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RUNX1 mutations in acute myeloid leukemia

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To my family

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ABBREVIATIONS

µl	Microliter
ALL	Acute lymphoblastic leukemia
AML	Acute myeloid leukemia
AMLSG	Austrian German-Austrian AML Study Group
APL	Acute promyelocytic leukemia
Ara-C	Cytosin-arabinosid
ASXL1	Additional sex combs like 1 gene
ATRA	All-trans retinoic acid
BCL6	B-cell CLL/lymphoma 6
BCOR	BCL6 co-repressor
BCR-ABL1	Breakpoint cluster region-c-abl oncogene 1
C/EBPα	CCAAT/enhancer-binding protein α
CALGB	Cancer and Leukemia Group B
CBFß	Core binding factor beta
CD	Cluster of differentiation
CGH	Comparative genomic hybridization
CMML	Chronic myelomonocytic leukemia
CN	Cytogenetically normal
CR	Complete remission
CSR	Cycle sequencing reaction
DNA	Deoxyribonucleic acid
DNMT3A/B	DNA (cytosine-5)-methyltransferase 3A/B
DOTL1	DOT1-like histone H3K79 methyltransferase
e.g.	Lat. *exempli gratia* (engl. *for example*)
ECOG	Eastern Cooperative Group
EDTA	Ethylene diamine tetra-acetic acid
EFS	Event-free survival
ELN	European LeukemiaNet
et al.	lat. *et alii* (engl. *and others*)
EZH2	Enhancer of zeste homolog 2
FAB	French American British
FLT3	FMS-like tyrosine kinase 3
FPD	Familial Platelet Disorder
G-CSF	Granulocyte colony stimulating factor

GM-CSF	Granulocyte-macrophage colony stimulating factor
HAM	High-dose cytarabine and mitoxantrone
HSCT	Hematopoietic cell transplant
HSCT-CI	Hematopoietic cell transplantation specific comorbidity index
HLA	Human leukocyte antigen
HPLC	High performance liquid chromatography
IC	Idarubicin-cytarabin
IDH1/2	Isocitrate dehydrogenase 1/2
IE	Idarubicine-Etoposide
inv	Inversion
ISCN	International System for Cytogenetic Nomenclature
ITD	Internal tandem duplication
KIT	v-kit Hardy Zuckerman 4 feline sarcoma viral oncogene homologue
KMT2A	Lysine [K]-specific methyltransferase 2A
KRAS	v-K-ras 2 Kirsten rat sarcoma viral oncogene homolog
MAPK	Mitogen activated protein kinase
MDS	Myelodysplastic syndrome
MKL1	Megakaryoblastic Leukemia (Translocation) 1
ml	Milliliter
mM	Micromolar
MPN	Myeloproliferative neoplasm
MRD	Minimal residual disease
mut	Mutated
MYH11	Myosin heavy chain 11
NGS	Next generation sequencing
NPM1	Nucleophosmin 1
NSD1	Nuclear receptor binding SET domain 1
NUP98	Nucleoporin 98
ORF	Open reading frame
OS	Overall survival
PB	Peripheral blood
PBMC	Peripheral blood mononuclear cell
PHF6	Plant homeodomain (PHD)-like finger 6
PICALM	Phosphatidylinositol binding clathrin assembly protein
PML-RARA	Promyelocytic leukemia/retinoic acid receptor alpha fusion gene
PRDM16	PR domain containing 16

PTD	Partial tandem duplication
PTPN*X*	Protein tyrosine phosphatase, non-receptor type-*X*
RBM15	RNA binding motif protein 15
RD	Resistant disease
RFS	Relapse Relapse-free survival
RPN1	Ribophorin 1
RUNT	Runt homology domain
RUNX1	Runt-related transcription factor 1
RUNX1T1	Runt-related transcription factor 1-Translocation 1
s-AML	Secondary AML
SEER	Surveillance Epidemiology and End Results Program
SMC3	Structural maintenance of chromosomes 3
SRSF2	Splicing factor arginine/serine-rich 2
SRSF3	Splicing factor arginine/serine-rich 3
STAG1	Stromal antigen 1
STAG2	Stromal antigen 2
t(x;y)	Translocation (Chromosom x>y)
TAD	Transactivation domain
t-AML	Therapy-related AML
TET1	Ten-eleven translocation methylcytosine deoxygenase 1
TET2	Ten-eleven translocation methylcytosine deoxygenase 2
TF	Transcription factor
TKD	Tyrosinkinase domain
TP53	Tumorprotein 53
WHO	World Health Organization
wt	Wildtype
WT1	Wilms tumor 1

1. INTRODUCTION

1.1. Acute myeloid leukemia-general consideration

1.1.1 Definition and epidemiology

Acute myeloid leukemia (AML) is a clonal disease of early myeloid progenitor cells and represents the most frequent leukemia in adults with an incidence of 3 to 4 per 100000 men and woman per year with a median age at diagnosis of 67 years (SEER· 2015). At the molecular level acquired somatic mutations in hematopoietic progenitors impair normal mechanisms of self-renewal, differentiation and proliferation (*Schlenk R.F. et al. 2013*). Clinically and biologically, AML is characterized by great heterogeneity. The current classification of the World Health Organization (WHO 2008) defines distinct entities of AML based on cytogenetic alterations and for the first time two molecular genetic changes, that is, ”AML with mutated *CEBPA*” and “AML with mutated *NPM1*” have been included as provisional entities (*Vardiman J.W. et al. 2008*).

