut-off level. Even though these cut-offs (0.1% leukemic cells for qPCR and 0.05% VAF for ddPCR and deep-seq) showed high specificity, PPV and NPV, and a sensitivity between 73-87%, their prognostic relevance will need to be tested in future clinical trials.

In conclusion, in this paper we have shown that DNA-based MRD techniques can complement RT-qPCR and add important information for MRD assessment. By using DNA, some samples with residual leukemic transcripts will be missed but interestingly it is also the other way around. We demonstrated that as many as 10% of samples with undetectable residual disease by RT-qPCR had detectable mutations by the DNA-based techniques.

Table 3. MRD results and diagnostic accuracy of the different DNA-based MRD methods for quantification of the *NPM1* type A mutation with respect to 1) the RT-qPCR cut-off 2% mutant *NPM1*/*ABL* transcripts or 3 $\log_{10}$ reduction in BM samples as compared to the diagnostic level and 2) the RT-qPCR cut-off detectable transcripts in both BM and PB samples.

DNA-based MRD method / cut-off	Above cut-off for RT-qPCR and DNA-based method / Above cut-off for RT-qPCR and below for DNA-based method / Below cut-off for RT-qPCR and above for DNA-based method / Below cut-off for both RT-qPCR and DNA-based method / False negative rate (%)	Sensitivity / PPA (%)	Specificity / NPA (%)	PPV (%)	NPV (%)
BM (RT-qPCR cut-off 2% transcripts or 3 $\log_{10}$ reduction)					
qPCR cut-off 0.1% (95% CI)	11 / 4 / 1 / 51 / 26.7	73.3 (48.1 - 89.1)	98.1 (89.9 - 99.7)	91.7 (64.6 - 98.5)	92.7 (82.7 - 97.1)
ddPCR cut-off 0.05% (95% CI)	12 / 3 / 0 / 52 / 20.0	80.0 (54.8 - 93.0)	100 (93.1 - 100)	100 (75.8 - 100)	94.5 (85.2 - 98.1)
Deep seq cut-off 0.05% (95% CI)	13 / 2 / 0 / 52 / 13.3	86.7 (62.1 - 96.3)	100 (93.1 - 100)	100 (77.2 - 100)	96.3 (87.5 - 99.0)
BM (RT-qPCR cut-off detectable)					
qPCR cut-off detectable (95% CI)	38 / 7 / 4 / 18 / 15.6	84.4 (71.2 - 92.3)	81.8 (61.5 - 92.7)	90.5 (77.9 - 96.2)	72.0 (52.4 - 85.7)
ddPCR cut-off detectable (95% CI)	32 / 13 / 0 / 22 / 28.9	71.1 (56.6 - 82.3)	100 (93.1 - 100)	100 (89.3 - 100)	62.9 (46.3 - 76.8)
Deep seq cut-off detectable (95% CI)	27 /18 / 0 / 22 / 40.0	60.0 (45.5 - 73.0)	100 (93.1 - 100)	100 (87.6 - 100)	55.0 (39.8 - 69.3)
PB (RT-qPCR cut-off detectable)					
qPCR cut-off detectable (95% CI)	12 / 1 / 1 / 29 / 7.7	92.3 (66.7 - 98.6)	96.7 (83.3 - 99.4)	92.3 (66.7 - 98.6)	96.7 (83.3 - 99.4)
ddPCR cut-off detectable (95% CI)	9 / 4 / 1* / 29 / 30.8	69.2 (42.4 - 87.3)	96.7 (83.3 - 99.4)	90.0 (59.6 - 98.2)	87.9 (72.7 - 95.1)
Deep seq cut-off detectable (95% CI)	8 / 5 / 1* / 29 / 38.5	61.5 (35.5 - 82.3)	96.7 (83.3 - 99.4)	88.9 (56.5 - 98.0)	85.3 (69.9 - 93.6)

*Same sample. BM, bone marrow; CI, confidence interval; NPA, negative percent agreement; NPV, negative predictive value; PB, peripheral blood; PPA, positive percent agreement; PPV, positive predictive value.

Paper III

Subclonal patterns in follow-up of acute myeloid leukemia combining whole exome sequencing and ultrasensitive IBSAFE digital droplet analysis

The knowledge of the mutational content from the diagnostic NGS can be used to choose several mutations as MRD markers. These mutations can be followed during treatment and follow-up by various methods including ddPCR. In this study we applied the recently developed improved ddPCR-assay, IBSAFE, to identify and try to predict relapses by producing information on multiple subclones. IBSAFE offers high sensitivity due to a two-phase chemistry which enhances true-positive signals and simultaneously reduces false-positives.

Ten relapsing and four non-relapsing patients, regardless of *NPM1* status at diagnosis, were selected. The mutational profile of each leukemia was determined at diagnosis and at first relapse by WES. A total of 86 mutations (single nucleotide variations (SNVs) and small indels (like *NPM1*) were selected from the WES data with priority towards mutations in recurrently mutated genes, CHIP mutations included. This selection generated between 5-9 mutations per patient.

For all relapsing patients, molecular evidence of disease was detected prior to the clinical relapse. At least one follow-up time-point before the relapse displayed at least one mutation with VAF >0.1%.

Three distinct patterns could be discerned for the relapsing patients: 1, All followed mutations reappearing at relapse (regardless of the number of subclones). 2, Some of the followed mutations are lost at relapse with the relapsing leukemia carrying only some of the followed mutations, reflecting evolution of subclones. 3, Two or more of the mutations persisting during chemotherapy despite complete remission, i.e. persistent clonal haematopoiesis.

For the non-relapsing patients two patients had persisting mutations before SCT. For one of these patients only *DNMT3A* at 0.01% VAF was detected at the last follow-up time-point after SCT. For the other no mutation was detected after SCT. For the two non-relapsing patients without SCT, the mutations gradually declined and either disappeared or stabilized at low VAF levels below 0.08%.

As in paper I, a comparison between a molecular method, here ddPCR, and MFC was performed. For most time-points assessed by ddPCR, MFC was also performed, either as a part of the clinical routine or for research purposes (with data acquisition at diagnosis or follow-up with the analysis performed at a later time-point). ddPCR was shown to be a more sensitive method for MRD compared to MFC with no time-point being positive by MFC and negative by ddPCR. Concomitant VAF >0.1% and

positive MFC-MRD (>0.1%) were seen in three patients at a few time-points. The combination of detectable leukemia by ddPCR and MFC-negativity was common, even after exclusion of *DNMT3A* and *TET2*-mutations.

