Comparisons between New and Established Methods for Analysis of Response to Treatment in Acute Leukemia

Department of Laboratory Medicine

Institute of Biomedicine

Sahlgrenska Academy, University of Gothenburg

UNIVERSITY OF GOTHENBURG

Gothenburg 2025

Cover illustrations by Mattias Karlén

Comparisons between New and Established Methods for Analysis of Response to Treatment in Acute Leukemia

© Sofie Johansson Alm 2025
sofie.johansson.alm@gu.se

All reprints with permission from the publishers.

ISBN 978-91-8115-168-8 (PRINT)
ISBN 978-91-8115-169-5 (PDF)
http://hdl.handle.net/2077/85334

Printed in Borås, Sweden 2025
Printed by Stema Specialtryck AB

To my family ♥

Comparisons between New and Established Methods for Analysis of Response to Treatment in Acute Leukemia

Sofie Johansson Alm

Department of Laboratory Medicine, Institute of Biomedicine
Sahlgrenska Academy, University of Gothenburg
Gothenburg, Sweden

ABSTRACT

Acute leukemia is a severe hematologic malignancy that affects both adults and children. Although prognosis has improved over recent decades, relapse remains one of the major challenges. A key reason for relapse is that small amounts of leukemic cells, in numbers well below the sensitivity of routine morphology, have survived treatment and remain in the bone marrow: *measurable residual disease* (MRD). Presence of MRD after initial treatment is one of the strongest predictors of relapse, and sensitive MRD analysis is crucial for identifying patients at risk of relapse. MRD is routinely assessed at treatment of acute leukemia, using various methods depending on the specific leukemia subtype. However, none of the current techniques is fully optimal; many require further evaluation, and for some patients, no applicable method is available. This thesis aims to improve MRD diagnostics in acute leukemia by evaluating existing methods and developing novel techniques, with the goal of contributing to improved clinical management and, ultimately, a better prognosis for patients with acute leukemia. The main method used for MRD analysis in this thesis was deep sequencing, a highly sensitive next-generation sequencing-based technique that quantifies mutations present in leukemic cells at diagnosis. Other molecular methods, including reverse transcription quantitative polymerase chain reaction (RT-qPCR), were also applied, as well as flow cytometry for comparison and complementary MRD assessment. The material consisted of blood and bone marrow samples analyzed at diagnosis and during and after treatment, from adult and pediatric patients with acute

myeloid leukemia (AML), and from pediatric patients with precursor B-cell acute lymphoblastic leukemia (pre-B ALL). Paper I shows that RT-qPCR of *ETV6*::*RUNX1* fusion transcript can be used as an alternative and valuable complementary MRD method to flow cytometry in children with pre-B ALL. Paper II shows that there is a strong correlation between RNA- and DNA-based methods for MRD analysis of *NPM1* mutations in adults with AML, and that DNA-based methods can complement, or replace, RT-qPCR. Paper III shows that deep sequencing of mutated *NPM1* during and after treatment predicts relapse and poorer survival in adult AML. Paper IV shows that deep sequencing of *FLT3*-ITD is a highly sensitive method for MRD detection in pediatric AML, enabling monitoring of treatment response and early relapse detection. In conclusion, this thesis contributes new knowledge about MRD analysis in acute leukemia, highlighting the clinical value of deep sequencing. The implementation of these findings in clinical practice could support more precise risk stratification, guide treatment decisions, and may contribute to improved prognosis for patients with acute leukemia.

Keywords: Acute Myeloid Leukemia, Precursor B-cell Acute Lymphoblastic Leukemia, Measurable Residual Disease, Deep Sequencing, *ETV6*::*RUNX1, NPM1*, *FLT3*-ITD

ISBN 978-91-8115-168-8 (PRINT)
ISBN 978-91-8115-169-5 (PDF)

SAMMANFATTNING PÅ SVENSKA

Leukemi, blodcancer, är samlingsnamn för cancersjukdomar som drabbar blodbildande celler i benmärgen. Sjukdomen delas in i två huvudgrupper: akut och kronisk leukemi, som båda delas in i myeloisk och lymfatisk leukemi beroende på vilken cellinje som är drabbad. Trots att prognosen för akut leukemi har förbättrats under de senaste decennierna, är återfall fortfarande en av de största utmaningarna. En orsak till återfall är att små mängder leukemiceller har överlevt behandlingen och finns kvar i benmärgen – s.k. *measurable residual disease* (MRD). Dessa kvarvarande leukemiceller kan ge upphov till sjukdom på nytt, och risken för återfall har visats vara högre vid påvisad MRD. För att förbättra prognosen är det därför viktigt att man lyckas identifiera patienter med kvarvarande leukemiceller efter behandling. Analys av MRD utförs rutinmässigt vid behandling av akut leukemi, och förekomst av MRD har visats vara en av de starkaste prognosfaktorerna. Flera olika metoder finns tillgängliga för detektion och kvantifiering av MRD, och valet av metod beror på vilken typ av leukemi patienten har. Ingen av metoderna är dock optimal, flera behöver utvärderas ytterligare, och hos vissa patienter kan ingen av metoderna användas.

Det övergripande syftet med denna avhandling är att förbättra diagnostiken vid akut leukemi genom att utvärdera befintliga metoder samt utveckla nya metoder för detektion av kvarvarande leukemiceller. Detta för att i förlängningen kunna bidra till bättre behandlingsresultat med ökad långtidsöverlevnad.

I delarbete I visar vi att MRD-analys med den molekylära metoden RT-qPCR av fusionstranskriptet *ETV6*::*RUNX1* kan användas som ett alternativ, och värdefullt komplement, till standardmetoden flödescytometri hos barn med akut lymfatisk leukemi. I delarbete II visar vi att det finns en stark korrelation mellan RT-qPCR och DNA-baserade metoder för MRD-analys av *NPM1*-mutation, den vanligaste genetiska avvikelsen vid akut myeloisk leukemi (AML) hos vuxna, och att DNA-baserade metoder kan användas som ett komplement, eller alternativ, till RT-qPCR. I delarbete III visar vi att MRD-analys av *NPM1*-mutation med djupsekvensering under och efter konsolideringsbehandling är starkt prediktivt för återfall och förkortad överlevnad. Metoden kan användas på alla patienter med *NPM1*-mutation, oavsett mutationssubtyp. I delarbete IV visar vi att djupsekvensering är en högkänslig metod för MRD-analys av *FLT3*-ITD-mutation hos barn med

AML, och att metoden kan användas både för att utvärdera behandlingssvar och för att upptäcka återfall på ett tidigt stadium.

Sammanfattningsvis bidrar denna avhandling med ny kunskap om MRD-analyser vid akut leukemi och belyser det kliniska värdet av djupsekvensering. Användningen av denna information inom sjukvården kan komma att vägleda behandlingsbeslut samt bidra till förbättrad prognos för patienter med akut leukemi.

LIST OF PAPERS

This thesis is based on the following studies, referred to in the text by their Roman numerals.

I. **Alm SJ**, Engvall C, Asp J, Palmqvist L, Abrahamsson J, Fogelstrand L. Minimal residual disease monitoring in childhood B lymphoblastic leukemia with t(12;21)(p13;q22); *ETV6-RUNX1*: concordant results using quantitation of fusion transcript and flow cytometry. *International Journal of Laboratory Hematology 2017; 39: 121-128.*

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, H. Saal L, Fogelstrand L, Ehinger M. Comparison of RNA- and DNA-based methods for measurable residual disease analysis in *NPM1*-mutated acute myeloid leukemia. *First authors contributed equally. *International Journal of Laboratory Hematology 2021; 43: 664-674.*

III. **Johansson Alm S***, Orrsjö G*, Shah Barkhordar G, Rehammar A, Staffas A, Delsing Malmberg E, Andersson P-O, Garelius G, Hardling M, Palmqvist L, Fogelstrand L. Prognostic significance of deep sequencing for analysis of measurable residual disease in acute myeloid leukemia with *NPM1* mutation. *First authors contributed equally. *Leukemia and Lymphoma 2025; 31: 1-11.*

IV. **Johansson Alm S**, Tornberg B, Shah Barkhordar G, Soboli A, Saeed B, Staffas A, Delsing Malmberg E, Palmqvist L, Hasle H, Abrahamsson J, Fogelstrand L. Response evaluation and post-treatment monitoring in childhood acute myeloid leukemia using deep sequencing of *FLT3*-ITD. *Manuscript*.

ADDITIONAL PUBLICATIONS BY THE AUTHOR

The following publications by the author are not included in this thesis but are relevant to the research field.

I. Malmberg EBR, Ståhlman S, Rehammar A, Samuelsson T, **Alm SJ**, Kristiansson E, Abrahamsson J, Garelius H, Pettersson L, Ehinger M, Palmqvist L, Fogelstrand L. Patient-tailored analysis of minimal residual disease in acute myeloid leukemia using next-generation sequencing. *European Journal of Haematology 2017; 98: 26-37.*

II. Delsing Malmberg E, **Johansson Alm S**, Nicklasson M, Lazarevic V, Ståhlman S, Samuelsson T, Lenhoff S, Asp J, Ehinger M, Palmqvist L, Brune M, Fogelstrand L. Minimal residual disease assessed with deep sequencing of *NPM1* mutations predicts relapse after allogeneic stem cell transplant in AML. *Leukemia and Lymphoma 2019; 60: 409-417.*

III. Juul-Dam KL, Ommen HB, Nyvold CG, Walter C, Vålerhaugen H, Kairisto V, Abrahamsson J, **Alm SJ**, Jahnukainen K, Lausen B, Reinhardt D, Zeller B, von Neuhoff N, Fogelstrand L, Hasle H. Measurable residual disease assessment by qPCR in peripheral blood is an informative tool for disease surveillance in childhood acute myeloid leukaemia. *British Journal of Haematology 2020; 190: 198-208.*

CONTENTS

ABBREVIATIONS

ALL	Acute lymphoblastic leukemia
AlloSCT	Allogeneic hematopoietic stem cell transplantation
AML	Acute myeloid leukemia
B-ALL	B-cell lineage acute lymphoblastic leukemia
CR	Complete remission
ddPCR	Droplet digital polymerase chain reaction
DNA	Deoxyribonucleic acid
ELN	European LeukemiaNet
MFC	Multiparameter flow cytometry
MRD	Measurable residual disease
NGS	Next-generation sequencing
NOPHO	Nordic Society for Paediatric Haematology and Oncology
OS	Overall survival
Pre-B ALL	Precursor B-cell acute lymphoblastic leukemia
qPCR	Quantitative polymerase chain reaction
RFS	Relapse-free survival
RNA	Ribonucleic acid
RT-qPCR	Reverse transcription quantitative polymerase chain reaction
VAF	Variant allele frequency
WHO	World Health Organization

1 INTRODUCTION

Leukemia is a group of malignant hematologic neoplasms classified into acute and chronic forms. While chronic leukemia typically progresses more slowly and involve more differentiated blood cells, acute leukemia is characterized by the uncontrolled proliferation of immature blood cells, blasts, in the bone marrow, which disrupts the normal hematopoiesis. Acute leukemia is further classified based on the lineage of the affected progenitor cells:

- Acute myeloid leukemia (AML) originates from myeloid progenitors, which give rise to granulocytes, erythrocytes and thrombocytes.
- Acute lymphoblastic leukemia (ALL) originates from lymphoid progenitors, primarily B- and T-lymphocytes.

Although the prognosis for acute leukemia has improved, relapses still carry a high risk of treatment failure and inferior outcomes. In recent years, it has become clear that a patient's response to treatment, particularly the presence of measurable residual disease (MRD), is one of the strongest prognostic factors for identifying patients at risk of relapse. Today, there are several different MRD analysis methods available, although their use in clinical practice remains a challenge. This thesis focuses on improving MRD analysis methods for patients with acute leukemia, specifically AML in both adults and children, and precursor B-cell ALL (pre-B ALL) in children.

1.1 HISTORICAL PERSPECTIVE

The understanding of leukemia has evolved over centuries. Although the earliest documented report of cancer can be traced back to c. 1500 BC in the *Ebers Papyrus* (1), the medical literature up to c. 500 AD contains no descriptions of blood malignancies. Examination of the blood became possible with advances in microscopy by Robert Hooke (1635-1703) and Antonie van Leeuwenhoek (1632-1723), the latter of whom first described the red blood cells in 1674. The white blood cells were first described by the French physician Joseph Lieutaud (1703–1780) in 1749, laying the foundation for a more detailed study of the blood. Two decades later, the first description of lymphocytes and the lymphatic system was published by William Hewson (1739-1774) (2). Early in the 19th century, a few cases of patients with unusual blood abnormalities were published, where the blood was described as "milky" or "pus-like". There is no definitive agreement on who first identified leukemia, but several individuals contributed to understanding the disease. In 1811, the British surgeon Peter Cullen (1769-unknown) noted white, milk-like serum in a patient with splenomegaly, and in 1827, the French surgeon Alfred Velpeau (1795-1867) documented a case with fever and swollen abdomen and linked the symptoms to an increased number of white blood cells. In 1842, the French physician Alfred Donné (1801-1878) described a third component of the blood, which later turned out to be thrombocytes (3). Around this time, the German pathologist Rudolf Virchow (1821-1902) studied patients with increased leukocytes and suggested that the cells originated from the spleen and the bone marrow. In 1847, he coined the term "leukemia", meaning white blood in Greek, for this newly discovered disease, and has since been called the father of leukemia research. In 1852, the Scottish pathologist John Bennett (1812-1875) published a detailed report on 35 cases of abnormal blood compositions, describing the presence of mysterious white corpuscles and suggesting that the patients suffered from leukemia. Both Virchow and Bennett are credited with contributing to the identification of leukemia. The distinction between acute and chronic leukemia was introduced in 1857 by German pathologist Nikolaus Friedreich (1825-1882). A major breakthrough came in 1872 when Ernst Neumann (1834-1918) identified the bone marrow as the place where leukemia starts, which was in contrast to the general perception at that time where many believed that bone was an impenetrable mass. In 1877, Paul Ehrlich (1854-1915) introduced histochemical staining, which enabled detailed study of blood cell differentiation. The myeloblast was identified in 1900 by the Swiss hematologist Otto Naegeli (1871-1938), who also showed that the lymphoblast is the precursor of lymphocytes. By the early 20th

century, leukemia was increasingly classified as acute or chronic, and further subdivided into lymphoid and myeloid based on the affected progenitor cells – a system that laid the foundation for the modern leukemia classification.

1.2 HEMATOPOIESIS AND LEUKEMOGENESIS

1.2.1 NORMAL HEMATOPOIESIS

Hematopoiesis is the tightly controlled process in which all blood cells are formed from hematopoietic stem cells (HSCs) in the bone marrow. In early embryonic development, hematopoiesis starts in the yolk sac and, as development progresses, HSCs emerge from the dorsal aorta. After a few weeks, the fetal liver takes over as the main site of hematopoiesis, with the spleen also contributing. Around 20 weeks of gestation, the bone marrow gradually takes over this role and remains the primary site of hematopoiesis throughout life. In children, blood cells are made in the red marrow of almost all bones, both long and flat bones. In adults, this process occurs mostly in the flat bones such as the pelvis, sternum, and ribs, as well as in the vertebrae and the proximal ends of the femur and humerus. With aging, red marrow is gradually replaced by yellow (adipose) marrow, leading to decreased hematopoietic capacity.

HSCs make up a larger proportion of bone marrow cells in newborns than in adults, with an estimated frequency of 1 in 1,000 marrow cells in newborns, compared to 1 in 10,000 in adults. The total number of HSCs in an adult is estimated to be between 50,000 and 200,000, but only a small fraction of these cells is actively producing blood cells at any given time. HSCs have the unique ability to give rise to all types of blood cells. They can also self-renew, meaning that when they differentiate, some of their daughter cells remain as HSCs to preserve the stem cell pool. In a healthy adult, around 10^{10}–10^{11} new blood cells are produced every day to maintain hematopoietic homeostasis.

HSCs follow two main developmental pathways: the myeloid and the lymphoid lineages. The myeloid lineage gives rise to erythrocytes, thrombocytes, granulocytes (neutrophils, eosinophils, and basophils), monocytes, and some types of dendritic cells. The lymphoid lineage gives rise to B cells, T cells, natural killer cells, and some dendritic cells, such as plasmacytoid dendritic cells. The entire hematopoietic process is controlled by

a complex system of growth factors, cytokines, and transcription factors. Disruptions in this process can lead to hematologic disorders, including leukemia.

1.2.2 LEUKEMOGENESIS

Leukemogenesis is a multistep process initiated by genetic and epigenetic alterations in HSCs, leading to the formation of preleukemic stem cells. These cells typically maintain self-renewal capacity but lack full malignant potential. Additional events, such as mutations affecting cell proliferation, differentiation, or survival, are necessary for transformation into fully malignant leukemic stem cells. These cells then drive the progression to overt leukemia (4).

Genetic alterations are the main cause of acute leukemia and can be divided into chromosomal abnormalities and gene mutations. Chromosomal abnormalities are categorized as either numerical or structural. Numerical abnormalities involve gains or losses of whole chromosomes, known as aneuploidy, including hyperdiploidy (increased chromosome number) and hypodiploidy (decreased chromosome number). Structural abnormalities involve alterations in chromosome structure, including translocations, deletions, inversions, and duplications. Gene mutations refer to smaller changes in DNA, such as point mutations, deletions, or insertions, and can be either acquired (somatic) or inherited (germline). Somatic mutations are further divided into driver mutations, which give the malignant cells a selective growth advantage and contribute to leukemia development, and passenger mutations, which are genetic alterations found in malignant cells but that do not contribute to disease progression and may have originated in a precursor cell during earlier divisions.

A two-hit theory has been proposed to explain the development of AML, suggesting that two classes of mutations, Class I and Class II, must cooperate to induce leukemia:

- **Class I mutations** give hematopoietic cells a proliferative advantage by affecting cell signaling pathways, leading to increased proliferation and/or reduced apoptosis. These mutations include driver mutations and are typically point mutations, deletions, or insertions, for example in genes such as *FLT3*, *NRAS*, *KRAS*, or *c-KIT*.
- **Class II mutations** have limited effect on proliferation but instead disrupt transcription factors and other proteins needed for normal

blood cell development. They typically involve chromosomal translocations or other structural alterations, such as t(8;21)(q22;q22)/*RUNX1*::*RUNX1T1*, inv(16)(p13q22)/t(16;16)(p13;q22)/*CBFB*::*MYH11*, and *KMT2A* (11q23) rearrangements.

Together, Class I and Class II mutations create the conditions for AML by driving proliferation and impairing differentiation (5).

A distinct two-hit hypothesis has also been proposed for childhood pre-B ALL, based on a different biological mechanism. It suggests that leukemogenesis starts with a genetic mutation, followed by a postnatal trigger such as an infection later in life. The first mutation occurs in utero and leads to the formation of a preleukemic clone, seen in about 5% of newborns. However, only 1% of these children later develop the disease. Early-life infections may help train the immune system to eliminate such clones, but in rare cases, later infections may trigger additional mutations and lead to disease onset (6).

1.3 ETIOLOGY

For the majority of AML patients, the disease arises without an identifiable cause, referred to as *de novo* AML. In around one-fourth of cases, AML develops secondary to a pre-existing hematologic disorder, such as myelodysplastic syndrome (MDS) or a myeloproliferative neoplasm, or following prior chemo- or radiotherapy for another malignancy. These cases are referred to as *secondary* AML and generally carry a worse prognosis (7, 8). Known external risk factors for AML include high-dose exposure to ionizing radiation and certain chemicals, such as long-term exposure to benzene.

In children, approximately 95% of all acute leukemia cases have no known cause, but an increasing number of hereditary forms have been described in recent years, particularly among younger patients. Several congenital conditions predispose individuals to develop leukemia, for example Fanconi anemia, Li-Fraumeni syndrome, *GATA2*-related disorders, and telomerase-related diseases (8). Children with Down syndrome have a 10- to 20-fold increased risk of developing acute leukemia, primarily AML (9).

1.4 EPIDEMIOLOGY

AML is the most common type of acute leukemia, affecting approximately 350 adults in Sweden every year, corresponding to 3–4 cases per 100,000 (10). The disease is rare in children, with around 15-20 reported cases annually. The incidence of AML increases with age, peaking at 84 years (11), and the median age at diagnosis in adults is 72 years (12). In children, the median age at diagnosis is approximately 6 years (13). The gender distribution is balanced up to the age of 70, after which a male predominance is seen (14).

ALL is the most common cancer in children, accounting for around 25% of all pediatric malignancies in high-income countries, whereas the disease is rare in adults. The global incidence varies, with higher rates reported in Western countries and parts of East Asia (15). In Sweden, ALL affects around 100 children and 50 adults every year. The median age at diagnosis is approximately 5 years in children and 50 years in adults (16-18).

1.5 CLINICAL MANIFESTATIONS

Symptoms of acute leukemia arise due to the proliferation of leukemic blasts in the bone marrow (Figure 1), which suppresses normal hematopoiesis and leads to anemia, thrombocytopenia, and abnormal leukocyte counts. Patients with acute leukemia may present with leukocytosis and a high proportion of blasts in peripheral blood, while others have a normal or low white blood cell count, often with an abnormal differential count. Symptoms can be either subtle and develop over weeks to months, or present with an acute onset. The most common symptoms are nonspecific, such as fatigue and malaise, often with persistent fever. Symptoms related to anemia include pallor, palpitations, and shortness of breath, while symptoms related to thrombocytopenia include petechiae, easy bruising, and bleeding. Leukopenia increases susceptibility to infections. Bone pain is a relatively common symptom, particularly in younger patients, caused by the expanding bone marrow. Additionally, leukemic cells can infiltrate various organs, leading to hepatomegaly, splenomegaly, skin involvement, and gingival swelling. Organomegaly and lymphadenopathy occur in up to half of AML patients and are even more common in ALL (19).

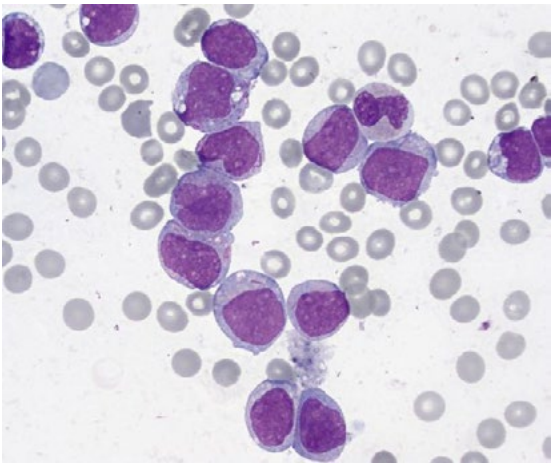

***Figure 1**. Light microscopy image of a bone marrow smear showing increased number of leukemic blasts in a patient with acute myeloid leukemia. Image by Animalculist, Wikimedia Commons, licensed under CC BY-SA 4.0.*

1.6 **PROGNOSIS**

AML is a heterogeneous disease with different subtypes, each associated with different prognostic outcomes. In general, the prognosis of AML is strongly age dependent, with survival rates decreasing with increasing age. Younger adults (under 50 years of age) have a significantly better prognosis than older patients, with 5-year overall survival (OS) rates often exceeding 50%. Among patients aged 50 to 70 years, the 5-year OS is approximately 30–40%. A major challenge in AML is the high relapse rate, where nearly half of younger patients and the majority of older patients experience relapse within 1–3 years from diagnosis (20). The prognosis following relapse is generally poor but varies depending on factors such as the timing of relapse and the patient's eligibility for allogeneic stem cell transplantation (alloSCT) (21). The prognosis for children with AML has improved significantly, with cure rates now close to 80% (22). However, about one-third of pediatric patients experience relapse, which significantly worsens the prognosis, with only 30–40% achieving cure after relapse (23).

The prognosis for childhood ALL has improved dramatically over the past decades, with a 5-year OS increasing from approximately 10% in the 1960s to around 90% today (18). The relapse rate is lower than in AML, approximately 15–20%, with a subsequent OS of 40-70% (24). In adults, the OS is comparable to that of AML and declines with increasing age.

1.7 DIAGNOSIS AND CLASSIFICATION

The most important examination for establishing a diagnosis of acute leukemia is a bone marrow aspiration, typically obtained from the posterior superior iliac crest. While the distribution of cell populations is generally consistent across sampling sites, minor variations may occur with age (25). If the aspiration yields insufficient material, commonly due to fibrosis or a highly packed bone marrow, a bone marrow trephine biopsy is performed. A lumbar puncture is used to assess central nervous system involvement, but it is not routinely performed in adults with AML unless there is suspicion of neurological involvement.

The bone marrow sample is used for multiple diagnostic assessments. Morphological examination is performed to evaluate cell morphology and blast percentage. Immunophenotyping using multiparameter flow cytometry (MFC) is used to determine cell lineage of origin and to characterize the leukemic cells for upcoming MRD analysis. Genetic analyses serve several purposes, primarily for accurate disease classification. Certain genetic aberrations influence relapse risk and thus guide treatment intensity, while others enable treatment with targeted agents. Also, some genetic markers allow for monitoring of MRD during and after treatment. Cytogenetic analyses, including karyotyping and fluorescence in situ hybridization (FISH), are used to detect specific chromosomal rearrangements. In addition, molecular techniques such as reverse transcription quantitative polymerase reaction (RT-qPCR) can identify fusion transcripts resulting from these rearrangements. In AML, FISH and karyotyping are used to detect t(8;21), inv(16), t(15;17)(q24;q21)/*PML*::*RARA* and *KMT2A* rearrangements, while PCR-based methods are used to detect mutations in genes such as *NPM1*, *FLT3* and *CEBPA*. In pediatric ALL, FISH and RT-qPCR are primarily used to detect important fusion genes, including, among others, t(9;22)(q34;q11)/*BCR*::*ABL1,* t(12;21)(p13;q22)/*ETV6*::*RUNX1*, and *KMT2A* rearrangements. To complement these analyses, next-generation sequencing (NGS) is performed in both AML and ALL, using multigene panels covering the most frequently mutated genes. Whole genome sequencing (WGS) and whole transcriptome sequencing (WTS) are now part of standard diagnostic care for pediatric patients in Sweden, although this is not yet the case in many other countries.

The diagnosis of acute leukemia is established based on the World Health Organization (WHO) classification (8), which has replaced the morphology-

based French-American-British (FAB) classification (26). In the WHO system, the former FAB subtypes are now largely represented under the category "acute leukemias defined by differentiation". Since 2022, the International Consensus Classification (ICC) is also used for classification in parallel with the most recent edition of the WHO classification (the fifth edition, 2022) (7).

According to the WHO classification, the general diagnostic criterion for AML is the presence of ≥20% blasts among nucleated cells in the bone marrow or peripheral blood. In AML with defining genetic abnormalities, a diagnosis can be established regardless of the blast count for most subtypes. In contrast to the WHO classification, the ICC sets a myeloid blast threshold of 10% for AML with recurrent gene mutations. However, in both the WHO and ICC classifications, the 20% blast requirement still applies for AML with *BCR*::*ABL1* and *CEBPA* mutations. Cases with 10-19% blasts are classified by the ICC as MDS/AML-overlap.