1.1.2 Pathogenesis of AML

AML develops as a consequence of serial acquisition of genetic changes in hematopoietic precursor cells. These changes alter the normal hematopoietic growth and differentiation leading to a differentiation block, which results in the accumulation of abnormal and immature myeloid cells (blasts) in the bone marrow, peripheral blood and occasionally extramedullary sites. Recent studies identified clonal hematopoiesis in approximately 4 % of the general population with increasing incidence with age with more than 10 % in persons older than 70 years (*Jaiswal S. et al. 2014*). Most frequent mutated genes were *DNMT3A*, *TET2* and *ASXL1*. Clonal hematopoiesis was associated with an increased risk of hematologic cancers but also with an increased overall mortality (*Genovese G. et al. 2014*). Progression to AML requires a series of genetic events starting with clonal expansion of transformed leukemic stem cell. One hypothesis has been the “*two hit model*” according to which leukemogenesis occurs as a consequence of at least 2 types of mutations. One type

confers a proliferative advantage (class I), such as mutations of *FLT3*, *KIT*, *K-RAS* or *NF1*, and the second type impairs normal hematopoietic differentiation (class II), such as *CEBPA, RUNX1-RUNX1T1*, *CBFß-MYH11*, *PML-RARA (Fröhling S. et al. 2005, Gilliland G. et al. 2002).* More recently, by using new technologies such as whole exome and whole genome sequencing, a large number of genes recurrently mutated in AML have been identified. Based on their biologic function, genes were grouped into 9 categories (**Table 1**). Some gene mutations occurred more frequently together indicating a synergic mode of action while others were mutually exclusive indicating duplicative pathways (*Ley T.J. et al. 2013*).

Table 1. Representation of the 9 categories of recurrently mutated genes in AML based on their biologic function (*Ley T.J. et al. 2013*)

Transcription factors fusions	*PML-RARA, MYH11-CBFß, RUNX1-RUNX1T1, PICALM-MLLT10*
Nucleophosmin 1	*NPM1*
Tumor suppressor genes	*TP53, WT1, PHF6*
Genes involved in DNA methylation	*DNMT3A, DNMT3B, TET1, TET2, IDH1, IDH2*
Genes involved in activation signaling	*FLT3, KIT, KRAS, NRAS, PTPN11, PTPRT, PTPN14*, other tyrosine serine-threonine kinases
Myeloid transcription factors	*RUNX1, CEBPA*
Chromatin modifiers	*KTM2A*-X-fusions, *KTM2A-PTD, NUP98-NSD1, ASXL1, EZH2*
Genes involved in the cohesion complex	*STAG1, STAG2, SMC3*
Genes involved in the splicing machinery	*SRSF2*

Abbreviations: **AML**, acute myeloid leukemia| ***DNMT3A and DNMT3B***, DNA (cytosine-5)-methyltransferase 3A/B| ***PML-RARA,*** Promyelocytic leukemia/retinoic acid receptor alpha fusion gene| ***MYH11-CBFß***, Myosin heavy chain 11- CCAAT/enhancer-binding protein α gene| ***RUNX1-RUNX1T1***, Runt-related transcription factor 1-Runt-related transcription factor 1 translocation| ***PICALM-MLLT10*** Phosphatidylinositol binding clathrin assembly protein-Myeloid/lymphoid or mixed-lineage leukemia translocated to 10| ***NPM1*** Nucleophosmin 1| ***TP53*** Tumorprotein 53| ***WT1*** Wilms tumor 1| ***PHF6*** Plant homeodomain (PHD)-like finger 6| ***TET1/TET2*** Ten-eleven translocation methylcytosine deoxygenase 1/2| ***IDH1/IDH2*** Isocitrate dehydrogenase 1/2 |***FLT3*** FMS-like tyrosine kinase 3 |***KIT*** v-kit Hardy Zuckerman 4 feline sarcoma viral oncogene homologue| ***KRAS/NRAS,*** v-K/N-ras 2 Kirsten rat sarcoma viral oncogene homolog| ***PTPNX*** protein tyrosine phosphatase, non-receptor type X |***KTM2A*-X-fusions** Lysine [K]-specific methyltransferase 2A |***CEBPA*** CCAAT/enhancer-binding protein α |***KTM2A-PTD*** Lysine [K]-specific methyltransferase 2A-*partial tandem duplication* |***NUP98-NSD1*** Nucleoporin 98- Nuclear receptor binding SET domain 1 |***ASXL1*** Additional sex combs like 1 gene |***EZH2*** Enhancer of zeste homolog 2 |***STAG1/ STAG2*** Stromal antigen 1/2 |***SMC3*** Structural maintenance of chromosomes 3| ***SRSF2*** Splicing factor, arginine/serine-rich 2.

1.1.3 Clinical presentation

The clinical picture of AML is dominated by symptoms caused by bone marrow failure. Inhibition of normal hematopoiesis can occur as a consequence of bone marrow replacement by leukemic blasts or inhibition of normal myeloid progenitors to differentiate and maturate. As a consequence, patients present with symptoms of anemia, thrombocytopenia with increased risk of bleeding and neutropenia which prone the patient to infections. In approximately 5 % of patients, extramedullary involvement (chloromas or myeloid sarcomas) can occur at various sites (skin, bones, gastrointestinal tract, lymph nodes). AML with extramedullary manifestations more often associates with trisomy 8, t(v;11q23), inv(16)(p13.1q22), t(8;21)(q22;q22) or *NPM1* mutation (*Pileri S.A. et al. 2007)*. Organ infiltration by leukemic blasts is usually present in AML with a high white blood cell count exceeding 50 G/I and predominantly affects the lungs and the brain (*Zuckerman T. et al. 2012*). The association of clinical, morphological and molecular genetic manifestations in AML is summarized in **Table 2.**

Table 2. Representation of the association between genetic subgroups of AML with morphologic classification according to the FAB classification and their incidence (*Estey H. and Döhner H. 2006, Pileri S.A. et al. 2007)*