For the *NPM1* type A mutated patients a comparison was performed between ddPCR and qPCR. This was a rather small survey preceding the larger one in paper II. In both studies, qPCR detected low amounts of residual disease undetected by ddPCR in a few samples.

Furthermore, our results showed the limitation of using *NPM1*-mutations as sole marker for MRD, with two patients being negative by ddPCR before the relapse, one of which even was *NPM1*-negative.

In summary, this proof-of-concept study demonstrates the ability of our multiplexing ddPCR-strategy to identify molecular-MRD prior to relapse, at a higher sensitivity compared to MFC, and to generate information on multiple subclones. Besides, ddPCR can be used on virtually any newly diagnosed adult with AML. However, these results need to be further validated before entering the clinical routine.

Paper IV

Mutational spectrum of de novo NPM1-mutated acute myeloid leukemia patients older than 75 years

Genetic markers such as *NPM1* are important not only for classification but also for risk stratification. However, the latter is primarily applicable for younger patients, below 75 years, since older patients have not been included in clinical trials to the same extent as younger patients. This is contradictory since AML is mainly a disease of the elderly with a median age at diagnosis of 71 years. Therefore, we decided to address this limitation and set out to explore the mutational landscape in older (>75 years) patients with AML. We chose *NPM1*-mutated AML since it typically presents *de novo*, deliberately aiming for exclusion of patients with secondary AML, which would have added complexity to the analysis. The aim was to compare the mutational landscape between younger and older patients with *de novo* AML with mutated *NPM1* and also try to explore the clonal evolution of the disease in this older cohort.

Diagnostic samples from 22 patients, with a median age of 84, were sequenced using Illuminas Trusight Myeloid Panel. For comparison with younger patients data was retrieved from two large studies [60, 134]. To validate we also compared our results with a study of elderly AML patients, where we selected the *NPM1*-mutated patients [243].

76 mutations (50 different variants) in 16 recurrently mutated genes were identified in our study. The most common mutated genes involved the DNA methylation genes, including *TET2*, *DNMT3A* and *IDH2*. They were followed by *FLT3*-mutations and mutations in the spliceosome gene *SRSF2*.

Compared to younger patients we found a significant enrichment of *TET2* and *SRSF2* mutations and a significantly reduced frequency of *DNMT3A* mutations. Also, *IDH2* mutations seemed to be more frequent in the elderly, although not reaching statistical significance.

As expected from the current knowledge of leukemogenesis, most patients (86%) had a coexisting mutation in a gene involved in DNA methylation (*TET2*, *DNMT3A* or *IDH2*) associated with clonal hematopoiesis. The allele frequencies (VAF%) were consistently higher for these DNA methylation genes than for *NPM1* in the majority of cases (89%), suggesting that the *NPM1*-mutation was acquired in a clone with a pre-existing mutation in DNA methylation genes. For the remaining 11%, VAF% was at the same level for the *NPM1* mutation and the DNA methylation genes. Unfortunately, follow-up samples were only available for three patients, precluding meaningful subclonal pattern analysis after therapy.

In conclusion, the results from paper IV indicate that the mutational pattern may differ between younger patients and the very old. However, more and larger studies need to be performed to elucidate the prognostic relevance of these differences.

Discussion

The results presented in this thesis have contributed to the research field of DNA-based MRD methods in AML. In paper I and paper III we showed superior sensitivity of DNA-based molecular MRD for the *NPM1*-mutation compared to MFC-MRD. With the well-established knowledge of superior risk assessment by molecular MRD compared to MFC-MRD for certain fusion gene transcripts and *NPM1* transcripts, it is likely that molecular MRD will be recommended also for other genes in the future. This vision was corroborated in paper II, demonstrating the high accuracy of DNA based methods as compared to RT-qPCR.

Current sequencing techniques can identify at least one molecular mutation in more than 90% of adult AML patients at diagnosis [26, 60, 64]. In paper III we demonstrated the power of personalised MRD-assays by following several mutations. This will probably be of utmost importance in the future when both less expensive and more sensitive sequencing and ddPCR techniques have been developed. The findings in paper IV strengthen the belief that elderly AML-patients have a disease that might differ from younger patients regarding both classification and prognostic relevance of detected mutations.

Aims of measuring residual leukemia

The major aim of MRD analysis is to identify individuals at risk of relapse who may need further consolidation treatment. Another aim is of course to identify low-risk patients who may not stand in need of intensified treatment. Choosing optimal cut-offs, time-points and tissue for MRD analysis is not trivial. Rather, what has become clear over the last decade of research is that there is no single assay, threshold, time-point or material (BM vs PB) that is consistently the most predictive of disease relapse across clinical trials. “The perfect MRD assays does not (yet) exist” [206]. It is also important to keep in mind that not all patients with MRD will relapse, and not all patients without MRD will remain in remission [244]. This might not only be due to shortcomings of techniques and optimal thresholds or time-points; however, we must better understand how to act on the MRD results from the different subtypes of AML. Sensitivity is an important issue, but only one of the problems to be resolved for accurate MRD determination. To move towards more harmonised MRD assays, it is important to include MRD in future clinical trials.

This may also entail a more accurate assessment of the therapeutic efficacy [195]. The inclusion of MRD as a surrogate endpoint marker (surrogate for overall survival or event-free survival) in clinical trials may accelerate the development of AML treatments, as the long duration of AML trials (~8 years) is a limitation [171].

Challenges in measuring residual leukemia

There are several ways to measure residual disease and due to the heterogeneity of AML there is no method, threshold or time-point that suits all [54]. Two different approaches are used for the detection of MRD in routine clinical diagnostic work-up: either multicolor flow cytometry (MFC) or molecular techniques such as RT-qPCR. Newer molecular techniques are emerging including digital PCR and next-generation sequencing (NGS).