The fifth edition of the WHO classification (pediatric volume) introduces for the first time a separate classification of childhood AML (27). As the pediatric volume covers tumors across various organ systems, the information of AML is somewhat limited, making the hematolymphoid classification a valuable complement. The classification of ALL is largely similar between the WHO and ICC systems. The WHO 2022 classifications of B-cell lineage ALL (B-ALL) and AML are presented in Tables 1 and 2.

Table 1. *Diagnostic classification of B-cell lineage acute lymphoblastic leukemia (B-ALL) according to the World Health Organization 2022.*

B-ALL with defining genetic abnormalities
B-ALL with high hyperdiploidy
B-ALL with hypodiploidy
B-ALL with iAMP21
B-ALL with *BCR*::*ABL1* fusion
B-ALL with *BCR*::*ABL1*-like features
B-ALL with *KMT2A* rearrangement
B-ALL with *ETV6*::*RUNX1* fusion
B-ALL with *ETV6*::*RUNX1*-like features
B-ALL with *TCF3*::*PBX1* fusion
B-ALL with *IGH*::*IL3* fusion
B-ALL with *TCF3*::*HLF* fusion
B-ALL with other defined genetic alterations[a]
B-ALL, not otherwise specified

[a] B-ALL with *DUX4* rearrangement, B-ALL with *MEF2D* rearrangement, B-ALL with *ZNF384* rearrangement, B-ALL with *PAX5* alteration, B-ALL with *PAX5* p.P80R, B-ALL with *NUTM1* rearrangement, B-ALL with *MYC* rearrangement

Table 2. *Diagnostic classification of acute myeloid leukemia (AML) according to the World Health Organization 2022.*

AML with defining genetic abnormalities
Acute promyelocytic leukemia with *PML*::*RARA* fusion[1]
AML with *RUNX1*::*RUNX1T1* fusion[1]
AML with *CBFB*::*MYH11* fusion[1]
AML with *DEK*::*NUP214* fusion[1]
AML with *RBM15*::*MRTFA* fusion[1]
AML with *BCR*::*ABL1* fusion[2]
AML with *KMT2A* rearrangement[1]
AML with *MECOM* rearrangement[1]
AML with *NUP98* rearrangement[1]
AML with *NPM1* mutation[1]
AML with *CEBPA* mutation[2]
AML, myelodysplasia-related[2,a,b]
AML with other defined genetic alterations[1,c]
AML defined by differentiation
AML with minimal differentiation[2]
AML without maturation[2]
AML with maturation[2]
Acute basophilic leukemia[2]
Acute myelomonocytic leukemia[2]
Acute monocytic leukemia[2]
Acute erythroid leukemia
Acute megakaryoblastic leukemia[2]
Myeloid sarcoma
Myeloid neoplasms and proliferations associated with antecedent or predisposing conditions
Myeloid neoplasm post cytotoxic therapy
Myeloid neoplasms associated with germline predisposition[d]
Myeloid proliferations associated with Down syndrome

[1] AML regardless of blast count

[2] AML with ≥ 20% blasts

[a] Defining cytogenetic abnormalities: Complex karyotype (≥3 abnormalities), 5q deletion or loss of 5q due to unbalanced translocation, Monosomy 7, 7q deletion, or loss of 7q due to unbalanced translocation, 11q deletion, 12p deletion or loss of 12p due to unbalanced translocation, Monosomy 13 or 13q deletion, 17p deletion or loss of 17p due to unbalanced translocation, Isochromosome 17q, idic(X)(q13)

[b] Defining somatic mutations: *ASXL1, BCOR, EZH2, SF3B1, SRSF2, STAG2, U2AF1, ZRSR2*

[c] AML with *CBFA2T3*::*GLIS2* fusion, AML with *KAT6A*::*CREBBP* fusion, AML with *FUS*::*ERG* fusion, AML with *MNX1*::*ETV6* fusion, AML with *NPM1*::*MLF1* fusion

[d] Myeloid neoplasms with germline predisposition without a pre-existing platelet disorder or organ dysfunction: Germline *CEBPA* P/LP variant (*CEBPA*-associated familial AML), Germline *DDX41* P/LP variant, Germline *TP53* P/LP variant (Li-Fraumeni syndrome); Myeloid neoplasms with germline predisposition and pre-existing platelet disorder: Germline *RUNX1*P/LP variant (familial platelet disorder with associated myeloid malignancy, FPD-MM), Germline *ANKRD26* P/LP variant, Germline *ETV6* P/LP variant; Myeloid neoplasms with germline predisposition and potential organ dysfunction: Germline *GATA2* P/LP variant (*GATA2*-deficiency), Bone marrow failure syndromes (Severe congenital neutropenia, Shwachman-Diamond syndrome, Fanconi anaemia), Telomere biology disorders, RASopathies (Neurofibromatosis type 1, CBL syndrome, Noonan syndrome or Noonan syndrome-like disorders), Down syndrome, Germline *SAMD9* P/LP variant (MIRAGE Syndrome), Germline *SAMD9L* P/LP variant (SAMD9L-related Ataxia Pancytopenia Syndrome), Bi-allelic germline *BLM* P/LP variant (Bloom syndrome). P = pathogenic, LP = likely pathogenic

1.8 COMMON GENETIC ABERRATIONS

Advances in genomic technologies, especially with the development of NGS, have significantly improved our understanding of the genomic landscape in AML. AML was the first cancer to be analyzed using WGS in 2008 (28). In 2013, The Cancer Genome Atlas (TCGA) Research Network published a landmark study analyzing 200 cases of *de novo* AML using WGS or whole exome sequencing (WES), along with RNA sequencing and DNA methylation profiling. The study found that AML has relatively few mutations compared to other adult cancers, with an average of 13 mutated genes per case, including five recurrently mutated genes. A wide range of significantly mutated genes were identified, and nearly all cases (99.5%) harbored at least one mutation in key pathogenic categories, grouped into nine functional classes (Table 3) (29). By analyzing variant allele frequency (VAF), the researchers were also able to map the clonal structure of AML. More than half of the patients harbored at least one subclone in addition to a dominant leukemic clone. The findings suggest that mutations in epigenetic regulators, such as *DNMT3A*, *TET2*, and *ASXL1*, often act as early initiating events in preleukemic precursor cells, preceding leukemia-driving mutations in genes such as *NPM1* or signaling genes.

***Table 3**. Categorization of genes involved in AML development, adapted from Ley et al. 2013. A single sample may contain multiple mutations and can thus be classified into more than one category, resulting in a total percentage exceeding 100%.*

Gene category	Included genes	% of cases	Function in AML pathogenesis
Transcription factor fusions	t(8;21), inv(16)/t(16;16), t(15;17)	18	Interference with transcriptional regulation, leading to impaired differentiation.
Nucleophosmin 1	*NPM1*	27	Abnormal accumulation of *NPM1* in the cytoplasm.
Tumor suppressors	*TP53*, *WT1*, *PHF6*	16	Loss of tumor suppressor function, affecting genome stability and transcription.
DNA methylation	*DNMT3A*, *TET1*, *TET2*, *IDH1*, *IDH2*	44	Aberrant DNA methylation and accumulation of oncometabolites.
Activated signaling	*FLT3*, *KIT*, *KRAS, NRAS*, *PTPN11*	59	Constitutive activation of proliferative signaling pathways.
Myeloid transcription factors	*RUNX1*, *CEBPA*	22	Impaired hematopoietic differentiation due to transcriptional dysregulation.
Chromatin modifiers	*KMT2A* fusions, *NUP98*::*NSD1*, *ASXL1*, *EZH2*, *KDM6A*	30	Disruption of histone modification and chromatin structure.
Cohesin complex	*STAG1*, *STAG2*, *RAD21*	13	Impaired chromosomal segregation and dysregulated transcription.
Spliceosome	*SRSF2*, *SF3B1*, *U2AF1*	14	Abnormal RNA splicing and transcript processing.

In 2016, Papaemmanuil *et al*. published a landmark study in which they analyzed 1,540 AML patients from multiple cohorts, including the TCGA project and clinical trials, using WGS, WES, and targeted sequencing. They introduced a genomics-based classification of AML, identifying 14 molecular subtypes based on recurrent driver mutations and cytogenetic abnormalities. Their findings showed that AML is even more genetically heterogeneous than previously thought. They also identified key driver mutations, with 96% of patients harboring at least one and 86% harboring at least two. The results from this study contributed to updates in the WHO and, in particular, the European LeukemiaNet (ELN) classification systems (30).

The molecular aberrations seen in pediatric AML differ from those in adults. Unlike adult AML, pediatric AML is characterized by a high frequency of structural variations, such as chromosomal translocations and gene fusions. Fusion oncogenes are detected in nearly two thirds of pediatric AML patients. The most common are *KMT2A* rearrangements (15–20%), followed by t(8;21) (10–15%) and inv(16) (5–10%). Additionally, cryptic rearrangements such as t(5;11)(q35;p15.5)/*NUP98*::*NSD1* and inv(16)(p13q24)/*CBFA2T3*::*GLIS2* are relatively frequent in young children, though being less common with increasing age (25). These translocations are not detectable using standard cytogenetic methods.

A large WGS study of nearly 1,000 pediatric AML patients has provided further insights into the unique molecular landscape of pediatric AML (31). Mutations that are rare in adult AML, such as *GATA2* and *CEBPA*, were more common in children. Conversely, mutations typically seen in adults, such as *DNMT3A*, were rarely seen in children. Older children developed mutations more commonly found in adult AML, such as *NPM1* and *FLT3*-ITD. Additionally, mutations affecting signaling pathway mutations, including *NRAS*, *KRAS*, and *PTPN11*, were significantly more prevalent in pediatric patients.

In childhood ALL, genetic abnormalities are identified in the vast majority of cases, many of which have prognostic significance. Aneuploidy, particularly hyperdiploidy, is seen in nearly one-third of patients and is generally associated with a favorable prognosis. In contrast, hypodiploidy is rare and associated with a worse prognosis (32).

Recurrent chromosomal rearrangements are also common and define distinct biological subtypes. The *ETV6*::*RUNX1* fusion transcript is the most frequent,

which is discussed below. Other recurrent fusions include t(9;22)(q34;q11.2)/*BCR*::*ABL1* (3–5%), which is associated with poor outcomes unless treated with tyrosine kinase inhibitors, and t(1;19)(q23;p13.3)/*TCF3*::*PBX1* (~5%), which is linked to an intermediate prognosis (33). *KMT2A* rearrangements, found especially in infants, are associated with an unfavorable prognosis. In addition to these structural changes, somatic mutations in genes involved in lymphoid development and differentiation, such as *PAX5*, *IKZF1*, *EBF1*, *ETV6*, and *LMO2*, are frequently observed in pediatric ALL. These mutations often cooperate with chromosomal alterations to promote leukemogenesis (34). Advances in genomic profiling have also uncovered novel, often cryptic, gene fusions involving genes such as *DUX4*, *MEF2D*, *ZNF384*, and *NUTM1*. Some cases of ALL show gene expression patterns similar to known subtypes, such as *BCR*::*ABL1*-like and *ETV6*::*RUNX1*-like, even though they do not carry the typical fusion genes. These so-called phenocopies are genetically heterogenous, and identifying them often requires gene expression profiling (35).

1.8.1 NPM1

Nucleophosmin 1 (NPM1) is the most frequently mutated gene in AML, found in about 30% of all AML cases and up to 60% of patients with a normal karyotype (36, 37). Due to its unique characteristics, *NPM1*-mutated AML is classified as a distinct entity in both the WHO and ICC classifications (7, 8). NPM1 was originally identified in the 1970s as a nucleolar phosphoprotein in rat liver and hepatoma cells. The *NPM1* mutation associated with AML was first described by Falini *et al.* in 2005 and has since been extensively studied (38). The mutation is slightly more common in women than in men, a pattern also observed in pediatric patients (39-41). *NPM1*-mutated AML typically presents with high blast counts, elevated leukocyte and thrombocyte levels compared to other subgroups, and a higher frequency of extramedullary manifestations (41). Patients with *NPM1* mutations are usually slightly younger than the average AML patient, with a median age of 65 years at diagnosis. Although the frequency declines with age, around 20% of AML patients over 70 years still harbor the mutation (42).

NPM1 is a multifunctional phosphoprotein mainly localized in the nucleolus of the cell, where it plays a central role in numerous cellular processes. Its major functions include:

- **Ribosome biogenesis and transport** – essential for ribosomal RNA processing, subunit assembly, and nuclear export.
- **Genomic stability maintenance** – helps protect DNA by participating in damage response pathways.
- **Regulation of the p53-dependent stress response** – contributes to cell cycle control and apoptosis through stabilizing and modulating p53 activity.
- **Modulation of growth-suppressive pathways** – interacts with Arf to regulate cell proliferation and tumor suppression.
- **Histone chaperone activity** – helps organize chromatin structure by binding to histones and assisting in nucleosome formation.
- **Chromatin remodeling** – influences transcriptional regulation by altering chromatin structure and accessibility.
- **DNA repair** – contributes to DNA damage response and repair mechanisms, supporting genome integrity.
- **Apoptosis regulation** – plays a role in both pro- and anti-apoptotic signaling (43-45).

Despite extensive knowledge of its cellular functions, the exact role of NPM1 in leukemogenesis remains incompletely understood.

Wild-type NPM1 is a 37-kDa protein composed of three main structural regions that enable its broad functionality:

1. **N-terminal domain**: hydrophobic region that enables NPM1 to form pentamers and interact with other proteins. It contains two nuclear export signals (NES) which facilitate shuttling of NPM1 between the nucleus and the cytoplasm.
2. **Central domain**: includes two acidic subregions which contribute to histone binding and chaperone activity. It also contains two nuclear localization signals (NLS) that direct NPM1 from the cytoplasm to the nucleus, as well as RNA- and DNA-binding regions essential for ribosome biogenesis.
3. **C-terminal domain**: interacts with nucleic acids and contains two tryptophan residues (W288 and W290), essential for ribosomal DNA binding and nucleolar localization. It also contains two nucleolar localization signals (NoLS) which guide NPM1 to the nucleolus, where it primarily resides in the wild-type form (37) (Figure 2).

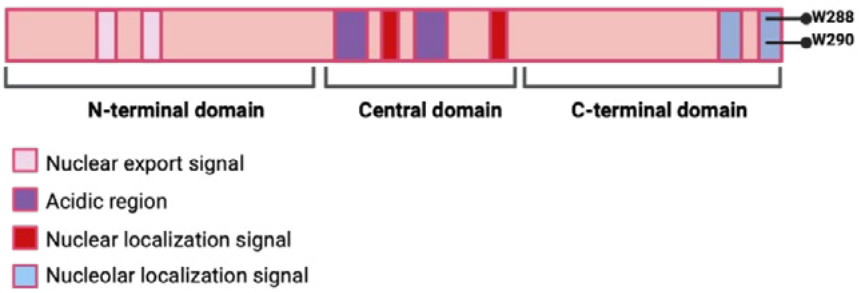

***Figure 2**. Schematic overview of the NPM1 protein and its functional domains. Created with BioRender.com.*

The *NPM1* gene is located on chromosome 5q35.1, and more than 99% of *NPM1* mutations occur in the last exon – exon 11 (previously referred to as exon 12) (43). These mutations are always heterozygous and result from insertions that cause a frameshift at the C-terminal domain of the protein. This leads to the loss of tryptophan residues (W288 and/or W290) and the acquisition of new NESs, which disrupt normal protein localization. As a result, the NoLSs are functionally lost, shifting NPM1 from the nucleus to the cytoplasm (46). This aberrant localization impairs NPM1's normal cellular functions and is believed to contribute to leukemia development. A key mechanism in the leukemogenic process involves the interaction between mutant NPM1 and exportin 1 (*XPO1*), a nuclear export protein that facilitates its translocation to the cytoplasm (Figure 3). Additionally, mutant NPM1 is associated with increased expression of homeobox (*HOX*) genes and their cofactors *MEIS* and *PBX3*, which enhance the self-renewal capacity of leukemic cells (47).

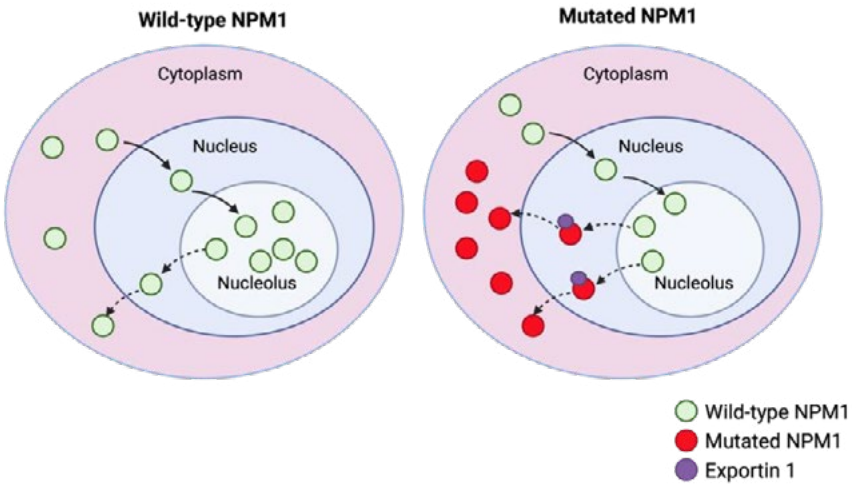

***Figure 3**. Subcellular localization of NPM1 in wild-type (left) and mutated (right) states. Created with BioRender.com.*

NPM1 mutations consist of insertions of varying lengths, most commonly of 4 base pairs (bp). To date, around 90 different mutation types have been described, where type A (c.860_863dupTCTG, p.(Trp288CysfsTer12)) is the most common, accounting for 70-85% of cases, followed by types B (c.863_864insCATG, p.(Trp288Cysfs*12)) and D (c.863_864insCCTG, p.(Trp288Cysfs*12)), which each constitutes 2-7% (48). Other mutation types are very rare in individual patients (47-49).

NPM1 mutation status is routinely assessed at diagnosis of AML and plays both an important diagnostic and prognostic tool. The prognosis for *NPM1*-mutated AML largely depends on the presence of co-mutations, as *NPM1* almost always co-occurs with other mutations. The most common co-mutations include *DNMT3A* (~50%), *FLT3*-ITD (~40%), Cohesin complex genes (~20%), *NRAS* (~20%), *TET2* (~15%), *IDH1*/*IDH2* (~15%), and *PTPN11* (~15%) (50). Patients with a normal karyotype and no *FLT3*-ITD mutation generally have a favorable prognosis, with improved survival and lower relapse risk. In these cases, *NPM1*-mutated leukemic cells are particularly susceptible to chemotherapy (51, 52). Although all *NPM1* mutation types are classified as favorable risk, data on subtype-specific prognostic relevance are limited. An earlier study did not find a significant difference (53), but a recent study reported that the type D mutation is associated with a poorer prognosis in children and young adults (54).

According to the 2022 ELN guidelines, *NPM1*-mutated AML is classified as favorable risk, except when accompanied by *FLT3*-ITD or intermediate-/high-risk cytogenetics. A concurrent *FLT3*-ITD mutation is a negative prognostic factor, classifying patients as intermediate risk. Unlike in the 2017 ELN guidelines, where *NPM1* with a low *FLT3*-ITD allelic ratio (<0.5) was considered favorable risk, the current guidelines classify all *NPM1*/*FLT3*-ITD cases as intermediate risk, regardless of allelic ratio (50).

The combination of *NPM1*, *FLT3*-ITD and *DNMT3A* mutations, found in approximately 6% of AML cases, is associated with poor outcomes. This three-way interaction is linked to a high leukemic stem cell burden and chemotherapy resistance (55, 56). Although *DNMT3A* mutations are present in nearly half of *NPM1*-mutated AML cases, their prognostic impact remains controversial (57), and they do not currently affect risk stratification.

1.8.2 FLT3

Mutations in the gene encoding the Fms-like tyrosine kinase 3 (FLT3), located on chromosome 13q12.2, are found in approximately 30% of adult and 10-15% of pediatric AML patients. FLT3 encodes a receptor tyrosine kinase expressed on the surface of early hematopoietic stem and progenitor cells, where it plays a central role in regulating proliferation, survival, and differentiation. Upon ligand binding, the receptor dimerizes and activates downstream signaling pathways, primarily Ras/MAPK, and to a lesser extent STAT5 and P13K/AKT/mTOR, which together promote controlled hematopoiesis (Figure 4). FLT3 mutations are classified into two main types: internal tandem duplications (*FLT3*-ITD), which are the most frequent (20-25%), and point mutations in the tyrosine kinase domain (*FLT3*-TKD), accounting for 5-10% of AML cases (58). Both mutations lead to constitutive, ligand-independent receptor activation, but differ in signaling intensity and clinical impact. *FLT3*-ITD causes strong and sustained downstream activation and is associated with an adverse prognosis. In contrast, *FLT3*-TKD mutations, typically affecting the activation loop, induce more limited and transient signaling and are generally not associated with the same poor outcomes (59). *FLT3*-ITD mutations usually arise from in-frame duplications of 3-400 bp in exons 14 and 15, resulting in an elongation of the juxtamembrane domain and disruption of the receptor's normal auto-inhibitory control, thereby contributing to leukemia development (60). *FLT3*-ITD-mutated cells are often subclonal, meaning they exist in a subset of leukemic cells with varying VAFs

within the same patient. Over time, different subclones may gain a growth advantage and, through clonal expansion, become the dominant clone at relapse. *FLT3*-ITD mutations are present at relapse in around 75-85% of patients who had the mutation at diagnosis (61). *FLT3*-ITD is routinely analyzed at diagnosis of AML using PCR with fragment analysis. Its prognostic impact depends on co-occurring mutations; in the absence of an *NPM1* mutation, it is associated with poor outcomes in both adults and children (62, 63). This was further demonstrated in a recent study in pediatric AML, where *FLT3*-ITD-positive patients with favorable co-mutations, such as *NPM1*, *CEBPA*, t(8;21), or inv(16), had significantly better outcomes, whereas poor-risk mutations were *WT1*, *UBTF* and *NUP98*::*NSD1* (64).

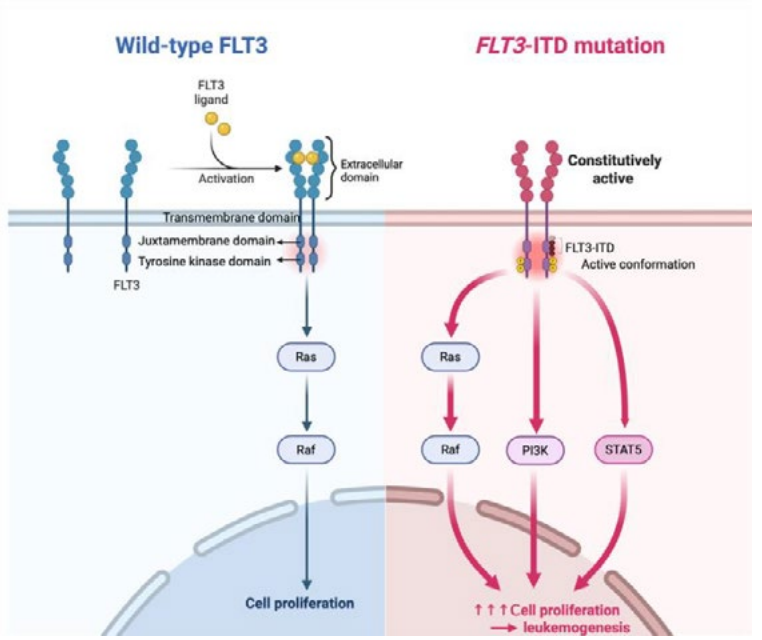

Figure 4. *FLT3 signaling in wild-type and FLT3-ITD mutated states. In normal hematopoiesis (left), ligand binding leads to transient activation of downstream pathways, including Ras (illustrated), PI3K, and STAT5. In FLT3-ITD mutated cells (right), the receptor is constitutively active, leading to sustained activation of downstream pathways and thereby leukemogenesis. Created with BioRender.com.*

1.8.3 ETV6::RUNX1

The translocation t(12;21)(p13;q22), resulting in the fusion gene *ETV6*::*RUNX1*, is the most common genetic abnormality in childhood B-ALL, seen in approximately 25% of patients, especially between 2 and 10 years of age. The fusion transcript is extremely rare in adults, with only isolated cases reported (65, 66). According to the WHO classification, *ETV6*::*RUNX1* is recognized as a distinct entity and confers standard risk. The translocation is often cryptic at the cytogenetic level but can be detected with FISH or RT-qPCR.

Both *ETV6* and *RUNX1* encode transcription factors, and the resulting chimeric protein disrupts normal hematopoiesis by promoting self-renewal and impairing differentiation through oncogenic pathways such as STAT3/MYC and PI3K/AKT/mTOR. The translocation is an early, prenatal event, and additional postnatal genetic changes are required for leukemia development (67). An earlier study performed WGS and WES on diagnostic samples from 57 pediatric ALL patients with the *ETV6*::*RUNX1* fusion and revealed an average of 11 structural variants and 14 coding point mutations per case. The main mutational mechanism was RAG-mediated genomic rearrangements, which targeted and inactivated genes essential for normal B-cell development. These rearrangements remained active throughout leukemic evolution, often producing recurrent, clustered deletions (68).

Additional recurrent genetic abnormalities in *ETV6*::*RUNX1*-positive ALL include *ETV6* deletions, *CDKN2A/B* deletions, *PAX5* deletions, and copy number changes such as 6q and 11q deletions, as well as gains of chromosome 21 (69). The fusion has been detected in neonatal blood samples (Guthrie cards) and in both monozygotic twins when one twin develops *ETV6*::*RUNX1*-positive ALL, supporting its prenatal origin. However, only around 5% of such twin pairs both develop leukemia, and there can be a latency of up to 14 years before disease onset, supporting a two-hit hypothesis of leukemogenesis (67).

ETV6::*RUNX1*-positive ALL is associated with a favorable prognosis, with an OS of 90-95%. However, this subtype has an increased risk of late relapses (>5 years post-diagnosis), likely due to the persistence of quiescent pre-leukemic clones (67).

1.9 RISK STRATIFICATION AND PROGNOSTIC FACTORS

1.9.1 ADULT AML

Risk stratification aims to identify patients with an increased risk of relapse. In adult AML, risk groups are defined according to the ELN risk stratification, which is based on cytogenetic and molecular genetic findings at diagnosis, and categorizes patients into three groups: favorable, intermediate, and adverse risk (52) (Table 4). For patients in the intermediate- and adverse-risk group, allogeneic stem cell transplantation (alloSCT) is recommended to reduce the risk of relapse, given that the patient's condition allows it. In addition to genetic characterization, the 2022 ELN recommendations emphasize the importance of initial treatment response and early MRD status. This means that a patient with favorable-risk genetics may be reclassified to the intermediate-risk group based on poor treatment response, and vice versa. According to the Swedish National Care Program for AML, patients with >15% blasts after the first cycle of chemotherapy or those requiring more than two cycles to achieve complete remission (CR) are considered high-risk, regardless of genetic findings (12). The role of MRD in risk stratification will be discussed in more detail further ahead in this thesis.

In addition to genetic findings at diagnosis and response to treatment, other factors also influence prognosis. These include high age (typically over 75 years), comorbidities, poor performance status, antecedent hematologic disorders, extramedullary involvement, and baseline leukocyte count.