Translocations and Inversions	Gene fusions or rearrangements	Morphologic association	Incidence
t(8;21)(q22;q22)	*RUNX1-RUNX1T1*	M2 with Auer rods	5 %
inv(16)(p13.1q22) or t(16;16)(p13.1;q22)	*CBFß-MYH11*	M4Eo	5-8 %
t(15;17)(q22;q12)	*PML-RARA*	M3 or M3v	2 %
t(9;11)(p22;q23)	*KMT2A-AF9*	M5	1 %
t(6;11)(q27;q23)	*KMT2A-AF6*	M4 or M5	1-2 %
inv(3)(q21q26.2) or t(3;3)(q21;q26.2)	*GATA2-EVI1 (MECOM)*	M1, M4, M6, M7?	2 %
t(6;9)(p22;q34)	*DEK-NUP214*	M2, M4	2 %
Chromosomal imbalances			
-5/5q-	n.a.	No FAB preference	7 %
-7/7q-	n.a.	No FAB preference	7 %
+8	n.a.	M2, M4, M5	9 %

Table 2 continued on page 4

Continuation of table 2 from page 3

9q-	n.a.	No FAB preference	3 %
+11	*KMT2A?*	M1, M2	2 %
+13	n.a.	M0, M1	2 %
-17/17q-	*TP53*	No FAB preference	55
-20/20q-	n.a.	No FAB preference	3 %
+21	n.a.	No FAB preference	2 %
+22	n.a.	M4, M4Eo	3 %
Complex karyotype	n.a.	No FAB preference	10 %
Normal karyotype	n.a.	No FAB preference	45 %

Abbreviations: AML, acute myeloid leukemia| **FAB**, French American British Classification| **Eo**, Eosinophilia| **n.a**. not available ***RUNX1-RUNX1T1*** Runt-related transcription factor 1-Runt-related transcription factor 1 translocation |***CBFß-MYH11*** Myosin heavy chain 11- CCAAT/enhancer-binding protein α gene| ***PML-RARA*** Promyelocytic leukemia/retinoic acid receptor alpha fusion gene| ***KMT2A-AF6/9,*** Lysine [K]-specific methyltransferase 2A-ALL1 fused gene from chromosome 6/9| ***GATA2,MECOM (EVI1*)**, GATA binding protein 2-Ecotropic Viral Integration Site 1 **(EVI1)** and Myelodysplastic Syndrome 1| ***DEK-NUP214*** DEK proto oncogene-Nucleoporin 214| **"-"** and **"+"**, loss or gain of a whole chromosome or chromosome region| **p** and **q**, short and long arms of chromosomes| **M1-7**, FAB morphologic categories of AML

1.1.4 Diagnosis of AML

Diagnosis of AML is based on cytomorphology, histology, flow cytometry and genetic markers. A minimum of 20 % blasts in the bone marrow and/or peripheral blood is required for the diagnosis of acute leukemia. A trephine biopsy is not routinely indicated except for those cases with dry tap. For cases with t(8;21)(q22;q22), inv(16)(p13.1q22) or t(15;17)(q22;q22) the presence of the cytogenetic abnormality is sufficient for the diagnosis and the cut off of 20 % blasts is not mandatory for defining myeloid leukemia. Cytochemistry (using myeloperoxidase, nonspecific esterase, or Sudan Black) or flow cytometry are used for precise lineage specification. In the initial work up, cytogenetic and molecular genetic characterization is mandatory for prognostic and treatment decision. The current 2008 World Health Organization (WHO) of Tumors of Hematopoietic and Lymphoid Tissues defines 4 major categories of AML: *AML with recurrent genetic abnormalities, AML with myelodysplasia-related changes, therapy-related AML and AML otherwise not specified* **(Table 3)**. In the upcoming WHO Classification, the provisional entities "*AML with mutated NPM1*" and "*AML with mutated CEBPA*" will become entities

(*Döhner H. et al. 2015*). AML with *CEBPA* mutation will include only biallelic *CEBPA* mutation, as only this form is associated with distinct clinicopathologic features and favorable prognosis. In addition, 2 new provisional entities "*AML with RUNX1 mutation*" and "*AML with BCR-ABL1 gene fusion"* and a new section of *Familial myeloid neoplasm* will be added (*Döhner H. et al. 2015*).

Table 3. The current WHO Classification of acute myeloid leukemia and related neoplasms (*Vardiman J.W. et al. 2008*)

Acute myeloid leukemia with recurrent genetic abnormalities
AML with t(8;21)(q22;q22); *RUNX1-RUNX1T1*
AML with inv(16)(p13.1q22) or t(16;16)(p13.1;q22); *CBFß-MYH11*
APL with t(15;17)(q22;q12); *PML-RARA*
AML with t(9;11)(p22;q23); *MLLT3-KMT2A*
AML with t(6;9)(p12;q34); *DEK-NUP214*
AML with inv(3)(q21q26.2) or t(3;3)(q21;q26.2); *RPN1-EVI1*
AML (megakaryoblastic) with t(1;22)(p13;q13); *RBM15-MKL1*
Provisional entity: AML with mutated *NPM1*
Provisional entity: AML with mutated *CEBPA*
Acute myeloid leukemia with myelodysplasia-related changes
Therapy-related myeloid neoplasm
Acute myeloid leukemia otherwise not specified
AML with minimal differentiation
AML without maturation
AML with maturation
Acute myelomonocytic leukemia
Acute monoblastic/monocytic leukemia
Acute erythroid leukemia
• Pure erythroid leukemia
• Erythroleukemia, erythroid/myeloid
Acute megakaryoblastic leukemia
Acute basophilic leukemia
Acute panmyelosis with myelofibrosis
Myeloid sarcoma
Myeloid proliferations related to Down syndrome