The experiments in paper I were planned and performed prior to the main breakthrough of NGS. Nowadays it is not necessary to screen for the *NPM1*-mutation at diagnosis since all patients considered for treatment in Sweden have sequencing performed on their diagnostic sample. The panel currently in use is a nationally designed (by Genomic Medicine Sweden) myeloid panel including the *NPM1* gene. The results in paper I and III, implying that quantification of the *NPM1* type A mutation by qPCR or ddPCR is more sensitive than MFC for MRD analysis is no longer up for discussion, but a rather established fact. qPCR was chosen as MRD-method for paper I partly because the hematopathology section at the Department of Pathology in Lund was familiar with the technique due to its high sensitivity and routine use in ALL-MRD. Shortly after the discovery of *NPM1*-mutations in AML, it became clear that these mutations are potential markers of MRD. In the beginning, several studies used genomic DNA [119, 120, 210, 211] besides cDNA (RNA). Paper I was also prior to the ELN recommendations of RT-qPCR as the gold standard for MRD assessment of *NPM1*-mutated AML [41]. Without detracting the high sensitivity of RT-qPCR and its use in most clinical trials, there are several advantages of using DNA instead of cDNA. The most important is perhaps that DNA-based methods can be applied independently of gene expression levels or fusion transcripts, which limits the applicability of RT-qPCR. Moreover, DNA-based methods more accurately measure residual leukemic cells. qPCR based on genomic DNA is highly sensitive, as clearly demonstrated by us in paper I, II and III and by others [119, 185, 186]. In addition, neither ddPCR nor deep seq require standard curves or reference genes [187, 188], which make them attractive alternatives. Also, due to the inherent instability of RNA, DNA protocols are more flexible than are RNA protocols.

Previous studies comparing cDNA and genomic DNA for MRD detection are rather scarce with few samples analysed, but have shown good agreement between RT-

qPCR and NGS on the one hand [190, 213, 240], and RT-qPCR and qPCR on the other [245]. An important result from Paper II was that RT-qPCR was negative, i.e. failed to detect leukemic transcripts in as many as 10% of samples detected by any of the DNA-based methods. This is novel information that may change the prevailing view on interpretation of molecular MRD results. RT-qPCR is indeed a method with very high sensitivity, but this does not necessarily mean that other methods should be scorned. In the original publication by Gorello *et al*. for qPCR dependent quantification of *NPM1* mutated DNA [120], detectability was defined as one leukemic signal in a duplicate analysis, contrasting to the stricter criteria applied for our qPCR method in paper I, where both replicates of the same sample needed to be detectable to qualify as positive. If instead applying Gorellos definition to our cohort, there would have been four additional positive samples by qPCR also detectable by RT-qPCR, one of which was also measurable by ddPCR. Furthermore, an additional nine RT-qPCR negative samples would have been regarded as positive with qPCR with this definition. Thus, even more samples would have been considered as false negative with RT-qPCR if adhering to the criteria for *NPM1* qPCR defined by Gorello *et al*. In other words, molecular DNA methods, in particular qPCR, are highly sensitive and even comparable to RT-qPCR. qPCR appeared somewhat more sensitive than ddPCR and deep seq. However, this can probably be explained by the higher DNA input in the qPCR assay (500ng) compared to ddPCR and deep seq (100ng each). The exact input-level of RNA is of less importance for the sensitivity level. Analysed samples in our RT-qPCR assay should contain between 10^3 (optimal $>10^4$) and 10^6 transcripts for the analysis to be performed.

Another interesting finding in paper II was the considerable fluctuation of the *NPM1* transcript level in the leukemic cells in follow-up samples, when combining data from the simultaneous RT-qPCR and qPCR measurements. The ratio of transcripts versus leukemic DNA varied markedly both between individuals and within individuals at different follow-up time-points, suggesting that interpretation of RT-qPCR results is more complex than just reflecting the number of residual cells. Different expression levels may also depend on the AML subtype. For example, the low sensitivity of *KMT2A-MLLT3* assays (10^{-3}) compared to *NPM1* assays (10^{-6} or below) can probably be at least partly explained by the inherent expression levels of the respective leukemias, emphasizing that RT-qPCR does not measure the number of leukemic cells, but rather their gene expression. Clearly, both MFC and DNA-based methods more directly measure the number of leukemic cells. However, whether these cells also have the capacity to cause relapse of the leukemia is not always obvious.

Both PB and BM samples can be used to analyse molecular MRD. For certain time-points and for some AML subtypes, MRD results from either PB or BM may be the most useful prognostic variable for relapse risk. The main concern about PB MRD is the lower sensitivity compared to BM MRD [176]. However, the specificity of

PB can be higher due to the lower frequency of background noise caused by normal myeloid progenitors [207]. There are conflicting results concerning the correlation between PB and BM MRD discussed in a recent paper [167]. When comparing matched PB and BM samples in paper II (data not shown), several PB-BM pairs had detectable levels of *NPM1* in the BM but not in the PB-sample, reflecting the higher sensitivity of BM analyses. RT-qPCR never showed a positive PB-sample together with a negative BM-sample, which contrasted to the DNA-based methods. Here, qPCR detected mutated *NPM1* in one PB-sample undetectable by all other DNA-based methods and RT-qPCR. The corresponding BM-sample was negative with all techniques including qPCR. Another patient had detectable mutated *NPM1* in PB by both ddPCR and deep seq, while qPCR and RT-qPCR were negative. Interestingly, in the corresponding BM-sample, qPCR detected mutated *NPM1* whereas the remaining methods were negative. These discordant results strongly indicate the presence of residual leukemia at the border of what we can measure with any of our techniques.

Different cut-offs for *NPM1*-mutated AML have been studied for BM samples. It is not necessarily the mere presence of measurable leukemic disease that provides the best prognostic cut-off. For AML with mutated *NPM1*, there are good reasons to consider low positive and negative MRD states together in BM-samples, supported by an abundance of studies demonstrating different outcomes when certain cut-offs for MRD are applied in BM. Thus, other cut-offs than detectable versus undetectable have been shown to be superior for identifying patients at high risk of relapse, which was the reason for dichotomizing the RT-qPCR results into MRD high versus MRD low/undetectable patient groups for comparison with the DNA-based methods in paper II. Kronke *et al.* have shown that higher transcript levels are associated with higher risk of relapse and shorter remission duration and overall survival both during and after completion of therapy [175]. They discovered that all patients with >200 *NPM1*-mutated transcripts / 10^4 *ABL* transcripts during follow-up experienced relapse. Other important cut-offs to identify patients at high risk of relapse include a less than 3 log10 reduction of *NPM1* transcripts as compared to the diagnostic level after induction therapy [177] and an increase of more than 1% *NPM1*-mutated transcripts / *ABL* transcripts after chemotherapy [176]. Also, an increase of more than 10% *NPM1*-mutated transcripts / *ABL* transcripts is associated with a high risk of relapse after allo-SCT [176]. As for other molecular targets, it is important to continuously monitor MRD for early relapse detection. A persistent high-level positivity or a rising level of transcripts is a strong sign of approaching relapse [17, 41, 54, 197].