***Table 4**. European LeukemiaNet (ELN) 2022 risk classification based on genetic findings at AML diagnosis.*

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 • bZIP in-frame mutated *CEBPA*‖
Intermediate	• Mutated *NPM1*†,§ with *FLT3*-ITD • Wild-type *NPM1* with *FLT3*-ITD (without adverse-risk genetic lesions) • t(9;11)(p21.3;q23.3)/*MLLT3*::*KMT2A*†,¶ • Cytogenetic and/or molecular abnormalities not classified as favorable or adverse
Adverse	• t(6;9)(p23.3;q34.1)/*DEK*::*NUP214* • t(v;11q23.3)/*KMT2A*-rearranged# • t(9;22)(q34.1;q11.2)/*BCR*::*ABL1* • t(8;16)(p11.2;p13.3)/*KAT6A*::*CREBBP* • inv(3)(q21.3q26.2) or t(3;3)(q21.3;q26.2)/*GATA2*, *MECOM(EVI1)* • t(3q26.2;v)/*MECOM*(*EVI1*)-rearranged • −5 or del(5q); −7; −17/abn(17p) • Complex karyotype,** monosomal karyotype†† • Mutated *ASXL1, BCOR, EZH2, RUNX1, SF3B1, SRSF2, STAG2, U2AF1, and/or ZRSR2*‡‡ • Mutated *TP53*[a]

†Mainly based on results observed in intensively treated patients. Initial risk assignment may change during the treatment course based on the results from analyses of measurable residual disease.
‡Concurrent *KIT* and/or *FLT3* gene mutation does not alter risk categorization.
§AML with *NPM1* mutation and adverse-risk cytogenetic abnormalities are categorized as adverse-risk.
‖Only in-frame mutations affecting the basic leucine zipper (bZIP) region of *CEBPA*, irrespective whether they occur as monoallelic or biallelic mutations, have been associated with favorable outcome.
¶The presence of t(9;11)(p21.3;q23.3) takes precedence over rare, concurrent adverse-risk gene mutations.
#Excluding *KMT2A* partial tandem duplication (PTD).
**Complex karyotype: ≥3 unrelated chromosome abnormalities in the absence of other class-defining recurring genetic abnormalities; excludes hyperdiploid karyotypes with three or more trisomies (or polysomies) without structural abnormalities.
††Monosomal karyotype: presence of two or more distinct monosomies (excluding loss of X or Y), or one single autosomal monosomy in combination with at least one structural chromosome abnormality (excluding core-binding factor AML).
‡‡For the time being, these markers should not be used as an adverse prognostic marker if they co-occur with favorable-risk AML subtypes.
[a]*TP53* mutation at a variant allele fraction of at least 10%, irrespective of the *TP53* allelic status (mono- or biallelic mutation); *TP53* mutations are significantly associated with AML with complex and monosomal karyotype.

1.9.2 CHILDHOOD AML

According to the treatment protocol used in Sweden since 2013, the Nordic Society for Paediatric Haematology and Oncology (NOPHO)-DBH AML 2012 protocol, pediatric AML patients are stratified into two risk groups: standard- or high-risk, primarily based on response to initial treatment. Patients are assigned to the high-risk group if they achieve CR after two cycles of induction treatment, and at least one of the following criteria is met:

1. Poor response after the first cycle, defined as ≥15% leukemic cells in bone marrow by MFC.
2. ≥0.1% leukemic cells by MFC after the second cycle.
3. Presence of *FLT3*-ITD mutation without a concurrent *NPM1* mutation.

Inv(16) influences treatment intensity and is associated with a favorable prognosis, although it is not used for risk stratifying in the current NOPHO protocol (70). In contrast, other international study protocols, such as the BFM (Germany) and MyeChild (UK and France), include additional genetic aberrations for risk stratification. In these, t(8;21), inv(16), *NPM1* mutations, and *CEBPA* bZIP mutations define standard-risk, while other alterations such as t(3;21)(q26;q22)/*RUNX1*::*MECOM*, t(6;9)(p22;q34)/*DEK*::*NUP214*, *CBFA2T3*::*GLIS2*, *NUP98*::*NSD1*, and t(4;11)(q21;q23)/*KMT2A*::*AFF1* are classified as high-risk (25).

1.9.3 CHILDHOOD ALL

Risk stratification of pediatric ALL patients at the time of Paper I was based on the NOPHO-ALL 2008 protocol, which classified patients into three risk groups: standard risk, intermediate risk, and high risk. In B-ALL, standard risk was defined as a leukocyte count <100 x 10^9/L, MRD <0.1% on day 29, and absence of central nervous system (CNS) involvement at diagnosis. Intermediate risk included patients with leukocyte count <100 x 10^9/L and MRD 0.1–5% on day 29, or leukocyte count ≥100 x 10^9/L with MRD <0.1% on day 29. Certain cytogenetic abnormalities, including dic(9;20), iAMP21, and t(1;19), also placed patients in the intermediate-risk group. High risk was defined as a leukocyte count <100 x 10^9/L and >5% blasts in bone marrow on day 29, or MRD >0.1% on day 79. Additionally, patients with hypodiploidy or *KMT2A*-rearranged ALL were classified as high risk. The currently used treatment protocol for children and young adults with ALL is the ALLTogether protocol, which incorporates a more refined and comprehensive risk stratification system.

1.10 TREATMENT

For decades, the cornerstone of treatment for acute leukemia has been a combination of chemotherapeutic drugs, with the primary goal of achieving CR. According to the ELN, CR is defined by the following criteria (71):

- <5% blasts in bone marrow, and presence of regenerating hematopoiesis
- absence of extramedullary leukemia and no peripheral blasts
- neutrophil count >1 x 10^9/L (≥0.5 for pediatric AML patients)
- platelet count >100 x 10^9/L (≥80 for pediatric AML patients)
- no ongoing need for erythrocyte transfusions

For the majority of AML patients up to 75 years of age, remission-inducing therapy is generally recommended. However, age alone is not a sufficient reason to withhold intensive treatment, as several studies have demonstrated that older patients benefit more from intensive treatment than dose-reduced regimens (72-74). All treatment administered prior to achieving CR is referred to as induction therapy, and consists of a combination of an anthracycline, most commonly daunorubicin, together with cytarabine (75). This regimen has remained standard since its introduction in the early 1970s (76). Around 75-80% of patients under 65 years of age achieve CR following intensive induction treatment (77), although remission rates vary among genetic and chromosomal subgroups. In pediatric AML, etoposide is often added as a third agent during the first induction cycle. Patients who achieve CR receive post-remission therapy, referred to as consolidation, which typically consists of one to three additional cycles of daunorubicin and cytarabine.

The treatment approach for pediatric ALL differs from that of pediatric AML and is also considerably longer, with a total duration of over two years. Induction therapy typically includes dexamethasone, vincristine, and pegylated asparaginase, with the addition of daunorubicin for high-risk and T-cell ALL patients. Intrathecal methotrexate is also administered during induction. Consolidation therapy is adapted based on MRD status: patients in the standard-risk group without detectable MRD continue with vincristine and a reduced number of intrathecal doses, while all other patients receive full-dose treatment including vincristine, cyclophosphamide, and intensified intrathecal therapy. Following consolidation, maintenance therapy is given for about two years and consists of methotrexate and 6-mercaptopurine (78).

1.10.1 ALLOGENEIC HEMATOPOIETIC STEM CELL TRANSPLANTATION

An alloSCT is recommended as consolidation therapy for patients in remission, including adult patients in the intermediate- or high-risk groups and pediatric patients in the high-risk group. Studies have shown that alloSCT in first CR reduces the risk of relapse in AML by more than 60% compared to intensive chemotherapy alone (79).

The procedure involves intensive conditioning chemotherapy to eradicate residual leukemic cells and suppress the recipient's immune system, followed by infusion of donor stem cells from a human leukocyte antigen (HLA)-matched sibling or an unrelated donor from a registry. For patients without an HLA-matched donor, a haploidentical donor may be used as an alternative (80). The curative potential of alloSCT relies on both the conditioning regimen and the graft-versus-leukemia (GvL) effect, in which donor immune cells attack remaining leukemic cells. The GvL effect can be enhanced through donor lymphocyte infusion (DLI) or by tapering immunosuppressive therapy. However, donor cells may also trigger graft-versus-host disease (GvHD), which can be acute or chronic. Acute GvHD typically occurs within the first 100 days post-transplant and primarily affects the skin, liver, and gastrointestinal tract. Severe cases of acute GvHD can lead to multi-organ failure. Chronic GvHD develops beyond day 100 and may persist for months or years, affecting multiple organs and contributing to long-term morbidity and reduced quality of life.

AlloSCT is also associated with other significant risks, including transplant-related mortality (approximately 10% in adults), infections, and late complications such as secondary malignancies, infertility, and cardiac toxicity (81). Given these risks, an individualized risk-benefit assessment is essential to determine whether the benefits of transplantation outweigh the potential harms. In pediatric leukemia, treatment is generally tailored to minimize long-term toxicity, and alloSCT is avoided whenever possible. This contrasts with adult AML treatment, where alloSCT is more frequently used in first remission, mainly due to the higher relapse risk and the fact that chemotherapy alone is often insufficient for long-term disease control.

1.10.2 TARGETED AGENTS

During the last decade, several novel therapies – including targeted therapies – have been introduced into the treatment arsenal for AML. The first FLT3

inhibitor, the multitargeted kinase inhibitor midostaurin, was approved in 2017 following the RATIFY trial, a phase III study that demonstrated prolonged OS and event-free survival (EFS) when added to standard chemotherapy in patients under 60 years of age (82). Since the introduction of midostaurin, several FLT3 inhibitors with differing selectivity and mechanisms of action have been developed, commonly classified as type I or type II inhibitors:

- **Type I inhibitors** bind to the active state of FLT3 and are effective against both ITD and TKD mutations, these include midostaurin and gilteritinib.
- **Type II inhibitors** bind to the inactive state of FLT3 and are mainly effective against ITD mutations, these include quizartinib and sorafenib (83).

For adult AML patients, FLT3 inhibitors are used during induction therapy, as post-alloSCT maintenance, and at relapse (including post-alloSCT relapse). These agents are also soon being incorporated into the treatment protocols for pediatric AML. Several clinical trials have demonstrated improved outcomes when FLT3 inhibitors are added to standard therapy (84-87). However, resistance development is a relatively common problem and occurs through different mechanisms such as clonal evolution, protection within the bone marrow microenvironment, or activation of alternative signaling pathways (88).

Another targeted agent is gemtuzumab ozogamicin, a CD33-directed antibody-drug conjugate, which is used in patients with core binding factor (CBF)-AML (including t(8;21) and inv(16)). It is typically administered during induction treatment together with intensive chemotherapy (12).

Menin inhibitors are a promising new class of targeted agents for patients with *KMT2A* rearrangements or *NPM1* mutations. These drugs inhibit menin, a cofactor that facilitates interaction between *KMT2A* and chromatin, thereby stabilizing the transcription of *HOXA9* and *MEIS1*, two key regulators of leukemogenesis. Disrupting this interaction downregulates *HOXA9* and *MEIS1*, leading to apoptosis of leukemic cells and promoting normal myeloid differentiation. Several clinical trials are currently ongoing, showing promising results, especially in patients with both *NPM1* and *FLT3* mutations (89, 90).

1.10.3 LOW-INTENSITY TREATMENT

Venetoclax is a relatively new agent that induces apoptosis in leukemic cells by inhibiting BCL-2, a protein that protects leukemic cells from apoptosis. It is primarily used in elderly or unfit patients who are ineligible for intensive chemotherapy, either in combination with azacitidine or low-dose cytarabine. Venetoclax is also an option for patients with relapsed or refractory AML (91-93).

Azacitidine is a hypomethylating agent that inhibits DNA methylation and thereby reactivates silenced tumor suppressor genes. Similar to venetoclax, it is primarily used in elderly or unfit patients as an alternative to intensive chemotherapy and can also be administered as maintenance therapy following remission or alloSCT. It can be given either alone or in combination with venetoclax, with synergistic effects observed especially in patients with *NPM1* or *IDH* mutations (94-97).

1.10.4 REFRACTORY AND RELAPSED DISEASE

In adult AML, refractory disease is defined as failure to achieve CR after two cycles of intensive induction chemotherapy (52). In such cases, where initial induction with cytarabine and daunorubicin is unsuccessful, there is no clearly established second-line treatment. Examples of salvage regimens that have shown efficacy include a combination of amsacrine, cytarabine and etoposide (ACE), as well as a combination of fludarabine, low-dose cytarabine, asparaginase, and idarubicin (FLA-IDA). Additionally, alloSCT can be performed in patients who achieve a second remission (CR2), with relatively favorable outcomes – particularly in patients with low-risk genetics (98).

In pediatric AML, resistant disease is defined as the presence of ≥5% leukemic cells in bone marrow after the second induction cycle. These patients are not treated according to standard protocols, but FLA-based regimens have demonstrated effectiveness.

For patients who experience relapse, treatment strategies depend on factors such as prior treatment response, genetic profile, and time to relapse. Standard options include re-induction chemotherapy and targeted agents. In recent years, combinations such as azacitidine and venetoclax have shown promising efficacy in selected patient groups, especially among older or unfit patients. For eligible patients, alloSCT in CR2 is recommended. If the patient has previously undergone alloSCT, DLI is a potential treatment option at relapse.

1.11 MEASURABLE RESIDUAL DISEASE

1.11.1 DEFINITION OF MRD

Measurable (previously referred to as minimal) residual disease is defined as the presence of leukemic cells in bone marrow or peripheral blood during or after treatment, in numbers that are well below the sensitivity of routine morphology (i.e. <5%). The presence of MRD indicates that leukemic cells have survived treatment and is associated with an increased risk of relapse (Figure 5).

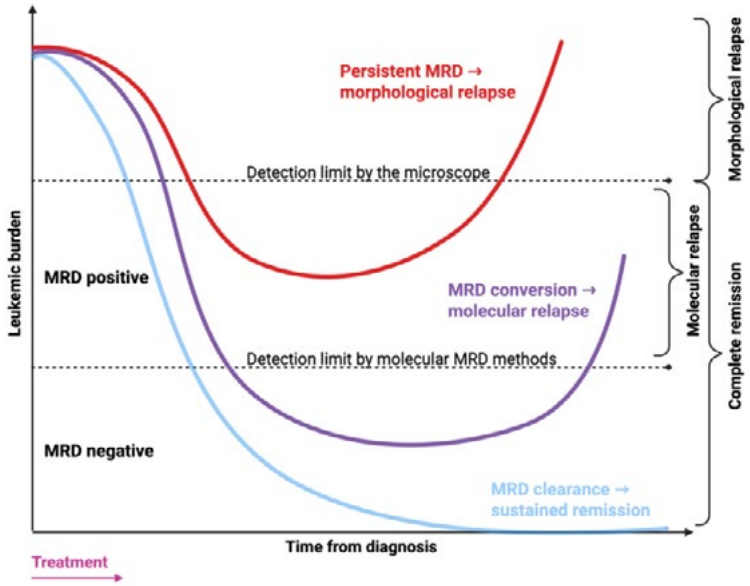

***Figure 5**. Illustration of the concept of measurable residual disease (MRD) and how different clinical trajectories can evolve after treatment. The red curve shows persistent MRD above the molecular detection limit, eventually leading to morphological relapse, while the purple curve illustrates MRD conversion after initial clearance. The light blue curve represents successful MRD clearance and sustained remission. Dashed lines indicate the detection limits of morphology and molecular MRD methods. Created with BioRender.com.*

Traditionally, diagnosis and treatment monitoring in acute leukemia have relied on morphological analysis, where leukemic blasts are identified via light microscopy and assessed based on size, nuclear morphology, and cytoplasmic properties. Morphological assessment has been a cornerstone of acute leukemia diagnostics since the disease was first described. However, this method has a limited sensitivity of approximately 5%, meaning that a

considerable amount of residual leukemic cells may go undetected. Also, the assessment is subjective and may vary between performing persons.

The term "minimal residual disease" was introduced into the medical literature during the 1980s. In 1981, a landmark study showed that ALL-patients in morphological remission could still harbor residual malignant cells that were undetectable by the light microscope but identifiable using a new immunofluorescence technique based on monoclonal antibodies directed against cell surface markers. The study showed that patients with residual leukemic cells were more prone to relapse (99). Over the next decade, methods for detecting residual leukemic cells were improved, and especially with the development of MFC and PCR-based techniques (100). While the concept of MRD was first established and clinically implemented in ALL, where it has long been part of risk stratification and treatment decisions, its role in AML has gradually become more prominent. Growing evidence of its clinical value, together with advancements in methodology, have now established MRD as one of the most important prognostic factors, guiding clinical decisions.

In 2018, ELN incorporated MRD into its official AML guidelines as a standardized biomarker, and recommended that patients with *NPM1* type A, t(8;21), inv(16) or t(15;17) should be monitored with a molecular technique, and the remaining should be monitored with MFC (101). The guidelines were updated in 2021 with several important revisions, including more detailed technical recommendations for NGS-based MRD analysis, as well as recommendations on combining different methods. However, while the updates focus on *how* MRD should be measured, the clinical interpretation of MRD results remains less clearly defined in the current ELN guidelines.

The primary main objectives of MRD analysis are:

- To identify genetically low-risk patients who have a higher risk of relapse and may therefore benefit from alloSCT in the first remission.
- To identify genetically intermediate-risk patients who have a relatively low risk of relapse, where avoidance of alloSCT may be advantageous.
- To identify patients at increased risk of relapse post-alloSCT.
- To enable early detection of molecular relapse after treatment completion, allowing for intervention to prevent morphological relapse (12).

1.11.2 TIME POINTS FOR MRD ASSESSMENT

MRD is measured both during and after treatment, with different time points applied in adult and pediatric AML, as well as in pediatric ALL. The specific time points used clinically at the time of writing this thesis are detailed below.

Adult AML:

- After the second cycle of chemotherapy: for assessment of treatment response and decision on alloSCT.
- Before alloSCT: to evaluate disease burden and assess relapse risk.
- After alloSCT: to allow early detection of relapse and guide potential interventions.
- After completion of treatment: to monitor for early detection of relapse in patients with an RT-qPCR marker. Blood samples are typically analyzed monthly during the first year and every second month during the second year.

Childhood AML:

- Day 22 after the first induction cycle: to guide risk stratification and treatment intensity. If >5% leukemic cells – the second cycle of induction is given immediately without waiting for the bone marrow to regenerate. If <5% leukemic cells – MRD analysis is performed weekly during bone marrow regeneration.
- Day 22 after the second induction cycle: to guide risk stratification and treatment intensity. If >5% leukemic cells – the patient is classified as having resistant disease. If <5% leukemic cells – MRD is measured weekly during bone marrow regeneration.
- Before consolidation: to guide decisions on treatment intensity, including alloSCT.
- Before and alloSCT (2 to 4 weeks prior): to assess relapse risk.
- After alloSCT: to guide immunological intervention.
- After treatment completion: to monitor for early relapse in patients with an RT-qPCR marker.

Childhood ALL:

- Day 15 during induction: for early assessment of treatment response, primarily to detect immunophenotypic shifts during treatment.
- Day 29, i.e. at the end of induction: to determine risk stratification and adjust treatment intensity.
- Day 78, i.e. after consolidation: to refine treatment strategies.

1.11.3 MRD ANALYSIS METHODS

1.11.3.1 MULTIPARAMETER FLOW CYTOMETRY

MFC is the most frequently used method for MRD analysis in Sweden, both in adult and pediatric AML, as well as in B-ALL. It is applicable in approximately 90% of AML cases and in over 98% of pediatric ALL cases (102, 103). MFC is based on the principle of detecting cluster of differentiation (CD) antigens on the surface of leukemic cells, while simultaneously analyzing cell size and complexity. It offers a sensitivity of 0.1-0.01% in AML, an as low as down to 0.001% in ALL. For MRD analysis, bone marrow aspirate is processed with a lysis buffer to remove erythrocytes. In AML, around 500,000 to 1 million cells per sample are analyzed, and in ALL, 4-5 million cells are typically required. Monoclonal antibodies conjugated to fluorochromes are added to target relevant CD markers, using MRD-panels with specific combinations of myeloid or lymphoid markers. MFC enables simultaneous analysis of 8-12 (or more) fluorochromes per cell, allowing for detailed immunophenotypic profiling.

Leukemic blasts often express a leukemia-associated immunophenotype (LAIP), defined by aberrant expression of specific CD markers that distinguishes them from normal hematopoietic cells. The LAIP is patient-specific and generally remains stable during the course of the disease, making it suitable for MRD detection. An example of an AML-LAIP can be a combination of typical blast markers (e.g. CD34, CD117), together with an abnormal expression of myeloid markers (CD13, CD33), stem cell markers (HLA-DR, CD133), and/or aberrant expression of lymphoid markers (CD7, CD19, CD56). During analysis, stained cells pass through the flow cytometer one by one, guided by a hydrodynamic focusing system that ensures that the cells move in a single, uniform stream. As each cell passes a laser beam, the fluorochromes are excited and emit fluorescence signals detected by photodetectors. At the same time, the emitted light is measured in forward scatter (FSC) and side scatter (SSC), providing information of cell size and granularity. The combination of fluorescence data and light-scattering patterns is used to identify and distinguish leukemic blasts from normal cells, using a gating strategy based on both scatter properties and CD marker expression (Figure 6).

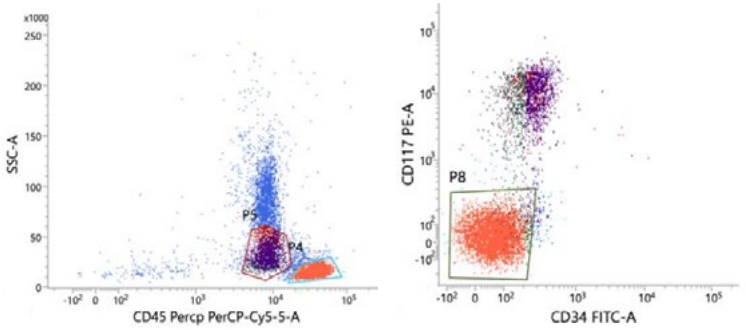

***Figure 6**. Multiparameter Flow Cytometry plots of bone marrow at diagnosis from a patient with NPM1-mutated AML. Leukemic blasts (dark purple) are here identified by low side scatter (SSC), low CD45 expression, and CD117 positivity. Lymphocytes (orange) show high CD45 expression and low SSC and are negative for both CD34 and CD117.*

An alternative to the LAIP approach is the different-from-normal (DfN) strategy, which focuses on aberrant patterns of differentiation and maturation by analyzing antigen expression. DfN enables identification of new aberrancies during treatment, which may reflect clonal evolution or shifts in antigen expression. In contrast to the LAIP-approach, DfN does not require a diagnostic sample for comparison, making it particularly valuable in cases where no diagnostic sample is available. To optimize MRD detection, ELN recommends a combination of these two methods (104).

One limitation with MFC is its relatively low negative predictive value (NPV), where several studies have shown that a considerable proportion of MRD-negative patients still experience relapse (105, 106). This could be explained by the heterogeneous nature of leukemic cells, meaning that not all malignant cells can be distinguished from normal ones. Additionally, leukemic cells can alter their antigen expression during treatment, a phenomenon known as immunophenotypic shift, allowing them to evade detection. Beyond these limitations, MFC is costly and requires extensive standardization across laboratories, as well as significant expertise among the performers. One proposed strategy to improve both NPV and sensitivity is to use MFC for MRD detection specifically targeting leukemic stem cells, using aberrant markers such as CD123 or CLL-1. However, due to antigenic overlap with normal hematopoietic stem cells, this approach remains investigational (107, 108).

MOLECULAR METHODS

Molecular techniques are an alternative to MFC in MRD analysis in acute leukemia and generally offer a higher sensitivity (109, 110), although they are applicable to a smaller subset of patients. These methods are well established in AML but are currently less widely implemented in ALL. Molecular methods can be either RNA-based, such as RT-qPCR, or DNA-based, such as quantitative PCR (qPCR), droplet digital PCR (ddPCR) or NGS-based methods, including deep sequencing.

RT-qPCR

RT-qPCR measures leukemic RNA molecules and is the standard method for MRD analysis in AML patients with *NPM1* type A mutation, t(8;21) and inv(16) – alterations found in approximately one third of patients. RT-qPCR enables MRD detection with a sensitivity of 0.01-0.001%, depending on the target gene analyzed (111). For patients with an RT-qPCR marker, MRD monitoring using monthly blood samples is recommended for 1-2 years post-treatment for both adults and children to detect impending relapse. However, as two-thirds of AML patients lack a standardized RT-qPCR marker, this large group is currently not offered post-treatment monitoring for early relapse detection.

For the RT-qPCR analysis, mRNA is extracted from bone marrow or peripheral blood and converted into complementary DNA (cDNA) using reverse transcription. The target sequence is then amplified using specific primers and a fluorescently labeled probe. During amplification, the probe is cleaved by the polymerase and releases a fluorescent signal that increases proportionally with the amount of amplified target DNA. The fluorescence is measured in real-time, cycle by cycle, and provides a quantitative assessment of the number of leukemic cells. The cycle threshold (Ct) value represents the PCR cycle at which the fluorescence signal surpasses a predefined threshold. A lower Ct value indicates a higher number of leukemic cells, while a higher Ct value indicates lower disease burden. To quantify MRD levels, Ct values are compared against a standard curve generated from serial dilutions of a sample with known target transcript concentration. The target gene expression is normalized to a reference gene (commonly *ABL1*), which serves as an internal control to adjust for variations in RNA across different samples. MRD levels are expressed as the ratio of target gene expression to that of the reference gene.

MRD analysis of *NPM1*

NPM1 mutations usually arise *de novo,* remain stable over the course of the disease, and are present in the whole leukemic population, making them suitable molecular MRD markers. However, around 10% of patients have lost the *NPM1* mutation at relapse (112, 113). The primary recommended method for MRD analysis is RT-qPCR, which is the most sensitive and also well-established and validated method. Other techniques such as ddPCR, qPCR and deep sequencing, can also be used depending on the specific *NPM1* mutation type and the available technology. The findings from Paper II have contributed to the clinical implementation of DNA-based methods for MRD analysis of *NPM1* (114). Compared to RT-qPCR, DNA-based methods offer several advantages, including broader mutation coverage and independence from gene expression variability. These methods allow for more accurate quantification of the proportion of cells harboring the mutation. qPCR is highly precise and offers a high sensitivity (115, 116). An additional advantage for ddPCR and deep sequencing is that neither of them require standard curves or reference genes (117, 118). From a practical perspective, DNA extraction is more flexible and less technically demanding than RNA extraction. Furthermore, DNA is more stable, which simplifies sample handling and transport. The primary limitation of RT-qPCR is that it is mutation-specific, and since there are around 90 different *NPM1* mutation types, the clinical laboratory needs to set up multiple assays to enable MRD analysis for all patients with *NPM1* mutations. Due to the requirement for strict quality control, the majority of clinical laboratories therefore only analyze the most common *NPM1* mutation(s), leading to unequal care for patients with *NPM1*-mutated AML.