Table 3 to be continued on page 6

Continuation of table 4 from page 5

Transient abnormal myelopoiesis
Myeloid leukemia associated with Down syndrome
Blastic plasmacytoid cell neoplasm
Abbreviations: **AML**, acute myeloid leukemia| **APL**, acute promyelocytic leukemia| ***RUNX1-RUNX1T1*** Runt-related transcription factor 1-Runt-related transcription factor 1 translocation | ***DEK-NUP214*** DEK proto oncogene-Nucleoporin 214| ***RPN1-EVI1*** Ribophorin 1-Ecotropic Viral Integration Site 1 and Myelodysplastic Syndrome 1|***CBFß-MYH11*** Myosin heavy chain 11- Core binding factor beta | ***RBM15-MKL*** RNA binding motif protein 15 **-** Megakaryoblastic Leukemia (Translocation) 1| ***NPM1*** *Nucleophosmin 1*| ***CEBPA*** CCAAT/enhancer-binding protein α|**WHO** World Health Organisation

1.1.5 Cytogenetics and molecular genetics of AML

Since the discovery of the genomes pivotal role in cancer development by the work of David von Hansemann and Theodor Boveri in the early twenties and the identification of the reciprocal translocation t(9;22)(q34;q11.2) associated with a particular type of cancer by Janet Rowley in the seventies, an immense progress has been made in elucidating the underlying mechanisms of oncogenesis, mainly due to implementation of new technologies. Recent data based on gene sequencing studies showed that among various cancer genomes, AML is characterized by a relatively low number of mutations with a median of 0.5 somatic mutations per Mb as compared with other hematologic cancers (e.g. multiple myeloma with over 1 somatic mutation per Mb) or solid tumors (e.g. malignant melanoma with the highest frequency of mutations of more than 10 per Mb) (*Alexandrow L.B. et al. 2013*). This translates into an average of only 13 mutations per AML patient with an average of 5 recurrently mutated genes in AML *(Ley T.J. et al. 2013).* At the microscopic level using conventional cytogenetic analysis approximately 55 % of AML cases harbor chromosomal abnormalities and 45 % of the cases have a normal karyotype (*Mrozek K. et al. 2004, Grimwade D. 2001).* To date, more than 300 recurrent chromosomal abnormalities have been described in AML (*Mitelman F. et al. 2007*). Pretreatment cytogenetic findings are the most important independent prognostic factors for achievement of complete remission (CR), event-free survival (EFS) and overall survival (OS) (*Mrozek K. et al. 2004, Grimwade D. 2006*). Among AML with normal karyotype outcome is very different due to the molecular heterogeneity. Thus, further classification and prognostication systems based on molecular markers are important for treatment decision in these patients.

1.1.6 Risk stratification and prognostic markers

Prognostic factors in AML can be divided into patient-specific and disease-associated factors. The most important patient-related prognostic factors are age and performance status (*Juliusson G. et al. 2009*). Cytogenetic and molecular genetic markers represent the most powerful disease-specific prognostic factors for outcome and treatment decisions (*Mrozek K. et al. 2004, Grimwade D. et al. 2006*). The current European LeukemiaNet (ELN) Classification system integrated cytogenetic and molecular genetic markers to divide cases into 4 genetic groups (*Döhner H. et al. 2010, Döhner H. et al. 2015*) **(Table 4)**.

Table 4. The genetic risk stratification of AML into 4 risk groups based on the European LeukemiaNet Classification **(***Döhner H. et al. 2010, Döhner H. et al. 2015)*

Genetic risk group	Subset
Favorable	t(8;21)(q22;q22); *RUNX1-RUNX1T1*
	inv(16)(p13.1q22 or t(16;16)(p13.1;q22); *CBFß-MYH11*
	Normal karyotype with mutated *NPM1* without *FLT3*-ITD mutation
	Normal karyotype with biallelic* mutated *CEBPA*
Intermediate I	Normal karyotype with mutated *NPM1* and *FLT3*-ITD mutation
	Normal karyotype with wild type *NPM1* and *FLT3*-ITD mutation
	Normal karyotype with wild type *NPM1* and without *FLT3*-ITD mutation
Intermediate II	t(9;11)(p22;q23); *MLLT3-KMT2A*
	Cytogenetic abnormalities not classified as favorable or adverse **
Adverse	inv(3)(q21q26.2) or t(3;3)(q21;q26.2); *GATA2,MECOM (EVI1)**
	t(6;9)(p23;q34); *DEK-NUP214*
	t(v;11)(v;q23); *KMT2A*-rearranged
	-5 or del(5q), -7, abnl(17p), complex karyotype***

Abbreviations: **AML**, acute myeloid leukemia| **APL**, acute promyelocytic leukemia| ***RUNX1-RUNX1T1*** Runt-related transcription factor 1-Runt-related transcription factor 1 translocation| ***CBFß-MYH11*** Myosin heavy chain 11- Core binding factor beta| ***NPM1*** *Nucleophosmin 1*| ***FLT3***-ITD| ***CEBPA*** CCAAT/enhancer-binding protein| ***KTM2A-PTD*** Lysine [K]-specific methyltransferase 2A-*partial tandem duplication*|| ***GATA2,MECOM (EVI1),*** GATA binding protein 2-Ecotropic Viral Integration Site 1 **(EVI1)** and Myelodysplastic Syndrome 1|| ***DEK-NUP214*** DEK proto oncogene-Nucleoporin 214| ***MLL*** Myeloid/lymphoid or mixed-lineage leukemia| **"-"** and **"+"**, loss or gain of a whole chromosome or chromosome region| **p** and **q**, short and long arms of chromosomes

*****AML with *CEBPA* mutation is restricted to biallelic *CEBPA* mutation; for inv(3)/t(3;3) *RPM1-EVI1* has been changed in *GATA2-MECOM (EVI1*) (*Gröschel S. et al. 2014)*; the current official gene symbol for MLL is *KMT2A* (lysine-[K]-specific methyltransferase 2A*)*

**For most abnormalities adequate numbers have not been studied to draw firm conclusions regarding their prognostic significance

***A complex karyotype is defined as 3 or more chromosome abnormalities in the absence of one of The WHO designated recurring translocations or inversions: t(15;17), t(8;21), inv(16) or t(16;16), t(9;11), t(v;11)(v;q23), t(6;9) and inv(3) or t(3;3).