Also for PB, prognostically relevant cut-offs other than detectable versus undetectable have been studied. Balsat *et al.* have shown strong prognostic significance of postinduction *NPM1*-MRD in PB using the cut-off 4-log reduction of transcripts as compared to the diagnostic level [200]. In a recent publication, Dillon *et al.* presented cut-offs for MRD in PB in the setting of allo-SCT, separating

the MRD detectable group into 'MRD high' and 'MRD low' using the cut-off ≥200 *NPM1* mutated copies/10^5 *ABL* copies for the 'MRD high'-group. This cut-off was used in combination with a cut-off for BM (≥1000 *NPM1*mutated copies/10^5 *ABL* copies) to define the 'MRD high'-group. The 'MRD low' group (between these PB and BM cut-offs and undetectable transcripts) was further subdivided according to the presence of *FLT3*-ITD. 'MRD low' samples with *FLT3*-ITD were classified as high risk and 'MRD low' without *FLT3*-ITD as low risk. With this strategy they found implications for prognosis and risk stratification [246]. In our study (Paper II), 10 of the PB detectable samples would have been classified as 'MRD high' using the PB cut-off alone and 3 as 'MRD low'. Of these, all 10 'MRD high' samples were detected by qPCR, 7 by ddPCR and 8 by deep seq, implying good sensitivity for the DNA-based methods, particularly qPCR. In the future, more knowledge is likely to be gained about cut-offs for MRD in PB. Further studies are needed to test the prognostic relevance of DNA-based MRD-methods and their optimal thresholds, time-points and whether they should be applied on BM, PB or both.

Challenges in interpreting MRD results

Several difficulties may arise during MRD assessment depending both on the specific method applied and the inherent biology of AML. Examples include immunophenotypic shift, clonal evolution with multiple subclones and CHIP mutations.

MRD using MFC can be applied on most patients. There are two major strategies, either Leukemia associated immunophenotype (LAIP) or the different from normal (DfN) approach. The most common defines leukemia-specific surface markers at diagnosis (LAIP) that are tracked in subsequent MRD analyses. With the different from normal (DfN) approach, aberrant populations with respect to normal hematopoiesis are searched for in the follow-up sample. This method has the potential to identify emerging clones with shifted immunophenotype. Limitations of MFC for MRD include the sensitivity of the assay. In paper I we demonstrated that the most sensitive LAIPs (e.g. CD117+/HLA-DR-/CD99++) could detect 0.01% leukemic cells while other LAIPs (e.g. HLA-DR++/CD33+/CD13+/CD56+) more resembled normal or regenerating BM cells conferring an inferior sensitivity level at 1%. The LAIP approach depends on the stability of the immunophenotype after therapy, and it is known that it can change i.e. immunophenotypic shift [169]. Consequently, it is advised to combine information from both diagnostic LAIP and DfN analysis to minimize false negatives [247, 248]. An example of immunophenotypic shift with clonal evolution was seen in paper III, where one of the patients with persistent clonal hematopoiesis (pattern 3) showed a significant change of the immunophenotype at relapse.

In an attempt to address the problem of clonal evolution, we targeted several mutations identified at diagnosis, and monitored them during follow-up. Even in this rather small proof-of-concept study, we were able to demonstrate some of the different ways in which the main clone and subclones can evolve. This was achieved by following 5-9 mutations per patient with a multiplexed, personalised ddPCR MRD technique. A shortcoming of this study was its retrospective nature. Samples were not collected at specific time-points according to guidelines after treatment, but rather when the patient had an appointment with the clinician. If all patients had been monitored according to current recommendations it is possible that pending relapses would have been identified earlier. In a similar study, this multi-target MRD strategy was much more informative and reliable than monitoring single genes alone [64]. Another approach to circumvent the problem of clonal evolution and sensitivity would be to combine immunophenotypical and genetic MRD methods. In a recent study, Jongen-Lavrencic *et al*. demonstrated that when both MFC and molecular MRD were negative, the four-year relapse rate was 26.7% compared to 73.3% if both assays were positive. When residual disease was detected using either method, the relapse risk was around 50% [249]. Thus, by combining two entirely different approaches, in this case sequencing and MFC, the false negative rate decreased with improved identification of residual leukemia during complete remission. Hence, the inherent weakness of each method to identify residual disease can be partly overcome by applying both.

We could also confirm that CHIP-mutations are not suitable as markers of MRD [41, 64, 137]. For example, one of the non-relapsing patients had clonal hematopoiesis, as judged by persistence of *DNMT3A* mutation around 5% VAF, before SCT. At the only MRD-assessment after SCT, around 80 days post-SCT, the level had decreased to 0.01% VAF. This patient was alive, without any clinical suspicion of relapse, almost five years after diagnosis. Unfortunately, no further sample was available to monitor the *DNMT3A* mutation during this long period of time. One of the relapsing patients had persistent clonal hematopoiesis, including *DNMT3A* and *TET2* mutations at VAF around 40%, during two years before the first relapse, with complete morphological and immunophenotypical remission. In this patient, the leukemia relapsed without the founding clone driven by *NPM1*, illustrating the risk of following a single mutation, even *NPM1*, since it may be lost during the evolution of the relapsing leukemia.

Some patients will not relapse despite being MRD positive. A possible explanation for some cases could be that the MRD analysis measures differentiated leukemic cells that have lost the leukemic proliferation potential, or that a mutation involved in clonal hematopoiesis has been measured. In many cases residual disease is probably controlled by immunological mechanism residing within the tumor microenvironment [250]. On the other hand, some MRD-negative patients will relapse. This can probably sometimes be explained by insufficient eradication of leukemic cells. Another explanation could be insufficient sensitivity of the MRD

assay applied. Sometimes, clonal evolution with loss of the initiating mutation occurs allowing escape of MRD detection with single target strategies. In other cases, a new leukemia appears e.g. therapy-related leukemia (tAML). AMLs with marked intratumoral genetic heterogeneity are particularly challenging and may prove difficult to track in MRD samples [247].