Next-generation sequencing

NGS has emerged as a highly sensitive approach for MRD detection in acute leukemia, offering several advantages over traditional methods and enabling analysis in almost all patients. NGS is a high-throughput sequencing technology that allows simultaneous analysis of millions of DNA fragments. Unlike the traditional method Sanger sequencing, which processes one DNA fragment at a time, NGS enables massively parallel sequencing. The technology includes various platforms and methodologies, including short-read sequencing, long-read sequencing and targeted approaches such as amplicon sequencing. Depending on the application, NGS can be used for WGS, WES, WTS, or targeted gene panel sequencing. Today, the majority of Swedish laboratories perform diagnostic NGS using a myeloid gene panel developed by Genomic Medicine Sweden. This panel includes around 200 genes associated with myeloid neoplasms, as well as genes linked to hereditary

forms of AML. In addition to reporting the variants identified in the gene panel analysis, the VAF of each gene is reported which describes the proportion of sequencing reads in which a specific variant is detected. Germline variants are generally present in all cells, typically resulting in a VAF of ~50% for heterozygous variants and ~100% for homozygous or hemizygous variants. In contrast, somatic variants usually show VAFs below 50% and reflect the tumor burden in the sample. However, VAF alone cannot determine whether a variant is germline or somatic. To distinguish between germline and somatic variants, a matched normal sample from the patient (such as skin or buccal swab) is required for comparison.

Mutations detected in the myeloid gene panel at diagnosis may be used for MRD monitoring – except for germline mutations and the so called DTA-mutations (*DNMT3A*, *TET2* and *ASXL1*), which are common in clonal hematopoiesis and therefore not reliable MRD markers (119-121). Mutations in genes involved in signaling pathways, such as *FLT3*, *KIT*, *NRAS*, and *KRAS,* indicate residual disease, but since they are often subclonal and have a low NPV, the ELN recommends that they be used in combination with an additional MRD marker (104). Currently, there are insufficient data to support the use of NGS-MRD as a stand-alone technique, underscoring the need for further standardization and clinical validation before it can replace conventional MRD methods.

Deep sequencing

We and others have developed deep sequencing, an NGS-based technique that enables high-coverage sequencing of specific genomic regions. Deep sequencing of *NPM1* has been shown, by us and others, as a feasible method for MRD analysis (117, 122, 123). Since it sequences the entire mutation hotspot region of the gene, it allows for the detection of all *NPM1* mutation types, including rare variants. This is a major advantage compared to RT-qPCR, which requires mutation-specific assays tailored for each individual variant. Since 2021, deep sequencing has been implemented in clinical routine at Sahlgrenska University Hospital for patients with rare *NPM1* mutations. Over time, both the number of incoming samples and the diversity of specific *NPM1* variants have increased steadily, showing the clinical need for this assay. In 2022, deep sequencing of *FLT3*-ITD was also implemented into clinical routine diagnostics at Sahlgrenska. In adult AML patients, this analysis is used to evaluate treatment response, monitor during ongoing treatment with FLT3 inhibitors, as well as monitor after completed treatment (58, 83). Its clinical value in pediatric AML has not yet been established.

1.11.4 CLINICAL SIGNIFICANCE OF MRD

In recent years, mounting evidence has confirmed the clinical significance of MRD in acute leukemia. A meta-analysis of 81 trials, including over 11,000 AML patients, demonstrated a strong association between MRD status and prognosis: MRD-negative patients had a 5-year OS of 68%, compared to 34% for MRD-positive patients – regardless of disease characteristics, age, MRD analysis method, or time point (124).

MRD by MFC in adult and pediatric AML

The prognostic value of MRD analysis using MFC in adult AML has been described in multiple studies (125-129). The AML17 trial, which included nearly 2,500 patients under 60 years of age without an *NPM1* mutation, found that presence of MRD ≥0.1% after the second treatment cycle was associated with an 89% risk of relapse (128). Post-alloSCT MRD assessment using MFC also carries prognostic value: Shah *et al*. reported that presence of MRD within one month after transplantation was associated with a 78% relapse incidence (130).

The value of MFC-based MRD has been confirmed in various clinical studies dating back to the early 2000s, in both ALL and AML (131-135). In pediatric AML, MRD ≥0.1% measured by MFC after induction treatment is one of the strongest prognostic factors (106, 136-138). In the NOPHO-AML 2004 study, patients with MRD ≥0.1% at the start of consolidation had an EFS of only 11% (106). Results from the latest NOPHO protocol showed that patients with ≥15% leukemic cells after the first induction or ≥0.1% after the second induction who received alloSCT had a 5-year OS of 80% – one of the best cure rates reported in pediatric AML (22).

Molecular MRD analysis of *NPM1* mutations

The prognostic value of MRD analysis of mutated *NPM1* using molecular methods has been described in multiple clinical studies (52, 139-145). Detection of *NPM1* transcripts in blood after the second cycle, or a $<3\log_{10}$ reduction (i.e. >0.1% relative to the diagnostic level) in bone marrow either after the second cycle or post-treatment, has been associated with a high risk of relapse (141, 142). In a study of 346 *NPM1*-mutated AML patients, Ivey *et al.* reported a cumulative incidence of relapse of 82% in MRD-positive patients after two cycles of treatment, compared to 30% among MRD-negative patients (139). MRD analysis of *NPM1* is also playing an increasingly important role in the decision-making on alloSCT and its timing, although no definitive clinical guidelines currently exist (146).

NGS-based MRD assessment

In the last years, multiple studies have evaluated the clinical utility of NGS-based MRD approaches. However, the lack of methodological standardization, combined with the absence of well-defined MRD thresholds and clinically validated time points for decision-making, remains a challenge. To date, there are no universally accepted clinical guidelines for the use of NGS-MRD in routine clinical practice.

NGS for *NPM1* and *FLT3*-ITD monitoring

Several studies have demonstrated that NGS can serve as a reliable alternative to established MRD methods, particularly for certain molecular markers such as *NPM1* and *FLT3*-ITD. Thol *et al.* were among the first to show that NGS-based analysis of *NPM1* and *FLT3*-ITD mutations is a robust MRD method, with high concordance compared to RT-qPCR (122). Patkar *et al.* also reported nearly 80% concordance between MRD assessment using NGS and MFC (147). In one study, 12 different *NPM1* mutations were analyzed in 83 AML patients using ultra-deep sequencing after induction and consolidation. Patients were categorized as MRD-positive or MRD-negative based on a >1-log reduction in VAF between time points. MRD positivity by deep sequencing (i.e. failure to clear >1-log) was strongly predictive of worse outcomes and was an independent predictor of relapse and mortality in multivariable analysis. The study also showed that the method provided prognostic value comparable to qPCR (49).

Grob *et al*. examined 161 AML patients with *FLT3*-ITD using NGS to detect patient-specific *FLT3*-ITD sequences at diagnosis and in CR. They found that 29% of patients had detectable residual *FLT3*-ITD in remission. MRD-positive patients had a 4-year relapse rate of 75% compared to 33% in MRD-negative patients, and a 4-year OS of 31% versus 57%. These findings validated *FLT3*-ITD as a clinically relevant MRD marker when analyzed using sensitive sequencing techniques (148). Other recent studies have also demonstrated the prognostic value of MRD analysis of *FLT3*-ITD using NGS (149-151).

NGS-based MRD panels

In parallel, several studies have investigated the use of broad NGS-MRD panels targeting multiple AML-associated genes, most commonly single nucleotide variants (SNVs), and have shown that NGS-based MRD assessment has strong prognostic value (152-154). Jongen-Lavrencic *et al.* analyzed 430 AML patients in CR using targeted NGS of multiple AML-related genes. MRD was detected in 28% of patients after the second chemotherapy cycle, where

MRD-positive patients had a 4-year relapse rate of 55%, compared to 32% among MRD-negative patients. Importantly, they found that patients with only DTA-mutations did not have an inferior prognosis. These findings support the use of molecular MRD to refine post-remission risk stratification (155). Tsai *et al.* performed sequential NGS-MRD monitoring in 335 AML patients using a 54-gene panel in first remission and after the first consolidation cycle. They found that 46% of patients had detectable MRD after induction and 29% after consolidation. Presence of MRD at either time point predicted worse outcomes, and MRD detected after consolidation was the strongest prognostic predictor. At both time points, NGS detected MRD in a subset of patients who were MRD-negative by MFC, highlighting the ability of NGS to detect otherwise undetected high-risk patients. These findings underscore the value of serial NGS-MRD analyses and suggest that post-consolidation MRD status is particularly relevant for risk stratification (156).

In another study, Hirsch *et al.* performed error-corrected NGS with a sensitivity of 0.1% in 189 patients to track all diagnostic mutations through remission, including DTA mutations (157). The persistence of any mutation in CR was associated with worse outcomes, but the effect was mainly driven by the presence of multiple residual mutations. Patients with at least two mutations detected in CR had a significantly worse prognosis, whereas presence of a single mutation, especially if it was a DTA mutation, was not predictive of relapse.

Molecular MRD assessment in pediatric ALL

In pediatric ALL, molecular techniques serve as a complementary approach to the well-established MFC for MRD detection. For ALL patients treated according to the ALLTogether protocol, MRD analysis is routinely performed using both MFC and allele-specific oligonucleotide qPCR (ASO-qPCR) targeting clonal immunoglobulin (Ig) or T-cell receptor (TCR) rearrangements. Over 90% of ALL patients harbor unique Ig/TCR rearrangements that can serve as molecular MRD markers. ASO-qPCR typically provides a sensitivity of 0.01-0.001%, and standardized protocols are available (158). In the AIEOP-BFM ALL 2000 study, MRD was measured in nearly 3,200 children with pre-B ALL at day 33 and day 78 using ASO-qPCR. Patients with MRD <0.01% at day 33 had a 5-year EFS of 92%, while those with MRD >0.1% at day 78 had significantly worse outcomes, with an EFS of only 50% (134). These results underscore the strong prognostic value of ASO-qPCR for MRD assessment. However, despite its clinical utility, ASO-qPCR has some limitations: it requires patient-specific assays, is labor-intensive, and may have reduced

sensitivity in cases with low Ig/TCR expression. To overcome these limitations, NGS-based amplicon sequencing has emerged as a promising alternative, offering standardized detection of Ig/TCR rearrangements without the need for patient-specific primers (159-163). In a recent study of 93 children with B-ALL, NGS-based MRD analysis identified IGH or IGK clonality in 98% of patients. Concordance with MFC and RT-qPCR was seen in 75% and 71% of cases, respectively. Also, higher levels of IGH/IGK clones were associated with an increased risk of relapse (164). In a larger cohort, Svaton *et al.* compared MRD levels using NGS and qPCR in 432 pediatric B-ALL patients, analyzing 780 Ig/TCR markers with both methods. Results were concordant in 82% of cases. Interestingly, 13% of markers detected by qPCR were classified as false positives by NGS due to shared Ig/TCR rearrangements in unrelated samples. NGS reclassified 19% of patients to a lower risk group, demonstrating its ability to reduce overtreatment based on false-positive qPCR findings. Taken together, the study showed that NGS-based MRD analysis can improve risk stratification, especially in patients with undetectable MRD by conventional methods, potentially allowing for treatment reduction in low-risk cases (165).

2 AIM

The overall aim of this thesis was to evaluate existing and develop new methods for the detection of MRD in acute leukemia. By advancing MRD analysis methods, this research aims to improve and provide deeper insights into disease monitoring and ultimately contribute to better patient outcomes.

The specific aims of the individual papers included in this thesis were:

Paper I: To investigate whether analysis of *ETV6*::*RUNX1* fusion transcript with RT-qPCR is useful for evaluating treatment response in children with pre-B ALL, and whether the results correlate with results from MRD analysis by MFC.

Paper II: To compare RT-qPCR with DNA-based methods for MRD analysis in adults with *NPM1*-mutated AML, and to determine whether DNA-based methods provide clinically relevant information and could serve as an alternative to RT-qPCR.

Paper III: To evaluate the clinical value of MRD analysis using deep sequencing in adults with *NPM1*-mutated AML, to verify the MRD cut-off proposed in Paper II, and to assess whether MRD status during chemotherapy provides prognostic information.

Paper IV: To assess the clinical value of MRD analysis using deep sequencing of *FLT3*-ITD in children with AML.

.

3 METHODOLOGICAL CONSIDERATIONS

3.1 PATIENTS AND SAMPLES

This thesis included samples collected between 2006 and 2024 from adult and pediatric patients with AML, as well as pediatric patients with pre-B ALL. In Papers I and III, only bone marrow samples were analyzed, whereas both bone marrow and peripheral blood samples were used in Papers II and IV. All studies included diagnostic and follow up-samples, and in Papers III and IV, relapse samples were also analyzed. Samples were either analyzed as part of routine clinical diagnostics or retrospectively from biobanked material. Most samples were from the Västra Götaland Region, with additional samples from Skåne University Hospital in Lund (Paper II) and from Denmark (Paper IV).

There are important considerations when using biobanked samples instead of fresh material. One potential issue is nucleic acid degradation during long-term storage. While DNA is generally highly stable and significant degradation is less likely to occur, RNA is more susceptible to degradation over time. In Paper III, the DNA concentration extracted from biobanked aspirate slides remained stable over time, suggesting that the DNA concentration was preserved despite many years of storage – although a slightly higher limit of detection was observed in these samples, which may reflect minor storage-related effects. Another factor to consider when using biobanked bone marrow is the sequence of aspiration. During bone marrow aspiration, sequential pulls differ in composition: the first pull tends to be the most representative of the marrow, while later pulls are more diluted with peripheral blood. In both Paper I and II, RT-qPCR analyses were performed on bone marrow samples obtained after MFC and morphology, typically from the second or third pull. As a result, the material used for molecular analysis may have been slightly hemodiluted compared to the initial pull. In Paper III, the smear material was collected first, indicating that the samples used in this study were likely the most representative. While there is a theoretical risk of contamination or background "noise" in smear samples due to air exposure and processing steps, this risk is generally considered low. In pediatric AML, the first pull is routinely used for MRD analysis by MFC, followed by samples for morphology and subsequently for biobanking. Therefore, the biobanked material used in Paper IV was obtained from the last pull, which was likely more hemodiluted

compared to the first aspirate. As a result, MRD levels may have been underestimated in these samples.

3.2 ETHICAL CONSIDERATIONS

All included studies in this thesis were approved by the Regional Ethics Review Board in Gothenburg and conducted in accordance with the Declaration of Helsinki. Informed consent was obtained from all patients or their guardians for storage of samples in a local biobank and for use in research. Throughout all projects, ethical principles such as voluntary participation, confidentiality, and anonymity have been carefully observed. All analyses presented in this thesis were performed retrospectively, meaning that the results did not influence clinical decision-making or patient treatment. When analyzing genetic mutations, the analyses were designed to target somatic variants only, not germline variants, thereby avoiding potential ethical concerns.

3.3 STATISTICS

The following statistical test were used in the included papers:

Spearman's rank correlation was used to assess associations between continuous variables. This test was chosen over Pearson's correlation due to the non-normal distribution of the data. In Paper I, it was used to evaluate the correlation between *ETV6*::*RUNX1* transcript levels and MRD levels measured by MFC. In Paper II, it was applied to assess correlations between RNA- and DNA-based methods, as well as between different DNA-based methods. In Paper IV, it was used to assess the correlation between ITD length and VAF.

Bland-Altman analysis was used in Paper II to visualize the agreement between DNA-based methods. The plots showed mean values on the x-axis and differences between methods on the y-axis, including 95% confidence intervals.

Cohen's kappa was used to evaluate categorical agreement between methods. In Paper II, it was used to evaluate the concordance between bone marrow and peripheral blood results from DNA-based methods compared with RT-qPCR. It was also used to evaluate agreement between RT-qPCR and DNA-methods when applying the chosen MRD cut-offs. In Papers III and IV, it was used to assess agreement between deep sequencing and MFC. Kappa values between

0.41-0.60 were considered moderate, 0.61-0.74 substantial, and ≥0.75 as excellent agreement.

Receiver operating characteristic (ROC) curve analysis was used to determine optimal MRD cut-off values. In Paper II, ROC curves were used to define cut-offs for DNA-based methods. In Paper III, it was used to determine the optimal cut-off for deep sequencing, where the values with the highest Youden's index were selected. Youden's index (sensitivity + specificity – 1) was chosen as it balances sensitivity and specificity in a single measure and avoids subjective interpretation of the ROC curve.

The log-rank test was used in Paper III to compare survival between groups, and survival probabilities were estimated using the **Kaplan-Meier method**.

Cox proportional hazard regression was performed in Paper III using both univariable and multivariable analysis to identify factors associated with RFS and OS. RFS was chosen over EFS, as the aim was to specifically assess the impact of MRD status on relapse risk.

Mann-Whitney U test was used in Paper III to compare VAF levels between subgroups (with or without *FLT3*-ITD mutations, *DNMT3A* mutations, or relapse). This test was chosen due to the non-normal distribution of VAF levels; if the data had been normally distributed, a t-test could have been used.

Wilcoxon signed-rank test was used in Paper IV to compare VAFs between bone marrow and peripheral blood, as the samples were paired (collected from the same patient at the same time point).

3.4 MULTIPARAMETER FLOW CYTOMETRY

MRD analysis using MFC was performed in Papers I, III, and IV. In Paper I, MFC was conducted at diagnosis and on days 15, 29 and 78 during treatment, using antibodies targeting 4-6 antigens per tube. All markers were analyzed in a single tube according to the NOPHO ALL-2008 protocol, with the addition of CD13 and CD33. In Papers III and IV, MFC was performed during consolidation (Paper III) and at multiple timepoints during treatment (Paper IV), using a standardized MRD panel with monoclonal antibodies targeting eight antigens per tube, analyzed in five tubes, according to the NOPHO-DBH AML 2012 protocol, and described in a previous study (166).

An antigen was considered positive when expressed by ≥10% of leukemic cells compared with normal cells. MRD positivity was defined as ≥0.1% cells with a LAIP in bone marrow. Although lower MRD levels can be technically detected, particularly in ALL where sensitivity can reach 0.01% or lower, values below 0.1% were not considered MRD-positive in the analyses. The threshold of 0.1% was used since it is an established risk-stratifying threshold in AML. The ability to detect MRD below this level depends on the specific LAIP. At the time of Paper I, 0.1% was also the only MRD risk stratifying threshold used in pediatric ALL.

3.5 REVERSE TRANSCRIPTION QUANTITATIVE PCR

In Papers I and II, RT-qPCR was used for MRD analysis of *ETV6*::*RUNX1* fusion transcript (Paper I) and *NPM1* type A mutation (Paper II). This analysis included RNA extraction, cDNA synthesis, and quantification of the respective targets. Transcript levels were expressed in relation to a reference gene: *GUSB* for *ETV6*::*RUNX1*, and *ABL1* for *NPM1*. For *ETV6*::*RUNX1*, results were expressed as the percentage ratio between the number of fusion transcript copies and the number of copies of *GUSB*. The MRD level in follow-up samples was then expressed relative to the diagnostic ratio. For *NPM1*, MRD was calculated as the ratio between number of mutated *NPM1* copies and number of copies of *ABL1*. MRD levels were also assessed using the thresholds of a $3\log_{10}$ reduction in *NPM1* transcript levels compared to the diagnostic level, and/or a level exceeding 2%. All samples were analyzed in triplicate, and results were considered quantifiable if ≥10 copies of *ETV6*::*RUNX1* or ≥15 copies of mutated *NPM1* were detected in ≥2/3 replicates. The threshold of 10 copies for *ETV6*::*RUNX1* was based on the calibration curve, where 10 copies represented the lowest quantifiable point. For *NPM1*, the threshold of 15 copies was defined from the limit of blank, calculated by analyzing 25 samples with wild-type *NPM1*. The mean background signal was 3.3 copies, resulting in ≥15 copies (mean + 3 standard deviations (SD)) being considered positive.

Since the time of Paper I, the RT-qPCR analysis of *ETV6*::*RUNX1* in the clinical lab has undergone some modifications. The standard curve is now based on absolute copy numbers, starting from 10 copies and upwards. In the previous method, absolute quantification of the plasmid was not performed, and a “copy” represented an arbitrary unit. Another change is that the input material previously consisted of mRNA, whereas total RNA is now used.

3.6 DEEP SEQUENCING

MRD analysis using deep sequencing of genomic DNA was performed for *NPM1* in Papers II and III, and for *FLT3*-ITD and patient-specific leukemic mutations in Paper IV. A schematic overview of the deep sequencing workflow is illustrated in Figure 7. When using NGS for MRD detection, strategies such as error-correcting algorithms, unique molecular indexes, and deep sequencing of targeted genomic regions can be used to achieve the required high sensitivity. In this thesis, an amplicon-based deep sequencing approach was used for analysis of *NPM1* and *FLT3*-ITD due to its previously demonstrated high sensitivity (117, 166), and because it enables sequencing of the entire mutation hotspot regions of the genes. This allows for detection of both known and novel genetic variants. In contrast, techniques such as RT-qPCR, ddPCR or qPCR require mutation-specific assays, making them considerably more labor-intensive. Insertions like *NPM1* are well suited for deep sequencing analysis since they are easily detected and have a lower risk of sequencing errors compared to point mutations, which are more prone to false positives. In Paper IV, additional patient-specific mutations were analyzed alongside *FLT3*-ITD to capture a broader measure of the leukemic clone. Somatic mutations were identified at diagnosis using a myeloid gene panel and selected for MRD analysis based on the highest VAF and the likelihood that the mutations were non-subclonal in most cases.

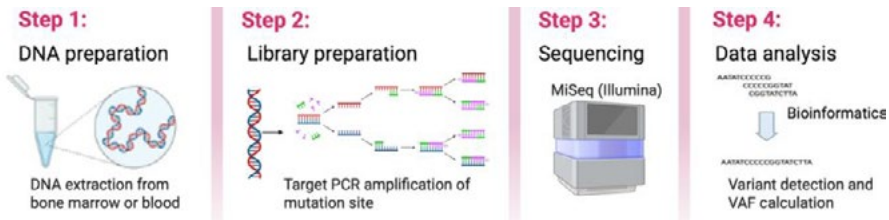

***Figure** 7. Overview of the deep sequencing workflow used for MRD analysis in this thesis. Created with BioRender.com.*

DNA was extracted from biobanked blood and bone marrow samples in Papers II and IV, and from biobanked bone marrow aspirate slides in Paper III, using the QIAamp DNA Micro Kit (Qiagen, Hilden, Germany). For detection of *NPM1* mutations, 100 ng DNA was used for PCR amplification, while 200 ng was used for *FLT3*-ITD and patient-specific mutations, as this higher input theoretically increases sensitivity. The PCR-based library preparation included the addition of index sequences for sample multiplexing and adapter sequences required for binding to the flow cell during sequencing. PCR products were

purified using AMPure XP beads (Beckman Coulter, Brea, CA), and amplicon lengths were assessed using the TapeStation 4200 system (Agilent Technologies, Santa Clara, CA). Paired-end sequencing was performed on a MiSeq (Illumina, San Diego, CA), where 16 samples (including positive and negative controls) were analyzed per run. A 12.5 pM PhiX Control (Illumina) was added to the pool to increase sequence diversity. Sequencing was performed using a sequencing-by-synthesis approach, a highly accurate technique in which specific regions of genomic DNA are first amplified with primers targeting mutation sites. The prepared libraries are loaded onto a flow cell that is coated with oligonucleotides, where the DNA strands bind and undergo clonal amplification through bridge amplification, generating millions of clusters on the surface of the flow cell. Sequencing then proceeds with primer annealing followed by repeated cycles where fluorescently labeled nucleotides are added. Only one nucleotide is incorporated per cycle, and after each incorporation, a fluorescent signal is emitted and recorded. This cyclic process is repeated base-by-base and occurs in parallel across hundreds of millions of clusters. Index sequences are used to distinguish samples multiplexed in the same run. After sequencing, reads were analyzed using bioinformatic tools to detect the presence of target mutations and calculate their VAF.

For calling of mutated and wild type *NPM1*, an in-house script was used containing all currently known *NPM1* mutations, around 90, reported in the clinical laboratory and/or in the literature. A complete list of the *NPM1* mutations included in the script is presented in Table 5. The VAF of *NPM1* was calculated as the number of reads with the mutation divided by the total number of reads with the mutated and the wild-type sequence, expressed as a percentage. For *FLT3*-ITD, a slightly modified version of the publicly available software tool *getITD*, as described by Blätte *et al*. (167), was used to detect and quantify ITDs from the sequencing data. *getITD* identifies ITD lengths up to 150 bp, and in some cases up to 242 bp. For patient-specific leukemic mutations, an error-corrected bioinformatics approach was used, where sequencing data from normal samples were subtracted to correct for technical errors.

In paper II, sequencing coverage was >7 x 10^5 reads in all samples. The limit of detection (LOD), defined as the highest signal measurable in a negative sample (i.e. background noise), was calculated as the mean + 3 SD of 21 normal samples. This corresponded to a VAF of 0.001% and 8 mutated reads. A previously performed dilution experiment demonstrated linearity down to

0.02% (117), thereby defining the limit of quantification (LOQ), the lowest level at which a signal can be reliably quantified. In paper III, sequencing coverage was >5 x 10^5 in all samples except one. The LOD was calculated based on 44 normal samples, corresponding to a VAF of 0.005% and 50 mutated reads (calculated as the mean + 3 SD). In Paper IV, coverage was >5 x 10^5 in all samples except two. The theoretical LOD was based on the number of DNA molecules analyzed and was calculated as 1 divided by the number of haploid genome copies, where one haploid genome equals 3.3 picogram of DNA. Based on the DNA input, this corresponded to an approximate LOD of 0.0015%. This level was supported by analysis of background noise in negative controls, where the signal remained below this threshold. By processing a sample with 0.01% *FLT3*-ITD VAF eight times and sequencing it in four separate runs, the coefficient of variation was 27%, which is acceptable at this low mutation burden. For patient-specific mutations, the LOD was 0.02%, which was described in a previous study (166).