Currently, for patients with normal karyotype mutations in *NPM1*, *FLT3* and *CEBPA* are used in clinical practice to further stratify patients (*Döhner K. et al. 2014*). Other markers such as *DNMT3A, ASXL1, IDH1, IDH2, TET2* and *RUNX1* have already been shown prognostic relevance but so far have not entered clinical practice.

AML with *NPM1* mutation

NPM1 mutations are one of the most frequent mutation found in adult AML. They are detected in ~ 25-35 % of AML and in ~ 45-65 % of cytogenetically normal AML (CN-AML) (*Falini B. et al. 2005*). Mutations in exon 12 cause abnormal cytoplasmic localization of the protein by loss of the tryptophan residues at the N-terminus generating a nuclear export signal. *NPM1* mutations occur frequently as single mutations or in association with *FLT3*-ITD mutations (~32.5 %) (*Falini B. et al. 2005*). Chromosomal abnormalities frequently associated with *NPM1* mutations are trisomy 8 and deletion 9q. *NPM1* mutations have been shown to be mutually exclusive of other recurrent genetic abnormalities (*Falini B. et al. 2005*). Clinically they are associated with distinct features like female predominance, high bone marrow blast count, elevated lactate dehydrogenase (LDH) serum levels, myelomonocytic differentiation as well as a specific immunophenotype with low or absent CD34 and high CD33 expression (*Falini B. et al. 2005).* Patients with the *NPM1*^{mut}/*FLT3*-ITD$^{negative\ (neg)}$ genotype do significantly better than patients with *NPM1* wildtype status (*Schlenk R.F. and Döhner K. et al. 2008*). So far, no molecular therapy has proven its efficiency for *NPM1* mutated AML but there are data showing a potential benefit of all-trans retinoic acid in this subset of patients (*Schlenk R.F. and Döhner K. et al. 2008*). Recently Falini (*Falini B. et al. 2015*) reported successful treatment of AML with *NPM1* mutation with dactinomycin, a drug used in the treatment of nephroblastoma. Up to now 7 patients received treatment with dactinomycin, 1 patient as first line and 6 patients as second line treatment. Three patients achieved complete hematologic remission. Two groups showed that the combination of all-trans retinoic acid (ATRA) with arsenic trioxide (ATO) acts synergistically in *NPM1* mutated cell lines and primary patients cells to induce apoptosis via proteasome degradation. In vivo, the combination of ATO/ATRA reduced blast counts in the bone marrow and restored subnuclear localization of NPM1 and PML (*El Hajj H. et al.*

2015). Regarding the role of allogeneic cell transplantation (alloHSCT) for the *NPM1*$^{mut/}$ /*FLT3*-ITD^{neg} genotype, there appears to be no benefit when performed in first complete remission *(Schlenk R.F. and Döhner K. et al. 2008, Döhner K. and Döhner H. 2008*).

AML with *FLT3* mutation

FLT3 belongs to the class III receptor tyrosine kinases family and plays an important role in proliferation, survival and differentiation of normal hematopoietic cells. *FLT3* mutations are identified in approximately 40-48 % of CN-AML and are frequently associated with *NPM1* mutations (*Falini B. et al. 2005, Schlenk R.F. et al. 2008).* In AML, *FLT3* mutations lead to ligand independent constitutive activation of the receptor and activation of downstream signaling. Mutations affect the juxtamembrane domain (JM) or the tyrosine kinase domain (TKD). Mutations in the JM domain are detected in about 28-34 % of CN-AML most frequently as internal tandem duplications (ITD) of various sizes and insertion sites. Point mutations in the JM are rare. Mutations affecting the TKD are point mutations affecting codons 835 or less frequent 836 and 842 and are detected in 11-14 % of CN-AML. *FLT3* mutations are associated with inferior outcome (*Levis M. and Small D. 2005*). The *FLT3* ITD mutant/wild type ratio was identified as a negative prognostic factor. Patients with allelic ratios ≥0.5 have lower CR rates irrespective of the *NPM1* status (*Kayser S. et al. 2013).* To date several *FLT3* inhibitors are tested in clinical trials, some of them with promising therapeutic effects (*Wander S.A. et al. 2014).* The addition of the multikinase inhibitor Midostaurin to chemotherapy improved outcome in younger patients with AML with activating *FLT3* mutations (5 year OS 50.8 % vs 43.1 %, *P*=0.007, 5 year EFS 26.7 % vs 19.1 %, *P*=0.0044) (*Stone R.M. et al. 2015*).

AML with *CEBPA* mutations

CCAAT/enhancer binding protein alpha (C/EBP α) functions as a myeloid transcription factor. Disruption of the gene leads to selective granulocyte differentiation block (*Zhang D.E. et al. 1997*). Somatic mutations in the *CEBPA* gene are found in 5-10 % of adult AML (*Fröhling S. et al. 2007)* with the majority in the subgroup of CN-AML. Mutations affecting the N-terminal transactivation domain generate a truncated isoform with dominant negative properties and mutations affecting the C-terminal region leucine zipper domain generate proteins with decreased DNA binding capacity (*Fröhling S. et al. 2004).* Epigenetic silencing by the *CEBPA* promoter hypermethylation has been recently reported. In one third of the cases a single mutation is identified (single mutant, *CEBPA*^{sm}) while in two thirds both the

N- and C-terminus are affected by mutations (biallelic mutated, *CEBPA*^{dm}). Only biallelic *CEBPA* mutations harbor a specific gene signature and have a favorable prognosis (*Taskesen E. et al. 2011, Wouters B.J. et al. 2009).* Similar to the *NPM1* mutations, there appears to be no benefit for alloHSCT in first CR. In addition to somatic mutations, germline *CEBPA* mutations were identified. Familial AML with *CEBPA* mutations carry biallelic mutations of which one is inherited in autosomal dominant manner with high penetrance and the second one is acquired (*Tawana K. et al. 2015*).

