From the DEPARTMENT OF MEDICINE
Karolinska Institutet, Stockholm, Sweden

CHARACTERIZATION OF SECONDARY AND THERAPY-RELATED ACUTE MYELOID LEUKEMIA

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Stockholm 2020

Published by Karolinska Institutet.

ISBN 978-91-7831-690-8
Printed by Eprint AB 2020

Characterization of secondary and therapy-related acute myeloid leukemia
THESIS FOR DOCTORAL DEGREE (Ph.D.)

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To Sonja and Felix

ABSTRACT

Secondary acute myeloid leukemia (s-AML) refers to patients with either therapy-related AML (t-AML), that is, AML after treatment with chemo- and/or radiation for a prior disease, or AML progressing from an antecedent hematologic disorder (AHD-AML), typically a myelodysplastic syndrome (MDS) or a myeloproliferative neoplasm. Patients with s-AML present with higher rates of adverse cytogenetic aberrations and higher frequencies of adverse mutations and, subsequently, respond worse to therapy, and have poorer outcome compared to *de novo* AML. To identify clinical and molecular factors that may improve prognostication is therefore of importance to guide clinical decision-making.

The general aim of this thesis was to broaden the real-world knowledge of s-AML, regarding disease properties and outcome, using population-based registries, and additionally to investigate the importance of mutations in the transformation from MDS to AML. The research papers presented herein cover mutational screening in MDS and s-AML (**study I**), general characteristics and outcome of s-AML (**study II**), the role of allogeneic hematopoietic cell transplantation (HCT) in patients with s-AML (**study III**), and the epidemiology and treatment outcome specifically for t-AML (**study IV**).

In **study I**, high-throughput methods were used to find mutations in 22 genes in 100 MDS and 92 AML patients. The AML cohort consisted of t-AML, AHD-AML and AML with MDS-like cytogenetics. In AML, mutations were most commonly seen in oncogenes and cell signaling genes, and in MDS in splicing factor genes and epigenetic regulators. A key finding was the overrepresentation of mutated *U2AF1* in cases with MDS progressing to AML. Furthermore, in addition to established risk scores, mutational status improved prognostication.

In **study II** we used the Swedish AML registry (SAMLR) to characterize s-AML in a population-based setting. Of the 3263 AML patients included, 19% were AHD-AML and 8% t-AML. Differences between the subtypes were found in age, gender distribution and cytogenetic risk. Compared to *de novo* AML, complete remission rates were lower in s-AML, but early death rates were similar. In multivariable analysis, both t-AML and AHD-AML emerged as independent prognostic factors, with a more pronounced negative impact in younger age groups.

HCT is a potentially curable consolidation treatment in eligible patients. In **study III**, data on 3337 intensively treated patients in SAMLR were combined with data from the Swedish Cancer Registry (SCR) and the Swedish transplantations centers to investigate the role of HCT in s-AML. HCT in first remission was superior to consolidation treatment with chemotherapy only. Long-term survivors with s-AML were rare without HCT.

In **study IV** we studied 686 patients with t-AML in detail using SAMLR, SCR and the Swedish Rheumatology Quality Register. We found an increasing incidence of t-AML over time, and an increasing proportion with t-AML of AML in total. Survival was overall dismal,

but comparable to *de novo* AML in patients with favorable cytogenetic risk and in patients with mutated *NPM1* in combination with absence of *FLT3*-ITD.

In conclusion, secondary AML is a highly heterogeneous disease with a particularly poor outcome. However, the clinical and genetic differences within the disease enable risk stratification of patients and may thus aid in treatment recommendations.

POPULÄRVETENSKAPLIG SAMMANFATTNING

Akut myeloisk leukemi, AML, är en cancersjukdom i de blodbildande cellerna i benmärgen. Sjukdomen beror på förvärvade molekylära avvikelser i blodets stamceller i form mutationer, kromosomrubbningar och epigenetiska förändringar. AML är den vanligaste akuta leukemin hos vuxna och drabbar framförallt äldre. Ca 350 individer insjuknar i AML årligen i Sverige. Prognosen är dyster med en 5-årsöverlevnad på endast 20%. Behandlingen består av intensiva cytostatikakurer och för långtidsöverlevnad och bot krävs oftast allogen blodstamcellstransplantation, vilket i regel är förbehållet yngre patienter utan större samsjuklighet.

Ungefär en fjärdedel av sjukdomsfallen är av typen sekundär-AML. Då har det antingen skett en övergång från en annan blodcancer, så kallad AHD-AML (*AML with an antecedent hematologic disorder*). Eller så har patienten tidigare behandlats med cytostatika eller strålning för en annan sjukdom, oftast cancer, och har då terapirelaterad AML (*therapy-related AML*). Båda typerna av sekundär-AML har ännu sämre prognos än *de novo-AML*, som nyinsjuknadende i AML kallas.

Den dystra prognosen belyser ett ouppfyllt behov i behandlingen av patienter med sekundär-AML. Målet med denna avhandling var att fördjupa karaktäriseringen av sekundär-AML, framförallt för att öka förståelsen av de faktorer som påverkar behandlingsutfallet. Syftet är att i förlängningen förbättra möjligheten att välja optimal behandling för enskilda individer. Avhandlingsarbetet har resulterat i fyra studier som sammanfattas nedan.

I **studie I** användes så kallad next-generation-sekvensering för att upptäcka mutationer i 22 gener i 92 patienter med sekundär-AML och 100 patienter med myelodysplastiskt syndrom (MDS, en besläktad blodcancer som kan övergå i AML). Till stor del var det olika gener som var muterade i AML och MDS, vilket återspeglar den underliggande biologiska funktionen generna har i respektive sjukdom. Ett huvudfynd var associationen mellan sjukdomsprogress från MDS till AML och samtidig förekomst av en muterad gen kallad *U2AF1*.

I **studie II** karaktäriserades sekundär-AML med hjälp av data från svenska AML-registret. Bland totalt 3263 patienter hade 8% terapirelaterad AML och 19% AHD-AML. Sekundär-AML skilde sig från *de novo* AML framförallt avseende genetisk riskprofil. De fick i mindre utsträckning intensiv cytostatikabehandling än *de novo*-patienter och de med sekundär-AML svarade sämre på behandling. Att ha sekundär-AML visade sig ha negativ påverkan på överlevnad oberoende av andra etablerade riskfaktorer. Den prognostiska betydelsen av sekundär-AML var tydligare hos yngre än hos äldre patienter.

Även i **studie III** användes svenska AML-registret, där tillsammans med data från Socialstyrelsens cancerregister och de svenska transplantationscentren. Huvudsyftet var att undersöka hur allogen blodstamscellstransplantation används och påverkar utfallet vid sekundär-AML. Det visade sig att ytterst få patienter med sekundär-AML blev

långtidsöverlevare utan transplantation. Att genomgå transplantation i s.k. första remission var överlägset behandling med enbart cytostatika.

Studie IV fokuserade på patienter med terapirelaterad AML. Data från svenska AML-registret på 686 patienter kombinerades med data från Socialstyrelsens cancerregister och Svensk Reumatologis Kvalitetsregister. Huvudsakliga fynd var att incidensen av t-AML tydligt ökar över tid och att andelen terapirelaterad AML av AML-fallen totalt blev allt större. Överlevnaden vid terapirelaterad AML var generellt mycket dyster, men i vissa genetiska undergrupper var den bättre och jämförbar med patienter med motsvarande genetisk riskprofil som nyinsjuknat i AML.

Sammanfattningsvis är sekundär-AML en ovanlig sjukdom som är mycket svårbehandlad. Nya läkemedel kommer att krävas för att förbättra överlevnaden, men förhoppningsvis kommer sådana att utvecklas hand i hand med den ökade förståelsen av sjukdomsbiologin. Behandlingen av AML har i stort sett varit oförändrad i årtionden, men nyligen har läkemedel som bl.a. utnyttjar specifika genetiska avvikelser introducerats. Inom andra cancerformer har immun- och cellterapi fått stort genomslag och dessa är potentiellt användbara även mot AML. För gruppen med sekundär-AML är det därför av stor betydelse att sjukdomshistorik, hög ålder och samsjuklighet inte utgör hinder för deltagande i kommande läkemedelsstudier.

LIST OF SCIENTIFIC PAPERS

I. High-throughput mutational screening adds clinically important information in myelodysplastic syndromes and secondary or therapy-related acute myeloid leukemia.
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Manuscript

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I. Incidence and prognostic significance of karyotypic subgroups in older patients with acute myeloid leukemia: the Swedish population-based experience.
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J Natl Cancer Inst. 2018 Oct 1;110(10):1094-1101.

CONTENTS

LIST OF ABBREVIATIONS

AA	Aplastic anemia
AHD-AML	AML transformed from an antecedent hematologic disorder
AML-MRC	AML with myelodysplasia-related changes
AML	Acute myeloid leukemia
APL	Acute promyelocytic leukemia
BM	Bone marrow
CBF	Core binding factor
CHIP	Clonal hematopoiesis of indeterminate potential
CMML	Chronic myelomonocytic leukemia
CPRT	Conventional post remission therapy
CR	Complete remission
CR1	First complete remission
DA	Daunorubicin and Cytarabine (Ara-C)
EBMT	European Society for Blood and Marrow Transplantation
ELN	European LeukemiaNet
FISH	Fluorescence in situ hybridization
GvHD	Graft-versus-host disease
HCT	Allogeneic hematopoietic cell transplantation
HMA	Hypomethylating agent
HR	Hazard ratio
ICD	International classification of diseases
IPSS-R	Revised international prognostic scoring system (for MDS)
MAC	Myeloablative conditioning
MDS	Myelodysplastic syndrome
MPN	Myeloproliferative neoplasm
MRD	Measurable residual disease
NGS	Next generation sequencing
OS	Overall survival
PCR	Polymerase chain reaction
RAEB	Refractory anaemia with excess blasts

RARS	Refractory anemia with ring sideroblasts
RARS-T	RARS with thrombocytosis
RCMD	Refractory cytopenia with multilineage dysplasia
RIC	Reduced intensity conditioning
RS	Ring sideroblasts
RT-PCR	Reverse transcriptase PCR
RT-qPCR	Real-time quantitative PCR
SAMLR	The Swedish AML Registry
SCR	The Swedish Cancer Register
SNP	Single nucleotide polymorphism
SRQ	The Swedish Rheumatology Quality Register
s-AML	Secondary AML
t-AML	Therapy-related AML
t-MN	Therapy-related myeloid neoplasm
TRM	Transplant-related mortality
WBC	White blood cell (count)
WHO	World Health Organization

1 INTRODUCTION

Acute myeloid leukemia is the most common type of acute leukemia in adults with more than 300 new cases every year in Sweden [1]. The disease is characterized by high mortality and a low cure rate with a median survival of less than 7 months [1]. The molecular basis of AML is highly heterogeneous, with multiple recurrent genetic alterations such as specific chromosomal aberrations, gene mutations and DNA methylation patterns [2, 3]. Accumulations of these alterations lead to block in the differentiation of myeloid stem and progenitor cells, and to clonal proliferation of leukemic cells in the bone marrow. Despite recent advances in the knowledge about the molecular pathogenesis in AML, the leukemogenic process remains largely unclear.

Until recently, the treatment of AML had basically not changed for almost 50 years [4], with a remaining poor prognosis . However, during the last few years, concurrent to the growing understanding of the genetic basis of AML, a number of new drugs complementing the standard therapy have shown promising results in clinical trials. This is a hopeful advancement, but it remains to be seen how big the improvement will be in clinical reality.

The majority of patients present with *de novo* AML, without any preceding hematologic disease or other cancer. However, 20% have an antecedent hematologic disorder (AHD-AML), typically a disease progression from myelodysplastic syndromes (MDS), myeloproliferative neoplasms (MPN) or chronic myelomonocytic leukemia (CMML) [5]. In yet another 5-10%, the patients have therapy-related AML (t-AML) [5-7], with a history of cytotoxic therapy or radiation therapy for a prior malignant or non-malignant disease. Together these two entities, AHD-AML and t-AML, make up a loosely defined group called secondary AML (s-AML).

These definitions are not part of the World Health Organization (WHO) classification system, and the usage varies in the literature. Some authors use s-AML to describe both t-AML and AHD-AML, while others use it only referring to AML with a preceding hematological disease. The WHO defines the disease entity therapy-related myeloid neoplasms (t-MNs) as myeloid neoplasms secondary to cytotoxic treatment, in which t-AML is categorized along with therapy-related MDS and therapy-related MDS/MPN [8].

Secondary AML frequently harbors unfavorable genetic aberrations, and the outcome is poorer than in *de novo* AML, both in response to chemotherapy and survival [5, 9]. Historically, these patients often escape inclusion in clinical trials [10], and are thus less thoroughly studied than those with *de novo* AML.

1.1 EPIDEMIOLOGY

The incidence rate of AML in Europe has been reported to 3.6 cases per 100,000 inhabitants per year during 2000-2002 [11]. Recent U.S. data from 2012-2016 shows a rate of 4.3 per 100,000 [12]. Worldwide, the incidence is elevated in highly developed areas, with the highest rates in Australia, USA and parts of Europe [13]. Prevalence is less known, however

in Sweden, during a snapshot in 2014, the overall prevalence of AML was 13.7 per 100,000, while the prevalence of patients surviving three or more years was 9.0 per 100,000 [14]. Although AML occurs in all ages it is considerably more common in the elderly, with a median age at diagnosis of 71 years [1]. Correspondingly, the incidence rate steadily increases by age to a peak at 80-85 years, where the incidence rate is >15 cases per 100.000 compared to <5 in the age <60. There is no gender difference in the total number diagnosed each year, but as a consequence of the age structure in western countries, AML is more common in males than females above the age of 70 [1, 11].

S-AML accounts for 20-25% of all AML cases, whereof AHD-AML constitutes about two thirds and t-AML one third of s-AML in total [5-7]. The median age at diagnosis is higher in AHD-AML than in *de novo* and t-AML [5]. Females are predominant in t-AML, since breast cancer and gynecological cancers are more common as primary malignancies than male cancers [6, 15], and males are more common in AHD-AML, reflecting the male predominance in MDS [16].