With a single cut-off level defining “MRD positive” and “MRD negative” patient groups, there is always a risk of oversimplification, since reality shows that “MRD positive” patients sometimes remain in remission and conversely, some “MRD negative” patients do relapse as mentioned above [170]. False positive samples may cause overtreatment, which is an important aspect to consider given the high risk of mortality associated with allo-SCT. A higher threshold defining MRD positivity will better identify patients at risk; however, this will come at the cost of increasing the false negative rate with possible undertreatment and inferior prognosis in the MRD-negative group. The risk stratification can be fine-tuned by applying two cut-offs instead of one thus creating three risk groups. Other well-known prognostic parameters can be included for the middle group, for which the MRD results provide less guidance for the treatment decision [170, 200]. It can also be problematic to rely on a single measurement that will not capture the kinetics of an evolving disease; hence, it is recommended to continuously monitor MRD. There is a strong need for standardization of molecular and immunophenotypic MRD assessments, as emphasized in a recent review [223]. This will allow for identification of meaningful MRD thresholds for the different AML subtypes, as the molecular heterogeneity of AML has made it clear that a "one size fits all" approach for MRD detection is probably not feasible [54].

Currently, MRD-tailored therapy primarily focuses on the choice of consolidation therapy including allo-SCT. However, in the future, MRD has the potential to guide the clinician in the choice of conditioning treatment, the choice of donor in the consolidation phase (some donors may increase the graft versus leukemia effect which can be used in MRD-positive patients), and strategies to prevent relapse in the maintenance phase [171].

AML-associated mutations in the elderly

AML is primarily a disease of the elderly with a median age at diagnosis of approximately 71 years. For accurate disease classification according to WHO and prognosis, the mutational profile of the leukemia is of utmost importance. However, there are surprisingly few reports that actually studied the mutational landscape in elderly AML patients, implying a need for better characterization to improve patient care.

Our results in paper IV, demonstrating more *TET2* and *SRSF2* mutations in patients above 75 years compared to younger *de novo NPM1*-mutated AML patients, are supported by other studies even if the inclusion criteria might have been different to ours [71, 72, 243, 251]. Interestingly, and contrasting to the increased frequency of *TET2* mutations (another CHIP-mutation), *DNMT3A* mutations were less frequent in the elderly *NPM1* mutated AML patients. This was a surprise considering the common finding of *DNMT3A* mutations in younger *NPM1*-mutated AML patients, but also the increased frequency of CHIP-mutations, including *DNMT3A*, in older individuals without AML.

Mutations in *SRSF2* and *ASXL1* are both associated with secondary AML, found in 20% and 32% of the cases, respectively [252], but also with CHIP [35, 253]. However, while *SRSF2* mutations were increased in the elderly *NPM1*-mutated cases compared to the younger control groups, mutations in *ASXL1* were infrequent in both groups. Even though mutations in *SRSF2* are associated with CHIP, our data showed an allele frequency lower or equal to the CHIP-associated mutation *TET2*, suggesting that the *SRSF2* mutation was a secondary event in the clonal evolution of the leukemia. Co-mutations of *SRSF2* and *NPM1* are rarely described in the literature. This may be explained by their importance as drivers of secondary AML (*SRSF2*) and *de novo* AML (*NPM1*) [252].

Also illustrating the potential different biology between younger and older AML patients, is the fact that the negative prognostic impact of secondary-AML, seen in younger patients, is not seen in the elderly, according to a report from the Swedish Acute Leukemia Registry [16]. Others have shown that subgroups of elderly patients, carrying certain mutations, like *IDH1*, have an inferior prognosis [71].

Thus, it is not only important to remember that the mutational spectrum is different in younger and older AML patients, but also that the applicability of genetic markers for risk stratification may vary. Therefore, it is important to continue to increase the inclusion of elderly AML patients in clinical trials.

Personalised MRD

Both ddPCR (paper II and III) and deep seq (paper II) are well suitable for personalised MRD-assays. Following several mutations allows for better detection of the evolution of different subclones and appropriate timing of MRD sampling will capture the kinetics of the evolving recurrent leukemia. In addition, a multi-target strategy may circumvent the potential problem of loss of an MRD marker such as *NPM1*, lost in approximately 10% of relapses. Also, different patterns of clonal evolution may be important for the relapse risk. Studies have shown conflicting results regarding the prognostic relevance of persisting clones, including those characterised by CHIP-mutations. Some studies concluded inferior prognosis

with perseverance of CHIP-mutations [254, 255] while others have shown the opposite [137]. One study showed no prognostic relevance of persisting isolated *DNMT3A* mutations, while persistence of *DNMT3A* together with other mutations, or acquisition of new AML-associated mutations, conferred an inferior prognosis [256]. These apparently contradictory conclusions can perhaps be explained by the uncertain biological consequences of persisting mutations, i.e. if they reflect leukemic cell burden or precursor clonal hematopoiesis [52]. The potential role of digital PCR for MRD-assessment has recently been reviewed [257]. The main advantages of ddPCR as MRD method are the ability to exactly quantify the number of mutant DNA molecules without need for a standard curve, its applicable on virtually all newly diagnosed AML patients and that both cDNA and genomic DNA can be used as starting material. An important issue to resolve is how many mutations that are needed to be targeted for optimal capturing of subclones, taking both risk assessment and costs into account. Another problem is that there may be additional relevant subclones that can be missed even with the multi-target approach presented in paper III. Also, acquired new mutations will not be targeted. Indeed, for the two patients in paper III where we backtracked mutations detected at relapse, we found the new emerging clones in the retrospective ddPCR MRD analysis. Nevertheless, it may be sufficient to use the diagnostic samples to select an appropriate number of mutations; the backtracking approach is of less interest for clinical purposes.

To be able to incorporate all relevant clinical, genetic (on the global genomic and transcriptomic level instead of focusing on single aberrations), epigenetic, immunophenotypic, and MRD information without undue simplification for personalised risk predictions may be beyond human comprehension. Therefore, artificial intelligence (AI) is likely to be used in the future for diagnostic, prognostic, as well as therapeutic decisions. AI may assist in MRD detection and relapse prediction, which also can pave the way for more personalised treatment of patients suffering from AML. The optimistic scenario is cure for most AML patients in the future [223].