DNMT3A mutations in AML

The *DNA (cytosine-5)-methyltransferase 3A* (*DNMT3A*) gene is located on 2p23.3. Mutations in *DNMT3A* are detected in 18-22 % of all AML and in 30-37 % of CN-AML *(Ley T.J. et al. 2010).* About 80 % of mutations cluster in exon 23 at codon R882 that is located in the DNA-binding domain. *DNMT3A* has a crucial function in stem cells; it was demonstrated that Dnmt3a-/- hematopoietic stem cells have a selective advantage over other cells in the bone marrow *(Yang L. et al. 2015)*. Furthermore, *DNMT3A* plays a role in epigenetic modifications necessary for mammalian development and cell differentiation. The prognostic impact of *DNMT3A* mutations varies across studies. The negative impact on outcome is restricted to the unfavorable molecular subgroup of CN-AML as defined by the ELN Classification (*FLT3*-ITD mutated, *NPM1* wildtype) (*Döhner H. et al. 2010, Döhner H. et al. 2015).* In addition, the impact of different mutations types needs further investigations as mutations in codon R882 were associated with a better OS in younger patients with CN-AML (*Gaidzik V.I. et al. 2013). DNMT3A* mutations were identified also in clonal hematopoiesis in healthy elderly persons (*Genovese G. et al. 2014).*

IDH1 and *IDH2* mutations in AML

Somatic mutations in the genes encoding isocitrate dehydrogenase 1 and 2 (*IDH1* and *IDH2*) are present in approximately 25-30 % percent of newly diagnosed AML (*Mardis E.R. et al. 2009*). Mutant IDH1 can heterodimerize with wild type IDH1 to create a mutant enzyme that converts α-ketoglutarate to 2-hydroxyglutarate (2-HG) which acts as an oncometabolite that blocks differentiation *(Dang L. et al. 2009).* The data regarding the prognostic value of *IDH1*/*2* mutations are conflicting. In one study, *IDH1* and *IDH2* mutations were associated with an unfavorable prognostic in the subtype of AML with *NPM1* mutation without *FLT3* ITD mutation. In another study, *IDH2* mutations were associated with improved outcome but the benefit was restricted to *IDH2* R140Q mutations (*Patel J.P. et al. 2012).* In one study,

patients with *IDH1/IDH2* mutations had significantly increased serum 2-HG levels and normalization of 2-HG levels after induction therapy was associated with better OS and DFS (*Janin M. et al. 2014).* Clinical trials with agents targeting IDH1 and IDH2 are ongoing (*Stein E.M. et al. 2014).*

***ASXL1* mutations in AML**

The additional sex combs gene (*ASXL1*) is a human analog of the Drosophila gene located on chromosome 20q11. Mutations in the *ASXL1* gene are present in 5-12 % of CN-AML (*Gelsi Boyer V. et al. 2012).* The incidence of *ASXL1* mutations increases with age and is higher in patients with secondary AML. Mutations in *ASXL1* have been associated with poor prognosis in particular the $ASXL1^{mut}/RUNX1^{mut}$ genotype (*Paschka P. et al. 2015).*

***TET2* mutations in AML**

*TET*2 mutations are identified in 9-23 % of CN-AML with an increasing incidence with age (*Delhommeau F. et al. 2009).* The prognostic impact of *TET*2 mutations in AML is not clearly established. For example, in one study (*Metzeler K.H. et al. 2011) TET2* mutations conferred an unfavorable prognosis in terms of achieving CR and EFS only among molecular favorable CN-AML as defined by the ELN Classification (*Döhner H. et al. 2010*). In contrast, the study by Gaidzik et al (*Gaidzik V.I. et al. 2012*) failed to show any prognostic effect of *TET*2 mutations in either CN-AML patients or ELN subgroups.

1.1.7 Treatment of AML

The basic concept of treatment in AML has not changed over the last 20-30 years (*Döhner H. et al. 2015*). In patients deemed eligible for intense chemotherapy treatment is divided in 2 phases, induction therapy followed by consolidation therapy.

Induction therapy. The backbone of induction is the combination of an anthracycline (daunorubicin, idarubicin or mitoxantrone) with cytarabine (Ara-C). Currently used doses are Ara-C given as 100-200 mg/m^2 intravenous (i.v.) continuous infusion (c.i.) for 7 days in combination with daunorubicin 60 mg/m^2 i.v. for 3 days in the so-called "3+7" induction regime. Higher doses of daunorubicin did not prove superiority in achieving CR or OS. For patients who do not achieve CR after one induction cycle a second cycle is usually given (*Döhner H. et al. 2015*). The response rates after induction therapy vary between 60-80 %

in patients younger than 60 years and 40-60 % in patients over 60 years (*Schlenk R.F. and Döhner H. 2013).* The strongest prognostic factor for achieving remission is the pretreatment karyotype (*Mrozek K. et al. 2004, Grimwade D. 2011).* **Table 5** summarizes response rates to therapy according to different cytogenetic and molecular genetic risk groups.

Table 5. Response to therapy showing complete remission rates according to molecular and cytogenetic risk factors for AML patients younger and older than 60 years who received intensive treatment and for AML patients older than 60 years not-intensively treated (*Schlenk R.F. et al. 2015*)

Complete remission rate			
	Age <60 years Intensive induction	**Age ≥60years Intensive induction**	**Age ≥60 years Non-intensive treatment**
Favorable marker			
t(8;21)(q22;q22)	80-90 %	70-80 %	n.a
inv(16)/t(16;16)	80-90 %	70-80 %	n.a.
NPM1 mutation	80-90 %	80-90 %	50 %
Biallelic *CEBPA* mutation	80-90 %	n.a.	n.a.
Unfavorable marker			
Monosomal karyotype	30-35 %	30-35 %	n.a.
TP53 alterations	25-30 %	25-30 %	n.a.
inv(3) or t(3;3)	31 %	n.a.	n.a.