Table 5. *NPM1 mutant sequences included in the script, grouped into four categories based on their respective wild-type sequence. The table displays each mutant sequence along with its corresponding Human Genome Variation Society (HGVS) nomenclature, based on the NPM1 reference sequence NM_002520.6. The mutation/insertion is highlighted in red.*

***NPM1* mutation group 1** *Wild-type sequence: AAGATCTCTGGCAGTG (c.854 to c.869)*	
HGVS nomenclature	**Mutant sequence**
c.860_863dupTCTG, p.(Trp288CysfsTer12)	TCTCTGTCTGGCAGTG
c.863_864insCATG, p.(Trp288CysfsTer12)	TCTCTGCATGGCAGTG
c.863_864insCCTG, p.(Trp288CysfsTer12)	TCTCTGCCTGGCAGTG
c.863_864insAGGA, p.(Trp288Ter)	TCTCTGAGGAGCAGTG
c.863_864insCTTG, p.(Trp288CysfsTer12)	TCTCTGCTTGGCAGTG
c.863_864insTTTG, p.(Trp288CysfsTer12)	TCTCTGTTTGGCAGTG
c.863_864insCCAG, p.(Trp288CysfsTer12)	TCTCTGCCAGGCAGTG
c.863_864insTCGG, p.(Trp288CysfsTer12)	TCTCTGTCGGGCAGTG
c.863_864insTATG, p.(Trp288CysfsTer12)	TCTCTGTATGGCAGTG
c.863_864insTTCC, p.(Trp288CysfsTer12)	TCTCTGTTCCGCAGTG
c.863_864insCCGA, p.(Trp288CysfsTer12)	TCTCTGCCGAGCAGTG
c.863_864insTAGG, p.(Trp288CysfsTer12)	TCTCTGTAGGGCAGTG
c.863_864insCAGG, p.(Trp288CysfsTer12)	TCTCTGCAGGGCAGTG
c.863_864insCGTG, p.(Trp288CysfsTer12)	TCTCTGCGTGGCAGTG
c.863_864insCAAA, p.(Trp288CysfsTer12)	TCTCTGCAAAGCAGTG
c.863_864insCTCG, p.(Trp288CysfsTer12)	TCTCTGCTCGGCAGTG
c.863_864insCCGG, p.(Trp288CysfsTer12)	TCTCTGCCGGGCAGTG
c.863_864insTTCG, p.(Trp288CysfsTer12)	TCTCTGTTCGGCAGTG
c.863_864insCTGG, p.(Trp288CysfsTer12)	TCTCTGCTGGGCAGTG
c.863_864insCAGA, p.(Trp288CysfsTer12)	TCTCTGCAGAGCAGTG
c.863_864insTGTG, p.(Trp288CysfsTer12)	TCTCTGTGTGGCAGTG
c.863_864insTCAG, p.(Trp288CysfsTer12)	TCTCTGTCAGGCAGTG
c.863_864insTAAG, p.(Trp288CysfsTer12)	TCTCTGTAAGGCAGTG
c.865_866insCAGC, p.(Gln289ProfsTer11)	TCTCTGGCCAGCAGTG
c.863_864insCAAG, p.(Trp288CysfsTer12)	TCTCTGCAAGGCAGTG
c.864delinsCCGTT, p.(Trp288CysfsTer12)	TCTCTGCCGTTCAGTG
c.863_864insTAGC, p.(Trp288CysfsTer12)	TCTCTGTAGCGCAGTG
c.863_864insTCAT, p.(Trp288CysfsTer12)	TCTCTGTCATGCAGTG
c.863_864insCTTG, p.(Trp288CysfsTer12)	TCTCTGCTTGGCAGTG
c.863_864insTACG, p.(Trp288CysfsTer12)	TCTCTGTACGGCAGTG
c.863_864insCGGA, p.(Trp288CysfsTer12)	TCTCTGCGGAGCAGTG
c.863_864insCGCC, p.(Trp288CysfsTer12)	TCTCTGCGCCGCAGTG
c.859_860insGCTG, p.(Leu287ArgfsTer13)	AAGATCGCTGTCTGGC
c.861_862insACAA, p.(Trp288ThrfsTer12)	GATCTCACAATGGCAG
c.861_863delinsATGC, p.(Trp288CysfsTer11)	AGATCTATGCGCAGTG
c.863_864insTGCT, p.(Trp288CysfsTer12)	TCTCTGTGCTGCAGTG
***NPM1* mutation group 2** *Wild-type sequence: TCTGGCAGTGGAGGAA (c.860 to c.875)*	
HGVS nomenclature	**Mutant sequence**
c.867_868insAGGA, p.(Trp290ArgfsTer10)	TGGCAGAGGATGGAGG
c.867_868insAGAA, p.(Trp290ArgfsTer10)	TGGCAGAGAATGGAGG
c.867_868insAGAC, p.(Trp290ArgfsTer10)	TGGCAGAGACTGGAGG
c.867_868insCGCT, p.(Trp290ArgfsTer10)	TGGCAGCGCTTGGAGG
c.867_868insCGCA, p.(Trp290ArgfsTer10)	TGGCAGCGCATGGAGG
c.867_868insCGGA, p.(Trp290ArgfsTer10)	TGGCAGCGGATGGAGG
c.867_868insCGGC, p.(Trp290ArgfsTer10)	TGGCAGCGGCTGGAGG
c.868delinsCGTTC, p.(Trp290ArgfsTer10)	TGGCAGCGTTCGGAGG
c.868_869insCCAT, p.(Trp290SerfsTer10)	TGGCAGTCCATGGAGG

c.867_868insAGGC, p.(Trp290ArgfsTer10	TGGCAGAGGCTGGAGG
***NPM1* mutation group 3** *Wild-type sequence: TCTCTGGCAGTGGAGGAAGTCTCTT (c.858 to c.882)*	
HGVS nomenclature	**Mutant sequence**
c.869_870insTTTTTCTC, p.(Trp290CysfsTer13)	CTGGCAGTGTTTTTCTCGAGGAAGT
c.869_870insCATGGCTC, p.(Trp290CysfsTer13)	CTGGCAGTGCATGGCTCGAGGAAGT
c.869_873delinsCTCTTGCCC, p.(Trp290SerfsTer10)	CTGGCAGTCTCTTGCCCAAGTCTCT
c.869_873delinsCCCTGGAGA, p.(Trp290SerfsTer10)	CTGGCAGTCCCTGGAGAAAGTCTCT
c.869_873delinsCCCTCGCCC, p.(Trp290SerfsTer10)	CTGGCAGTCCCTCGCCCAAGTCTCT
c.870_873delinsCTTCGCC, p.(Trp290_Arg291delinsCysPheAla)	CTGGCAGTGCTTCGCCAAGTCTCTT
c.870_873delinsTTTTTCAA, p.(Trp290CysfsTer10)	CTGGCAGTGTTTTTCAAAAGTCTCT
c.869_873delinsCTCTTTCTA, p.(Trp290SerfsTer10)	CTGGCAGTCTCTTTCTAAAGTCTCT
c.869_873delinsCCCTTTCCA, p.(Trp290SerfsTer10)	CTGGCAGTCCCTTTCCAAAGTCTCT
c.868_870delinsCGTTTCC, p.(Trp290ArgfsTer10)	CTCTGGCAGCGTTTCCAGGAAGTCT
c.870_873delinsCTGCTCCC, p.(Trp290CysfsTer10)	TGGCAGTGCTGCTCCCAAGTCTCTT
c.869_873delinsATTTTCCC, p.(Trp290LeufsTer10)	CTGGCAGTTATTTTCCCAAGTCTCT
c.869_873delinsCTTTCTCCC, p.(Trp290SerfsTer10)	CTGGCAGTCTTTCTCCCAAGTCTCT
c.869_875delinsCTTTCGCTCAC, p.(Trp290SerfsTer10)	TGGCAGTCTTTCGCTCACGTCTCTT
c.870_873delinsTTTTGCTC, p.(Trp290CysfsTer10)	TGGCAGTGTTTTGCTCAAGTCTCTT
c.870_873delinsTTTTTCCC, p.(Trp290CysfsTer10)	TGGCAGTGTTTTTCCCAAGTCTCTT
c.868_872delinsCGGATGGCC, p.(Trp290ArgfsTer10)	CTGGCAGCGGATGGCCGAAGTCTCT
c.868_871delinsCGGATTCC, p.(Trp290ArgfsTer10)	CTGGCAGCGGATTCCGGAAGTCTCT
c.868_871dup, p.(Arg291MetfsTer9)	CTGGCAGTGGATGGAGGAAGTCTCT
c.866_871delinsTCCGATTTGC, p.(Gln289LeufsTer11)	CTCTGGCTCCGATTTGCGGAAGTCT
c.864_873delins14, p.(Trp288CysfsTer12)	CTCTGTCAAGACTTTCTTAAAGTCT
c.864_876delins17, p.(Trp288CysfsTer12)	TCTGTCGGAGTCTCGGCGGACTCTC
c.868_876delins13, p.(Trp290GlyfsTer10)	TGGCAGGGGGTGGGGAATCTCTCTT
c.869_876delinsATCTGGGGGCCC, p.(Trp290TyrfsTer10)	CTGGCAGTATCTGGGGGCCCTCTCT
c.867_875delins14, p.(Trp290AspfsTer12)	CTGGCAAGATTTCTTAATTCGTCTC
c.868_875delinsGGGTTGGCCCGG, p.(Trp290GlyfsTer10)	CTGGCAGGGGTTGGCCCGGGTCTCT
c.871delinsTTGGC, p.(Arg291LeufsTer9)	CTGGCAGTGGTTGGCGGAAGTCTCT
c.868_876delins13, p.(Trp290ArgfsTer10)	TGGCAGCGTTTCGGGGACATCTCTT
c.869_876delinsCGGTTTCTTTGC, p.(Trp290SerfsTer10)	CTGGCAGTCGGTTTCTTTGCTCTCTTT
c.868_874delinsCGGTTCGGGGC, p.(Trp290ArgfsTer10)	CTGGCAGCGGTTCGGGGCAGTCTCT
c.864_875delins13, p.(Trp288CysfsTer11)	TCTCTGCCACGCAGTGGAGGTCTCT
c.863_873delinsCCCGGGCAGT, p.(Trp288SerfsTer12)	ATCTCTCCCGGGCAGTAAGTCTCTT
***NPM1* mutation group 4** *Wild-type sequence: AAGATCTCTGGCAGTGGAGGAAGTC (c.854 to c.878)*	
HGVS nomenclature	**Mutant sequence**
c.867_868insCTTCTCCA, p.(Trp290LeufsTer13)	ACTCTGGCAGCTTCTCCATGGAGGA
c.867_868insCGGATGGC, p.(Trp290ArgfsTer13)	CTCTGGCAGCGGATGGCTGGAGGAA
c.863_867delinsCCATGCTCC, p.(Trp288SerfsTer12)	AGATCTCTCCATGCTCCTGGAGGAA
c.864_867delinsTACCTTCC, p.(Trp288CysfsTer12)	AGATCTCTGTACCTTCCTGGAGGAA
c.864_865delinsCCGCGG, p.(Trp288CysfsTer12)	AAGATCTCTGCCGCGGAGTGGAGGA

c.864_865delinsTCACCT, p.(Trp288CysfsTer12)	AGATCTCTGTCACCTAGTGGAGGAA
c.864_865delinsCAGAAA, p.(Trp288CysfsTer12)	AGATCTCTGCAGAAAAGTGGAGGAA
c.867delinsAAAAA, p.(Trp290LysfsTer10)	ATCTCTGGCAAAAATGGAGGAAGT
c.864_867delinsCACAGTTA, p.(Trp288CysfsTer12)	AGATCTCTGCACAGTTATGGAGGAA
c.864_865delinsCAAGAA, p.(Trp288CysfsTer12)	AGATCTCTGCAAGAAAGTGGAGGAA
c.862_867delinsACGTGCAAAA, p.(Trp288ThrfsTer12)	AAGATCTCACGTGCAAAATGGAGGAA

3.7 DROPLET DIGITAL PCR

Analysis of MRD using ddPCR of mutated *NPM1* types A, B and DD5 at the genomic level was performed in Paper II in the laboratory of the Ehinger research group at Skåne University Hospital, Lund.

ddPCR is a method that partitions a standard PCR reaction into thousands of nanoliter-sized droplets using oil emulsion. Each droplet ideally contains one copy of the target DNA, along with primers and probes. A PCR reaction is then carried out, where each droplet functions as an individual reaction chamber. If the target DNA is present, a fluorescent signal is generated within the droplet. After amplification, a droplet reader detects fluorescence in each droplet and classifies them as either positive (signal present) or negative (no signal). By counting the number of positive and negative droplets and applying Poisson statistics, the number of target DNA molecules in the original sample can be calculated. The method is highly sensitive and enables accurate quantification of both mutant and wild-type DNA, allowing for calculation of the VAF.

3.8 QUANTITATIVE PCR

MRD analysis using qPCR for *NPM1* type A at the genomic level was performed in Paper II in the laboratory of the Ehinger research group. qPCR quantifies the amount of target DNA in real time during amplification. Fluorescent probes or dyes bind to the DNA, and fluorescence is measured cycle by cycle. The earlier a signal is detected, the higher the amount of target DNA in the original sample, allowing for sensitive quantification. In Paper II, the method was performed as previously described (115, 116). Samples were analyzed in duplicate using 125-500 ng of DNA per reaction. A standard curve was generated for each experiment using a dilution series of the *NPM1* type A-positive cell line OCI-AML3. Quantification and determination of the LOD followed established criteria for ALL (168). Using 500 ng of input DNA, the LOD reached at least 0.001%.

3.9 PCR WITH FRAGMENT ANALYSIS

PCR with fragment analysis was used in Paper III to analyze *FLT3*-ITD and its allelic ratio at diagnosis, as well as *NPM1* mutations at diagnosis and relapse. Target DNA sequences were amplified using gene-specific primers, one of which was labeled with a FAM fluorochrome. The resulting PCR products were separated and detected using capillary electrophoresis, and the data were analyzed using GeneMapper (ThermoFisher Scientific, Waltham, MA). For *FLT3*-ITD, the allelic ratio was determined by calculating the ratio between the area under the curve of the mutant and wild-type peaks in diagnostic samples.

3.10 GENERATIVE ARTIFICIAL INTELLIGENCE

Generative artificial intelligence (AI) in the form of ChatGPT was used for proof reading and minor text editing in parts of this thesis.

4 RESULTS

4.1 MINIMAL RESIDUAL DISEASE MONITORING IN CHILDHOOD B LYMPHOBLASTIC LEUKEMIA WITH T(12;21)(P13;Q22); *ETV6-RUNX1*: CONCORDANT RESULTS USING QUANTITATION OF FUSION TRANSCRIPT AND FLOW CYTOMETRY (PAPER I)

The *ETV6*::*RUNX1* fusion transcript is detected in approximately one in four children with pre-B ALL. While the prognosis is generally favorable with current treatment protocols, accurate risk stratification remains crucial to optimize outcomes and minimize treatment-related toxicity. At the time of the study, risk stratification was based on MRD levels on day 29 and 78 during treatment, assessed by MFC. In cases with uncertain MFC results, qPCR of Ig/TCR rearrangements was used. RT-qPCR offers advantages over qPCR, being faster and more easily standardized. RT-qPCR of fusion transcripts like *ETV6*::*RUNX1* could potentially serve as an additional method for MRD assessment in pre-B ALL cases harboring this fusion.

The aim of this study was to determine whether RT-qPCR analysis of *ETV6*::*RUNX1* fusion transcript is useful for treatment monitoring in children with pre-B ALL, and whether it could be a reliable alternative to MFC.

The study included 29 children diagnosed with pre-B ALL with *ETV6*::*RUNX1* fusion transcript, treated in Gothenburg between 2006 and 2013. The median age at diagnosis was 4 years (range 1-17). Three patients were treated according to the NOPHO ALL-2000 protocol and 26 according to NOPHO ALL-2008. Bone marrow samples were collected at diagnosis and on days 15, 29, and 78 during treatment. In total, 78 samples were analyzed using both MFC and RT-qPCR. RT-qPCR results were expressed as fusion transcript MRD (ftMRD), calculated as the *ETV6*::*RUNX1*/*GUSB* ratio at follow-up divided by the ratio at diagnosis. The cut-off for quantifiable ftMRD was set at >0.1%. Samples with detectable but non-quantifiable *ETV6*::*RUNX1* levels were defined as having any detectable number of *ETV6*::*RUNX1* transcripts without meeting the criteria for quantifiable levels (i.e. ≥10 copies in ≥2 of 3 replicates). MFC results were expressed as the percentage of leukemic cells (MFC MRD), with MRD positivity defined as ≥0.1%.

ETV6::*RUNX1* transcript levels decreased gradually over time during treatment, with detectable ftMRD in 28/29 patients (quantifiable in 20/29) at day 15, 21/26 (quantifiable in 9/26) at day 29, and 2/23 at day 78. When comparing RT-qPCR with MFC, a strong correlation between the methods was observed. At day 15, three cases showed quantifiable ftMRD but MFC MRD <0.1%. At the risk stratifying time points (days 29 and 78), total concordance was seen between the two methods (Table 6).

The limitations of the study include the relatively small sample size and the single-center design, which may limit generalizability. Also, a comparison with qPCR of Ig/TCR rearrangements would have added value.

In conclusion, this study shows a strong correlation between MRD analysis with MFC and RT-qPCR of *ETV6*::*RUNX1* fusion transcript, with perfect concordance at clinically relevant time points. RT-qPCR of *ETV6*::*RUNX1* could serve as a valuable addition or alternative to MFC for MRD monitoring, especially in cases where MFC is not feasible.

***Table 6**. Comparison of MRD results from RT-qPCR of ETV6::RUNX1 fusion transcript and multiparameter flow cytometry (MFC) at day 15, 29 and 78 during treatment.*

	Positive ftMRD*		**Negative ftMRD**	
	ETV6::*RUNX1* quantifiable, ftMRD ≥0.1%	*ETV6*::*RUNX1* quantifiable, ftMRD <0.1%	*ETV6*::*RUNX1* detectable, but not quantifiable**	*ETV6*::*RUNX1* not detectable
MFC MRD day 15 ≥0.1%	17	0	0	0
<0.1%	3	0	8	1
MFC MRD day 29 ≥0.1%	3	0	0	0
<0.1%	0	6	13	4
MFC MRD day 78 ≥0.1%	0	0	0	0
<0.1%	0	0	2	21

*ftMRD: fusion transcript MRD, expressed as *ETV6*::*RUNX1*/GUSB ratio divided with the ratio at diagnosis (%).
**Detectable, but not quantifiable: any number of *ETV6*::*RUNX1* transcripts, but not meeting the criteria for quantifiable levels (≥10 copies in ≥2 of 3 replicates).

4.2 COMPARISON OF RNA- AND DNA-BASED METHODS FOR MEASURABLE RESIDUAL DISEASE ANALYSIS IN *NPM1*-MUTATED ACUTE MYELOID LEUKEMIA (PAPER II)

Mutations in the gene *NPM1* represent the most common genetic alteration in adult AML and define a specific diagnostic entity. *NPM1* mutation status is routinely assessed at diagnosis and serves both diagnostic and prognostic purposes. *NPM1* mutations are ideal MRD targets and numerous studies have shown their prognostic significance. RT-qPCR, performed on RNA, is recommended for MRD assessment of *NPM1* during treatment to evaluate treatment response and post-treatment for early relapse detection. Although RT-qPCR remains the gold standard due to its high sensitivity, DNA-based methods offer several advantages.

The aim of this study was to compare three DNA-based MRD methods – qPCR, ddPCR and deep sequencing – with RT-qPCR, to evaluate whether DNA-based methods provide relevant MRD information and could potentially replace RT-qPCR.

A total of 132 bone marrow and peripheral blood samples from 32 AML patients with *NPM1* mutations, diagnosed between 2013 and 2019, were analyzed. This included 110 *NPM1* type A samples from 30 patients analyzed with all three DNA-methods, and 2 *NPM1* type B samples and 20 *NPM1* type DD5 samples from two patients analyzed with ddPCR and deep sequencing. Follow-up samples from remission were selected, including those with detectable transcripts and the first undetectable sample after a previously positive time point.

Analysis of *NPM1* type A showed a significant correlation between DNA-based methods and RT-qPCR, with qPCR showing the highest correlation, followed by ddPCR and deep sequencing. Agreement with RT-qPCR was 84% for qPCR, 81% for ddPCR, and 73% for deep sequencing in bone marrow, and even higher in blood (95% for qPCR, 88% for ddPCR and 86% for deep sequencing). Sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) were assessed for each method. All DNA-based methods demonstrated high specificity and PPV in bone marrow, and all reached >96% specificity in blood. qPCR demonstrated the highest sensitivity, PPV and NPV overall. However, the DNA-based methods missed leukemic

signals in a number of RT-qPCR-positive bone marrow samples, highlighting the superior sensitivity of RT-qPCR. Conversely, DNA-based methods, especially qPCR, detected leukemic DNA in around 10% of RT-qPCR-negative samples. An excellent correlation was also observed between the DNA-based methods themselves, with qPCR showing the highest sensitivity, due to a higher DNA input in the assay.

To determine optimal thresholds for the DNA-based methods in bone marrow, established RT-qPCR cut-offs were used as reference (a 3-$\log_{10}$ reduction of mutated *NPM1* compared to the diagnostic level or 2% mutated *NPM1*/10^4 *ABL* copies). ROC curve analysis identified the following thresholds: 0.1% for qPCR, and 0.05% for ddPCR and deep sequencing, reflecting the mutation's heterozygous nature. These cut-offs were selected to prioritize high specificity, and resulted in PPV and NPV >90%, although sensitivity was slightly lower (Table 7).

Potential limitations of the study include heterogeneity of patient samples used for ddPCR and deep sequencing, as well as the lack of assessment of whether our RT-qPCR assay yielded results fully comparable to the originally described assay, something that may influence interpretation of the findings.

In conclusion, this study shows strong agreement between RT-qPCR and DNA-based methods, although DNA-based methods are slightly less sensitive. The proposed cut-offs for risk stratification can be used when RT-qPCR is not possible to perform.

***Table 7**. Diagnostic accuracy of the chosen cut-offs for qPCR, ddPCR and deep sequencing, tested against RT-qPCR. Values in parentheses represent 95% confidence intervals for sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV).*

	Cut-off	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
qPCR	0.1%	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	0.05%	80.0 (54.8-93.0)	100 (93.1-100)	100 (75.8-100)	94.5 (85.2- 98.1)
Deep sequencing	0.05%	86.7 (62.1-96.3)	100 (93.1-100)	100 (77.2-100)	96.3 (87.5- 99.0)

4.3 PROGNOSTIC EVALUATION OF DEEP SEQUENCING FOR ANALYSIS OF MEASURABLE RESIDUAL DISEASE IN ACUTE MYELOID LEUKEMIA WITH *NPM1* MUTATION (PAPER III)

In *NPM1*-mutated AML, RT-qPCR is the recommended method for MRD analysis, but since RT-qPCR is mutation-specific, clinical laboratories restrict MRD monitoring to the most common *NPM1* mutation type(s). With around 90 known variants, this excludes some patients from monitoring, leading to unequal care. Deep sequencing is an alternative method for MRD monitoring of *NPM1*, that covers all different types of *NPM1* in one assay. The aim of this study was to evaluate the clinical utility of deep sequencing for MRD monitoring in *NPM1*-mutated AML, and to assess whether it can provide prognostic information during chemotherapy.

This retrospective study included all adult patients diagnosed with *NPM1*-mutated AML in the Västra Götaland Region between 2006 and 2016, with available biobanked bone marrow aspirate slides. In total, 97 patients were included, with a median age at diagnosis of 64 years (range 19-82). All patients received chemotherapy with curative intent and achieved remission; 67 patients received intensive treatment and 30 received reduced-intensity regimens. Thirty-one patients underwent alloSCT. During follow-up, 43 patients relapsed and 49 died, including 10 from non-relapse mortality. A total of 257 bone marrow aspirates slides were analyzed with deep sequencing for detection of mutated *NPM1* after first cycle of treatment, during consolidation (after two cycles, or after three if no bone marrow examination was performed after two), and at the end of treatment (after the last cycle of treatment). *FLT3*-ITD and *DNMT3A* mutation status were determined from diagnostic samples in all patients. MRD positivity was defined as *NPM1* VAF ≥0.05% and MRD negativity as <0.05%, based on the threshold established in Paper II.

Patients that were MRD-positive during consolidation had a significantly shorter RFS and OS compared to MRD-negative patients. The 3-year RFS was 23.1%±11.7% for MRD-positive patients versus 70.8%±6.1% for MRD-negative patients, and the 3-year OS was 30.8%±12.8% versus 71.9%±6.0% (Figure 8). The effect of MRD status was also seen when assessed at the end of treatment. In multivariable analysis including MRD status, age, *FLT3*-ITD status, and treatment intensity, MRD positivity during consolidation was the

only significant predictor of RFS. For OS, *FLT3*-ITD status and treatment intensity remained significant.

The prognostic impact of MRD status was next evaluated within the two treatment groups. Among patients who received intensive chemotherapy, few were MRD-positive, and no significant difference in prognosis was observed between MRD-positive and MRD-negative patients. In contrast, among patients receiving reduced-intensity treatment, MRD positivity was associated with both a shorter RFS and OS.

Limitations of the study include its retrospective design and the relatively limited number of study participants. Additionally, the cohort was treated prior to the introduction of FLT3 inhibitors, which may influence outcomes.

In conclusion, MRD status by deep sequencing of *NPM1* is a strong predictor of RFS and OS when assessed during or after treatment. This study confirms the MRD threshold of VAF ≥0.05%, previously proposed in Paper II through comparison with RT-qPCR. Since deep sequencing enables detection of all *NPM1* variants, including rare types, it offers broader applicability for MRD monitoring and refines risk stratification beyond what is possible with mutation-specific methods.

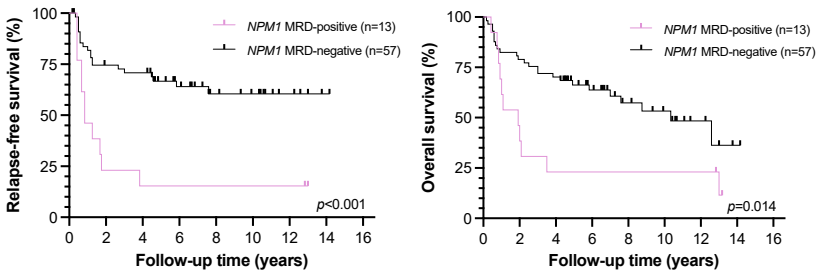

Figure 8. *Kaplan-Meier plots showing relapse-free survival (left) and overall survival (right) in patients with acute myeloid leukemia with mutated NPM1 based on measurable residual disease (MRD) status analyzed with deep sequencing during consolidation. MRD positivity was defined as NPM1 VAF ≥0.05%.*

4.4 RESPONSE EVALUATION AND POST-TREATMENT MONITORING IN CHILDHOOD ACUTE MYELOID LEUKEMIA USING DEEP SEQUENCING OF *FLT3*-ITD (PAPER IV)

FLT3-ITD mutations are present in 10-15% of pediatric AML patients and are associated with increased relapse risk and poorer outcomes. *FLT3*-ITD is measured at diagnosis and plays an important role in treatment decisions. According to the current treatment protocol used in Sweden, *FLT3*-ITD (without a concurrent *NPM1* mutation) is the only genetic marker that stratifies patients into the high-risk group, for whom alloSCT is recommended as consolidation therapy. In adult AML, FLT3 inhibitors are used during induction and at relapse with promising results, and they will soon be incorporated into pediatric treatment protocols. MRD analysis of *FLT3*-ITD using NGS-based methods has shown prognostic value in adults, including the ability to detect relapse both before and after alloSCT. However, due to the subclonal nature of the mutation, the NPV is limited, and a complementary MRD marker is recommended by the ELN. In pediatric AML, the clinical utility of *FLT3*-ITD MRD analysis has not yet been established. The aim of this study was therefore to evaluate the clinical value of MRD analysis using deep sequencing of *FLT3*-ITD in children with AML.

The study included 17 children diagnosed with AML with *FLT3*-ITD mutation between 2013 and 2024, treated according to NOPHO-DBH AML 2012 protocol. The median age at diagnosis was 11 years (range 4-17). A total of 173 biobanked bone marrow and blood samples were analyzed with deep sequencing of *FLT3*-ITD during and after treatment. Samples with detected ITD at or above the LOD of 0.0015% VAF were classified as MRD-positive, and those below as MRD-negative. MRD was also assessed by MFC during treatment as part of clinical routine.