1.2 CLINICAL PRESENTATION

Different manifestations of bone marrow failure related to blast infiltration lead to the initial symptoms in AML. These are fatigue caused by anemia, bleeding due to thrombocytopenia, and infections explained by neutropenia. Patients may present debilitated with severe infections or bleeding, but more commonly with mild symptoms, and cases are sometimes discovered unexpectedly through routine blood tests. In patients with MDS or MPN, a gradual developing anemia or other alterations in the peripheral blood counts could be signs of progress to s-AML.

1.3 DIAGNOSIS

The basis of diagnosis is a bone marrow aspirate with > 20% myeloid blasts (myeloblasts, monoblasts or megakaryoblasts), with the exception of the cytogenetic abnormalities t(8;21), inv(16) and t(15:17), which all qualify as AML independent of blast count [3]. Consequently, cytogenetic analysis is performed to aid classification and prognostication. Flow cytometry is run to ensure correct diagnosis, and to find an immunophenotypic profile that can be used during treatment to measure residual disease [17]. Fluorescence in situ hybridization (FISH) and reverse transcriptase polymerase chain reaction (RT-PCR) are rapid molecular methods commonly used to find specific chromosomal rearrangements of importance in certain clinical situations [3]. In addition, screening for mutations using NGS-based gene panels adds prognostic information [18].

1.4 CLASSIFICATION

The current WHO-classification (2016 revision) divides AML into subgroups based on recurrent cytogenetic and mutational aberrations, presence of morphologic dysplasia, history of prior MDS, or history of prior cytotoxic therapy [8, 19]:

- *AML with recurrent genetic abnormalities* includes a number of cytogenetic and mutational aberrations that have proven to be of clinical relevance. For instance, the favorable risk core binding factor (CBF) leukemias with t(8;21), inv(16) and t(16;16) along with the PML-RARA fusion defining favorable risk acute promyelocytic leukemia (APL) belong in this group, as well as adverse risk aberrations such as t(9;11) and inv(3). Mutations in *NPM1*, *RUNX1* (provisionally) and biallelic mutations in *CEBPA* also define disease entities in the group with recurrent aberrations.

- *AML with myelodysplasia-related changes* (AML-MRC) encompasses AML either progressed from MDS or MDS/MPN, or cases with morhological dysplasia in >50% of cells in at least 2 cell lines, along with cases with certain MDS-related cytogenetic abnormalities (Table I).

- *Therapy-related myeloid neoplasms*, as already described, include t-AML, t-MDS and t-MDS/MPN following cytotoxic therapy, and should be seen as a clinical syndrome defined by iatrogenic mutagenic exposure.

- *AML, not otherwise specified*, is used when the other classification criteria are not met.

- The WHO-classification also includes the two AML manifestations myeloid sarcoma and myeloid proliferations of Downs syndrome as distinct subgroups

Thus, proper classification requires cooperation between hematopathologists, geneticists and clinicians. Accordingly, while there is no specific WHO category for s-AML, patients with s-AML fall into any of the three WHO subcategories t-MN, AML-MRC or AML with recurrent genetic abnormalities.

Related to the classification of AML, there is a special section on myeloid neoplasms, including MDS and AML, with germ line predisposition in the 2016 WHO revision, due to the raising awareness that a subset of cases are not sporadic but inherited, which is highly important for patient management and for informing the affected families [19, 20].

Table I. List of the MDS-related cytogenetic abnormalities.

Complex karyotype (>= 3 abnormalities)
Unbalanced abnormalities
-7/del(7q)
del(5q)/t(5q)
i(17q)/t(17p)
-13/del(13q)
del(11q)
del(12p)/t(12p)
idic(X)(q13)
Balanced abnormalities
t(11;16)(q23.3;p13.3)
t(3;21)(q26.2;q22.1)
t(1;3)(p36.3;q21.2)
t(2;11)(p21;q23.3)
t(5;12)(q32;p13.2)
t(5;7)(q32;q11.2)
t(5;17)(q32;p13.2)
t(5;10)(q32;q21.2)
t(3;5)(q25.3;q35.1)

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1.5 PROGNOSIS

Multiple factors contribute to the outcome of AML and the genetic properties inherent in the disease have the most impact. However, prognosis also depends on patient-related factors such as age, fitness and comorbidities. Additional post-treatment prognostication is enabled by assessing treatment response and measurable residual disease.

1.5.1 Genetic risk classification

Cytogenetic evaluation has traditionally been, and still is, the basis of prognosis and treatment decisions in AML [3]. Large studies have shown that cytogenetic aberrations stratify AML by overall survival, and three distinct prognostic groups are used clinically: favorable, intermediate and adverse cytogenetic risk [21]. The value of gene mutations to further refine the classification has become evident, and current 2017 European LeukemiaNet (ELN) classification requires the mutational status of *NPM1*, *FLT3*-ITD, *CEBPA*, *RUNX1*, *ASXL1* and *TP53* [3].

Table II shows a simplified summarization of the genetic risk stratification according to ELN. Risk classification is mainly used as a tool to identify individuals with a low risk of long-term

survival after standard treatment, who would benefit from allogeneic stem cell transplantation. Patients in the intermediate and adverse risk groups have high and very high risk of relapse, respectively, and should be considered for HCT if otherwise eligible [22, 23].

Table II. Genetic risk stratification according to ELN 2017 (footnotes omitted).

Risk	Genetic abnormality
Favorable	t(8;21)(q22;q22.1); RUNX1-RUNX1T1
	inv(16)(p13.1q22) or t(16;16)(p13.1;q22); CBFB-MYH1
	Mutated *NPM1* without *FLT3*-ITD or with *FLT3*-ITD^{low}
	Biallelic mutated *CEBPA*
Intermediate	Mutated *NPM1* and *FLT3*-ITD^{high}
	Wild-type NPM1 without *FLT3*-ITD or with *FLT3*-ITD^{low} (without adverse-risk genetic lesions)
	t(9;11)(p21.3;q23.3); MLLT3-KMT2A
	Cytogenetic abnormalities not classified as favorable or adverse
Adverse	t(6;9)(p23;q34.1); DEK-NUP214
	t(v;11q23.3); KMT2A rearranged
	t(9;22)(q34.1;q11.2); BCR-ABL1
	inv(3)(q21.3q26.2) or t(3;3)(q21.3;q26.2); GATA2,MECOM(EVI1)
	-5 or del(5q); 27; 217/abn(17p)
	Complex karyotype, monosomal karyotype
	Wild-type *NPM1* and *FLT3*-ITD^{high}
	Mutated *RUNX1*
	Mutated *ASXL1*
	Mutated *TP53*

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1.5.2 Secondary AML as a prognostic factor

Secondary AML is a well-known adverse prognostic factor [6, 7, 9]. Patients with t-AML and AHD-AML are less responsive to intensive induction treatment, reflected in lower complete remission (CR) rates than in *de novo* AML, and they have shorter overall survival compared to novo AML [5, 21, 24]. Whether secondary AML confers a negative impact on outcome independently from the karyotype and age has been debated, but at least for cytogenetics several studies point in that direction [9, 25, 26]. However, the adverse prognostic impact of both t-AML and AHD-AML seems stronger in younger than in elderly patients [5, 6, 27]. In t-AML patients, this might be explained by increased mortality in younger due to the cumulative toxicity of treatment [6].

It is possible to identify subsets within s-AML with particularly dismal outcomes. Among patients with AHD-AML, a prior MPN or CMML confers worse outcome than a diagnosis of

MDS, regardless of age or cytogenetics [5]. Furthermore, patients with AHD-AML that have been treated for their prior MDS, MPN or aplastic anemia (AA) do worse than untreated patients, and define a distinct high-risk subgroup of AHD-AML [28]. In t-AML, scoring models to enhance risk stratification by integrating factors related to the treatment for the primary disease (e.g. type of chemotherapy) have been developed [29].

1.5.3 Additional prognostic factors

Other prognostic parameters are age, performance status, comorbidity, hyperleukocytosis, extramedullar manifestations and multilineage dysplasia.

- Treatment results of older patients are considerable worse than younger. Partly explained by a larger proportion of high-risk cytogenetics in the elderly and that a preceding diagnosis or phase of MDS is more common at higher age [30, 31].
- Poor performance status at the time of diagnosis is strongly associated with poor short- and long-term survival [32].
- Systematic comorbidity assessment can predict early death and survival after induction therapy [33].
- Elevated white blood cell counts (WBC), hyperleukocytosis, is seen in 5-10% of newly diagnosed patients and is a risk factor for early death by bleeding, thromboembolic events and pulmonary or CNS complications [34].
- Extramedullary disease is present in more than 20% of patients and is associated with shorter OS but lacks independent prognostic value [35].
- Multilineage dysplasia confers poor survival but is highly correlated to adverse MDS-related cytogenetics, and thus has no independent prognostic value [36].

1.5.4 Novel prognostic methods

In recent years the knowledge about the molecular basis of AML has been vastly increased, mainly due to the rapid development of sequencing techniques. The findings in disease mechanism go hand in hand with new predictive markers. In addition to mutations, profiles of gene expression [37, 38], signatures of non-coding RNAs [39, 40] and DNA methylation patterns [41, 42] all have proved to add prognostic information. Even though the molecular-based risk stratification models (for instance combining gene expression profiling with either or both cytogenetic and mutational data) may improve established prognostic models, most of them are not validated or replicated in larger cohorts [43]. Furthermore, the molecular methods required may be expensive or technically challenging. Thus, so far, apart from cytogenetics, only mutational profiling has been incorporated in guidelines and clinical routine.

1.6 ETIOLOGY

The reason why AML occurs *de novo* is not completely known. The only known lifestyle risk factor is smoking. In meta-analysis it has been estimated that smokers have a 40% higher risk of AML than non-smokers [44]. Recently it has also been suggested that smoking is

associated with genetically high-risk AML and poorer outcome [45]. Prolonged exposure to high levels of benzene is also a known causative factor, however the statistical evidence for an association between work-related exposure and increased risk is weak, and it is not considered a concern in developed countries [46, 47].

Among children and young adults there is a considerably elevated risk of AML in a number of, mostly very rare, inherited syndromes, e.g. Down's syndrome, Shwachman–Diamond syndrome, Fanconi anemia, dyskeratosis congenita and severe congenital neutropenia [48]. However due to increased use of diagnostic genome sequencing, genetic predisposition to AML and MDS is found in adults well, with our without other clinical signs [49]. Germ line predisposition, with mutations in *RUNX1*, *GATA2* and *CEBPA* among others, is now well recognized and incorporated into the WHO classification [50]. In addition, through genome wide association studies, there is some evidence suggesting existence of low-penetrance risk alleles for AML [51].

1.6.1 AML secondary to other myeloid neoplasms

AHD-AML accounts for the majority patients with s-AML, and the myeloid neoplasms preceding AML are MDS (~60%), MPN (~30%) and CMML (~10%) [5]. The progression to AML is explained by a gradual clonal evolution and one can argue that the term secondary is somewhat misleading since the disease evolvement genetically covers a continuous spectrum of conditions [52].

1.6.1.1 AML progressing from MDS

The myelodysplastic syndromes are clonal myeloid malignancies characterized by morphologic dysplasia in the hematopoiesis and bone marrow failure, resulting in peripheral blood cytopenias and risk of AML progression. The incidence is 5 cases per 100,000 per year and it is considerably more common in the elderly [53]. MDS is classified into subgroups based on degree of dysplasia and blast counts on morphology, ring sideroblast percentages, and presence of specific cytogenetic aberrations (e.g. del(5q)). Cytogenetics and clinical parameters divide patients into prognostic risk groups using the revised international prognostic scoring system (IPSS-R), with a variable risk of disease progression and death [54]. The median time to AML transformation varies from only a couple of months to ~10 years in high and low risk disease respectively. There is a considerable overlap between MDS and AML in cytogenetic abnormalities and mutations, particularly in the higher risk groups, and incorporation of mutational status enhances the prognostic scoring systems [55-57]. The most commonly mutated genes in MDS are *TET2*, *SF3B1*, *ASXL1*, *SRSF2*, *DNMT3A*, and *RUNX1* [55]. During the progression from MDS to AML, either mutations (e.g. in *FLT3, NRAS, IDH2*) or additional chromosome abnormalities, or both, are gained [58, 59]. This process can be described as a genetic evolution, where multiple mutational clones evolve over time, forming subclones, of which some are selected and persist over time, until progression to AML, where an increased number of mutations and enlarged clone sizes are seen [59, 60].

1.6.1.2 AML progressing from myeloproliferative neoplasms

The myeloproliferative neoplasms are a group of clonal myeloid diseases characterized by proliferation of mature blood cells. In polycythemia vera (PV), there is an excess of red blood cells, in essential thrombocytosis (ET) platelets, and in primary myelofibrosis (PMF) bone marrow fibrosis [61]. The risk of AML/MDS transformation over a 10-year period is 2-5%, 5-10% and 8-20% in ET, PV and PMF, respectively [62]. A mutation in *JAK2* is seen in the majority of the MPN patients, and drives the disease by activating the *JAK2*-*STAT* signaling pathway. Mutated *JAK2* defines PV, whereas mutated *CALR* or *MPL* are found in *JAK2* negative ET and PMF. Post MPN-AML is associated with a very poor prognosis with a median OS of less than 6 months [63] where mutations in *TP53*, *SRSF2* and *TET2* have been shown to have an adverse impact on survival [64].

1.6.1.3 AML progressing from CMML

CMML belongs in the WHO category myelodysplastic/myeloproliferative neoplasms (MDS/MPN) and shares clinical and genetic features with MDS but displays a myeloproliferative phenotype [19]. CMML patients have monocytosis in the peripheral blood and morphologic dysplasia in the BM. The most commonly mutated genes are *TET2*, *SRSF2* and *ASXL1* [19, 65]. CMML is a rare disease with an incidence of 1 in 100.000 per year, and the risk of progression to AML varies from 0% to 50% depending on risk group, and is associated with the presence of high-risk cytogenetics and high-risk mutations, e.g. *ASXL1*, *RUNX1*, *SETBP1* and *NRAS* [65, 66].