Conclusions

- The newly established qPCR method for *NPM1* type A mutation quantification was highly sensitive and more reliable than was flow cytometry for the determination of MRD in *NPM1*-mutated AML.
- The limit of detection for the qPCR *NPM1* type A assay was approximately 0.001% (10^{-5}) leukemic cells.
- DNA-based MRD techniques may complement RT-qPCR for the assessment of residual leukemia.
- RT-qPCR was demonstrated to be the most sensitive MRD method when compared to three DNA-based methods (qPCR, ddPCR, and deep-seq); however, RT-qPCR failed to detect mutations in 10% of samples with detectable leukemic DNA.
- For BM samples, MRD results obtained by qPCR, ddPCR and deep seq agreed with the established RT-qPCR cut-offs in 95% of the analyses.
- Clinically relevant cut-offs for DNA-based MRD methods that can be tested in future trials are 0.1% leukemic DNA for qPCR and 0.05% VAF for ddPCR and deep seq in *NPM1*-mutated AML.
- The IBSAFE ddPCR MRD method appears to be applicable to virtually all newly diagnosed AML patients and is more sensitive than flow cytometry.
- Monitoring a small number of mutations by ddPCR captures the kinetics of the evolving recurrent leukemia.
- *NPM1*-mutation alone may not be a reliable MRD-marker, as approximately 10% of relapses appear as *NPM1* wild-type leukemia.
- The mutational spectrum of *NPM1*-mutated AML in patients older than 75 years displays distinct features.
- A significant enrichment of *TET2* and *SRSF2* mutations, together with a reduced frequency of *DNMT3A* mutations, was observed in the very old patients.
- *NPM1* mutation is a secondary event in the clonal evolution and development of AML in patients older than 75 years.

Limitations

AML is a rather uncommon disease, and therefore a large amount of time is required to recruit patients. Our patients are predominantly from the southern healthcare region with a population of 1.9 million. This generated a rather small sample size that is a consistent limitation throughout this thesis. Hence, the prognostic relevance of our findings has not yet been evaluated.

When paper I was first published, it became clear that most clinical trials used RT-qPCR instead of qPCR based on genomic DNA. Therefore, RT-qPCR has become the gold standard for MRD assessment of *NPM1*-mutated AML [41]. At the start of the *NPM1*-MRD era, several studies were performed using DNA [119, 120, 210, 211] instead of cDNA. Nevertheless, our qPCR method possesses a high sensitivity (10^{-5}) that is comparable to that of RT-qPCR (10^{-5}-10^{-6}) [217].

In paper II, only the type A mutation was included in the comparison between RT-qPCR and DNA-based methods due to the only nationally available RT-qPCR assay. Although the second most common mutations are similar (4 bp insertions) to the type A mutation, it would have been informative to include more mutations for comparison. It is also important to determine if our RT-qPCR assay provides similar results to the one originally described by Kronke *et al.* [175], as this may bias the proposed DNA cut-offs presented in this study. Before applying the proposed cut-offs for DNA-based methods in clinical practice, they must be tested with respect to their prognostic impact. This was hampered by both the sample size and the time frame.

In paper III, the prognostic relevance of monitoring ddPCR-based personalised assays has not been analysed. Although our study was small and included few patients, we did observe a patient with *NPM1*-mutated leukemia who relapsed with *NPM1* wild-type leukemia. This phenomenon is well established, and it also highlights the need for improvements in the single-assay-MRD methods used today. Our multiplexed, personalised ddPCR assay could provide a means to improve sensitivity and prognostication; however, this must be further studied.

A limitation when performing a retrospective study of the very old (paper IV) is that these patients tend to undergo fewer diagnostic procedures [258] than do younger patients. This was also the case in the present study. In almost one-third of the patients (27%), the diagnosis was made on PB without BM aspiration. Almost half of the patients lacked investigations necessary for a correct diagnosis according to

WHO. For example, 41% lacked a diagnostic karyotype. Only 14% of patients provided follow-up samples. Another limitation of paper IV is that the exact ages of the patients in one of the published control groups were uncertain. Information on the age of the *NPM1*-mutated patients could thus not be retrieved from the published cohorts used for the comparison; however, given that only a minor percentage of the included patients were above 75 years, the entire control group was regarded as young. The prognostic significance of the observed differences, including the increase in secondary-type mutations such as *SRSF2*, is important to consider but could not be studied with the small number of patients examined.

Future considerations

The first three studies in this thesis have shed some light on the possible use of DNA-based methods for MRD assessment in AML patients. In paper I, we set up a highly sensitive DNA-based method (qPCR) for *NPM1* type A mutation quantification, but shortly thereafter, RT-qPCR was established as the gold standard method for molecular MRD in patients with *NPM1*-mutated AML [41]. In paper II, we were able to determine DNA-based cut-offs of potential use for clinical assessment of relapse risk. These cut-offs showed high positive and negative predictive values with respect to clinically relevant MRD-levels as determined by measuring *NPM1* transcripts. Finally, in paper III, we used ddPCR for MRD assessment on a wide range of AML-associated mutations. Using DNA instead of RNA as marker of residual leukemia has several advantages. Indeed, as demonstrated in paper II, the DNA-based MRD techniques may complement RT-qPCR for the assessment of residual *NPM1*-mutated leukemia, even if further studies are needed to establish their role in everyday MRD diagnostics. Therefore, a natural step is to continue with a prognostic study. Samples could be collected from a few university hospitals in Sweden and one (*NPM1*) or more selected mutations could be followed by both RNA and DNA-based methods. This thesis has added important knowledge to the field of DNA-based MRD assessment and my belief is that DNA-based assays will be used together with RNA-based techniques in the future.

When we started to work with paper III in 2015/2016, there was much less knowledge regarding clonal hematopoiesis and evolution of subclones than is available today. An interesting investigation would be to monitor 3-4 mutations in recurrently mutated genes that were detected at diagnosis by ddPCR and to study the prognostic relevance of these personalised assays. Using a multi-target MRD approach [64], rather than evaluating single genes, is likely to improve the accuracy of MRD assessments. For this purpose, both ddPCR and NGS can be used. An advantage of ddPCR is that it is less expensive and an advantage of NGS is that it more easily enables personalised leukemia surveillance.

The combination of MFC-MRD and molecular MRD can improve the accuracy of MRD assessments compared to using either method alone [249]. With faster turn-around times and lower costs for molecular MRD, this is, in my opinion, likely to be recommended in the future. Using different techniques in parallel will better capture the heterogeneity of AML, as one method does not fit all [54, 219].

Additionally, better standardization and optimal timing and frequency of testing and identification of test-specific thresholds for the different subtypes of AML must be clarified. Besides, we must also better establish when and how to treat.

Regarding the elderly AML patients described in paper IV, further studies must be performed to elucidate the prognostic impact of the observed differences of the mutational spectrum between younger patients and the very old. It is of vital importance to include also these patients in clinical trials; AML is indeed mainly a disease of the elderly, our population is getting older and new treatment options are rapidly becoming available for those not fit for high dose regimens. The outcomes of such studies have the potential to affect the therapeutic approach in this patient group.