To investigate the kinetics of *FLT3*-ITD during treatment, the level of *FLT3*-ITD were analyzed in 12 patients. In most patients with multiple subclones, a gradual reduction of *FLT3*-ITD levels was observed. However, in some patients, the levels fluctuated over time, indicating heterogeneity in the clonal response to therapy.

To assess whether deep sequencing of *FLT3*-ITD in blood could detect impending relapse, blood samples obtained after completion of treatment from

11 patients were analyzed (median 10 per patient, range 1-17). Among non-relapsing patients, 8/9 remained MRD-negative at all time points. One patient showed low-level MRD only at the first time point, with all subsequent samples being negative. In the two relapsing patients, increasing *FLT3*-ITD levels were detected prior to morphological relapse – 23 and 255 days before relapse, respectively (Figure 9).

To compare deep sequencing with the gold standard method MFC, 32 bone marrow samples from 12 patients were analyzed in parallel. The two methods yielded concordant MRD classification in 21/32 samples. Among the discordant samples, 10 were positive by deep sequencing but negative with MFC, while one was positive by MFC but negative by deep sequencing.

The main limitations of the study are the small number of participants and the fact that none of the patients were treated with FLT3 inhibitors, which reflects the standard of care for pediatric AML during the study period. Larger studies including FLT3 inhibitor treated patients are needed before deep sequencing can be used as a stand-alone method for clinical decision-making.

In conclusion, deep sequencing of *FLT3*-ITD is a highly sensitive MRD monitoring method in pediatric AML. It shows strong potential for assessing treatment response and detecting relapse at an early stage, and may serve as a valuable complement to MFC.

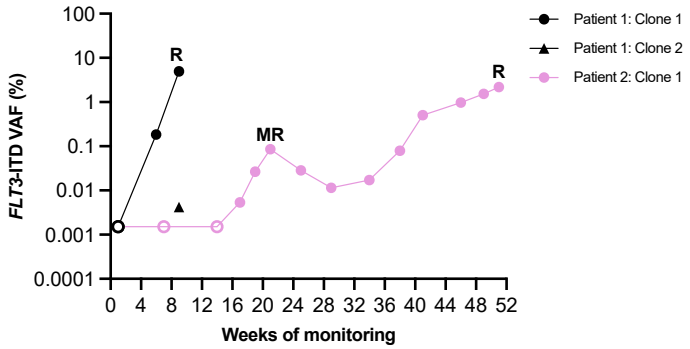

Figure 9. *Variant allele frequency (VAF) of FLT3-ITD over time in the two relapsing patients. MRD-positive samples are indicated by filled symbols, and MRD-negative samples by open symbols at 0.0015%. Abbreviations: MR, molecular relapse; R, morphological relapse.*

5 DISCUSSION

5.1 SUMMARY OF MAIN FINDINGS

By highlighting the clinical value of molecular methods, in particular deep sequencing, this thesis contributes new knowledge about MRD analyses in acute leukemia. The included studies demonstrate that MRD assessment can be optimized through a combination of established and emerging techniques, supporting the development of more personalized treatment strategies.

Paper I showed a strong correlation between MRD results from RT-qPCR of *ETV6*::*RUNX1* fusion transcript and MFC in pediatric pre-B ALL. The two methods demonstrated absolute concordance at risk stratifying time points using a 0.1% cut-off. These findings support the use of RT-qPCR not only as a valuable complement to MFC, providing an additional measure of the leukemic burden, but also as a reliable alternative in patients where MFC is not feasible.

Paper II demonstrated a strong agreement between DNA-based MRD methods (qPCR, ddPCR and deep sequencing) and the gold standard RT-qPCR for the detection of mutated *NPM1* in adult AML, although the DNA-based methods showed somewhat lower sensitivity. Based on consistent results across methods, clinically relevant thresholds were proposed for risk stratification: 0.1% leukemic DNA for qPCR, and 0.05% VAF for ddPCR and deep sequencing.

Paper III validated the clinical utility of MRD analysis with deep sequencing of *NPM1* in adult AML, using the 0.05% cut-off established in Paper II. MRD status during and after consolidation was predictive of outcome, especially of relapse. Since deep sequencing can detect all *NPM1* variants, including rare types, it enables MRD monitoring in patients who would otherwise not be offered such surveillance, thus promoting more equitable care.

Paper IV evaluated deep sequencing of *FLT3*-ITD in pediatric AML and showed that the method is highly sensitive for MRD analysis, with strong potential for assessing treatment response and for identifying impending relapse. These findings suggest that deep sequencing of *FLT3*-ITD could serve as a valuable complement to MFC, with potential applications similar to those already established in adult AML.

5.2 ADVANTAGES OF MOLECULAR METHODS FOR MRD ANALYSIS

MRD analysis using RT-qPCR offers several advantages. For specific fusion transcripts and *NPM1* mutations, standardized protocols are available through the Europe Against Cancer program, facilitating harmonized implementation across different laboratories. RT-qPCR is also widely available and relatively inexpensive. Another advantage of RT-qPCR for fusion transcripts like *ETV6*::*RUNX1* is that these transcripts are present in all leukemic cells and persist at relapse.

MRD analysis using deep sequencing in acute leukemia offers several advantages. One of the main benefits is its multiplexed design, where multiple samples, each tagged with a unique index, and multiple genes, including rare variants and patient-specific mutations, can be analyzed simultaneously in one assay. Deep sequencing also enables detection of clonal heterogeneity, such as multiple *FLT3*-ITD clones with different lengths and allele frequencies. These factors are major advantages compared to RT-qPCR, which requires mutation-specific primers and probes. This was demonstrated in Paper III, where deep sequencing detected all *NPM1* variants in one assay. Similarly, Paper IV, showed that deep sequencing could detect all *FLT3*-ITD clones, enabling a detailed analysis of clonal diversity – a process that would be very cumbersome using RT-qPCR due to the variability in ITD length and insertion sites.

Furthermore, deep sequencing is applicable in almost all AML patients and in over 90% of ALL patients (through Ig/TCR analysis) (169). This is particularly advantageous in AML, where 40-50% of patients lack a traditional PCR target. For these patients, deep sequencing allows MRD monitoring by tracking patient-specific mutations, thus extending the availability of molecular MRD monitoring to the vast majority.

When it comes to sensitivity, deep sequencing generally achieves equal or superior sensitivity compared to MFC and RT-qPCR. This was shown in Paper III, where deep sequencing could quantify *NPM1* mutations down to 0.05% VAF, and in Paper IV, where the limit of quantification for *FLT3*-ITD was as low as 0.01%.

Another important factor to consider is the starting material used in the analyses. Deep sequencing is performed on genomic DNA, which is more stable compared to RNA (used in RT-qPCR), and is unaffected by differences

in gene expression levels. As a result, the measurements are likely to be more reproducible and comparable between patients. Additionally, DNA is less prone to degradation during transport and handling, facilitating logistics and enabling reliable analysis even for patients located far from a central laboratory.

Another advantage of deep sequencing is that it does not require a standard curve, reference gene, or baseline diagnostic sample for quantification, all of which are needed for RT-qPCR. Unlike MFC, which require fresh, viable cells, deep sequencing can be performed on both newly collected and biobanked samples. This enables retrospective analyses, as demonstrated in Paper III, where biobanked slides up to fifteen years old were successfully analyzed.

In contrast to MFC – which is operator-dependent and requires highly experienced staff – deep sequencing provides more objective results. A further benefit is that sequencing data can be stored and reanalyzed as new mutations are discovered or as improved analytical methods become available. While MFC data can also be stored and reanalyzed, deep sequencing allows for a more detailed reanalysis – for example, the ability to search for newly identified mutations or to apply updated bioinformatic tools.

5.3 CHALLENGES OF MRD ASSESSMENT

Currently, there is no universal consensus on the optimal marker, method, time point, cut-off or sample type for MRD analysis using NGS in acute leukemia. There is also a lack of standardization in how laboratories perform NGS-based MRD analysis. Variations in gene panels, sequencing depth, and bioinformatic pipelines contribute to variability in results, potentially leading to inconsistency and reduced comparability of MRD results between different laboratories. This contrasts with MFC and RT-qPCR for certain fusion transcripts, where standardized protocols and international guidelines are well established. However, this field is rapidly evolving, and established guidelines for NGS-MRD in clinical practice are likely to be expected in the near future.

A key consideration is that the different methods used in this thesis measure different parameters. Deep sequencing quantifies the VAF of a specific mutation. For heterozygous mutations – present in one allele of a diploid genome – the VAF corresponds to half the proportion of leukemic cells carrying the mutation. Thus, multiplying the VAF by two provides an indirect estimate of the leukemic cell percentage. This means that deep sequencing

offers a direct correlation with the fraction of mutated cells. In contrast, RT-qPCR measures gene expression at the RNA level, where mRNA expression can vary considerably between cells and is influenced by transcriptional regulation and RNA stability. This variability was shown in Paper III, where the ratio between *NPM1* transcripts (measured by RT-qPCR) and leukemic cells (measured by qPCR) showed notable fluctuations both between and within individual patients. This highlights that there is no absolute relationship between transcript levels and the percentage of leukemic cells, meaning that RT-qPCR provides an estimate of the mutated transcript levels rather than an absolute quantification of mutated cells. MFC, on the other hand, identifies the presence of specific surface or intracellular antigens at the single-cell level. These markers reflect immunophenotypic features of leukemia but are not linked to genetic mutations, which means that MFC does not directly correspond to the percentage of cells carrying a specific mutation. Consequently, results from these different MRD methods are not directly interchangeable or comparable, as they assess distinct aspects of the leukemic clone – genetic, transcriptional and phenotypic.

The fact that different MRD methods measure different biological parameters became evident when comparing results obtained by different techniques. In Paper I, three samples were MRD-positive by RT-qPCR but negative with MFC. Similarly, in Papers III and IV, 2/20 and 10/32 samples, respectively, were MRD-positive by deep sequencing while negative with MFC. Several factors may explain these discrepancies. One possibility is a partly informative immunophenotype, which may result in an underestimation of MRD by MFC. Antigen modulation (immunophenotypic shift) can alter the expression of surface markers, reducing the sensitivity of MFC. Another possible explanation may be that molecular methods detect early mutations that persist in pre-leukemic or more mature hematopoietic cells that are not part of the active leukemic clone, potentially leading to false-positive MRD results. Additionally, MFC is generally less sensitive than both RT-qPCR and deep sequencing, which could also contribute to the observed discrepancies. However, false positives with MFC can also occur, especially when regenerating healthy blasts are difficult to distinguish from malignant blasts.

One of the main challenges in MRD assessment lies in interpreting the clinical significance of positive results. Not all patients with detectable MRD will relapse. For instance, a study by Tiong *et al*. showed that 42% of AML patients who were *NPM1*-positive in bone marrow after completed treatment had not relapsed after one year of follow-up (170). Similar observations have been

made in CBF-AML, where persistent transcripts can be detectable in follow-up samples without an imminent relapse (171). There may be several explanations for this, one possibility is that the residual leukemic clone is controlled by the immune system or lacks proliferative capacity. In other cases, low-level MRD may represent a clinically irrelevant subclone that does not contribute to disease progression. This was seen in Paper III where two patients were MRD-positive after two cycles of treatment, but did despite this not relapse. The first of these patients had a *NPM1* VAF of 0.08% after cycle two, but no detectable MRD after cycle three. The second patient remained MRD-positive after both cycles two (0.07%) and three (0.28%), with no further samples available. This patient was also *FLT3*-ITD-positive, but did despite this not undergo alloSCT and was still alive at the last follow-up, thirteen years from diagnosis. In Paper IV, three patients had detectable *FLT3*-ITD at very low levels prior to alloSCT but did not relapse or had resistant disease, further supporting that very low levels of residual leukemic cells may not always be of clinical relevance.

Conversely, not all MRD-negative patients remain relapse-free. False-negative results may arise due to limited assay sensitivity, sampling errors, or clonal evolution where the leukemic clone has evolved and is no longer detectable with the original MRD marker. Relapse may also emerge from a previously undetected subclone that escaped initial detection and proliferated over time. However, this is less likely when using early and stable MRD markers such as *ETV6*::*RUNX1*, t(8;21), inv(16) or *NPM1*, which are typically present in all leukemic cells. Taken together, these findings highlight the complexity of MRD interpretation and underscore the importance of integrating MRD results with other factors, such as clinical, genetic and treatment-related, to inform clinical decisions.

Another relevant consideration in MRD assessment is the choice of sample type. Bone marrow is generally recommended for response evaluation during treatment, as it provides higher sensitivity than peripheral blood – a finding supported by previous studies (140, 141, 166, 172). For adult AML patients with t(8;21), inv(16) or *NPM1* mutation type A, current guidelines recommend peripheral blood sampling after the second cycle of treatment and at the end of treatment. In paper IV, *FLT3*-ITD levels were consistently higher in bone marrow than in blood, likely due to the fact that myeloblasts reside and proliferate in the bone marrow which results in a higher concentration of leukemic cells within this compartment. Despite its superior sensitivity, bone marrow aspiration is invasive and often associated with patient discomfort. In

pediatric patients, the procedure is performed under general anesthesia. Also, persistent low-level MRD in bone marrow does not always signal impending relapse. In contrast, peripheral blood sampling is minimally invasive, generally well tolerated, more suitable for frequent monitoring, and can be performed in local health care centers. Taken together, while bone marrow remains the preferred sample type for MRD evaluation during treatment, peripheral blood represents a valuable alternative, especially when highly sensitive molecular techniques are used.

Practical aspects, such as turnaround time (TAT) and cost, are also crucial when implementing MRD analysis in the clinical setting. Among the methods used in this thesis, MFC offers the shortest TAT, with results often available within a few business days. RT-qPCR is also fast, and once primers and probes are set up and the laboratory logistics allow, the analysis can be done within 1-2 days. In contrast, deep sequencing is more time-consuming, typically requiring 1-2 weeks, which could potentially delay clinical decision-making. Cost is another important factor. RT-qPCR is relatively less expensive, while both MFC and deep sequencing are associated with higher costs – MFC due to the need for experienced staff and time-consuming analysis, and deep sequencing because of expensive sequencing reagents and platforms. These factors may represent a practical barrier for implementing NGS-based MRD techniques, particularly in resource-limited settings. However, as NGS technologies become more integrated in routine diagnostics, both cost and TAT will likely improve.

5.4 CLINICAL IMPLICATIONS OF STUDY FINDINGS

The findings from this thesis offer several relevant insights into how MRD analysis can be used in the clinical setting to support more precise risk stratification and guide treatment decisions.

Paper I showed that RT-qPCR of *ETV6*::*RUNX1* fusion transcript is a reliable alternative to MFC for MRD monitoring in pre-B ALL, expanding MRD monitoring possibilities for patients in whom MFC is not feasible. These findings contributed to the implementation of RT-qPCR of *ETV6*::*RUNX1* at Sahlgrenska University Hospital, where it is performed in parallel with MFC and qPCR when sufficient sample material is available. In this setting, it can be used as a complementary tool in cases where there is discrepancy between MFC and qPCR results.

Since the publication of Paper I, NGS has emerged as a promising tool for MRD monitoring in pediatric ALL (164). Several studies have demonstrated its prognostic value, especially through analysis of Ig/TCR rearrangements, as previously described in the MRD section. A study by Mai *et al*. analyzed 236 bone marrow samples from 64 children with ALL, comparing MRD analysis using NGS, qPCR and MFC, and showed that NGS was the most sensitive method for detecting MRD post-treatment (173). In a recent study, Huang *et al*. compared NGS-based MRD monitoring of Ig/TCR rearrangements with RT-qPCR of three fusion transcripts (*ETV6*::*RUNX1*, *BCR*::*ABL1* and *TCF3*::*PBX1*) using 104 bone marrow samples from 56 patients (174). The overall concordance between the methods was high. In the *ETV6*::*RUNX1* subgroup, concordance was 83%; five samples from five patients were MRD-negative by NGS but positive by RT-qPCR at the end of treatment. For the three patients with available follow-up data, all remained in long-term remission (>10 years). Although based on a small cohort, these findings suggest that NGS-based MRD assessment may offer stronger prognostic value than RT-qPCR, especially in cases with discordant results.

Paper II demonstrated that DNA-based methods can serve as a valuable alternative to RT-qPCR for MRD monitoring of mutated *NPM1* in AML. This is a clinically important finding, since not all *NPM1* mutation types can be monitored by RT-qPCR assays. By providing reliable alternatives, DNA-based methods expand MRD monitoring options, contributing to a more equitable and personalized care. The findings from this study have led to the implementation of MRD analysis with deep sequencing of *NPM1* into clinical routine at Sahlgrenska University Hospital, providing a monitoring option for patients who otherwise lack a suitable MRD marker. Since the introduction of the assay in our clinical laboratory in 2021, the number of samples have increased gradually. To date, almost 450 samples from 42 patients with 23 different mutation types, including some novel, have been analyzed.

There are few earlier studies comparing RNA- and DNA-based methods, but those that exist have shown a significant correlation between MRD results obtained from RT-qPCR and targeted sequencing (49, 122). In AML with t(8;21), Duployez *et al*. showed a strong correlation between *RUNX1*::*RUNX1T1* levels measured by RT-qPCR and qPCR of the fusion at the genomic level (175). In a previous study in pediatric AML, we showed concordance between MRD levels measured by patient-tailored deep sequencing and RT-qPCR of *RUNX1*::*RUNX1T1* and *KMT2A*::*MLLT10* fusion transcripts (166). In Paper II, although DNA-based methods demonstrated

somewhat lower sensitivity than RT-qPCR, they detected MRD in 10% of samples that were negative by RT-qPCR. This indicates that DNA-based methods may find residual leukemic cells that are missed by transcript-based assays. These findings emphasize the complementary role of DNA-based MRD methods and suggest that combining molecular approaches could improve the accuracy of MRD assessment.

The results from Paper III add to the limited knowledge regarding the clinical relevance of *NPM1* MRD analysis using NGS (49, 176). We show that deep sequencing of *NPM1* is highly predictive of outcome, especially relapse, when assessed during and after consolidation. The study also confirms the clinical value of the 0.05% cut-off obtained in Paper II. While current guidelines recommend RT-qPCR as the primary method for *NPM1* MRD assessment, deep sequencing serves as a valuable alternative, particularly in cases with rare *NPM1* variants. In previous studies, we have shown that deep sequencing is a highly sensitive method for patient-tailored MRD analysis (117), and that it can predict relapse after alloSCT in patients with mutated *NPM1* (177). The clinical value of MRD analysis of *NPM1* in the transplant setting has been described in a few additional studies. Dillon *et al*. analyzed pre-transplant blood sample from 451 adult AML patients in first remission using ultra-deep NGS targeting *NPM1* and *FLT3*-ITD mutations, with a sensitivity of 0.01% (178). Patients with detectable MRD before transplant had a 3-year relapse rate of 68%, compared to 21% in MRD-negative patients, and a 3-year OS of 39% versus 63%. In a smaller study, Zhou *et al*. evaluated both NGS and MFC for MRD assessment before and after alloSCT in *NPM1*-mutated AML (179). While pre-transplant MRD positivity by NGS alone did not predict relapse, post-transplant NGS-MRD was highly predictive. The authors concluded that NGS adds prognostic value, particularly post-transplant, while pre-transplant positivity may require careful interpretation, as some patients with low-level positivity may be rescued by the transplantation.

The findings from paper IV highlight the clinical utility of deep sequencing of *FLT3*-ITD as a sensitive and reliable MRD method in pediatric AML. The results support its use as a complementary tool to MFC for evaluation of treatment response. In a recent study, Rücker *et al*. evaluated NGS-based *FLT3*-ITD MRD monitoring in 142 adult AML patients and found that 78% were MRD-negative after two cycles of chemotherapy in combination with midostaurin (151). MRD-negative patients had significantly better outcomes than MRD-positive, with a 4-year cumulative incidence of relapse of 26% versus 46%, and 4-year OS of 70% compared to 44%. In multivariable

analysis, MRD negativity was the strongest predictor of outcome. Similarly, Dillon *et al*. showed that persistence of *FLT3*-ITD in blood before alloSCT, measured by NGS in 537 adult patients, was associated with increased risk of relapse and death, using a cut-off of 0.01% VAF (150). A dose-dependent relationship was observed: higher pre-transplant MRD levels correlated with poorer outcomes. Given the growing evidence regarding the clinical value of *FLT3*-ITD MRD monitoring in adult AML, our findings from paper IV suggest that similar clinical applications may be valuable in pediatric patients, especially since FLT3 inhibitors will soon be incorporated into pediatric treatment protocols.

Paper IV also demonstrated that deep sequencing is a highly sensitive and specific method for relapse prediction. Among the two relapsing patients, increasing *FLT3*-ITD levels were observed well before morphological relapse – in one case, as early as 255 days prior. Conversely, in patients who remained in remission, all but one were MRD-negative at every follow-up time point. In the single exception, low-level positivity occurred at the first post-treatment time point only and did not meet the ELN definition of a molecular relapse, which requires two consecutive positive samples from the same tissue (52). These findings align with results from our previous study in pediatric patients with CBF-AML and *KMT2A* rearrangements, where molecular relapse (or conversion from negative to positive) in blood during follow-up predicted relapse in all 14 relapsing patients. In contrast, all 36 non-relapsing patients remained MRD-negative in all 253 blood samples from follow-up (180). Since molecular relapse can precede morphological relapse by weeks or months, early MRD detection provides a valuable window for preemptive intervention. Our findings suggest that deep sequencing could serve as an effective post-treatment surveillance tool in children with *FLT3*-ITD mutations, a group currently lacking standardized MRD monitoring. The method could be used in a similar way to RT-qPCR of fusion transcripts or *NPM1* type A mutations, potentially enabling preemptive treatment at the molecular level. This approach has been shown to improve outcomes in AML. Orvain *et al*. monitored 303 adults with CBF-AML or *NPM1* mutations using qPCR after intensive chemotherapy (without alloSCT in first CR) (181). Among them, 31% experienced relapse, and 18% had morphological relapse. Patients who received preemptive therapy (including alloSCT or chemotherapy) at molecular relapse had a 3-year OS of 71%, compared to 51% for those treated after morphological relapse. Similar findings have been reported in other studies, further underscoring the prognostic benefit of molecular monitoring and early intervention (182-185).

Taken together, the findings presented in this thesis support the use of deep sequencing as a powerful MRD monitoring tool in both adult and pediatric AML, with the potential to improve risk stratification, guide treatment decisions, and ultimately enhance patient outcomes.

6 CONCLUDING REMARKS AND FUTURE PERSPECTIVES

In recent years, MRD analysis has become increasingly important in acute leukemia and is now recognized as one of the strongest predictors of outcome in both adults and children. This thesis contributes new knowledge about MRD analysis methods and their clinical relevance.

The field of MRD diagnostics has developed rapidly, and since the start of Paper I in 2013/2014, significant progress has been made. In Paper I, we showed that RT-qPCR analysis of *ETV6*::*RUNX1* fusion transcript is a reliable alternative or complement to MFC in pediatric ALL. The method is now implemented in clinical routine at our laboratory alongside MFC and qPCR. In Paper II, we established clinically relevant MRD cut-offs for *NPM1* using deep sequencing, qPCR and ddPCR, all demonstrating high predictive values. These findings led to the clinical implementation of deep sequencing for MRD monitoring in patients with *NPM1* non-type A mutations. Paper III confirmed the prognostic value of deep sequencing of *NPM1*, using the cut-off established in Paper II. In Paper IV, we showed that deep sequencing of *FLT3*-ITD is a highly sensitive MRD method in pediatric AML, suggesting that *FLT3*-ITD MRD monitoring may be used to guide treatment decisions in this group of patients.

Despite major advances within MRD diagnostics, several challenges remain. There is a need for harmonization regarding optimal time points, clinically relevant cut-offs, and the choice of material for MRD analysis. Currently, these parameters vary between study groups and treatment protocols. Based on the findings from this thesis and previous literature, the most informative time point for MRD assessment in *NPM1*-mutated AML appears to be during consolidation (after two cycles), in both bone marrow and blood. For post-treatment monitoring, peripheral blood is likely the optimal material, as molecular relapse in blood has been shown to correlate strongly with clinical relapse. However, the ideal MRD time points may also vary depending on genetic subtype, as different mutations display different kinetics and response patterns.

A key question in clinical practice is at what MRD level to intervene – and how. MRD has the potential to move from a prognostic marker to a direct therapeutic target. This approach has already shown great success in both

pediatric ALL and AML. Whether it can be applied across all age groups remains to be explored with studies addressing this strategy in adult patients.

New immunotherapies, such as bispecific antibodies and CAR-T cells, have shown the ability to eradicate MRD in B-ALL and may be used in cases of molecular relapse. Their potential use in preemptive treatment, before morphological relapse, underscores the need to define MRD thresholds for intervention. To guide such strategies, it is crucial to determine which MRD levels that motivate therapeutic intervention. Should all MRD-positive patients receive preemptive treatment, or only those above a certain level? And at which time points is MRD most predictive? Answering these questions will require prospective clinical trials in which treatment decisions are based on MRD results. These are essential to determine whether MRD-guided therapy improves prognosis compared to standard treatment. While randomized trials may pose ethical challenges – such as withholding intensified treatment from MRD-positive patients – they remain crucial for establishing evidence-based protocols.

Another important area for future research is comparative studies between different MRD methods, since there are few studies that directly compare which method is most optimal in a given clinical situation. For example, should NGS replace qPCR in ALL, or serve as a complement? Is ddPCR more robust than RT-qPCR for certain fusion transcripts? In Paper II, we found that DNA-based methods failed to detect MRD in a proportion of RT-qPCR-positive samples – and vice versa. These discrepancies highlight the need for more comprehensive comparisons in larger cohorts to better understand the strengths and limitations of each method, and to assess whether combining methods could improve diagnostics.

In the coming years, NGS-based techniques for MRD detection are expected to become increasingly integrated in clinical practice. It is likely that clinical laboratories will routinely use both conventional and NGS-based methods, providing more information for clinical decision making. As sequencing technology advances, scalability increases, and costs decrease, NGS may become the standard method for MRD monitoring in several leukemia subtypes. At the same time, existing techniques will continue to be refined, and peripheral blood is expected to be used more frequently, especially for post-treatment surveillance. For some genetic markers, blood has already been shown to be a reliable surrogate for bone marrow and is incorporated in the ELN MRD guidelines.

Parallel to advances in sequencing, AI is expected to play an increasingly important role in MRD diagnostics. AI and machine learning algorithms could help automate MRD detection, integrate results with other prognostic data, and even provide real-time risk assessments. A possible future scenario is individualized treatment planning based on MRD levels and clinical and genetic features – where AI estimates relapse risk and recommends early intervention before overt relapse occurs.

For future research, it would be very interesting to build on our results by conducting a prospective study in *NPM1*-mutated AML, where deep sequencing is used to guide treatment decisions. Our data clearly showed the prognostic value of MRD status by deep sequencing, and the next step would be to test whether acting on MRD levels improves outcomes. One possible study could include prospective MRD monitoring during consolidation, where MRD-positive patients are randomized to either intensified treatment or standard care. This would allow evaluation of whether MRD-guided therapy improves prognosis. Another interesting future project would be a prospective study involving longitudinal monitoring of *FLT3*-ITD and co-mutations in pediatric AML. Using deep sequencing, both *FLT3*-ITD and co-mutations could be analyzed in parallel to track clonal dynamics over time. If molecular relapse is detected, preemptive treatment would be initiated. This approach could enable early, individualized treatment and improve prognosis by preventing morphological relapse, and it would also provide valuable information about clonal evolution.