1.6.2 Therapy-related AML

The most common primary cancers prior to t-AML are lymphoma, breast cancer, multiple myeloma, ovarian cancer and testicular cancer [5, 7, 15]. It may also arise after autoimmune diseases, such as rheumatoid arthritis, treated with cytotoxic agents with or without immunosuppression [67]. Furthermore, there is an increased risk of AML in patients treated with azathioprine after solid organ transplantations, suggesting that apart from directly cytotoxic effects, immunomodulatory mechanisms might also contribute to t-AML [68, 69]. The risk of AML in patients treated with chemotherapy is 1.5 to more than 10 times higher compared to the population in general [70, 71], and varies according to type of primary cancer, chemotherapy regimens and accumulated doses of chemotherapy.

1.6.2.1 t-AML following chemotherapy and radiation

Traditionally, t-AML patients have been divided into two subgroups depending on the causative agent. The first well-characterized group of t-AML followed treatment with alkylating agents (e.g. cyclophosphamide, melphalan etc.) or radiation therapy. Alkylating agents cause cell death by crosslinking DNA, causing DNA double strand breaks followed by mutations and chromosomal rearrangements [72], which are thought to be leukemogenic, in particular when DNA repair systems are dysregulated [73]. Typically, deletions of the whole or parts of chromosomes 5 or 7, together with other MDS-like properties such as multilinear

dysplasia, can be seen after a latency period of 5-7 years [74, 75]. The other classic group of t-AML arises secondary to treatment with topoisomerase II inhibitors (e.g. doxorubicin, etoposide etc.). They block the DNA resealing step in the process of unwinding DNA during replication, causing double strand breaks [76]. Chromosomal aberrations typically associated with topoisomerase II inhibitors are balanced translocations, most commonly involving MLL (e.g. KMT2A) at 11q23 and RUNX1/AML1 at 21q22, but also to a lesser extent CBFB at 16q22, as well as NUP98 at 11p15.5 [77, 78]. These patients have a shorter latency period of 2-3 years and do in general not show a preceding myelodysplastic phase, but rather an abrupt onset of overt disease. The WHO previously subclassified t-AML based on the type of treatment mentioned above. However, since most cancer patients receive combination therapy this definition has been revised. Furthermore, although uncommon, t-AML may also occur after treatment with radioiodine for malignant and non-malignant thyroid diseases [79].

Apart from the aberrations described above, other well-known molecular features of t-AML that differs from *de novo* AML are the substantially higher frequencies of *TP53* mutations [80, 81] and complex cytogenetics [6].

1.6.2.2 t-AML following autoimmune conditions

Evidence from large epidemiological studies show a clear elevated risk of AML in patients with autoimmune conditions compared to the population in general, with odds ratios between 1.3 - 1.7 [82-84]. Whether the reason is a shared genetic predisposition, immunological/inflammatory driven mechanisms or secondary to treatment is a complex issue. However, the over-risk has been shown to be confined to patients treated with cytotoxic therapy, indicating that immune mediated mechanisms have a minor role [84]. Treatment with azathioprine [85] and cyclophosphamide are associated with myeloid neoplasms while the role of methotrexate is uncertain [67]. On a related note, immunosuppression with azathioprine in the setting of organ transplantation shows a strong correlation with development of AML [69].

1.7 GENETICS

1.7.1 Chromosomal aberrations

Chromosomal translocations that give rise to fusion genes have long been known to mediate acute myeloid leukemogenesis [86]. Typically, the resulting hybrid proteins dysregulate transcription factors involved in myeloid differentiation or involved in cell survival and apoptosis. In Swedish population-based data 43% of the patients had normal karyotype, 24% complex (at least 3 abnormalities) and (18%) monosomal karyotype [30]. The most common recurrent abnormalities were -5/del(5q) (13%), -7/del(7q) (13%), -17/del(17p) (8,8%), inv(16)(p13q22)/t(16;16)(p13;q22) (2,2%) and t(8;21)(q22;q22) (1,9%). These figures do not include APL, which constitute about 3,5% of all AML in Sweden.

1.7.2 Somatic mutations

In 2008, AML was the first disease in which the whole cancer genome was sequenced [87]. The genetic diversity in AML is large, both regarding structural chromosomal variations and point mutations [2]; however, the median frequency of mutations is lower in AML than in most other cancers, but with large variations between individual patients [88]. AML is part of the Cancer Genome Atlas Program (TCGA), and in 2013 they published highly cited DNA and RNA sequencing data as well as DNA methylation results on 200 AML patients, although on *de novo* patients only [2]. Many comprehensive sequencing studies have been performed since then, including large numbers of patients with t-AML and AHD-AML, and it is reasonable to say that by now the mutational spectrum in the coding parts of the genome in AML is well characterized [89-91].

Since nearly half of the AML cases have normal cytogenetics, somatic point mutations and smaller copy number variations must play an important role in the disease pathogenesis. In *de novo* AML there is an average of 13 somatic mutations in the leukemic cells of each patient [2]. However, only 4-5 of these mutations are found in recurrently mutated genes [2, 89]. Virtually all cases have at least one driver mutation, and >85% at least two [2, 89]. However, the large published AML sequencing studies are not population-based, and age groups and subtypes are skewed. Furthermore, they use technologies with differences in gene coverage and sequencing depth. As a consequence, mutation patterns and frequencies are not always straightforward to compare between studies. Nevertheless, the most commonly mutated genes in AML overall are *FLT3 (30-35%)*, *NPM1* (~30%), *DNMT3A* (20-30%), *IDH1* or *IDH2* (~20%), *NRAS* or *KRAS* (~15-25%) and *TET2* (~10-15%), followed by a several genes with a frequency around 10% (*RUNX1, CEBPA, TP53, WT1, ASXL1, PTPN11, SRSF2* etc.) [2, 89, 90].

The number of driver mutations per individual is highest in AHD-AML and lowest in t-AML, with *de novo* AML in-between [89]. A reason for the subtype differences could be that in t-AML, structural chromosomal aberrations (rather than point mutations) are usually additional drivers, and in AHD-AML, with its older population, the higher frequency of mutations could be age acquired.

Mutations in AML can be grouped according to functional categories (such as DNA-methylation-related genes, tumor-suppressor genes and splice-factor genes) or grouped by patterns of cooperation and mutual exclusivity between genes. Either way, the groups correlate to clinical outcome and suggest specific patterns and underlying biology in disease development [2, 89, 90].

There has been an increasing interest in the order of genetic events leading to AML and to disease relapse. Initiating genetic lesions, division of driver mutations versus passenger mutations and mechanisms of clonal evolution have been outlined [60, 92, 93]. For instance, mutations that have shown to be early and present in preleukemic cells have been found in *DNMT3A*, *TET2* and *ASXL*1, genes that all are involved in epigenetic regulation [94-96].

1.7.3 Genetic properties of secondary AML

The division of AML into the three categories *de novo*, AHD- and t-AML is purely based on clinical information, that is, a history of either a antecedent hematological disorder or previous exposure to cytotoxic agents. While these subgroups have distinct differences in clinical characteristics and outcome at large, there is reason to believe that the underlying genetics explain the clinical phenotype even better than clinical history alone. By sequencing a large cohort of patients with *de novo*, AHD- and t-AML Lindsley and colleagues identified subtypes based on mutational signatures and clinical outcome: secondary-type (that is, secondary to MDS or CMML), *TP53*-mutated, *de novo*-type and PAN-AML-type [31].

The secondary-type mutations were in the genes *SRSF2*, *SF3B1*, *U2AF1*, *ZRSR2*, *ASXL1*, *EZH2*, *BCOR* and *STAG2* (Figure 1), and they were highly specific to AHD-AML, but also found in the elderly *de novo* patients, while infrequently in younger patients. Cases with t-AML were evenly distributed among the subtypes and were clinically more similar to non-t-AML cases within the same mutational subtype than to t-AML at large (Figure 2).

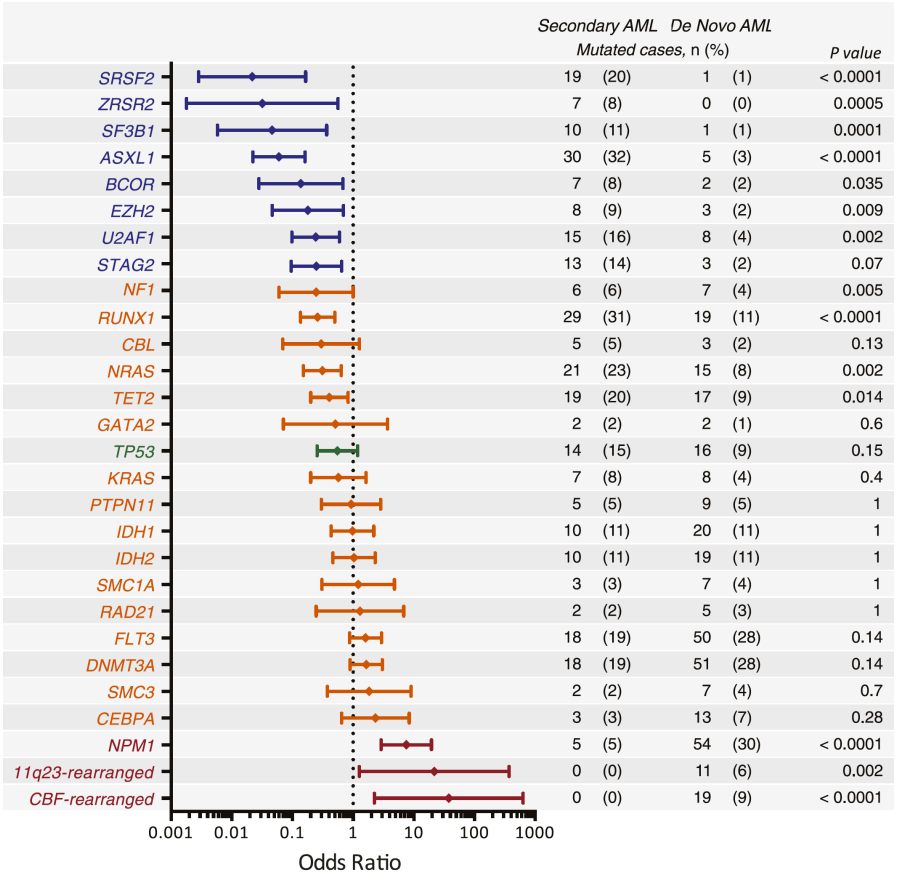

Figure 1. Specificity of myeloid driver mutations to secondary AML (AHD-AML). *Reprinted with permission from American Society of Hematology (ASH): Blood, Acute myeloid leukemia ontogeny is defined by distinct somatic mutations, R. Lindsley et al, copyright 2015.*

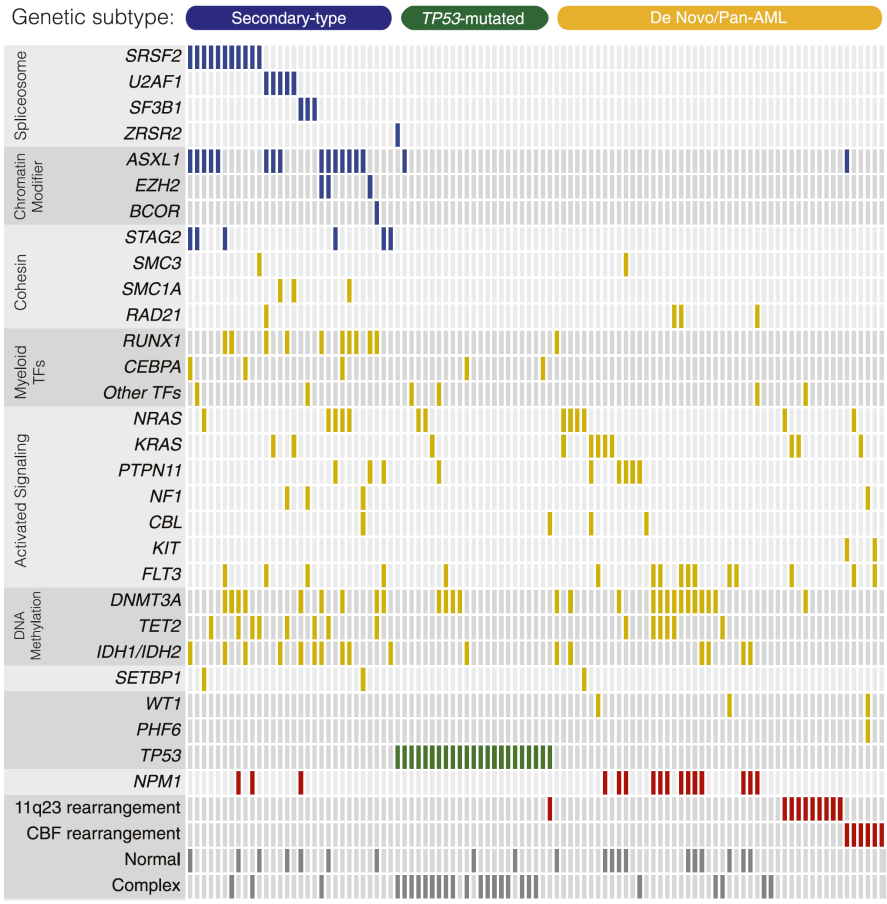

Figure 2. Mutations in therapy-related AML by mutational subtypes. *Reprinted with permission from American Society of Hematology (ASH): Blood, Acute myeloid leukemia ontogeny is defined by distinct somatic mutations, R. Lindsley et al, copyright 2015.*

Furthermore, compared to *de novo* AML, t-AML has a markedly higher rate of high-risk aberrations, e.g. del(5q), -7/del(7q), del(17p)/t(17p)/-17 and *TP53* mutations, while normal karyotype and favorable-risk abnormalities such as t(8;21) and mutations in *NPM1* and *DNMT3A* are significantly underrepresented [2, 6, 21, 31, 89, 97]. Only few large sequencing studies have analyzed t-AML separately, nevertheless, in addition to *TP53,* the most commonly mutated genes are *FLT3*, the family of *ABC* transporter genes, *PTPN11*, *IDH2* and *NRAS [97, 98]*. While there is a strong overrepresentation of high-risk cytogenetics in t-AML, there is a large overlap between *de novo* and t-AML on a mutational level, and the study by Lindsley et al suggests that when adjusting for karyotype, *de novo* and t-AML have quite similar clinical and mutational profiles [31].