Abbreviations: AML, acute myeloid leukemia| **CR**, complete remission| **n.a**., not available |***NPM1*** Nucleophosmin 1| ***CEBPA*** CCAAT/enhancer-binding protein α| ***TP53*** Tumorprotein 53|

Consolidation therapy. Postremission therapy consists of conventional chemotherapy and alloHSCT. Factors that guide decisions whether a patient should be offered conventional chemotherapy or alloHSCT are the genetic risk profile and the general health status as assessed for example by the hematopoietic cell transplantation comorbidity index (HSCT-CI) *(Sorror M.L. et al. 2005).* Consolidation with intensive chemotherapy consists of intermediate-dose Ara-C (1000-1500 mg/m^2 intravenously every 12 hours for 3 days or once daily for 6 days for 2-4 cycles). These regiments are used for patients younger than 60 years in the favorable ELN risk group or patients in the intermediate (intermediate I & II) or adverse risk group in whom an alloHSCT is not possible. Patients older than 60 years may be given reduced intensity chemotherapy regimens.

Allogeneic hematopoietic cell transplantation is indicated in patients in the ELN intermediate (intermediate I & II) and adverse genetic groups who are not likely to achieve durable remissions with conventional chemotherapy. In alloHSCT the antileukemic effect is the result of both conditioning chemotherapy and immunologic effect of graft versus leukemia (*Döhner H. et al. 2015*).

Patients who are ineligible for intensive chemotherapy are older patients with unfavorable genetic risk and comorbidities that contraindicate intensive treatment. Therapy options for these patients consists of low-dose Ara-C (20 mg s.c. every 12h for 7 days as 4 weeks cycles) with a median OS of 5-6 months, hypomethylating agents such as decitabine (20mg/m^2 i.v. on days 1-5 as 4 weeks cycles) with a median OS of 7.7 months or azacitidine (75mg/m^2 s.c. for 7 days, as 4 week cycle) with a median OS of about 10 months (*Döhner H. et al. 2015).*

In the last years, some molecular markers such as *NPM1, RUNX1-RUNX1T1, CBFß-MYH11 and KMT2A-MLLT3* have been established for measuring minimal residual disease. This allows a close disease monitoring and early salvage intervention before hematologic relapse (*Krönke J. et al. 2011, Corbacioglu A. et al. 2010, Grimwade D. and Freemann P. 2014).*

Treatment of relapsed AML. In most patients relapse occurs within the first 3 years after diagnosis (*Döhner H. et al. 2015*). Frail patients ineligible for intensive treatment are treated with best supportive care or may be enrolled in clinical trials with investigational agents. Fit patients can be offered intensive chemotherapy followed by alloHSCT if they achieve complete remission. Salvage chemotherapy regimens consist of cytarabine alone, cytarabine in combination with daunorubicin or with mitoxantrone and etoposide or as FLAG-IDA regimen (combination of fludarabine, cytarabine and idarubicin) (*Döhner H. et al. 2015).*

Novel therapies. A broad spectrum of new drugs targeting different leukemic pathways are currently being tested in clinical trials as single agents or in combination with chemotherapy. An overview of some of the current targeted agents is summarized in **Table 6**.

Table 6. Targeted therapies currently under investigation in AML (adapted from *Döhner H. et al. 2015*)

Drug Class	Agents
Epigenetic modifiers	AG-120 (IDH1 inhibitor), AG-221 (IDH 2 inhibitor), SGI-110 (2nd generation hypomethylating agent)
Tyrosine kinase inhibitors	Sorafenib, Midostaurin (1st generation), Quizartinib, Crenolanib (2nd generation), Dasatinib (KIT inhibitor)

Table 6 to be continued on page 14

Continuation of table 6 from page 13

Cell-cycle and signaling inhibitors	Volasertib (PLK inhibitor), Palbociclib (CDK inhibitor)
Nuclear export inhibitor	Selinexor (CRM1 inhibitor)
Antibody based therapies	Gemtuzumab Ozogamicin (anti-CD33), AMG 330 (anti-CD33 and anti-CD3 bispecific T cell engager), CART-123, Ipilimumab (immune check point blockade)
Cytotoxic agents	Vosaroxin (Quinolone derivate), Clofarabine & Cladribine (nucleoside analogues)
Other agents	Venetoclax (BCL2 inhibitor), ATRA, Lenalidomide (Immunomodulatory drug).

Abbreviations: **AML**, acute myeloid leukemia| ***IDH1/IDH2*** Isocitrate dehydrogenase 1/2| B-cell CLL-lymphoma 2 protein| **CRM1** inhibitor, chromosome region maintenance 1| **CD,** cluster of differentiation| **PLK,** polo like kinase| **CDK** cyclin dependent kinase| **CART** chimeric antigen receptor T cells| **ATRA** all-trans retinoic acid| **CD,** cluster of differentiation.