It would also be interesting to follow a few selected mutations in a group of elderly *de novo* AML patients by ddPCR as in paper III. This could generate information on treatment response and different evolving subclones.

I look forward to following the development of sequencing techniques and observing how they will influence both the primary diagnostic work-up and the MRD assessment. In five years from now, will we use both whole genome sequencing and whole transcriptome sequencing? Will a combination of MFC and molecular MRD be mandatory? As always, costs play an important role but incorrect treatments are also expensive. The future use of epigenomics for classification is also of interest especially if no leukemia-driving genetic event can be discerned. How will time frames and cost be dealt with? Will conventional cytogenetic analysis become outdated? Will we use artificial intelligence to incorporate all information for personalised risk predictions? And finally and most importantly, will all these efforts lead to improved well-being and survival for the individual patient?

Populärvetenskaplig sammanfattning på svenska

(General summary in Swedish)

Akut myeloisk leukemi (AML) är den vanligaste formen av akut leukemi hos vuxna. I Sverige insjuknar årligen ungefär 350 personer med en genomsnittlig ålder på 72 år. Vid AML sker en okontrollerad produktion av omogna celler, så kallade blaster, i benmärgen. Dessa blaster engagerar oftast även blodet och i vissa fall andra delar av kroppen. Ökningen av blaster i benmärgen gör att denna inte kan utföra sina normala uppgifter, vilket ofta leder till blodbrist, infektionskänslighet och blödningsbenägenhet. Orsaken till AML är inte helt klarlagd. Man vet att en del fall utvecklas från en tidigare benmärgssjukdom, eller efter tidigare given behandling (strålning eller cellgifter) mot en annan elakartad sjukdom. Vissa genetiska tillstånd hos barn som t ex Downs syndrom innebär en ökad risk att insjukna i AML. Prognosen vid AML är generellt dålig och försämras ytterligare med stigande ålder. Femårsöverlevnaden är drygt 60% för patienter under 40 år vid diagnos och under 10% för dem över 75 år. Behandlingen är i de flesta fall cellgifter (cytostatika). Obehandlad sjukdom leder till döden inom loppet av några veckor eller månader.

Det är viktigt att följa upp resultatet av behandlingen för att utvärdera effekten och för att identifiera patienter som behöver ytterligare tung behandling, inklusive benmärgstransplantation, för att klara sig. Det är emellertid lika viktigt att identifiera patienter som inte behöver ytterligare behandling, eftersom den kan ge svåra biverkningar och i värsta fall leda till döden. Denna utvärdering görs bland annat genom mätning av kvarvarande leukemiceller efter behandling, så kallad Measurable Residual Disease (MRD). Den gemensamma principen som ligger till grund för alla typer av bedömningar av kvarvarande leukemisjukdom (MRD) är att leukemiutvecklingen resulterar i ett antal förändringar i gener (mutationer; genotypen) eller i vad som uttrycks på cellytan hos leukemicellerna (fenotypen), som gör att dessa kan skiljas från normala blodbildande celler. Den vanligaste metoden för MRD-bedömning är flödescytometri, där man främst tittar på markörer (proteiner) på cellytan. Andra metoder, så kallade molekylära tekniker, kan upptäcka genetiska förändringar, t ex mutationer. En vanlig molekylär metod är PCR, som kan identifiera enskilda mutationer eller andra genetiska förändringar. En annan teknik är sekvensering, som kan bestämma den exakta DNA-sekvensen av

valda delar av arvsmassan. Flödescytometriska och molekylära tekniker är komplementära, var och en med sina inbyggda styrkor och svagheter. Fördelen med flödescytometri jämfört med molekylära tekniker är att den kan tillämpas på nästan alla leukemipatienter. Nackdelen är begränsad känslighet, att metoden kräver en hög nivå av expertkunnande för tolkning samt att förändringar hos leukemicellerna kan leda till falskt negativa resultat. Molekylära tekniker har i vissa fall högre känslighet och mindre inslag av subjektiv bedömning jämfört med flödescytometri. I min avhandling undersökte jag därför möjligheterna att vidareutveckla användningen av molekylära tekniker för MRD-bedömning. Det arbetet försvåras till viss del av att AML har många ansikten med sinsemellan olika förändringar av gener och proteiner, vilket gör det svårt att hitta en enda metod som fungerar som uppföljning för alla leukemipatienter. Kunskapen om de vanligast förekommande mutationerna i AML har ökat betydligt de senaste åren genom utveckling av avancerade sekvenseringstekniker, så kallad massiv parallell sekvensering eller nästa generations sekvensering (NGS). Trots att AML uppvisar ett spretigt, heterogent mutationsmönster så finns det några mutationer som regelbundet påträffas. En av dem är mutation i *NPM1*-genen som förekommer i ca 30% av AML hos vuxna. Det finns många olika typer av *NPM1*-mutationer där typ A är vanligast (70-80%).

Delarbete I redogör för uppsättandet av en ny molekylär MRD-metod, kvantitativ realtids-PCR (qPCR), för den vanligaste formen av *NPM1*-mutation. Data jämfördes med MRD baserat på flödescytometri och qPCR visade sig vara betydligt känsligare, dvs hittade fler positiva prover. Denna metod kan användas på en stor andel AML-patienter, upp mot 30%.

I delarbete II jämfördes olika DNA-baserade metoder (qPCR, NGS (djupsekvensering [deep seq]) och droplet digital PCR [ddPCR]) med en RNA-baserad metod (RT-qPCR) för MRD-analys av den vanligaste typen av *NPM1* mutation. Analys med RNA-baserad metod anses vara den gyllene standarden, men analys baserad på DNA har många fördelar. Resultaten visade att även om RT-qPCR är en känsligare metod, så upptäcks leukemiskt DNA i en del fall där RT-qPCR inte kan påvisa kvarvarande leukemisk sjukdom. Resultaten mynnade också ut i föreslagna gränsvärden för DNA-metoderna beträffande kliniskt och biologiskt relevant MRD, som kan användas i klinisk rutin som vägledning för behandlingsbeslut, eftersom det inte enbart handlar om detekterbara versus icke detekterbara MRD-nivåer.

Fokus i delarbete III låg på biologiska aspekter där flera olika leukemiska subkloner kunde identifieras och följas med droplet digital PCR efter behandling. Arbetet visade att AML är biologiskt komplex såtillvida att återfall kan orsakas av olika subkloner som förlorat sin MRD-markör, till exempel *NPM1* mutationen. Resultaten belyser således svagheten om man bara använder en ensam MRD-markör, men också styrkan att kunna monitorera flera olika subkloner med droplet digital PCR för att bättre kunna bedöma risk för återfall. Resultaten visade också

hur väl droplet digital PCR fungerar som MRD-metod med tillämplighet på i stort sett alla AML-patienter.