1.7.4 Epigenetics

Apart from mutations and chromosomal aberrations, epigenetic changes, i.e. DNA methylation by covalent modification of cytosine residues and various histone modifications, contribute to the disease development in AML [99]. In cancer in general, there is a global DNA hypomethylation and a hypermethylation of CpG islands of certain promoters. DNA

hypomethylation potentially causes chromosomal instability whereas promoter hypermethylation has been linked to silencing of tumor suppressor genes [100]. Genome wide DNA methylation profiling in AML has shown distinct patterns in different genetic subgroups and also that methylation pattern subtypes can classify AML into prognostic groups [41, 42, 101]. Epigenetics, chromosomal aberrations and somatic mutations are closely related. One example is recurrent chromosomal translocations leading to fusion proteins that alter the epigenome (e.g. *MLL*, a histone methyltranferase). Another example is that more than 70% of the AML cases have mutations in genes related to DNA methylation or histone modification [2].

1.7.4.1 Epigenetics in t-AML

Only few studies have specifically described epigenetic changes in therapy-related AML. Radiation causing DNA strand breaks has been shown to induce DNA hypomethylation that is stable even after the DNA damage has been repaired [102]. Benzene, even though not used as a chemotherapeutic agent, is an alkylating agent, which causes both hypo- and hypermethylation [103]. Several studies have found aberrant methylation status in specific genes in t-AML. For example, the p15 promoter is commonly hypermethylated, both in diagnostic and preleukemic t-AML samples, which is also correlated to -7/del(7q) [104, 105]. Another example is *DAPK1*, a known cancer pathway gene, which is more commonly methylated in t-AML than *de novo* AML [106]. The methylation status of certain genes has also been correlated to the latency between the primary treatment and t-AML [107].

1.8 RECENT ADVANCES IN THE UNDERSTANDING OF THE BIOLOGY OF T-AML

Evidence are accumulating that the pathogenesis of t-AML is not fully explained by the direct mutagenic effects of cytotoxic therapy, but rather by several coexisting mechanisms such as the clonal selection of somatic driver mutations existing already before the treatment of the primary disease, abnormal microenvironment induced by chemo/radiotherapy, and in some cases inherited mutations associated with susceptibility for cancer [108].

By using whole-genome-sequencing, Wong and colleagues [97] found the mutational burden of *TP53* to be similar in *de novo* and t-AML, and even though *TP53* mutations were significantly overrepresented in t-AML compared to *de novo* AML, the number of somatic mutations within the *TP53* gene was similar, implying that chemotherapy does not induce genome-wide DNA damage and does not cause the *TP53* mutations. Instead the authors found *TP53* mutations in preleukemic t-AML samples, even before start of treatment for the primary diagnosis, supporting the hypothesis that *TP53* mutations confer a competitive advantage during the pressure of chemotherapy.

Not only *TP53*, but several other genes usually associated with myeloid malignancies, most frequently *DNMT3A*, *TET2* and *ASXL1*, are commonly mutated in the blood of healthy individuals, a phenomenon with a strong correlation to increased age and increased risk of hematologic cancer [94, 95]. This acquirement of somatic mutations in aged healthy

individuals is known as clonal hematopoiesis of indeterminate potential (CHIP) and is defined as the presence of mutated clones (>2% variant allele frequency) in the hematopoietic cells without cytopenias or dysplasias or other signs of hematological disease [109].

Already in the 1990s it was known that patients treated for cancers had a higher frequency of clonality in the blood of patients that had received chemotherapy, although that early only with evidence on chromosomal level [110]. We now know that preleukemic clonal hematopoiesis is found at a high frequency at the time of diagnosis of the primary cancer, even before start of therapy, in patients who later develop t-MN. We also know that patients with clonal hematopoiesis are at higher risk of developing t-MN than those without. In a case-control study, Takashi and colleagues detected clonal hematopoiesis in the peripheral blood in more than 70% of the patients who later developed t-MN but only in about ~30% of the controls that did not [111]. Healthy individuals with clonal hematopoiesis at risk of AML development can be identified and distinguished from other individuals with benign clonal hematopoiesis years prior to the onset of AML based on point mutations, where the preleukemic cases have more mutations, mutations enriched in specific genes (e.g. *U2AF1*, *TP53* etc.) and higher allele frequencies than controls [112].

Thus, the pathogenesis of t-AML is most likely multifactorial. McNerney and colleagues propose an updated model of the development of t-AML (and t-MDS as well) that combines four different mechanisms (Figure 3) [108].

1. The direct cytotoxic effects of chemotherapy and radiation on the DNA do induce genomic instability, chromosomal aberrations and likely mutations.

2. As described above, pre-existing somatic mutations acquired before treatment, as part of clonal hematopoiesis, have a competitive advantage and are selected for by chemotherapy and/or radiation.

3. In a subset of patients inheritance also matters. Germline mutations in pathways of the DNA repair systems are associated development of t-MN, and there may also be a genetic susceptibility for the independent development of a secondary cancer [113].

4. Furthermore, cytotoxic treatment likely damages the bone marrow niche causing abnormalities in the microenvironment, resulting in aberrant cytokine levels and altered cross talk between hematopoietic stem cells and other cells in the niche, providing an advantage for preleukemic clones [114].

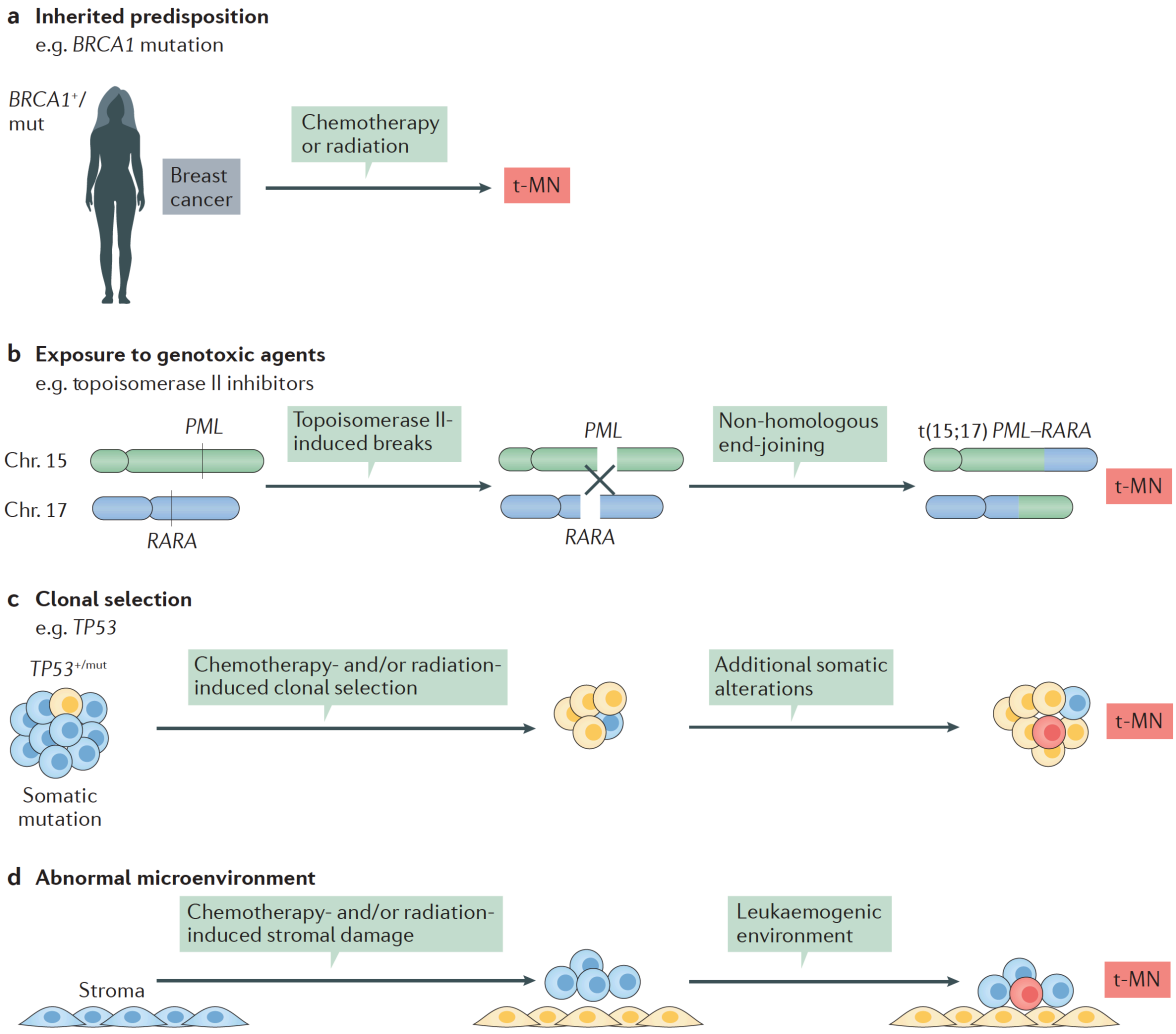

Figure 3. Contributions to the development of therapy-related myeloid neoplasms. *Reprinted by permission from Springer Nature: Nature Reviews Cancer, Therapy-related myeloid neoplasms: when genetics and environment collide, M. McNerney et al, copyright 2017.*

1.9 TREATMENT

1.9.1 Classical induction treatment

The principle of treatment of AML is to reduce the blast percentage below 5% through induction therapy and to reduce minimal residual disease by giving consolidation therapy. In the Swedish national guidelines the induction treatment consists of combination therapy with three days of daunorubicin and five days of cytarabine (DA3+5) [115]. This is comparable to the internationally more common 7+3 regimens, i.e. cytarabine continuously for 7 days with an anthracycline once a day for three days. Bone marrow is usually evaluated on day 25-28, and if complete remission is reached, 2-3 courses of consolidation therapy follow. If complete remission is not reached, reinduction with alternative drug combinations is given. In patients with intermediate and high risk cytogenetics, allogeneic hematopoietic stem cell

transplantation should be considered, but with careful consideration of other risk factors such as age, comorbidity and treatment response.

In elderly patients or patients otherwise not fit for intensive treatment there are other treatment options, which although not aim for cure, can prolong survival and/or alleviate symptoms. These options are low-dose cytarabine, hypomethylating agents (HMAs), best supportive care with the option of cytoreductive drugs and transfusions, or merely palliative care. [116]

1.9.2 Hypomethylating agents

The HMAs azacitidine and decitabine are widely used for the treatment of MDS. In AML, they are primarily a treatment option for patients not eligible for intensive induction. They can also be used as bridging to HCT in selected subgroups of patients [117, 118], and can potentially have a place as maintenance treatment after remission in elderly patients ineligible for HCT [119]. Treatment with HMAs in patients with s-AML is an appealing option due of the overrepresentation of typical adverse risk aberrations such as complex or monosomal karyotypes, abnormalities in chromosome 5, 7 and 17, and TP53 mutations that might predict response to HMAs [6, 120, 121].

1.9.3 Treatment aspects of s-AML

Boddu and colleagues compared treatment results of patients with s-AML treated with either cytarabine based intensive treatment, HMAs, low-dose cytarabine, CPX-351 (se below) and investigational agents [122]. The survival was overall poor, but the authors conclude that lower intensity approaches have lower rates of early death rates and improved OS compared to intensive treatment. Older data also supports the rationale to spare intensive treatment in high-risk disease even in fit patients, and only treat low-risk s-AML patients intensively [123].

Conversely, in Swedish population-based data intensive treatment is tolerable in the elderly, and improves early death rates and prolongs survival compared to palliative treatment [1, 124]. In view of the understanding that s-AML patients share genetic properties with elderly [31], they also ought to benefit from intensive treatment.

The challenges of treating s-AML and other AML high-risk groups, especially in the elderly, are not only to choose the treatment option that has the highest probability of prolonged survival, but also to decide when not to treat intensively or when not to proceed to HCT when chances of long-term survival are futile, and there is a high risk of treatment morbidity or reduced quality of life.

1.9.4 New treatment options

The classical induction treatment described above has been virtually unchanged for 40 years [4]. However, during the last few years several new drugs have shown efficacy in subpopulations of AML. Since 2017, midostaurin, gemtuzomab ozogamicin and CPX-351

have been approved in Europe, and in the U.S. also venetoclax, ivosidenib, and glasdegib (Figure 4). New therapy options are of course welcome, in particular for s-AML and the elderly, where current regimens are insufficient. However, criticism has been raised over issues in the approvals: lack of randomized studies, problematic endpoints, ill-defined inclusion criteria (e.g. "unfit"), and approvals for patients not included in the studies [125]. Hopefully, large randomized studies together with real-world data will eventually answer these questions.