I look forward to following the continued development of NGS-based MRD methods in acute leukemia, and to seeing how these techniques are increasingly implemented in clinical decision-making. In summary, MRD research holds many exciting opportunities. Through close collaboration between research and clinical practice, we can continue to refine and optimize MRD methods. With the ongoing advancements in standardized techniques and NGS, clinical trials, targeted therapies, and AI, I believe the future holds great promise for improving outcomes in acute leukemia, with MRD playing a central role in personalized care.

ACKNOWLEDGEMENT

Little did I know, when I first tried out summer research as a medical student back in 2013, that it would eventually lead to this thesis. It has been a journey during which I have learnt so much and got to know many fantastic people. I'm very grateful to everyone who has been part of this process. I would especially like to thank:

Linda Fogelstrand, my main supervisor. Thank you so much for giving me the opportunity to pursue this PhD. From the very beginning, you have believed in me and supported me every step of the way. Your encouragement, guidance, and everything you've taught me over the years have been invaluable. I'm so impressed by your drive, your work capacity, and your ability to manage everything with such grace – always with a sense of joy and optimism that lifts everyone around you. You are a superwoman with the warmest heart. I couldn't have wished for a better supervisor, and I feel very lucky to have the privilege of working with you.

Lars Palmqvist, my co-supervisor. Thank you for sharing your deep knowledge and extensive scientific experience. I truly appreciate your constructive feedback, which has greatly improved the quality of this work. And also – if it weren't for you, this thesis might never have been written, as you were the one I contacted in the first place and who introduced me to Linda. I'm forever thankful for that!

Erik Delsing Malmberg, my co-supervisor. Thank you for so generously sharing your knowledge and insights, and for supporting me along the years. Even with a busy schedule, you've always made time to help – answering my questions and offering thoughtful feedback. Your continuous encouragement and genuine interest in my progress have meant a lot to me!

Giti Shah Barkhordar, thank you for so patiently and thoroughly teaching me everything in the lab, and for sharing your broad expertise. This thesis would not have been possible without you. You have been there for me in every step of this journey – always taking the time to guide me, answer my questions, run countless deep sequencing experiments, and support me in so many ways, even if it meant staying late in the lab. Your kindness, generosity, dedication – and friendship – have meant more to me than I can say. Also, thank you for all the fun we had in between and all the laughs we've shared!

Anastasia Soboli, fellow PhD student and dear friend. Thank you for introducing me to flow cytometry and helping me understand it so well. It has been so much fun getting to know you and to go through this PhD journey together.

Gustav Orrsjö, fellow PhD student and dream team partner on Paper III! Thank you for a fantastic collaboration, I really appreciate your clinical expertise, sharp insights, and the positive energy you bring to every meeting.

Anna Rehammar, thank you for all your help with the statistics in Paper III, and for teaching me so much along the way. You've made statistics not only understandable, but genuinely enjoyable. Thank you also for your help with the bioinformatic analysis in Paper IV – handling it even during your vacation.

Anna Staffas, thank you for contributing with insightful comments and for generously sharing your knowledge, which has improved Papers III and IV. Your ability to explain complex things in a clear way has really helped me, and your dedication and sharp thinking have been a great source of inspiration.

Bea Tornberg, it was such a joy to be your co-supervisor on your bachelor thesis and to see how you grew and developed. Thank you for your valuable contributions to Paper IV, and for all the fun we had. You are so kind, talented, and full of positive energy, and I know that you will go far no matter what path you choose in the future.

Kajsa Olde, I was so happy when you joined our group, especially after having had the pleasure of being supervised by you during my internship a few years earlier. Thank you for your great company and for your thoughtful input that brings value to every discussion.

Anja Lidén Österberg, it has been fun and interesting to follow your work as a master's student in our group. Wishing you the best of luck with everything ahead!

Natalie Gevert, it was so nice to have you as a master's student in our group. Thank you for all the great chats and for being so positive and helpful!

Jenni Adamsson and **Susanna Jacobsson**, thank you for all the lovely everyday chats, the nice lunch breaks together, and for always being so kind and supportive. I'm so glad I got to know the two of you!

Eric Malmhäll-Bah, **Ahmed Waraky**, and **Gürcan Tunali**, my three office mates – thank you for valuable scientific (and non-scientific) discussions, and for your helpful advice on all things PhD-related.

Anders Östlund, it was so much fun sharing office with you during your time as a PhD student. Thank you for interesting discussions, and for being such a great travel companion in San Diego!

Mats Ehinger and **Louise Pettersson**, thank you both for a fruitful and enjoyable collaboration on Paper II. Louise, it was inspiring working with you – we made a great team, and I learned a lot from you.

Alvar Almstedt, thank you for your help with the bioinformatics, and especially for developing the *NPM1* script.

Angela Cheng Pettersson, in the very beginning, you were the one who introduced me to RT-qPCR – thank you for doing so with such encouragement, warmth, and positivity!

Julia Asp, thank you for your contribution to Papers I and II. I really appreciated your support and helpfulness during those early years – and it's always so nice talking to you!

Sara Ståhlman, you were the one who taught me how to work in the lab during my first years. Thank you so much for that, and for being there when I needed support. It's been a while since we last saw each other, and I miss you!

Pegah Johansson, thank you for enjoyable conversations and for giving me input on my work. Your positivity, kindness, and impressive research skills are inspiring!

To the co-authors on the papers not yet mentioned – **Jonas Abrahamsson**, **Charlotte Engvall**, **Yilun Chen**, **Li Zhou**, **Heike Kotarsky**, **Karina Vidovic**, **Vladimir Lazarevic**, **Lao Saal**, **Per-Ola Andersson**, **Hege Garelius**, **Mats Hardling**, **Borhan Saeed**, and **Henrik Hasle** – thank you for your valuable contributions and collaboration.

The studies presented in this thesis were made possible through generous financial support from the following sources: **Grants from the Swedish state under the ALF agreement**, **Stiftelsen Assar Gabrielssons Fond**, **Lions**

Cancerfond Väst, **Barncancerfonden**, and **Sahlgrenska Universitetssjukhusets fonder**.

I also wish to thank the **University of Gothenburg** and the **Department of Laboratory Medicine** for the opportunity to pursue my PhD and for providing an inspiring academic environment.

While research is never done alone, neither is life – and I'm truly thankful to my family and friends who are always there for me.

To all my **wonderful friends** – I'm so blessed to have you by my side. Thank you for all the laughter, fun times, and unforgettable moments.

Lilie and **Bengt**, my very dear parents-in-law. Thank you for your constant help and support, for being the best grandparents, and for bringing so much joy. We are so grateful to have you!

My brother **Carl Johan**, my wonderful sister-in-law **Mai**, and cutest little **Hugo**. Thank you for all the fun and joyful moments, I can't wait to make many more memories together!

My grandparents **Anette** and **Rolf**, thank you for your unwavering support throughout my life – your love and presence have meant so much to me.

My wonderful mother **Jeanette**, thank you for always believing in me and for supporting me in everything I do. I'm forever grateful for your never-ending love. You are the most amazing grandmother, and we are so lucky to have you in our lives.

The biggest thank you to my very own little family. You are my universe. **Ola**, the love of my life – thank you for always being by my side, for lifting me when I need it the most, and for making me so happy. Our sons, **Theodor** and **Gustaf** – the most precious gifts life has given me. Thank you for filling every day with laughter, wonder, and cuddles. You mean the world to me, and being your mamma is the greatest joy of all.

Lastly, I want to express my heartfelt thanks to all the **patients** who participated by allowing us to use their biobanked samples. I truly hope that this research will contribute valuable knowledge that can help improve care and outcomes for others in the future.

REFERENCES

1. Di Lonardo A, Nasi S, Pulciani S. Cancer: we should not forget the past. J Cancer. 2015;6(1):29-39.
2. Piller G. Leukaemia - a brief historical review from ancient times to 1950. Br J Haematol. 2001;112(2):282-92.
3. Kampen KR. The discovery and early understanding of leukemia. Leuk Res. 2012;36(1):6-13.
4. Pandolfi A, Barreyro L, Steidl U. Concise review: preleukemic stem cells: molecular biology and clinical implications of the precursors to leukemia stem cells. Stem Cells Transl Med. 2013;2(2):143-50.
5. Kelly LM, Gilliland DG. Genetics of myeloid leukemias. Annu Rev Genomics Hum Genet. 2002;3:179-98.
6. Greaves M. A causal mechanism for childhood acute lymphoblastic leukaemia. Nat Rev Cancer. 2018;18(8):471-84.
7. Arber DA, Orazi A, Hasserjian RP, Borowitz MJ, Calvo KR, Kvasnicka HM, et al. International Consensus Classification of Myeloid Neoplasms and Acute Leukemias: integrating morphologic, clinical, and genomic data. Blood. 2022;140(11):1200-28.
8. Khoury JD, Solary E, Abla O, Akkari Y, Alaggio R, Apperley JF, et al. The 5th edition of the World Health Organization Classification of Haematolymphoid Tumours: Myeloid and Histiocytic/Dendritic Neoplasms. Leukemia. 2022;36(7):1703-19.
9. Hasle H, Clemmensen IH, Mikkelsen M. Risks of leukaemia and solid tumours in individuals with Down's syndrome. Lancet. 2000;355(9199):165-9.
10. The Swedish Adult AML registry; Svenska nationella kvalitetsregistret för AML 2023 [https://statistik.incanet.se/AML/].
11. Juliusson G, Abrahamsson J, Lazarevic V, Antunovic P, Derolf Å, Garelius H, et al. Prevalence and characteristics of survivors from acute myeloid leukemia in Sweden. Leukemia. 2017;31(3):728-31.
12. Cancercentrum R. Nationellt vårdprogram Akut myeloisk leukemi. 2023.
13. Quessada J, Cuccuini W, Saultier P, Loosveld M, Harrison CJ, Lafage-Pochitaloff M. Cytogenetics of Pediatric Acute Myeloid Leukemia: A Review of the Current Knowledge. Genes (Basel). 2021;12(6).
14. Juliusson G, Antunovic P, Derolf A, Lehmann S, Möllgård L, Stockelberg D, et al. Age and acute myeloid leukemia: real world data on decision to treat and outcomes from the Swedish Acute Leukemia Registry. Blood. 2009;113(18):4179-87.

15. Ding F, Deng L, Xiong J, Cheng Z, Xu J. Analysis of global trends in acute lymphoblastic leukemia in children aged 0-5 years from 1990 to 2021. Front Pediatr. 2025;13:1542649.
16. Cancercentrum R. Nationellt vårdprogram Akut lymfatisk leukemi. 2023.
17. Svenska Barncancerregistret; Årsrapport 2022. 2022.
18. Björk-Eriksson T, Boström M, Bryngelsson IL, Lähteenmäki PM, Jarfelt M, Kalm M, Olsson DS. Mortality Among Pediatric Patients With Acute Lymphoblastic Leukemia in Sweden From 1988 to 2017. JAMA Netw Open. 2022;5(11):e2243857.
19. Greer JP. Wintrobe's clinical hematology. 13. ed. Philadelphia: Philadelphia : Wolters Kluwer Health; 2013.
20. Badar T, Ravandi F. Relapsed Acute Myeloid Leukemia: Need for Innovative Treatment Strategies to Improve Outcome. Clin Lymphoma Myeloma Leuk. 2015;15 Suppl:S104-8.
21. Thol F, Ganser A. Treatment of Relapsed Acute Myeloid Leukemia. Curr Treat Options Oncol. 2020;21(8):66.
22. Tierens A, Arad-Cohen N, Cheuk D, De Moerloose B, Fernandez Navarro JM, Hasle H, et al. Mitoxantrone Versus Liposomal Daunorubicin in Induction of Pediatric AML With Risk Stratification Based on Flow Cytometry Measurement of Residual Disease. J Clin Oncol. 2024:Jco2301841.
23. Karlsson L, Forestier E, Hasle H, Jahnukainen K, Jónsson Ó G, Lausen B, et al. Outcome after intensive reinduction therapy and allogeneic stem cell transplant in paediatric relapsed acute myeloid leukaemia. Br J Haematol. 2017;178(4):592-602.
24. Oskarsson T, Söderhäll S, Arvidson J, Forestier E, Montgomery S, Bottai M, et al. Relapsed childhood acute lymphoblastic leukemia in the Nordic countries: prognostic factors, treatment and outcome. Haematologica. 2016;101(1):68-76.
25. Tomizawa D, Kolb EA, Reinhardt D, Reinhardt D, Kolb EA, Tomizawa D. Acute Myeloid Leukemia in Children: Standard of Care and Future Perspectives. 1 ed. Cham: Cham: Springer; 2024.
26. Bennett JM, Catovsky D, Daniel MT, Flandrin G, Galton DA, Gralnick HR, Sultan C. Proposals for the classification of the acute leukaemias. French-American-British (FAB) co-operative group. Br J Haematol. 1976;33(4):451-8.
27. World Health Organization (WHO) Classification of Paediatric Tumours. 5th ed: International Agency for Research on Cancer; 2023.
28. Ley TJ, Mardis ER, Ding L, Fulton B, McLellan MD, Chen K, et al. DNA sequencing of a cytogenetically normal acute myeloid leukaemia genome. Nature. 2008;456(7218):66-72.

29. Ley TJ, Miller C, Ding L, Raphael BJ, Mungall AJ, Robertson A, et al. Genomic and epigenomic landscapes of adult de novo acute myeloid leukemia. N Engl J Med. 2013;368(22):2059-74.
30. Papaemmanuil E, Gerstung M, Bullinger L, Gaidzik VI, Paschka P, Roberts ND, et al. Genomic Classification and Prognosis in Acute Myeloid Leukemia. N Engl J Med. 2016;374(23):2209-21.
31. Bolouri H, Farrar JE, Triche T, Jr., Ries RE, Lim EL, Alonzo TA, et al. The molecular landscape of pediatric acute myeloid leukemia reveals recurrent structural alterations and age-specific mutational interactions. Nat Med. 2018;24(1):103-12.
32. Mullighan CG. Molecular genetics of B-precursor acute lymphoblastic leukemia. J Clin Invest. 2012;122(10):3407-15.
33. Jia M, Hu BF, Xu XJ, Zhang JY, Li SS, Tang YM. Clinical features and prognostic impact of TCF3-PBX1 in childhood acute lymphoblastic leukemia: A single-center retrospective study of 837 patients from China. Curr Probl Cancer. 2021;45(6):100758.
34. Zhang X, Rastogi P, Shah B, Zhang L. B lymphoblastic leukemia/lymphoma: new insights into genetics, molecular aberrations, subclassification and targeted therapy. Oncotarget. 2017;8(39):66728-41.
35. World Health Organization (WHO) Classification of Tumours Online 2022 [5th Edition:[Available from: https://tumourclassification.iarc.who.int/home.
36. Rau R, Brown P. Nucleophosmin (NPM1) mutations in adult and childhood acute myeloid leukaemia: towards definition of a new leukaemia entity. Hematol Oncol. 2009;27(4):171-81.
37. Sharma N, Liesveld JL. NPM 1 Mutations in AML-The Landscape in 2023. Cancers (Basel). 2023;15(4).
38. Falini B, Mecucci C, Tiacci E, Alcalay M, Rosati R, Pasqualucci L, et al. Cytoplasmic nucleophosmin in acute myelogenous leukemia with a normal karyotype. N Engl J Med. 2005;352(3):254-66.
39. Döhner K, Schlenk RF, Habdank M, Scholl C, Rücker FG, Corbacioglu A, et al. Mutant nucleophosmin (NPM1) predicts favorable prognosis in younger adults with acute myeloid leukemia and normal cytogenetics: interaction with other gene mutations. Blood. 2005;106(12):3740-6.
40. Brown P, McIntyre E, Rau R, Meshinchi S, Lacayo N, Dahl G, et al. The incidence and clinical significance of nucleophosmin mutations in childhood AML. Blood. 2007;110(3):979-85.
41. Thiede C, Koch S, Creutzig E, Steudel C, Illmer T, Schaich M, Ehninger G. Prevalence and prognostic impact of NPM1 mutations in 1485 adult patients with acute myeloid leukemia (AML). Blood. 2006;107(10):4011-20.

42. Nagel G, Weber D, Fromm E, Erhardt S, Lübbert M, Fiedler W, et al. Epidemiological, genetic, and clinical characterization by age of newly diagnosed acute myeloid leukemia based on an academic population-based registry study (AMLSG BiO). Ann Hematol. 2017;96(12):1993-2003.
43. Heath EM, Chan SM, Minden MD, Murphy T, Shlush LI, Schimmer AD. Biological and clinical consequences of NPM1 mutations in AML. Leukemia. 2017;31(4):798-807.
44. Patel SS, Kluk MJ, Weinberg OK. NPM1 Biology in Myeloid Neoplasia. Curr Hematol Malig Rep. 2020;15(4):350-9.
45. Lindström MS. NPM1/B23: A Multifunctional Chaperone in Ribosome Biogenesis and Chromatin Remodeling. Biochem Res Int. 2011;2011:195209.
46. Falini B, Brunetti L, Sportoletti P, Martelli MP. NPM1-mutated acute myeloid leukemia: from bench to bedside. Blood. 2020;136(15):1707-21.
47. Falini B, Nicoletti I, Martelli MF, Mecucci C. Acute myeloid leukemia carrying cytoplasmic/mutated nucleophosmin (NPMc+ AML): biologic and clinical features. Blood. 2007;109(3):874-85.
48. Shi Y, Chen X, Jin H, Zhu L, Hong M, Zhu Y, et al. Clinical prognostic value of different NPM1 mutations in acute myeloid leukemia patients. Ann Hematol. 2024;103(7):2323-35.
49. Patkar N, Kodgule R, Kakirde C, Raval G, Bhanshe P, Joshi S, et al. Clinical impact of measurable residual disease monitoring by ultradeep next generation sequencing in NPM1 mutated acute myeloid leukemia. Oncotarget. 2018;9(93):36613-24.
50. Döhner H, Estey E, Grimwade D, Amadori S, Appelbaum FR, Büchner T, et al. Diagnosis and management of AML in adults: 2017 ELN recommendations from an international expert panel. Blood. 2017;129(4):424-47.
51. Verhaak RG, Goudswaard CS, van Putten W, Bijl MA, Sanders MA, Hugens W, et al. Mutations in nucleophosmin (NPM1) in acute myeloid leukemia (AML): association with other gene abnormalities and previously established gene expression signatures and their favorable prognostic significance. Blood. 2005;106(12):3747-54.
52. Döhner H, Wei AH, Appelbaum FR, Craddock C, DiNardo CD, Dombret H, et al. Diagnosis and management of AML in adults: 2022 recommendations from an international expert panel on behalf of the ELN. Blood. 2022;140(12):1345-77.
53. Pastore F, Greif PA, Schneider S, Ksienzyk B, Mellert G, Zellmeier E, et al. The NPM1 mutation type has no impact on survival in cytogenetically normal AML. PLoS One. 2014;9(10):e109759.
54. Tregnago C, Benetton M, Ries RE, Peplinski JH, Alonzo TA, Stirewalt D, et al. Influence of Nucleophosmin (NPM1) Genotypes on

Outcome of Patients With AML: An AIEOP-BFM and COG-SWOG Intergroup Collaboration. J Clin Oncol. 2025;43(8):972-84.
55. Garg S, Reyes-Palomares A, He L, Bergeron A, Lavallée VP, Lemieux S, et al. Hepatic leukemia factor is a novel leukemic stem cell regulator in DNMT3A, NPM1, and FLT3-ITD triple-mutated AML. Blood. 2019;134(3):263-76.
56. Bezerra MF, Lima AS, Piqué-Borràs MR, Silveira DR, Coelho-Silva JL, Pereira-Martins DA, et al. Co-occurrence of DNMT3A, NPM1, FLT3 mutations identifies a subset of acute myeloid leukemia with adverse prognosis. Blood. 2020;135(11):870-5.
57. Oñate G, Bataller A, Garrido A, Hoyos M, Arnan M, Vives S, et al. Prognostic impact of DNMT3A mutation in acute myeloid leukemia with mutated NPM1. Blood Adv. 2022;6(3):882-90.
58. Daver N, Schlenk RF, Russell NH, Levis MJ. Targeting FLT3 mutations in AML: review of current knowledge and evidence. Leukemia. 2019;33(2):299-312.
59. Bacher U, Haferlach C, Kern W, Haferlach T, Schnittger S. Prognostic relevance of FLT3-TKD mutations in AML: the combination matters--an analysis of 3082 patients. Blood. 2008;111(5):2527-37.
60. Stirewalt DL, Radich JP. The role of FLT3 in haematopoietic malignancies. Nature Reviews Cancer. 2003;3(9):650-65.
61. Schmalbrock LK, Dolnik A, Cocciardi S, Sträng E, Theis F, Jahn N, et al. Clonal evolution of acute myeloid leukemia with FLT3-ITD mutation under treatment with midostaurin. Blood. 2021;137(22):3093-104.
62. Port M, Böttcher M, Thol F, Ganser A, Schlenk R, Wasem J, et al. Prognostic significance of FLT3 internal tandem duplication, nucleophosmin 1, and CEBPA gene mutations for acute myeloid leukemia patients with normal karyotype and younger than 60 years: a systematic review and meta-analysis. Ann Hematol. 2014;93(8):1279-86.
63. Wu X, Feng X, Zhao X, Ma F, Liu N, Guo H, et al. Prognostic significance of FLT3-ITD in pediatric acute myeloid leukemia: a meta-analysis of cohort studies. Mol Cell Biochem. 2016;420(1-2):121-8.
64. Tarlock K, Gerbing RB, Ries RE, Smith JL, Leonti A, Huang BJ, et al. Prognostic impact of cooccurring mutations in FLT3-ITD pediatric acute myeloid leukemia. Blood Adv. 2024;8(9):2094-103.
65. Jeon SJ, Park JH, Lim H, Won EJ, Choi H, Choi HJ, et al. First Case of ETV6-RUNX1 Fusion in Adult De Novo Acute Myeloid Leukemia Detected Using Targeted RNA Sequencing. Ann Lab Med. 2023;43(1):92-5.
66. Zhou MH, Gao L, Jing Y, Xu YY, Ding Y, Wang N, et al. Detection of ETV6 gene rearrangements in adult acute lymphoblastic leukemia. Ann Hematol. 2012;91(8):1235-43.

67. Sun C, Chang L, Zhu X. Pathogenesis of ETV6/RUNX1-positive childhood acute lymphoblastic leukemia and mechanisms underlying its relapse. Oncotarget. 2017;8(21):35445-59.
68. Papaemmanuil E, Rapado I, Li Y, Potter NE, Wedge DC, Tubio J, et al. RAG-mediated recombination is the predominant driver of oncogenic rearrangement in ETV6-RUNX1 acute lymphoblastic leukemia. Nat Genet. 2014;46(2):116-25.
69. Kaczmarska A, Derebas J, Pinkosz M, Niedźwiecki M, Lejman M. The Landscape of Secondary Genetic Rearrangements in Pediatric Patients with B-Cell Acute Lymphoblastic Leukemia with t(12;21). Cells. 2023;12(3).
70. NOPHO--DBH AML 2012 Protocol v.2.1. 2013.
71. Cheson BD, Bennett JM, Kopecky KJ, Büchner T, Willman CL, Estey EH, et al. Revised recommendations of the International Working Group for Diagnosis, Standardization of Response Criteria, Treatment Outcomes, and Reporting Standards for Therapeutic Trials in Acute Myeloid Leukemia. J Clin Oncol. 2003;21(24):4642-9.
72. Juliusson G. Most 70- to 79-year-old patients with acute myeloid leukemia do benefit from intensive treatment. Blood. 2011;117(12):3473-4.
73. Medeiros BC, Satram-Hoang S, Hurst D, Hoang KQ, Momin F, Reyes C. Big data analysis of treatment patterns and outcomes among elderly acute myeloid leukemia patients in the United States. Ann Hematol. 2015;94(7):1127-38.
74. Lin TL, Pagano L. The important role of intensive induction chemotherapy in the treatment of acute myeloid leukemia. Expert Rev Hematol. 2021;14(3):303-14.
75. Jaramillo S, Schlenk RF. Update on current treatments for adult acute myeloid leukemia: to treat acute myeloid leukemia intensively or non-intensively? That is the question. Haematologica. 2023;108(2):342-52.
76. Yates JW, Wallace HJ, Jr., Ellison RR, Holland JF. Cytosine arabinoside (NSC-63878) and daunorubicin (NSC-83142) therapy in acute nonlymphocytic leukemia. Cancer Chemother Rep. 1973;57(4):485-8.
77. Oñate G, Garrido A, Arnan M, Pomares H, Alonso E, Tormo M, et al. Diverse real-life outcomes after intensive risk-adapted therapy for 1034 AML patients from the CETLAM Group. Blood Cancer J. 2025;15(1):4.
78. ALLTogether1 – A Treatment study protocol of the ALLTogether Consortium for children and young adults (0-45 years of age) with newly diagnosed acute lymphoblastic leukaemia (ALL). 2023.
79. Loke J, Buka R, Craddock C. Allogeneic Stem Cell Transplantation for Acute Myeloid Leukemia: Who, When, and How? Front Immunol. 2021;12:659595.
80. Hong KT, Park HJ, Kim BK, An HY, Choi JY, Kang HJ. Post-Transplantation Cyclophosphamide-Based Haploidentical versus Matched

Unrelated Donor Peripheral Blood Hematopoietic Stem Cell Transplantation Using Myeloablative Targeted Busulfan-Based Conditioning for Pediatric Acute Leukemia. Transplant Cell Ther. 2022;28(4):195.e1-.e7.
81. Penack O, Peczynski C, Mohty M, Yakoub-Agha I, Styczynski J, Montoto S, et al. How much has allogeneic stem cell transplant-related mortality improved since the 1980s? A retrospective analysis from the EBMT. Blood Adv. 2020;4(24):6283-90.
82. Stone RM, Mandrekar SJ, Sanford BL, Laumann K, Geyer S, Bloomfield CD, et al. Midostaurin plus Chemotherapy for Acute Myeloid Leukemia with a FLT3 Mutation. N Engl J Med. 2017;377(5):454-64.
83. Ahn JS, Kim HJ. FLT3 mutations in acute myeloid leukemia: a review focusing on clinically applicable drugs. Blood Res. 2022;57(S1):32-6.
84. Burchert A, Bug G, Fritz LV, Finke J, Stelljes M, Röllig C, et al. Sorafenib Maintenance After Allogeneic Hematopoietic Stem Cell Transplantation for Acute Myeloid Leukemia With FLT3-Internal Tandem Duplication Mutation (SORMAIN). J Clin Oncol. 2020;38(26):2993-3002.
85. Röllig C, Serve H, Noppeney R, Hanoun M, Krug U, Baldus CD, et al. Sorafenib or placebo in patients with newly diagnosed acute myeloid leukaemia: long-term follow-up of the randomized controlled SORAML trial. Leukemia. 2021;35(9):2517-25.
86. Cortes JE, Khaled S, Martinelli G, Perl AE, Ganguly S, Russell N, et al. Quizartinib versus salvage chemotherapy in relapsed or refractory FLT3-ITD acute myeloid leukaemia (QuANTUM-R): a multicentre, randomised, controlled, open-label, phase 3 trial. Lancet Oncol. 2019;20(7):984-97.
87. Bazarbachi A, Labopin M, Battipaglia G, Djabali A, Passweg J, Socié G, et al. Sorafenib improves survival of FLT3-mutated acute myeloid leukemia in relapse after allogeneic stem cell transplantation: a report of the EBMT Acute Leukemia Working Party. Haematologica. 2019;104(9):e398-e401.
88. Eguchi M, Minami Y, Kuzume A, Chi S. Mechanisms Underlying Resistance to FLT3 Inhibitors in Acute Myeloid Leukemia. Biomedicines. 2020;8(8).
89. Swaminathan M, Bourgeois W, Armstrong SA, Wang ES. Menin Inhibitors in Acute Myeloid Leukemia-What Does the Future Hold? Cancer J. 2022;28(1):62-6.
90. Candoni A, Coppola G. A 2024 Update on Menin Inhibitors. A New Class of Target Agents against KMT2A-Rearranged and NPM1-Mutated Acute Myeloid Leukemia. Hematol Rep. 2024;16(2):244-54.
91. DiNardo CD, Rausch CR, Benton C, Kadia T, Jain N, Pemmaraju N, et al. Clinical experience with the BCL2-inhibitor venetoclax in combination therapy for relapsed and refractory acute myeloid leukemia and related myeloid malignancies. Am J Hematol. 2018;93(3):401-7.