I delarbete IV undersöktes diagnostiska och klonala aspekter på *NPM1*-muterad AML hos patienter äldre än 75 år. Mutationsmönstret hos äldre är mindre känt, inte minst eftersom de oftare hanteras utanför kliniska studier. Resultaten visade påfallande skillnader i mutationsförekomst vid diagnos mellan äldre och yngre patienter. Vidare studier får visa om detta påverkar riskbedömning och behandlingsresultat i denna åldersgrupp. Dessutom visade det sig att majoriteten av patienterna utvecklat sin *NPM1*-muterade leukemi från förstadier innehållande andra mutationer.

Sammanfattningsvis har avhandlingen belyst problematiken kring molekylär analys av mätbar kvarvarande leukemisk sjukdom (MRD) ur både ett metodologiskt och biologiskt perspektiv. DNA-baserade MRD-metoder visade samstämmigt en hög känslighet och ddPCR kommer att kunna tillämpas på i princip alla patienter med AML. En annan viktig nyhet är att DNA-teknikerna har en mycket god förmåga att påvisa kvarvarande leukemi av betydelse för att bedöma hur patienterna svarat på behandling. Avhandlingen har också bidragit till ökad förståelse för hur leukemi kan utvecklas, både före och efter behandling. Dessutom visar avhandlingsarbetet att AML hos äldre patienter uppvisar några genetiska särdrag.

Acknowledgements

I would like to express my sincere gratitude to all those who have supported me and in one way or another contributed to this work, with special thanks to:

Mats Ehinger, my main supervisor. Thank you for providing me with the opportunity to conduct this thesis and for your enthusiasm, support, patience, and commitment. And thanks for all the fun that we have had!

Gunnar Juliusson, my first co-supervisor. Thank you for sharing your great experience in hematology and for your statistical considerations. It has been a pleasure working with you.

Lao Saal, my co-supervisor during papers II-IV. Thank you for letting me into your group and for introducing me to the combination of research and business.

Annica Pontén, my co-supervisor. For being my local research network during the first part of my thesis. I miss you more than you know.

Per Levéen, my co-supervisor during paper I. Thank you for introducing me to PCR and to lab work, for lots of fun, "ruggugglor" and jalapeño cheese curls.

The professional staff at the molecular pathology division at the Department of Pathology in Lund for excellent technical assistance. Special thanks to Heike Kotarzky, Li Zhou and Karina Vidovic for helping me with the experiments.

The professional team at the Hematopathology unit at the Department of Pathology in Lund. Special thanks to Anna Porwit, Kerstin Torikka, Olof Axler and Despoina Violidaki.

Vladimir Lazarevic for sharing your knowledge and experience and for your contributions.

Yilun (Alan) Chen for fruitful collaboration and for introducing me to ddPCR and the design of primers and probes.

Linda Fogelstrand for interesting discussions and support. It has been inspiring to work with you. Linda's group at Sahlgrenska, especially to Giti Shah-Barkhordar, for taking good care of me and bringing me to nice places for lunch and to Sofie Johansson Alm for always being positive during our struggles.

Benjamin Holmgren, my roomie, for being my molecular biology support during the last part of the thesis, for your patience and for performing the bioinformatics in paper IV.

Thoas Fioretos, Christina Orsmark-Pietras and Henrik Lilljebjörn for your contributions and Marianne Rissler for the introduction to wet-lab NGS.

The Center for Translational Genomics, Lund University, for DNA sequencing in paper IV.

Peter Rådström and Johannes Hedman, Department of Chemistry, Division of Applied Microbiology, Lund University, for assistance with the PCR inhibition experiments in paper I.

All my colleagues at the Department of Pathology and Cytology in Halmstad and especially to Mariusz Trela for introducing me to the world of bone marrow cytology and for helping me whenever needed, to Hala Issa for answering my questions about PCR and NGS, to Hideki Yokoyama for your endless commitment, kindness and your chocolate and to Tomas Seidal, former Head of the Department of Pathology in Halmstad and my former clinical supervisor for making me a pathologist.

Colleagues and PhD students at the Department of Pathology in Lund for your encouragement.

Urban Gullberg and Magnus Hultdin for scrutinizing my work half way through.

FoU Halland for really good help in the struggles of being a PhD student outside the university environment. To Stefan Lönn, Amir Emamverdi Baigi, Anders Holmén and Ulf Strömberg for great statistical assistance and support and to Hanna Svensson for superb administrative support, understanding and encouragement.

Hematologists in Halland, no one mentioned no one forgotten, for answering my clinical questions.

Hematologists in Lund / Skåne who contributed to the sample collection.

Other co-authors than mentioned above: Dana Dvorakova, Anthony M. George, Robert Rigo, Alvar Almstedt, Julia Asp and Gustav Orrsjö - thank you for fruitful cooperation and for your contributions.

My parents Ingrid and Silas for your love and constant support. Dad you are a true source of inspiration and I am not sure if I would have conducted this thesis if it was not for you.

My parents-in-law, Mariann and Nils Anders, for generosity and support in everything from childcare to preparing food for skiing vacations.

My brother Gustaf for love and understanding.

My friends outside the pathology-world for support.

Erik and Gustaf, my wonderful children for bringing so much happiness into my life. I am so proud of you.

Johan, my husband, biggest support and best friend for your understanding and patience with me during this last year. I love you.

This work was supported by Region Halland, Vetenskapliga rådet Halland and Södra sjukvårdsregionen.

Finally, I would like to thank all the patients who participated in this work and made it possible.

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Printed by Media-Tryck, Lund 2021 NORDIC SWAN ECOLABEL 3041 0903

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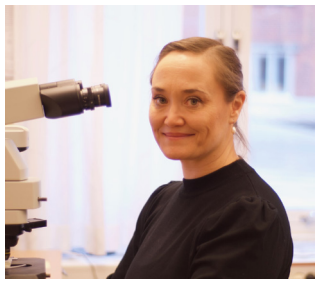

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FACULTY OF MEDICINE

Department of Clinical Sciences, Lund

Lund University, Faculty of Medicine
Doctoral Dissertation Series 2021:46
ISBN 978-91-8021-052-2
ISSN 1652-8220