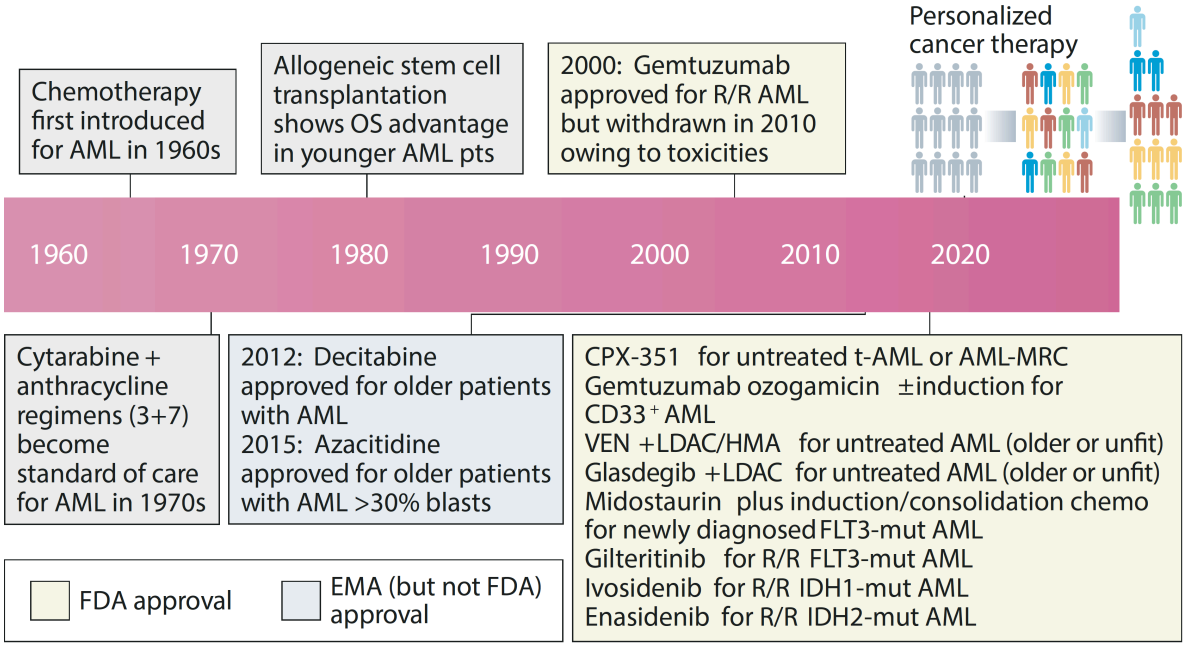

Figure 4. Developments in the treatment of AML. *Reprinted by permission from Springer Nature: Nature Reviews Clinical Oncology, Advances in patient care through increasingly individualized therapy, CD. DiNardo et al, copyright 2018.*

Liposomal cytarabine and daunorubicin for induction treatment

Aimed specifically at older patients with s-AML is CPX-351. This is a combination of cytarabine and daunorubicin in a liposomal encapsulations which has shown improved OS compared to conventional 7+3 induction and consolidation in a large phase III study including t-AML, AHD-AML and AML with dysplasia-related cytogenetic abnormalities [126].

Gemtuzomab ozogamicin in addition to standard induction

Gemtuzomab ozogamicin (GO), which is a conjugate between a CD33 antibody and cytotoxic drug, was in use already in the early 2000, but was withdrawn due to toxicity and no survival advantage. However, newer data has shown efficacy in favorable and intermediate risk groups [127]. As of 2019, GO is recommended as an addition to standard induction only in patients with CBF *de novo* AML. Thus, this is not an option for patients with s-AML.

Venetoclax for patients ineligible for intensive treatment
Venetoclax, an oral BCL-2 inhibitor, is approved in the US in combination with HMAs or low-dose cytarabine for first-line treatment of older patients or those unfit for intensive induction. Response rates and median survival (over 16 months) in this elderly group (including a large proportion of patients with s-AML and adverse risk cytogenetics) was impressive in the phase I study that lead to the US regulatory approval [128].

FLT3 inhibitors as addition to induction, maintenance or salvage therapy
The most commonly mutated gene in AML is *FLT3*, while highly enriched in *de novo* AML (24-28% of cases), it is less frequently mutated in t-AML (8-16%) and AHD-AML (19%) [2, 31, 97]. Multiple kinase inhibitors that target *FLT3*, along with other kinases with varying specificity, are available or being studied both in AML and other malignancies [129]. In the current Swedish guidelines (2019)[115], midostaurin is recommended as addition to standard induction therapy in *FLT3* mutated patients <70 years, based on the phase III study that showed a significant lower hazard ratio for death compared to placebo [130]. Several other *FLT3* inhibitors have been developed and are investigated for use during induction, as maintenance therapy or for relapsed or refractory AML [131, 132].

IDH inhibitors
IDH1/*2* mutations occur in 20% of AML, however the frequency in t- AML and s-AML is estimated to only 5-10%. Enasidenib targeting *IDH2* and ivosidenib targeting *IDH1* have shown promising results in Phase I and II studies, and both drugs are approved by the FDA for use in relapsed/refractory AML [133, 134]. It has also shown efficacy in older newly diagnosed patients, including patients with AHD-AML [135]. Ongoing studies are investigating *IDH* inhibitors in combination with azacitidine [136] and as addition to induction/maintenance treatment [137].

Glasdegib for elderly patients not fit for intensive treatment
The Hedgehog signaling pathway is important for leukemic stem cell maintenance and inhibition of this pathway increases sensitivity to chemotherapy [138]. Glasdegib is an oral Hedgehog pathway inhibitor and is approved in the U.S for use with low-dose cytarabine in older patients or in patients with comorbidities that prevent them from receiving intensive treatment. The approval study included patients with secondary AML and showed a median OS of 8.3 months compared to 4.3 months with low-dose cytarabine alone [139].

Oral azacitidine as maintenance therapy
Oral azacitidine as maintenance therapy has been presented as an effective maintenance therapy after complete remission in patients (both *de novo* and s-AML) ineligible for HCT with 24.7 months OS compared to 14.8 months in the placebo group [140].

To summarize, the current treatment approaches in s-AML patients are inefficient, and there is evidently a hitherto unmet need for improvement. However, even though the novel treatment options are interesting, they do not prolong survival more than a few months compared to control arms, and real-world data will eventually test their efficacy.

1.10 FOLLOW-UP / MEASURABLE RESIDUAL DISEASE

Morphological evaluation is a too imprecise method to detect imminent relapse or residual leukemia cells after treatment. Instead, measurable residual disease (MRD) by using either flow cytometry or molecular methods can identify patients at high risk of relapse and to guide treatment [17]. The flow cytometry approach is based on finding either a leukemia-associated immunophenotypes (LAIP) or a phenotype that is aberrantly differentiated compared to normal (DfN, different from normal), or a combination of both. Molecular methods using real-time quantitative PCR (RT-qPCR) to detect MRD can be even more sensitive than flow-based approaches and are currently used to analyze *NPM1* mutations, t(15;17), t(8;21) and inv(16) [141]. Additional methods with even higher sensitivity, e.g. digital droplet PCR and NGS, are likely to be used in the future [142]. MRD usage depends on the clinical situation, but is especially useful to guide HCT in low-risk patients with detectable MRD in CR1, to refrain HCT in intermediate-risk patients with low risk of relapse, to identify patients with elevated relapse risk post-HCT and to monitor patients after treatment [17].

1.11 ALLOGENEIC STEM CELL TRANSPLANTATION

HCT is a potentially curable treatment primarily used as post-remission therapy in younger patients in the intermediate or adverse risk groups [23, 143, 144], but is also an option in relapsed or refractory AML [145, 146]. Genetic risk, age, comorbidities, treatment response and availability of a donor form the basis of transplant decisions [3, 22]. The transplantation is preceded by a combination of chemotherapy and immunosuppression, and the conditioning can either me myeloablative (MAC) or reduced (RIC). HCT is a complex procedure with a significant risk of complications, including transplant related mortality (TRM) and graft-versus-host disease (GvHD), both acute and chronic [147, 148].

The role of HCT in s-AML is less studied than in *de novo* AML, however in the majority of patients with s-AML HCT should be considered, since these patients are characterized by non-favorable genetics. Data on transplant related factors of importance and outcome specific for s-AML has historically been scarce. However, during 2018-2019 the European Society for Blood and Marrow Transplantation (EBMT), mostly through the work of the Acute Leukemia Working Party, published a series of reports on various aspects of allogeneic transplantation based on large numbers of s-AML patients [149-156]. They show that s-AML, compared to *de novo* AML, is a risk factor for relapse and survival after HCT [150, 154]. Within AHD-AML, prior diagnosis of MPN rather than MDS is associated with worse OS, along with age, unrelated donor, CMV mismatch, Karnofsky index <= 80, remission status and peripheral blood as stem cell source [152]. Moreover, in patients with s-AML and similar to AML in total, limited chronic GvHD is associated with longer survival, while worse OS is seen in patients with grade II-IV acute and extensive chronic GvHD [149]. Regarding conditioning regimen, MAC is preferable to RIC in prolonging OS in patients with s-AML [155, 156]. In the subgroup of t-AML with a prior lymphoma, MAC was also preferable to RIC, and patients previously treated with autologous HCT had inferior leukemia-free survival [151]. Haploidentical transplantation seems feasible in s-AML with

similar impact of anti-thymoglobulin vs. post-transplant cyclophosphamide and choice of conditioning intensity as in *de novo* AML [153]. While the large EBMT studies are valuable sources of information, prospective trials about s-AML and HCT are still lacking.

1.12 HEALTH CARE AND QUALITY REGISTRIES

There is a long history of centrally administered health registries and disease specific healthcare quality registries in Sweden. The clinical data available therein and the possibility to cross-link them, are useful resources both for research and for improvement of quality of care [157]. In addition to the Swedish Acute Leukemia Registry (SAMLR), two other registries are of importance in the context of s-AML. The Swedish Cancer Register (SCR) and the Swedish Rheumatology Quality Register (SRQ). They can be used for data validation, give detailed information about prior diagnoses, including international classification of diseases (ICD) codes and dates of diagnoses and, in particular, aid the classification of s-AML.

All adult patients diagnosed with AML in Sweden are reported to SAMLR. The treating physician is responsible to report to the registry at several time-points during the time from diagnosis to a possible transplantation or relapse. The reporting system is web-based, and late or missing reports are actively requested. Since the start in 1997 the registry has grown to include over 250 variables. It has been validated against the SCR and found to have a coverage of 98% of all patients with a diagnosis of AML [1].

Both clinicians and pathologists are obliged to report all new cancer diagnoses to SCR. The diagnoses can be based on clinical, morphological and other laboratory exams. Data contained in SCR include basic patient data, diagnostic dates and ICD codes for tumor site and histological type. Since it was founded in 1958 it enables long follow-up of outcomes, including secondary malignancies [158].

SRQ was initiated in 1996 and encompasses 89,000 patients and more than 100 rheumatic diseases. Data include patient information, ICD codes, treatment and follow-up [159]

Studies using population-based registry data complement basic research and clinical trials. Although a clinical trial is the gold standard for evaluating an intervention, many trials have narrow inclusion criteria and are not generalizable to a broader population, and for some clinical questions randomized controlled trials will have issues with patient numbers, logistics or other resources [160].

2 AIMS

2.1 OVERALL AIM

The overall aim of this thesis was to define clinical, molecular and epidemiological characteristics of secondary AML.

2.2 SPECIFIC AIMS OF THE STUDIES

Study I

1. Test the feasibility of high-throughput mutation screening in MDS and AML
2. Identify mutational patterns with relevance for prognosis and progression from MDS to AML

Study II

1. Compare characteristics and outcome between s-AML and *de novo* AML
2. Validate, in a population-based setting, if s-AML in itself is a useful prognostic marker independent of established risk factors

Study III

1. Compare HCT with conventional post remission therapy (CPRT) in s-AML
2. Compare s-AML with *de novo* AML in patients undergoing HCT

Study IV

1. Investigate the incidence of t-AML over time
2. Identify prognostic factors in t-AML

3 METHODOLOGICAL APPROACHES

3.1 STUDY I

3.1.1 Patients and samples

Consecutive diagnostic bone marrow samples stored in the Karolinska Institutet MDS and AML research biobanks fulfilling the predetermined classification requirements were selected. Among the cases with MDS, the WHO 2008 [161] subtypes considered were RCMD-RS, RARS, RARS-T (grouped together as "RS"), 5q-, RCMD, RAEB-I, RAEB-II and CMML. In AML, only non-*de novo* cases were included, that is, AHD-AML, t-AML and AML with specific MDS associated cytogenetic aberrations (according to the definition of *AML with myelodysplasia-related changes*, WHO 2008 [161]). The patients were treated following national guidelines. Clinical data were collected from local registries, electronic patient records and the Swedish AML registry. Samples from 20 healthy donors were used as controls.

3.1.2 Mutational analyses

To screen for mutations, a three-step pipeline was followed. First the exons of the 22 selected genes were amplified using Halogenomics target amplification technology (Halogenomics AB, Uppsala, Sweden). To make this cost-effective, the samples were pooled in groups of 10. Each pool was marked and identified by a introducing a 6 base-pair barcode. Secondly, the pools were sequenced in two runs using Illumina Hiseq 2000 sequencing system. Next, sequencing data were filtered based on the barcodes and mapped onto a reference genome (The 1000 Genomes Project) and all the single nucleotide polymorphisms (SNPs) were filtered out based on SNP databases (SNP-DB). Third and last, the mutations in the individual patients were verified using the Sequenom system, which is a high-throughput system to analyze point mutations and SNPs. Additionally, hotspot mutations in the three splicing genes *SRSF2*, *SF3B1* and *U2AF1*, not included in the original gene list, where analyzed.

3.2 STUDY II - IV

3.2.1 Patients

These observational registry studies were based on adult (18 years or older) patients in the Swedish AML registry. Table III summarizes the numbers and subtypes of the included patients for each study.

Table III. Number and subtypes of patients included in studies II - IV.

	Study II	Study III	Study IV
Study description	Characterization and outcome in patients with s-AML	The impact of HCT in patients with s-AML	Characterization and outcome of patients with t-AML
Study period	1997-2007	1997-2013	1997-2015
Number of patients diagnosed with AML	3363	5873	6779
Included for analyses	All 3363 AML patients	All 3337 intensively treated non-APL AML patients	All 5492 patients with either *de novo* or t-AML
de novo AML	2474	2613	4806
AHD-AML	630	442	-
t-AML	259	282	686
Additional data sources	-	Swedish cancer registry The 6 transplantation centers in Sweden	Swedish cancer registry Swedish Rheumatology Quality registry

3.2.2 Data collection and definitions

Apart from the Swedish AML Registry, data were collected from the Swedish cancer registry, the Swedish Rheumatology Quality registry and local registries at all 6 transplantation centers in Sweden. Patients were classified into the three subgroups, *de novo* AML, AHD-AML and t-AML. AHD-AML was defined as cases diagnosed with a prior hematologic disorder, essentially the myeloid neoplasms MDS, CMML and MPN. T-AML was defined as AML with a prior diagnosis of a malignancy or a non-neoplastic disease treated with chemotherapy and/or radiation therapy, but not immunosuppressive treatment alone. Patients treated with chemotherapy for a myeloid antecedent hematologic disorder were classified as AHD-AML and patients treated with chemotherapy or radiation for a non-myeloid disease (e.g. lymphoma or multiple myeloma) were classified as t-AML. Patients

treated with chemotherapy/radiation for a primary disease who developed MDS or MPN in-between the therapy and the diagnosis of AML were classified as t-AML.