92. Wei AH, Montesinos P, Ivanov V, DiNardo CD, Novak J, Laribi K, et al. Venetoclax plus LDAC for newly diagnosed AML ineligible for intensive chemotherapy: a phase 3 randomized placebo-controlled trial. Blood. 2020;135(24):2137-45.
93. DiNardo CD, Jen WY, Takahashi K, Kadia TM, Loghavi S, Daver NG, et al. Long term results of venetoclax combined with FLAG-IDA induction and consolidation for newly diagnosed and relapsed or refractory acute myeloid leukemia. Leukemia. 2025.
94. Pratz KW, Jonas BA, Pullarkat V, Thirman MJ, Garcia JS, Döhner H, et al. Long-term follow-up of VIALE-A: Venetoclax and azacitidine in chemotherapy-ineligible untreated acute myeloid leukemia. Am J Hematol. 2024;99(4):615-24.
95. Cui J, Chen X, Li C, Yan Q, Yuan G. Reduced duration and dosage of venetoclax is efficient in newly diagnosed patients with acute myeloid leukemia. Hematology. 2024;29(1):2293512.
96. Xie Y, Wei X, Wang W, Liao C, Han P, Yu Y. Meta-analysis of the efficacy of venetoclax and azacitidine combination therapy and azacitidine monotherapy for treating acute myeloid leukemia. Exp Ther Med. 2024;27(4):164.
97. DiNardo CD, Tiong IS, Quaglieri A, MacRaild S, Loghavi S, Brown FC, et al. Molecular patterns of response and treatment failure after frontline venetoclax combinations in older patients with AML. Blood. 2020;135(11):791-803.
98. Craddock C, Tauro S, Moss P, Grimwade D. Biology and management of relapsed acute myeloid leukaemia. Br J Haematol. 2005;129(1):18-34.
99. Bradstock KF, Janossy G, Tidman N, Papageorgiou ES, Prentice HG, Willoughby M, Hoffbrand AV. Immunological monitoring of residual disease in treated thymic acute lymphoblastic leukaemia. Leuk Res. 1981;5(4-5):301-9.
100. van Dongen JJM, van der Velden VHJ, Brüggemann M, Orfao A. Minimal residual disease diagnostics in acute lymphoblastic leukemia: need for sensitive, fast, and standardized technologies. Blood. 2015;125(26):3996-4009.
101. Schuurhuis GJ, Heuser M, Freeman S, Béné MC, Buccisano F, Cloos J, et al. Minimal/measurable residual disease in AML: a consensus document from the European LeukemiaNet MRD Working Party. Blood. 2018;131(12):1275-91.
102. Theunissen P, Mejstrikova E, Sedek L, van der Sluijs-Gelling AJ, Gaipa G, Bartels M, et al. Standardized flow cytometry for highly sensitive MRD measurements in B-cell acute lymphoblastic leukemia. Blood. 2017;129(3):347-57.

103. Ouyang J, Goswami M, Tang G, Peng J, Ravandi F, Daver N, et al. The clinical significance of negative flow cytometry immunophenotypic results in a morphologically scored positive bone marrow in patients following treatment for acute myeloid leukemia. American Journal of Hematology. 2015;90(6):504-10.
104. Heuser M, Freeman SD, Ossenkoppele GJ, Buccisano F, Hourigan CS, Ngai LL, et al. 2021 Update on MRD in acute myeloid leukemia: a consensus document from the European LeukemiaNet MRD Working Party. Blood. 2021;138(26):2753-67.
105. Rosso A, Juliusson G, Lorenz F, Lehmann S, Derolf Å, Deneberg S, et al. Is there an impact of measurable residual disease as assessed by multiparameter flow cytometry on survival of AML patients treated in clinical practice? A population-based study. Leuk Lymphoma. 2021;62(8):1973-81.
106. Tierens A, Bjørklund E, Siitonen S, Marquart HV, Wulff-Juergensen G, Pelliniemi TT, et al. Residual disease detected by flow cytometry is an independent predictor of survival in childhood acute myeloid leukaemia; results of the NOPHO-AML 2004 study. Br J Haematol. 2016;174(4):600-9.
107. Zeijlemaker W, Kelder A, Cloos J, Schuurhuis GJ. Immunophenotypic Detection of Measurable Residual (Stem Cell) Disease Using LAIP Approach in Acute Myeloid Leukemia. Curr Protoc Cytom. 2019;91(1):e66.
108. Zeijlemaker W, Grob T, Meijer R, Hanekamp D, Kelder A, Carbaat-Ham JC, et al. CD34(+)CD38(-) leukemic stem cell frequency to predict outcome in acute myeloid leukemia. Leukemia. 2019;33(5):1102-12.
109. Cruz NM, Mencia-Trinchant N, Hassane DC, Guzman ML. Minimal residual disease in acute myelogenous leukemia. Int J Lab Hematol. 2017;39 Suppl 1(Suppl 1):53-60.
110. Kayser S, Walter RB, Stock W, Schlenk RF. Minimal residual disease in acute myeloid leukemia--current status and future perspectives. Curr Hematol Malig Rep. 2015;10(2):132-44.
111. Grimwade D, Freeman SD. Defining minimal residual disease in acute myeloid leukemia: which platforms are ready for "prime time"? Blood. 2014;124(23):3345-55.
112. Höllein A, Meggendorfer M, Dicker F, Jeromin S, Nadarajah N, Kern W, et al. NPM1 mutated AML can relapse with wild-type NPM1: persistent clonal hematopoiesis can drive relapse. Blood Adv. 2018;2(22):3118-25.
113. Cocciardi S, Dolnik A, Kapp-Schwoerer S, Rücker FG, Lux S, Blätte TJ, et al. Clonal evolution patterns in acute myeloid leukemia with NPM1 mutation. Nat Commun. 2019;10(1):2031.

114. Pettersson L, Johansson Alm S, Almstedt A, Chen Y, Orrsjö G, Shah-Barkhordar G, et al. Comparison of RNA- and DNA-based methods for measurable residual disease analysis in NPM1-mutated acute myeloid leukemia. Int J Lab Hematol. 2021;43(4):664-74.
115. Chou WC, Tang JL, Wu SJ, Tsay W, Yao M, Huang SY, et al. Clinical implications of minimal residual disease monitoring by quantitative polymerase chain reaction in acute myeloid leukemia patients bearing nucleophosmin (NPM1) mutations. Leukemia. 2007;21(5):998-1004.
116. 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.
117. Malmberg EB, Ståhlman S, Rehammar A, Samuelsson T, Alm SJ, Kristiansson E, et al. Patient-tailored analysis of minimal residual disease in acute myeloid leukemia using next-generation sequencing. Eur J Haematol. 2017;98(1):26-37.
118. Pettersson L, Chen Y, George AM, Rigo R, Lazarevic V, Juliusson G, et al. Subclonal patterns in follow-up of acute myeloid leukemia combining whole exome sequencing and ultrasensitive IBSAFE digital droplet analysis. Leuk Lymphoma. 2020;61(9):2168-79.
119. Godley LA. Germline mutations in MDS/AML predisposition disorders. Curr Opin Hematol. 2021;28(2):86-93.
120. Hasserjian RP, Steensma DP, Graubert TA, Ebert BL. Clonal hematopoiesis and measurable residual disease assessment in acute myeloid leukemia. Blood. 2020;135(20):1729-38.
121. Bowman RL, Busque L, Levine RL. Clonal Hematopoiesis and Evolution to Hematopoietic Malignancies. Cell Stem Cell. 2018;22(2):157-70.
122. Thol F, Kölking B, Damm F, Reinhardt K, Klusmann JH, Reinhardt D, et al. Next-generation sequencing for minimal residual disease monitoring in acute myeloid leukemia patients with FLT3-ITD or NPM1 mutations. Genes Chromosomes Cancer. 2012;51(7):689-95.
123. Salipante SJ, Fromm JR, Shendure J, Wood BL, Wu D. Detection of minimal residual disease in NPM1-mutated acute myeloid leukemia by next-generation sequencing. Mod Pathol. 2014;27(11):1438-46.
124. Short NJ, Zhou S, Fu C, Berry DA, Walter RB, Freeman SD, et al. Association of Measurable Residual Disease With Survival Outcomes in Patients With Acute Myeloid Leukemia: A Systematic Review and Meta-analysis. JAMA Oncol. 2020;6(12):1890-9.
125. Terwijn M, van Putten WL, Kelder A, van der Velden VH, Brooimans RA, Pabst T, et al. High prognostic impact of flow cytometric minimal residual disease detection in acute myeloid leukemia: data from the HOVON/SAKK AML 42A study. J Clin Oncol. 2013;31(31):3889-97.

126. Freeman SD, Virgo P, Couzens S, Grimwade D, Russell N, Hills RK, Burnett AK. Prognostic relevance of treatment response measured by flow cytometric residual disease detection in older patients with acute myeloid leukemia. J Clin Oncol. 2013;31(32):4123-31.
127. Ravandi F, Jorgensen J, Borthakur G, Jabbour E, Kadia T, Pierce S, et al. Persistence of minimal residual disease assessed by multiparameter flow cytometry is highly prognostic in younger patients with acute myeloid leukemia. Cancer. 2017;123(3):426-35.
128. Freeman SD, Hills RK, Virgo P, Khan N, Couzens S, Dillon R, et al. Measurable Residual Disease at Induction Redefines Partial Response in Acute Myeloid Leukemia and Stratifies Outcomes in Patients at Standard Risk Without NPM1 Mutations. J Clin Oncol. 2018;36(15):1486-97.
129. Zhang C, Gu R, Zhou C, Li Y, Liu Y, Wei S, et al. Prognostic Effect and Clinical Application of Early Measurable Residual Disease (MRD) By Flow Cytometry on De Novo Acute Myeloid Leukemia (AML). Blood. 2022;140(Supplement 1):2030-2.
130. Shah MV, Jorgensen JL, Saliba RM, Wang SA, Alousi AM, Andersson BS, et al. Early Post-Transplant Minimal Residual Disease Assessment Improves Risk Stratification in Acute Myeloid Leukemia. Biol Blood Marrow Transplant. 2018;24(7):1514-20.
131. Sievers EL, Lange BJ, Alonzo TA, Gerbing RB, Bernstein ID, Smith FO, et al. Immunophenotypic evidence of leukemia after induction therapy predicts relapse: results from a prospective Children's Cancer Group study of 252 patients with acute myeloid leukemia. Blood. 2003;101(9):3398-406.
132. Coustan-Smith E, Ribeiro RC, Rubnitz JE, Razzouk BI, Pui CH, Pounds S, et al. Clinical significance of residual disease during treatment in childhood acute myeloid leukaemia. Br J Haematol. 2003;123(2):243-52.
133. Björklund E, Mazur J, Söderhäll S, Porwit-MacDonald A. Flow cytometric follow-up of minimal residual disease in bone marrow gives prognostic information in children with acute lymphoblastic leukemia. Leukemia. 2003;17(1):138-48.
134. Conter V, Bartram CR, Valsecchi MG, Schrauder A, Panzer-Grümayer R, Möricke A, et al. Molecular response to treatment redefines all prognostic factors in children and adolescents with B-cell precursor acute lymphoblastic leukemia: results in 3184 patients of the AIEOP-BFM ALL 2000 study. Blood. 2010;115(16):3206-14.
135. Schrappe M, Valsecchi MG, Bartram CR, Schrauder A, Panzer-Grümayer R, Möricke A, et al. Late MRD response determines relapse risk overall and in subsets of childhood T-cell ALL: results of the AIEOP-BFM-ALL 2000 study. Blood. 2011;118(8):2077-84.
136. Inaba H, Coustan-Smith E, Cao X, Pounds SB, Shurtleff SA, Wang KY, et al. Comparative analysis of different approaches to measure

treatment response in acute myeloid leukemia. J Clin Oncol. 2012;30(29):3625-32.
137. Buldini B, Rizzati F, Masetti R, Fagioli F, Menna G, Micalizzi C, et al. Prognostic significance of flow-cytometry evaluation of minimal residual disease in children with acute myeloid leukaemia treated according to the AIEOP-AML 2002/01 study protocol. Br J Haematol. 2017;177(1):116-26.
138. Langebrake C, Creutzig U, Dworzak M, Hrusak O, Mejstrikova E, Griesinger F, et al. Residual disease monitoring in childhood acute myeloid leukemia by multiparameter flow cytometry: the MRD-AML-BFM Study Group. J Clin Oncol. 2006;24(22):3686-92.
139. Ivey A, Hills RK, Simpson MA, Jovanovic JV, Gilkes A, Grech A, et al. Assessment of Minimal Residual Disease in Standard-Risk AML. N Engl J Med. 2016;374(5):422-33.
140. Shayegi N, Kramer M, Bornhäuser M, Schaich M, Schetelig J, Platzbecker U, et al. The level of residual disease based on mutant NPM1 is an independent prognostic factor for relapse and survival in AML. Blood. 2013;122(1):83-92.
141. Krönke J, Schlenk RF, Jensen KO, Tschürtz F, Corbacioglu A, Gaidzik VI, et al. Monitoring of minimal residual disease in NPM1-mutated acute myeloid leukemia: a study from the German-Austrian acute myeloid leukemia study group. J Clin Oncol. 2011;29(19):2709-16.
142. Hubmann M, Köhnke T, Hoster E, Schneider S, Dufour A, Zellmeier E, et al. Molecular response assessment by quantitative real-time polymerase chain reaction after induction therapy in NPM1-mutated patients identifies those at high risk of relapse. Haematologica. 2014;99(8):1317-25.
143. Schnittger S, Kern W, Tschulik C, Weiss T, Dicker F, Falini B, et al. Minimal residual disease levels assessed by NPM1 mutation-specific RQ-PCR provide important prognostic information in AML. Blood. 2009;114(11):2220-31.
144. Balsat M, Renneville A, Thomas X, de Botton S, Caillot D, Marceau A, et al. Postinduction Minimal Residual Disease Predicts Outcome and Benefit From Allogeneic Stem Cell Transplantation in Acute Myeloid Leukemia With NPM1 Mutation: A Study by the Acute Leukemia French Association Group. J Clin Oncol. 2017;35(2):185-93.
145. Kayser S, Benner A, Thiede C, Martens U, Huber J, Stadtherr P, et al. Pretransplant NPM1 MRD levels predict outcome after allogeneic hematopoietic stem cell transplantation in patients with acute myeloid leukemia. Blood Cancer J. 2016;6(7):e449.
146. Othman J, Potter N, Ivey A, Jovanovic J, Runglall M, Freeman SD, et al. Postinduction molecular MRD identifies patients with NPM1 AML who benefit from allogeneic transplant in first remission. Blood. 2024;143(19):1931-6.

147. Patkar N, Kakirde C, Shaikh AF, Salve R, Bhanshe P, Chatterjee G, et al. Clinical impact of panel-based error-corrected next generation sequencing versus flow cytometry to detect measurable residual disease (MRD) in acute myeloid leukemia (AML). Leukemia. 2021;35(5):1392-404.
148. Grob T, Sanders MA, Vonk CM, Kavelaars FG, Rijken M, Hanekamp DW, et al. Prognostic Value of FLT3-Internal Tandem Duplication Residual Disease in Acute Myeloid Leukemia. J Clin Oncol. 2023;41(4):756-65.
149. Lee JM, Park S, Hwang I, Kang D, Cho BS, Kim HJ, et al. FLT3-ITD Measurable Residual Disease Monitoring in Acute Myeloid Leukemia Using Next-Generation Sequencing. Cancers (Basel). 2022;14(24).
150. Dillon LW, Gui G, Ravindra N, Andrew G, Mukherjee D, Wong ZC, et al. Measurable Residual FLT3 Internal Tandem Duplication Before Allogeneic Transplant for Acute Myeloid Leukemia. JAMA Oncol. 2024;10(8):1104-10.
151. Rücker FG, Bullinger L, Cocciardi S, Skambraks S, Luck TJ, Weber D, et al. Measurable residual disease monitoring in AML with FLT3-ITD treated with intensive chemotherapy plus midostaurin. Blood Adv. 2024;8(23):6067-80.
152. Press RD, Eickelberg G, Froman A, Yang F, Stentz A, Flatley EM, et al. Next-generation sequencing-defined minimal residual disease before stem cell transplantation predicts acute myeloid leukemia relapse. Am J Hematol. 2019;94(8):902-12.
153. Li Y, Solis-Ruiz J, Yang F, Long N, Tong CH, Lacbawan FL, et al. NGS-defined measurable residual disease (MRD) after initial chemotherapy as a prognostic biomarker for acute myeloid leukemia. Blood Cancer J. 2023;13(1):59.
154. Heuser M, Heida B, Büttner K, Wienecke CP, Teich K, Funke C, et al. Posttransplantation MRD monitoring in patients with AML by next-generation sequencing using DTA and non-DTA mutations. Blood Adv. 2021;5(9):2294-304.
155. Jongen-Lavrencic M, Grob T, Hanekamp D, Kavelaars FG, Al Hinai A, Zeilemaker A, et al. Molecular Minimal Residual Disease in Acute Myeloid Leukemia. N Engl J Med. 2018;378(13):1189-99.
156. Tsai CH, Tang JL, Tien FM, Kuo YY, Wu DC, Lin CC, et al. Clinical implications of sequential MRD monitoring by NGS at 2 time points after chemotherapy in patients with AML. Blood Adv. 2021;5(10):2456-66.
157. Hirsch P, Lambert J, Bucci M, Deswarte C, Boudry A, Lambert J, et al. Multi-target measurable residual disease assessed by error-corrected sequencing in patients with acute myeloid leukemia: An ALFA study. Blood Cancer J. 2024;14(1):97.

158. Saygin C, Cannova J, Stock W, Muffly L. Measurable residual disease in acute lymphoblastic leukemia: methods and clinical context in adult patients. Haematologica. 2022;107(12):2783-93.
159. Faham M, Zheng J, Moorhead M, Carlton VEH, Stow P, Coustan-Smith E, et al. Deep-sequencing approach for minimal residual disease detection in acute lymphoblastic leukemia. Blood. 2012;120(26):5173-80.
160. Cheng S, Inghirami G, Cheng S, Tam W. Simple deep sequencing-based post-remission MRD surveillance predicts clinical relapse in B-ALL. J Hematol Oncol. 2018;11(1):105.
161. Pulsipher MA, Carlson C, Langholz B, Wall DA, Schultz KR, Bunin N, et al. IgH-V(D)J NGS-MRD measurement pre- and early post-allotransplant defines very low- and very high-risk ALL patients. Blood. 2015;125(22):3501-8.
162. Kotrova M, Muzikova K, Mejstrikova E, Novakova M, Bakardjieva-Mihaylova V, Fiser K, et al. The predictive strength of next-generation sequencing MRD detection for relapse compared with current methods in childhood ALL. Blood. 2015;126(8):1045-7.
163. Wood B, Wu D, Crossley B, Dai Y, Williamson D, Gawad C, et al. Measurable residual disease detection by high-throughput sequencing improves risk stratification for pediatric B-ALL. Blood. 2018;131(12):1350-9.
164. Ahn WK, Yu K, Kim H, Lee S-T, Choi JR, Han JW, et al. Monitoring measurable residual disease in paediatric acute lymphoblastic leukaemia using immunoglobulin gene clonality based on next-generation sequencing. Cancer Cell International. 2024;24(1):218.
165. Svaton M, Skotnicova A, Reznickova L, Rennerova A, Valova T, Kotrova M, et al. NGS better discriminates true MRD positivity for the risk stratification of childhood ALL treated on an MRD-based protocol. Blood. 2023;141(5):529-33.
166. Delsing Malmberg E, Rehammar A, Pereira MB, Abrahamsson J, Samuelsson T, Ståhlman S, et al. Accurate and Sensitive Analysis of Minimal Residual Disease in Acute Myeloid Leukemia Using Deep Sequencing of Single Nucleotide Variations. J Mol Diagn. 2019;21(1):149-62.
167. Blätte TJ, Schmalbrock LK, Skambraks S, Lux S, Cocciardi S, Dolnik A, et al. getITD for FLT3-ITD-based MRD monitoring in AML. Leukemia. 2019;33(10):2535-9.
168. van der Velden VH, Cazzaniga G, Schrauder A, Hancock J, Bader P, Panzer-Grumayer ER, et al. Analysis of minimal residual disease by Ig/TCR gene rearrangements: guidelines for interpretation of real-time quantitative PCR data. Leukemia. 2007;21(4):604-11.
169. Dekker SE, Rea D, Cayuela JM, Arnhardt I, Leonard J, Heuser M. Using Measurable Residual Disease to Optimize Management of AML,

ALL, and Chronic Myeloid Leukemia. Am Soc Clin Oncol Educ Book. 2023;43:e390010.
170. Tiong IS, Dillon R, Ivey A, Kuzich JA, Thiagarajah N, Sharplin KM, et al. Clinical impact of NPM1-mutant molecular persistence after chemotherapy for acute myeloid leukemia. Blood Adv. 2021;5(23):5107-11.
171. Puckrin R, Atenafu EG, Claudio JO, Chan S, Gupta V, Maze D, et al. Measurable residual disease monitoring provides insufficient lead-time to prevent morphologic relapse in the majority of patients with core-binding factor acute myeloid leukemia. Haematologica. 2021;106(1):56-63.
172. Boeckx N, De Roover J, van der Velden VH, Maertens J, Uyttebroeck A, Vandenberghe P, van Dongen JJ. Quantification of CBFB-MYH11 fusion gene levels in paired peripheral blood and bone marrow samples by real-time PCR. Leukemia. 2005;19(11):1988-90.
173. Mai H, Li Q, Wang G, Wang Y, Liu S, Tang X, et al. Clinical application of next-generation sequencing-based monitoring of minimal residual disease in childhood acute lymphoblastic leukemia. J Cancer Res Clin Oncol. 2023;149(7):3259-66.
174. Huang YJ, Chen SH, Liu HC, Jaing TH, Yeh TC, Kuo MC, et al. Evaluation of next-generation sequencing for measurable residual disease monitoring in three major fusion transcript subtypes of B-precursor acute lymphoblastic leukaemia. Pathology. 2024;56(5):681-7.
175. Duployez N, Nibourel O, Marceau-Renaut A, Willekens C, Helevaut N, Caillault A, et al. Minimal residual disease monitoring in t(8;21) acute myeloid leukemia based on RUNX1-RUNX1T1 fusion quantification on genomic DNA. Am J Hematol. 2014;89(6):610-5.
176. Vonk CM, Grob T, Rijken M, Kavelaars FG, Konijnenburg JML, Ossenkoppele GJ, et al. Advantages of a genomic DNA-based next-generation sequencing assay for detection of mutant NPM1 measurable residual disease in AML. Blood Adv. 2025;9(5):1069-77.
177. Delsing Malmberg E, Johansson Alm S, Nicklasson M, Lazarevic V, Ståhlman S, Samuelsson T, et al. Minimal residual disease assessed with deep sequencing of NPM1 mutations predicts relapse after allogeneic stem cell transplant in AML. Leuk Lymphoma. 2019;60(2):409-17.
178. Dillon LW, Gui G, Page KM, Ravindra N, Wong ZC, Andrew G, et al. DNA Sequencing to Detect Residual Disease in Adults With Acute Myeloid Leukemia Prior to Hematopoietic Cell Transplant. Jama. 2023;329(9):745-55.
179. Zhou Y, Othus M, Walter RB, Estey EH, Wu D, Wood BL. Deep NPM1 Sequencing Following Allogeneic Hematopoietic Cell Transplantation Improves Risk Assessment in Adults with NPM1-Mutated AML. Biol Blood Marrow Transplant. 2018;24(8):1615-20.
180. Juul-Dam KL, Ommen HB, Nyvold CG, Walter C, Vålerhaugen H, Kairisto V, et al. Measurable residual disease assessment by

qPCR in peripheral blood is an informative tool for disease surveillance in childhood acute myeloid leukaemia. Br J Haematol. 2020;190(2):198-208.
181. Orvain C, Bertoli S, Peterlin P, Desbrosses Y, Dumas PY, Iat A, et al. Molecular relapse after first-line intensive therapy in patients with CBF or NPM1-mutated acute myeloid leukemia - a FILO study. Leukemia. 2024;38(9):1949-57.
182. Platzbecker U, Middeke JM, Sockel K, Herbst R, Wolf D, Baldus CD, et al. Measurable residual disease-guided treatment with azacitidine to prevent haematological relapse in patients with myelodysplastic syndrome and acute myeloid leukaemia (RELAZA2): an open-label, multicentre, phase 2 trial. Lancet Oncol. 2018;19(12):1668-79.
183. Bataller A, Oñate G, Diaz-Beyá M, Guijarro F, Garrido A, Vives S, et al. Acute myeloid leukemia with NPM1 mutation and favorable European LeukemiaNet category: outcome after preemptive intervention based on measurable residual disease. Br J Haematol. 2020;191(1):52-61.
184. Tiong IS, Dillon R, Ivey A, Teh TC, Nguyen P, Cummings N, et al. Venetoclax induces rapid elimination of NPM1 mutant measurable residual disease in combination with low-intensity chemotherapy in acute myeloid leukaemia. Br J Haematol. 2021;192(6):1026-30.
185. Løvvik Juul-Dam K, Ifversen M, Guldborg Nyvold C, Hansen M, Hasle H. Treatment of Molecular Relapse by Cessation of Immunosuppression After Hematopoietic Stem Cell Transplantation in Pediatric FLT3-ITD AML Monitored by WT1 Expression in Peripheral Blood. J Pediatr Hematol Oncol. 2019;41(5):417-9.