3.3 STATISTICAL CONSIDERATIONS

Throughout the studies, all tests were two-sided with a significance threshold of 0.05. Categorical data were compared using either Pearson's chi-squared test or Fisher's exact test (depending on sample size). Survival analyses were central in all four studies and estimation and visualization of survival was performed using the Kaplan-Meier method and comparisons between groups using the log-rank test. Although this method is adequate for crude comparisons, it does not accommodate analyses of multiple covariates. Instead, for multivariable analysis of survival, Cox proportional hazards regression was used. This method assumes that the rate of events is proportional between the groups compared, which was tested using inspection of the scaled Schoenfeld residuals. To limit immortal time bias in comparisons between HCT vs. CPRT in **study III** landmark techniques and Cox regression including HCT as a time-varying covariate was used. In addition, propensity score matching was used to balance groups when comparing HCT with CPRT. Multiple imputation was used in **study II** to test the impact of missing data.

3.4 ETHICAL CONSIDERATIONS

The benefits of increasing the knowledge about AML, with the goal of optimizing treatment methods of individuals, must be weighed against the cost and resources used for both the research in itself, but also for the clinical implications the results might have. Regarding personal integrity, all patient data used in this thesis were anonymized, without the possibility to identify specific individuals, and should not pose any risk of personal harm. The vast majority of patients included were deceased already at initiation of the studies, simply because of the short survival of AML. The possibility of renewed consent or possibility of changing their treatment was therefore impossible. Consequently, the patients included will not themselves benefit from any results. In **study I**, informed consent was obtained from all patients and healthy controls. There was a risk that the molecular analyzes could have detected hereditary genetic defects of importance to relatives to patients. However, although samples were unidentified they were traceable to the each individual. Regional ethical review boards approved all studies. In summary, the assumed benefits of the studies are judged to outweigh any risks.

4 RESULTS

4.1 STUDY I

We performed targeted exon sequencing of 22 genes in 100 patients with MDS and 92 with AML. The AML cases were either therapy-related, progressed from MDS or MPN, or had MDS-like-cytogenetics. MDS included both low hand high-risk patients with the subtypes MDS-RS, del(5q) syndrome, RCMD (MDS-MLD, WHO 2016), RAEB I and II (MDS EB-1 and EB-2, WHO 2016) and CMML.

Mutations were found in 61% of the MDS and 50% of the AML patients. Overall, the most frequently mutated genes in MDS were *SF3B1*, *TET2*, *SRSF2* and *IDH2* and in AML *TET2*, *SRSF2*, *U2AF1* and *IDH2*. Mutational patterns differed between subtypes in both MDS and AML. As expected, in MDS, del(5q) syndrome had the lowest frequency of mutations, and splice factor *SF3B1* mutations were dominant in RARS and RCMD-RS. The MDS-AML subgroup shared mutations with high risk MDS with frequent mutations in splicing and epigenetic genes. MPN-AML was different from the other AML subtypes with a higher incidence of mutations in signaling and oncogenes (Figure 5). T-AML had a relatively low mutational burden at 33%, but a high rate of cytogenetic aberrations (87%).

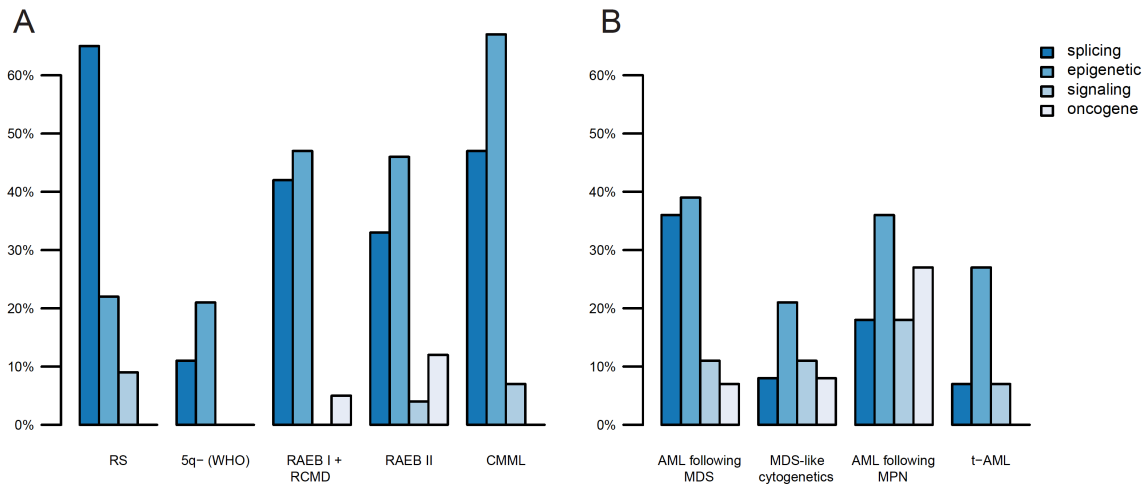

Figure 5. Proportion of patients with mutations in defined functional gene categories. (A) MDS subgroups (n=100) (B) AML subgroups (n=92).

To investigate mutations associated with progression from MDS to AML, we compared the 39 cases with either MDS with a subsequent AML progression or AML with an antecedent diagnosis of MDS with all the 89 MDS cases without AML progression. *U2AF1* was the only gene significantly associated with progression (21% of transformed vs. 4% non-transformed, p=0.008). In contrast, and as expected, there was a negative correlation between mutated *SF3B1* and leukemic progression (22% of non-transforming vs. 3% transformed, p = 0.004).

We then assessed if the presence of mutations added prognostic information. The MDS patients were divided into two groups, IPSS-R very low, low and intermediate risk versus high and very high risk. Overall and progression-free survival was compared between patients without any mutation and patients with one or more mutations (*SF3B1* excluded). Patients with mutations had worse OS and progression free survival in both risk groups ($p < 0.001$), log-rank. In the same manner we divided AML into favorable and intermediate risk versus adverse risk and compared outcome in patients with or without presence of mutations. No difference could be seen in the adverse risk group in contrast to favorable/intermediate risk, where mutational status separated the group ($p < 0001$, log-rank).

The influence of specific mutations on MDS to AML progression was explored using Cox regression analysis. In univariable models, *SF3B1* had a strong positive impact whereas *SRSF2*, *IDH2*, *U2AF1* and *RUNX1* were negative prognostic markers. However, when adjusting for age, sex and IPSS-R, only *SF3B1* and *U2AF1* remained statistically significant. Corresponding analysis in AML yielded *TP53* and *NRAS* as significant negative markers, also after adjusting for age, sex and cytogenetic risk.

4.2 STUDY II

Of the 3363 patients included in the study, 2474 (74%) were *de novo* AML, 630 (19%) were AHD-AML, and 259 (8%) were therapy-related. Median age was 70 in both t-AML and *de novo* AML, but slightly higher in AHD-AML at 71 years ($p <0.001$). The gender balance was equal in *de novo* AML, predominantly female in t-AML (165 female, 64%, $p <0.001$) and predominantly male in AHD-AML (363 male, 58%, $p <0.001$). Adverse cytogenetic risk was most common in t-AML (46%) but also more common in AHD-AML (40%) than in *de novo* AML (20%). Favorable cytogenetic risk was rare in AHD-AML but similar in t-AML and *de novo* AML (8-9%).

The prior diseases in AHD-AML were MDS in 404 (64%) patients and MPN in 187 (30%). The median latency period between the diagnosis of MDS and AML was 1.1 year and between MPN and AML over 7 years. The most common primary diseases in t-AML were breast cancer (n=55, 21%), non-Hodgkin lymphoma including CLL (n=50, 19%), uterine/cervical cancer (n=18, 7%), rheumatoid arthritis (n=18, 7%) and multiple myeloma (n=17, 7%). The median latency period between prior diseases and t-AML was 5.8 years for malignancies and 14.3 years for non-neoplastic diseases.

Intensive treatment was more common in *de novo* AML. Among patients younger than 65 years, 94% received intensive treatment compared to 69% in AHD-AML and 82% in t-AML. In addition, CR rates were lower in both AHD-AML and t-AML compared to *de novo* AML (39%, 54% and, 72% respectively, $p < 0.001$). Early death rates (death within 30 days of diagnosis) were similar between groups.

Table IV. Multivariable analysis for OS in intensively treated AML patients.

	Overall		After CR	
	HR (95% CI)	P	HR (95% CI)	P
Age—each 10 year increase	1.40 (1.33–1.47)	<0.001	1.46 (1.37–1.55)	<0.001
Male vs. female	1.31 (1.16–1.48)	<0.001	1.29 (1.11–1.50)	<0.01
AHD-AML vs. *de novo* AML	1.51 (1.26–1.79)	<0.001	1.59 (1.23–2.05)	<0.001
t-AML vs. *de novo* AML	1.72 (1.38–2.15)	<0.001	1.63 (1.20–2.23)	<0.01
Favorable vs. intermediate cytogenetic risk	0.51 (0.38–0.68)	<0.001	0.40 (0.28–0.56)	<0.001
Adverse vs. intermediate cytogenetic risk	1.66 (1.46–1.89)	<0.001	1.51 (1.26–1.80)	<0.001

In crude analyses, AHD-AML and t-AML had inferior OS compared to *de novo* AML. This was consistently seen in both intensively treated patients and overall, in both younger and older patients and across all cytogenetic risk groups. In the same manner, both types of secondary AML were inferior to *de novo* AML when comparing survival after the date of CR. In multivariable analysis (adjusting for age, sex, type of AML and cytogenetic risk group) both AHD-AML and t-AML had a significant negative impact on survival (t-AML vs. *de novo* HR 1.72; CI 1.38-2.15 and AHD-AML vs. *de novo* HR 1.51; CI 1.26-1.79) (Table IV). Subgroup analysis revealed that the prognostic effect of s-AML varied among age groups. In patients younger than 55, secondary AML had a markedly stronger negative impact on survival than in patients aged 55 or older. This age-dependent effect of s-AML on survival was apparent when comparing median OS between age groups: 158, 16 and 7 months in patients with *de novo* AML aged <55, 55-74 and >= 75 years respectively, compared to 14, 9 and 8 months in t-AML, and 7, 7 and 6 months in AHD-AML.

4.3 STUDY III

There were 3337 intensively treated AML patients (APL excluded) during 1997-2013. Of these, 282 (8%) had t-AML, 442 (13%) AHD-AML and 2613 (78%) *de novo* AML. The antecedent disorders in AHD-AML were MDS in 311 (9% of total) and MPN in 130 (4% of total) patients. Gender distribution and rates of CR were similar to **study II**. HCT, regardless of disease state, was performed in 22% (n=576) of patients with *de novo* AML, 20% (n=57) of t-AML and 17% (n=74) of AHD-AML. The proportion of patients in CR1 who underwent HCT was 23% in *de novo*, 28% in AHD-AML and 27% in t-AML.

Of patients transplanted in CR1, the median age was higher in AHD-AML than in *de novo* and t-AML (58 years vs. 48 and 51 years, respectively, $p < 0.001$). Moreover, in patients transplanted in CR1, adverse cytogenetic risk was more common in the two groups of s-AML compared to *de novo* AML. Myeloablative conditioning was given in 63% of s-AML patients who underwent HCT in CR1, 61% received a graft from an unrelated donor and 39% from a related donor, and in 89% of cases the stem cell source was peripheral blood. Transplanted

patients with AHD-AML and t-AML were similar regarding conditioning intensity, donor type, stem cell source, sex incompatibility, EBMT score and time from CR1 to HCT.

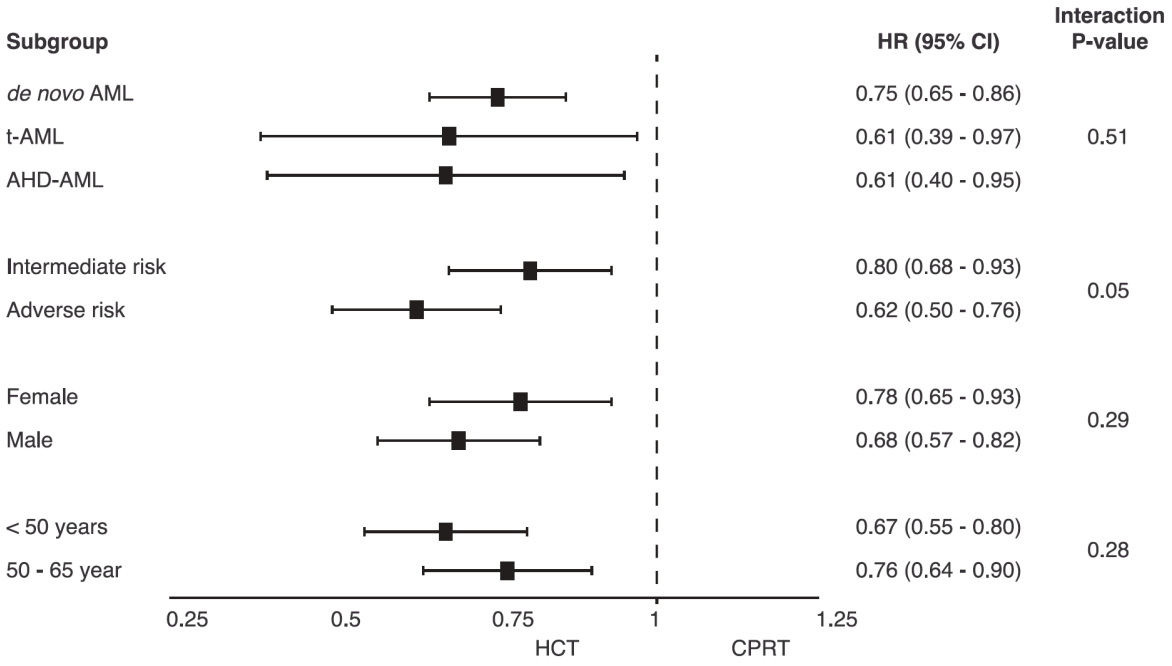

Figure 6. Subgroup analysis showing the impact of allogeneic HCT versus CPRT on survival.

In crude comparisons, s-AML had inferior OS compared to *de novo* AML, both overall and after the date of HCT in patients transplanted in CR1 ($p < 0.001$ and $p = 0.005$ respectively, log-rank). We compared HCT with chemotherapy only as postremission therapy in s-AML using three different statistical approaches. First, in a day 200 landmark analysis of non-favorable risk patients younger than 65 years who reached CR1, HCT was advantageous to CPRT ($p = 0.04$, log-rank). Similar results were found when choosing 300 days as the landmark cutoff. Secondly, in the same group of patients, we performed a multivariable Cox regression with HCT as a time-varying covariate, adjusting for subtype of AML, cytogenetic risk and stratifying by age. The hazard ratio for HCT vs. CPRT overall was 0.73 (CI 0.64-0.83), and in subgroup analysis both AHD-AML and t-AML had a significant negative impact on survival (Figure 6). Third, we performed a propensity score matching analysis comparing HCT and CRPT. Patients were matched on type of s-AML, cytogenetic risk, age and year of diagnosis and patients with CR1 < 90 days were excluded. 45 patients who underwent HCT were matched with 66 patients treated with CPRT. OS in the HCT group was longer than in the CPRT group ($p = 0.021$, log-rank). Altogether, the three methods all found HCT to outperform CPRT in patients with AHD- and t-AML.

Furthermore, in multivariable analyses, the only transplant-related factors associated with prognosis after HCT was mild chronic GvHD vs. no chronic GvHD and absence of acute GvHD grade < I.

4.4 STUDY IV

During the period 1997-2015 a total of 6779 individuals were diagnosed with AML of which 686 (10%) were t-AML, 4806 (71%) *de novo* AML and 1287 (19%) AHD-AML. Comparisons of patient characteristics between t-AML and *de novo* AML were consistent with **study II**, showing similar median age (71 vs. 70), similar performance status, female predominance in t-AML (57% vs. 49%), and a higher frequency of adverse but lower frequency of favorable risk in t-AML (46% vs. 28% and 12% vs. 16% in adverse and favorable risk respectively). The proportion of APL was similar in t-AML and *de novo* AML (4% vs. 5%). Both the rate of receiving intensive induction and the rate of CR were lower in t-AML than in *de novo* AML (60% vs. 70%, $p < 0.001$ and 58% vs. 75%, $p < 0.001$, respectively). Patients with t-AML were less likely to undergo HCT (9% vs. 16%, $p < 0.001$) than patients with *de novo* AML.

There was a steady rise in incidence of t-AML over study period. The mean age-standardized incidence rate increased from 0.39 cases per 100,000 during 1997-2006 to 0.63 during 2007-2015 ($p = 0.004$). Similarly, the estimated annual percentage change in incidence rate of t-AML was 4.5% (CI 2.8% - 6.2%). This was higher than the estimated annual percentage change in *de novo* AML (0.7%, CI 0.2% - 1.2%) and in AML in total (1.2%, CI 0.7% - 1.7%). Consequently, the proportion t-AML of AML in total increased from 8.3% 1997-2006 to 11.8% 2007-2015 ($p = 0.004$).

The most common primary diagnoses prior to t-AML were lymphoma (n=139), breast cancer (n=124), gynecological malignancies (n=60), prostate cancer (n=47), rheumatic and inflammatory disease (n=47), gastrointestinal cancer (n=36), and multiple myeloma (n=33). In total, 55% of the primary diseases were solid cancers, 25% hematological cancers and 18% non-malignant diseases. Multiple prior diseases were common; 19% of the patients had more than one malignancy reported and 17% had a diagnosis of MDS between the primary diagnosis and the onset of AML. There were large variations in the latency periods between the primary disease and AML. The median latency period after hematological malignancies was 57 months, after solid cancers 61 months and after non-malignant disease 173 months ($p < 0.001$).

Table V. Crude 5-year survival rates and median survival in patients with t-AML and *de novo* AML.

	t-AML		*de novo* AML	
	5-year OS	Median OS (months)	5-year OS	Median OS (months)
Overall	10%	5.0	23%	9.7
Intensively treated	17%	9.5	34%	20.7
Patients subjected to HCT	48%	48	57%	113

Crude (unadjusted) survival data are shown in Table V. The median OS in t-AML in total was 5.0 months, in intensively treated patients 9.5 months and in patients who underwent HCT 48 months (corresponding OS in *de novo* AML was 9.7, 20.7 and 113 months respectively). The difference in outcome between t-AML patients with or without an intermediate diagnosis of MDS that was found in univariable analyses (HR 1.52, CI 1.09 - 2.12, p = 0.013) was non-significant after adjustment for age and cytogenetic risk (HR 1.28, CI 0.89 - 1.87, p = 0.185). There were no significant survival differences between treatment modalities of the primary disease (radiation vs. chemo, HR 1.01, CI 0.72-1.41 and radiation/chemo vs. chemo alone, HR 1.36, CI 0.96-1.41, adjusted for age, risk and performance status).

There was no significant survival difference between t-AML and *de novo* AML among favorable risk patients (t-AML vs. *de novo* AML, HR 1.11 CI 0.62 - 1.97, p = 0.73) when adjusting for age and performance status. In contrast, with the same adjustments, t-AML had a strong negative impact in the intermediate and adverse risk groups (t-AML vs. *de novo* AML, HR 1.53, CI 1.25 - 1.87, p < 0.001 and HR 1.59, CI 1.31 - 1.93, p < 0.001, respectively). Additionally, t-AML patients with the combination of mutated *NPM1* and absence of *FLT3*-ITD had longer OS than patients with other combinations of *NPM1* and *FLT3*-ITD (p = 0.019, log-rank). In the subset of patients with the favorable combination *NPM1*+/*FLT3*-ITD-, OS was similar in t-AML and *de novo* AML, both in crude comparison (p = 0.58, log-rank) and after adjusting for age, performance status and risk (t-AML vs. *de novo* AML, HR 0.75, CI 0.36 - 1.58, p = 0.454).

5 DISCUSSION

5.1 NGS MUTATIONAL SCREENING

The question posed in **study I**, whether high-throughput mutational screening is feasible, is no longer debatable. NGS, usually implemented as gene panel assays, are already part of clinical routine for diagnostic profiling, prognostication and identification of possible drug targets in MDS and AML [162-164]. On the other hand, the other main finding, that mutated *U2AF1* is an adverse prognostic marker for OS and AML progression in MDS, is in hindsight not as certain. Other small series have found similar results, either for transformation only [165] or both transformation and OS [166], while others conclude that *U2AF1* has no prognostic value [167, 168]. Studies in lower-risk MDS have found *U2AF1* not to be of significance after adjusting for risk adapted prognostic scores [169], while more recent data suggests *U2AF1* (as well as several other genes) only to have independent prognostic value in patients with <5% blasts [170]. Finally, in a large meta-analysis of 3322 *de novo* MDS patients of which 390 patients had mutated *U2AF1*, the mutation did indeed have a prognostic impact on both OS and AML transformation [171].

This highlights the fact that in many cases different studies reach conflicting conclusion when it comes to the impact of specific mutations, not only *U2AF1*. The reason is probably a combination of sample size, varying inclusion criteria, ages and different subgroups of patients. Usually, most commonly mutated genes have a significant negative impact in univariable analyses, however when adjusting for other genes and known clinical risk factors the number of significant gene mutations are considerable fewer. Thus, the issue of prognostic impact of specific genes is highly complex, and it is important that the impact of specific gene mutations are thoroughly validated, preferable in large collaborative groups, before implementation in clinical guidelines that influence patient management.

5.2 AGE AND S-AML AS AN ADVERSE RISK FACTOR

It is well known that age, along with cytogenetic risk, is the most important prognostic factor in AML. **Study II** found both t-AML and AHD-AML to be additional negative prognostic factors for OS, also when adjusting for age, sex, and most important, cytogenetic risk. However, two age-related findings were notable. First, while CR rates were substantially higher in younger than older patients with *de novo* AML, they were similar between age groups in s-AML. Secondly, the adverse prognostic impact of s-AML, in particular of AHD-AML, was age-dependent. Having s-AML in older age did not add any prognostic information, while it was a strong negative marker in the younger, who had almost as poor outcome as the elderly. These results are in line with Danish population-based data have also shown that intensively treated patients >60 years old with t-AML and MDS-AML have similar outcomes as *de novo* AML when adjusting for performance status, comorbidities and cytogenetics [5]. And as mentioned in the introduction, others have also noted this lack of prognostic impact of s-AML in the elderly [5, 6, 27]. The obvious explanation is the high prevalence of both adverse-risk cytogenetic aberrations and high-risk mutations across ages

in s-AML, in contrast to AML in total, where high-risk genetics are much more common in the elderly [30, 172]. Moreover, on the mutational level, one third of elderly *de novo* patients share genetic features with AHD-AML, suggesting an undiagnosed MDS-phase before presenting as *de novo* AML [31].

5.3 SUPPORT FOR HCT IN S-AML

With such dismal survival rates without HCT as presented in **study III**, it is hard to convince physicians and patients to take part in a randomized trial comparing HCT with CPRT, even though the differences in outcome could be explained by unforeseen bias. However, it is likely that HCT in CR1 for s-AML patients with intermediate and high-risk confers a true advantage in the same manner as for *de novo* AML.

The comprehensive recent publications from EBMT, as referred to in the introduction, overshadow the results from **study III** regarding risk factors for outcome after HCT. Probably due to low number of patients in our study, only GvHD was significantly associated with outcome (mild chronic GvHD favorable, acute GvHD adverse). Owing to the EBMT reports, we now have strong data supporting not only the same GvHD findings [149], but also that MAC as opposed to RIC [155], related donor and BM as stem cell source [152] among other factors are beneficial for OS.

Nonetheless, the EBMT data on almost 5000 s-AML patients transplanted during 2000-2016 show a 2-year OS rate of 44.5% [156], which is in line and comparable to the 37% 3-year survival of the patients in our study, in particular since the patients in **study III** were transplanted earlier in time (1997-2015).

5.4 INCREASE IN T-AML INCIDENCE

With an aging population and increasing incidence of cancer overall, together with advances in treatment and care of the patients, the prevalence of cancer survivors is rising and is expected to do so also in the future [173-175]. Thus, the population at risk of t-AML is enlarging and a possible explanation of the steady increase of incidence of t-AML seen in **study IV**. However, the risk of t-AML following different cancers varies over time and is associated to changes in treatment protocols, which can be seen in comprehensive population-based U.S. data [71]. For instance, the risk of t-AML increased for non-Hodgkin lymphoma but decreased for myeloma and ovarian cancer during 1975-2008 [71]. Consequently, the prediction of the future incidence of t-AML is difficult. Moreover, incidence rates are highly dependent on age-structure, which makes crude comparisons with historical data across nations and time-periods difficult. Adjusting the data to an age distribution specific to a year or to a standard population, e.g. the world or European standard population, is one solution [176], but all data not coinciding with the reference will be skewed and may cause confusion.

5.5 FAVORABLE RISK T-AML

Study IV suggested that t-AML confers no prognostic information in patients with favorable karyotype and in patients with the favorable *NPM1*+/*FLT3*-ITD- signature. However, no subgroup analysis was performed to investigate the relation to specific chromosomal aberrations and no extensive mutation profiling was available. Conversely, in a study comparing t-AML vs. *de novo* AML in patients with CBF-AML, t-AML had poorer survival after adjusting for age, performance status and other cytogenetic abnormalities [177]. Kayser and colleagues [6] found t-AML to be a negative prognostic factor in patients with inv(16) or t(16;16), but not significantly in t(15;17), t(8;21) or mutated *NPM1*, though only adjusted for WBC, not age or performance status. A likely explanation of the difference in outcome between the studies could be presence of adverse mutations coexisting in CBF-leukemia patients, e.g. mutations in *KIT* and chromatin modifiers and cohesin [178, 179]. A small study found that t-AML patients harboring t(8;21) had poorer OS than their *de novo* counterparts. However, the t-AML patients were older and no statistical adjustments were performed [180].

Furthermore, in a cohort of 305 s-AML patients, mutated *NPM1* was an independent *negative* factor for prognosis adjusted for age, t-AML or AHD-AML, cytogenetic risk and *FLT3*-ITD status, however losing significance at age > 60 [181]. Moreover, only 72 patients had t-AML (of which only 3 were *NPM1* mutated) and the other 233 MDS-AML (27 *NPM1* mutated). Our cohort was t-AML only, which probably explains the discrepancy. On the other hand, the cytogenetic risk stratification used in our multivariable adjustment had *NPM1* already included, which makes the statistical interpretation intricate. Furthermore, one can simply argue there is no proof that the *NPM1* mutated t-AML patients in **study IV** truly are therapy-related and not *de novo*, as *NPM1* typically is associated with *de novo* and normal karyotype AML [182].

While **study IV** did not specifically focus on t-APL, OS was found to be similar in t-APL compared to *de novo* APL (poorer than *de novo* APL in crude analysis, but better after adjusting for age and performance status, both analyses non-significant however). Few large studies have compared t-APL and *de novo* APL, but most data support similar outcome in the two groups [183, 184], even though a higher rate of induction mortality in t-APL has been reported [185].

Aldoss and Pullarkat nicely reviews favorable cytogenetic t-AML [186], and our data supports their conclusion that t-AML per se is no reason to intensify treatment or perform HCT in patients with favorable cytogenetics.

5.6 CHANGES IN TREATMENT OVER TIME

During the era in which the present studies took place, 1997-2015, very few new drugs for AML were approved. Practically all intensively treated patients received similar DA regimens. While hypomethylating agents were introduced in clinical practice in Sweden 2008, only 142 patients (53 with AHD-AML, 61 with *de novo*, and 28 with t-AML) were

given HMAs as primary treatment until 2015 (*unpublished data*). HMAs are an option in patients not eligible for standard induction, and notably, only a handful actually reached CR according to the registry. The lack of new therapies introduced might seem gloom, however, as discussed in the introduction, new promising drugs are already underway. On the other hand, the standstill up to present has made the study of outcome in AML considerably simpler, since one of the most important variables has not changed over time. One can safely assume that a 40-year old Swede diagnosed in 2000 would get the same induction treatment if diagnosed 15 years later. In 2020, with the advent of new drugs, this is no longer certain. AML registries, including SAMLR, will have to be flexible enough to accommodate the multiple combinations of new treatment regimens, keep track of treatment periods and disease stages (not only remission/relapsed/refractory disease, but also relapse on MRD level). Altogether this will make coming clinical trials as well as observational studies highly complex with more and smaller subgroups of patients.

5.7 LIMITATIONS

Confounders and immortal-time bias

The most problematic issue with analyses of survival and prognosis in the studies in this thesis was to control for confounders (e.g. to handle to interrelationships between the different risk factors). Confounding is a general problem in observational studies trying to estimate effects, and the multivariable models used to limit confounding are only *models*. No matter how meticulous you are in adjusting for covariates or how well you try to balance your groups of comparison, you can never eliminate bias completely in retrospective analyses.

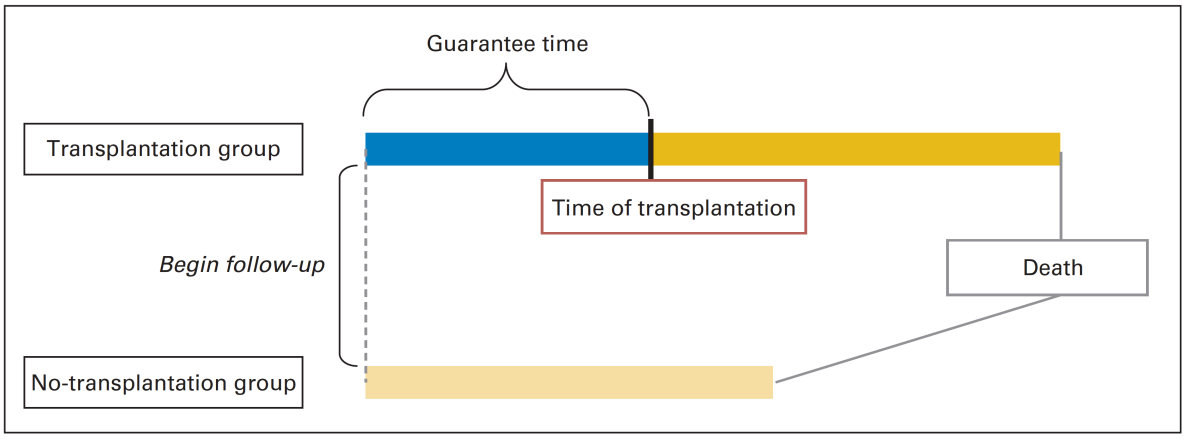

Figure 7. Illustration of immortal-time/guarantee-time bias. Survival from diagnosis (beginning of follow-up) appears longer in the transplantation group. However, in the transplantation group, patients are guaranteed to survive until date of transplantation, adding extra time to the transplantation group. *Reprinted with permission from American Society of Clinical Oncology: Journal of Clinical Oncology, Challenges of Guarantee-Time Bias, A. Giobbie-Hurder et al, copyright 2013.*

Guarantee-time bias or immortal time bias arises if two groups are compared and there is a period of time before a certain exposure when the event of outcome impossibly can occur in one of the groups [187] (Figure 7). This was a challenge in **study III**, where HCT was compared against CPRT and patients in the HCT-arm could not have experienced the event "death" between time zero and the time of the exposure "HCT". The patients in the CPRT-arm, on the other hand, were at risk of death already from time zero. Crude analyses between the groups would result in a strong bias in favor for HCT, since patients that might have been candidates for HCT but died before the eventual transplantation all would be allocated to the CPRT-arm.

In the comparison between HCT and CRPT in **study III**, several techniques were used to limit immortal time bias. First a landmark analysis in which follow-up started at day 200 and patients who died or relapsed before day 200 were excluded and the patients surviving to day 200 were assigned either to the HCT arm if they had undergone HCT by day 200, or to the CPRT arm if not (robustness was checked by using 300 days as a landmark cutoff). Secondly, a multivariable Cox regression analysis with HCT as a time-varying covariate was performed. In addition, to reduce selection bias and balance confounding factors between groups, a propensity score matching analysis was performed, in which patients who underwent HCT were matched against patients who received CPRT with regards to age, cytogenetic risk, AML subtype and year of diagnosis.

Limitations of registries

The strengths of the Swedish acute myeloid leukemia registry are the truly population-based coverage, the large sample size and the rigorous follow-up. However, while the coverage compared to the population and to the national cancer register has been validated, there has been no thorough systematic validation of the overall data quality in SAMLR. There are missing data, e.g. incomplete karyotyping, which can make the interpretation and management of data a challenge. Data might be misclassified; either through recall bias or by simple input errors by the reporting clinicians, but also when retrospectively analyzing the data. Furthermore, there are changes over time that might cause bias. The overall study period spans almost 20 years, and reclassification of disease subtypes do occur (e.g. blast count 20% or 30% to separate AML from MDS). Furthermore, there might have been a change over time in the awareness of s-AML and to what extent clinicians have reported prior diseases and therapy. With this in mind, the results and conclusions in this thesis should continuously be evaluated and compared with independent studies from others.

6 CONCLUSIONS AND CLINICAL IMPLICATIONS

- Mutational screening using targeted sequencing is a feasible method that adds prognostic information in addition to established risk-assessment tools in MDS and AML. Targeted sequencing of myeloid cancer genes has since the publication of **study I** been incorporated in the clinical routine for the diagnostic work-up of MDS and AML.

- Patients with AML progressing from a prior hematologic disease or arising after chemo- or radiation therapy have lower remission rates and shorter overall survival than *de novo* AML. The negative impact of s-AML is independent of other risk factors, and while pronounced in younger patients, the impact decreases in older patients. Thus, in clinical prognostic assessment, information about s-AML has less value in elderly patients.

- Virtually no long-term survival can be seen in s-AML without HCT. The outcome without HCT is especially poor for AHD-AML and in particular MPN-AML. HCT should be considered for all s-AML patients who are otherwise eligeble for HCT.

- The incidence of t-AML has increased steadily in Sweden during the last 20 years and the proportion t-AML of AML in total is becoming larger.

- A large proportion of patients with t-AML have multiple prior cancers and t-AML is commonly preceded by t-MDS.

- The outcome of t-AML patients with favorable risk cytogenetics or a favorable mutational profile with mutated *NPM1* in the absence of *FLT3*-ITD is similar to the outcome of patients with *de novo* AML with matching cytogenetic or mutational risk.

7 FUTURE PERSPECTIVES

For such a detrimental disease as AML the word exciting might be a bit inappropriate. However, this is probably what many clinicians and researchers do feel about the current development in disease understanding and drug development.

7.1 THE CHALLENGE OF INDIVIDUALIZED PROGNOSTICATION AND THERAPY

The mapping of the genetic abnormalities, especially the mutational spectrum, the last few years has confirmed the molecular heterogeneity of AML. Large sequencing studies have been able to stratify prognosis based on both single somatic mutations and combinations of mutations. In addition, there is increased knowledge of the importance of the variant allele frequency of certain mutations (e.g. *FLT3*-ITD [188], *TP53* [189]). Up until now, the cytogenetic aberrations and the somatic mutations making up the risk classification systems have been manageable knowledge for the individual clinician. However, due to the growing complexity of assessing genetic data, support from new computational prognostic tools might be required to aid clinical decision-making. These tools could not only use genetic data, but patient related and clinical factors as well. Proof-of-concept of knowledge banks to support individual prediction in this manner have already been developed, with a notable example of Gerstung and colleagues [190] who have made a predictive tool based on the sequencing 1540 intensively treated patients publicly available.

The abundance of potentially clinically relevant genetic subgroups is also a challenge when selecting therapy. One patient might have multiple drugable targets where it is not necessarily obvious which drug or combination of drugs to chose. Furthermore, since the current risk classification systems are based on outcome after standard intensive induction therapy, they will no longer be applicable if new effective drugs are introduced that change the outcome for certain subgroups. This highlights the importance of being able to dynamically update knowledge-banks and real-world registries to accommodate new and complex treatment protocols.

Furthermore, there is a multitude of clinical trials investigating combinations of old and new drugs, with the aim of enabling "precision medicine" or personalized treatment. These trials involve subgroups of patients in different disease stages and with diverse clinical and molecular properties. As a result, the data and results generated will be highly complex to compare and evaluate. Models to manage trials combining interventions with observational real-world data in multi-institutional master protocols, using standardized reporting methods to databases storing clinical and molecular data along with outcomes have been suggested [191].

7.2 SCREENING FOR PRELEUKEMIC MUTATIONS?

Since the combination of clonal hematopoiesis and cytotoxic therapy is a probable major factor in the pathogenesis of t-AML, the incentives should be strong to screen for high-risk

mutations in cancer patients about to start chemotherapy [97, 111], or to screen cancer patients post treatment as surveillance [192, 193]. Including inherited risk-alleles in these gene panels could also be of value to detect unnoticed syndromes at high risk for inherited AML [20]. However, to weigh the risk vs. benefit of adjusting chemotherapy regimens to prevent iatrogenic development of t-AML is obviously difficult and warrants more research. More controversial is the idea to monitor or screen for elevated risk of *de novo* AML in healthy individuals. Nonetheless, studies have shown that it is possible to risk-stratify clonal hematopoiesis, and that mutational signatures associated with increased risk of leukemia development can be detected several years before the onset of AML [112, 194]. The questions arising are many and complicated: Who should be monitored? What are the risks of over-diagnosis? Do we have any early interventions at hand?

8 ACKNOWLEDGEMENTS

These studies were supported by Region Stockholm (combined clinical residency and PhD training program).

This thesis has followed me for quite some years now. Many important things have happened in parallel, outside of research: I changed clinical residency from Södersjukhuset to Karolinska. I have moved twice. And I fractured the greater tuberosity of humerus once. I also met my wife Malin and got two incredible kids.

During these years, owing to this project, I have gotten to know and met so many great people, in particular at HERM and in the clinic, but many places elsewhere as well. I will surely forget to thank all of you, but this is a start.

A big heartfelt thank you to:

Sören, for being the most encouraging and positive supervisor imaginable. You are not only a big part of this thesis, you are also one of the reasons I chose to specialize in hematology, being inspired by you in medical school (SVK hematologi 2008). I'll never beat you in anything related to AML, and with your ability to practice and learn, you'll probably soon beat me at tennis as well.

The Lehmann Lab members in random order: **Sofia Bengtzén**, **Huthayfa Mujahed**, **Stefan Deneberg, Anna Eriksson**, **Anna Bohlin**, **My Björklund**, **Anne Neddermeyer**, **Ying Qu**, **Albin Österroos**, **Sylvain Mareschal**, **Christer Paul**, **Minna Suomela**.

Eva Hellström-Lindberg. For HERM and for always giving good advice. I cannot mention HERM without also thanking **Monika Jansson** and **Gunilla Walldin**.

Colleagues, nurses and secretaries at the department of hematology at Karolinska. You are too many too list here. However, I do need to mention **Kristina Sonnevi**, **Maria Ljungqvist** and **Johanna Ungerstedt**.

The Swedish AML group for keeping the AML community in Sweden together (and for creating the AML registry...). In particular **Gunnar Juliusson**, **Martin Höglund** and **Vladimir Lazarevic**.

Mohsen Karimi and **Marios Dimitriou** for the collaboration in study II.

The Stockholm-Gothenburg s-AML team members **Erik Hulegårdh**, **Hege Garelius**, **Lars Möllgård** and **Dick Stockelberg**. Not only am I glad to have gotten to know you; you are also an important part of this thesis. However, while we improved our knowledge about s-AML, we did not improve our skills in running electronic conferencing.

Andreas Lennartsson and his team **Anna Palau De Miguel**, **Sophia Miliara**, **Wenbo Dong** and **Farzaneh Shahin Varnoosfaderani**, for *big lab* meetings and fun retreats.

Juha Kere, my co-supervisor. One of only two people that I know who got their first thesis paper into the NEJM. Our methylation study on preleukemia is ongoing. We might need more advice...

The ClinSeq group at MEB: **Henrik Grönberg**, **Johan Lindberg**, **Mattias Rantalainen**, **Mei Wang** and **Arvind Mer** for a fruitful collaboration. I'm still impressed by your solid sequencing and biostatistical skills. Although none of our projects are part of this thesis, they surely were a big part of my PhD years.

Lina Benson for good advice and statistical input at the early stages.

Michael Grövdal, my research mentor, for all the "fikas". ;-) Hope to see you in the clinic soon.

All other coauthors on the papers: **Fredrika Linde**, **Mats Remberger**, **Petar Antunovic**, **Åsa Derolf**, **Bertil Uggla**, **Lovisa Wennström**, **Anders Wahlin**, **Hans Matsson**, **Per Unneberg**, **Mats Brune**, **Stig Lenhoff**, **Ulla Frödin**, **Jörg Cammenga**, **Martin Jädersten**, **Cecilia Kämpe Björkvall**, **Emma Ölander**.

My dear close friends who I'm immensely grateful to have around (in order of getting to know each other): **Fredrik Ulfström**, **Magnus Innala**, **Richard Nordqvist**, **Henrik Adolfsson**, **Pontus Persson**, **Joel Norevik**, **Johan Viberg**, **Joar Sundman** and **Buster** & **Alex Mannheimer**.

My kindest parents **Katarina** & **Lennart**.

And finally, thank you to my big love **Malin**. Your support made it all.
I can't describe how grateful I am to live my life with you!!!

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