1.2 *RUNX1* mutations in myeloid malignancies

1.2.1 *RUNX1* mutations-general considerations

RUNX1 located on chromosome band 21q22.12, belongs to the *RUNX* (*Runt-related transcription factor*) gene family that encodes transcription factors important for differentiation and development. In mammalians there are 3 family members encoded by *RUNX1*, *RUNX2* and *RUNX3* genes with no redundant tissue specific functions. The RUNX transcription factors are composed of an α subunit that binds DNA via the *Runt* domain and a CBFß subunit that increases the affinity of the α subunit for DNA without DNA binding itself. All proteins have a conserved 128 amino acid *Runt* domain. RUNX1 has a nuclear localization and is widely expressed in hematopoietic cells with an essential role in the development and maintenance of hematopoiesis. In mouse models, lack of Runx1 gene impairs definitive hematopoiesis and cause embryonic death. In adult hematopoiesis, disruption of the *Runx1* gene by intragenic mutations leads to a preleukemic state that predisposes to AML (*Ichikawa M. et al. 2004, Mangan J.K. et al. 2011).* Recent experiments addressing the role of *RUNX1* in hematopoiesis proved that *RUNX1* acts in a stage-dependent manner as a positive regulator of cell adhesion and migration associated genes, prior to the emergence of hematopoietic cells by down regulation of the endothelial program. Consistent with that, *RUNX1* controls the expression of CD61 integrin at the cell surface in the

hemogenic endothelium required for the surface expression of CD41, an early hematopoietic marker that mediates adhesion of hematopoietic progenitors to the marrow niches. In the absence of *RUNX1*, the early hematopoietic transition is blocked as demonstrated by the lack of CD41/CD61 expression (*Liakhovitskaia A. et al. 2014, Lie-A-Ling M. et al. 2014).*

RUNX2 is involved in bone formation and mutation in the *RUNX2* gene causes cleidocranial dysplasia, an inherited skeletal disorder. *RUNX3* deficiency has been associated with precancerous state as *Runx3* deficient mice are prone to spontaneous solid tumor formation, i.e., development of colon, breast, lung or bladder tumors, indicating a tumor suppression function for *RUNX3 (Ito Y. et al. 2015).*

In mammalians three functional domains of the RUNX1 protein are defined **(Figure 1)**:

1) The N-terminal domain or *Runt* homology domain (RHD) spans exons 3-5 with 128-amino-acid and is highly conserved within all family members with a homology of 90 % and also evolutionary from Drosophila to humans (*Yto I. et al. 2015).* Downstream to the RHD there is less homology between the RUNX family members explaining in part the different function of each protein. In all family members, via the *Runt* domain, RUNX proteins form heterodimers with the transcriptional co-activator CBFß and recognize the same DNA consensus sequence PyGPyGGTPy. The RHD molecule is made of 12 ß strains separated by loops that form an S-type immunoglobulin fold providing the scaffold for interaction with either DNA or the CBFß. The interaction with the CBFß stabilizes the CBFα–CBFß complex, enhances its DNA binding affinity and protects it from ubiquitination and degradation *(Yto Y. et al. 2015, Tahirov T.H. et al. 2001).*

2) The second well-characterized domain is the transactivation domain (TA) spanning exons 7-8 located between the C-terminus domain and the *Runt*-domain.

3) The third domain, the inhibitory domain (ID) at the C-terminus, is less well characterized and is thought to be involved in downregulation of gene expression *(Schmit J.M. et al. 2015).* The ID modulates the DNA binding potential of RUNX1. Deletion in the C-terminal sequences with loss of the DNA binding inhibitory domain produces a 40-fold increase in DNA binding capacity *(Speck N. et al. 2011, Matheney C.J. et al. 2007, Ichikawa M. et al. 2013).*

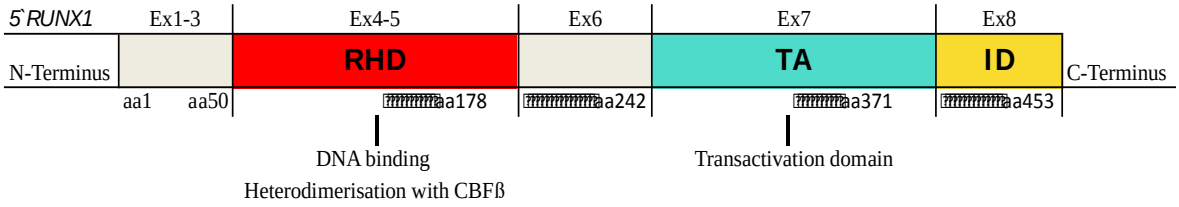


Figure 1. Schematic representation of the RUNX1 protein domains. The 8 exons of the *RUNX1* gene and the corresponding amino acid sequences are illustrated in the colored boxes from N-to C-Terminus. The red box shows the Runt homology domain, responsible for the DNA binding and heterodimerisation with the CBFß. The blue box shows the transactivation domain and the yellow box, the inhibitory domain. (aa1-453 amino acids 1-453| RHD Runt homology domain| TA Transactivation domain| ID Inhibitory domain| EX1-8 Exons 1-8|CBFß Core binding factor ß).

Each domain can be affected by mutations. Depending on the location and functional consequence of the mutation, they are divided into 4 categories (*Harada H. et al. 2009, Speck N. et al. 2011, Matheney C.J. et al. 2007 and Ichikawa M. et al. 2013)*:

1) N-terminal, truncation (Nt): nonsense mutations (arising when a premature nonsense or stop codon is introduced in the DNA sequence; the resulting protein is incomplete, shorter than normal and mostly nonfunctional) and frameshift mutations (arising when the normal sequence of codons is disrupted by the insertion or deletion of one or more nucleotides, provided that the number of nucleotides added or removed is not a multiple of three) and result in partial deletion of the RHD and total loss of the C-terminal region. Functionally, they induce loss of the DNA binding capacity and transactivation potential.

2) N-terminal missense mutations (arising when the change of a single base pair causes the substitution of a different amino acid in the resulting protein) or insertions (Ni) are located in the three protein loops, ß (A-B), ß (E-F) and ß G that mediate DNA binding potential. The functional consequence is reduced or loss of the DNA binding ability.

3) C-terminal truncation mutations (Ct) show enhanced DNA binding ability but no transactivation potential.

4) Chimera like mutations (Cc) in the C-terminal region are longer and display reduced DNA binding potential without transactivation potential.

1.2.2 *RUNX1* mutations in AML

In acute leukemia the *RUNX1* gene can be altered by chromosomal translocations, copy number variations (CNV) or point mutations. *RUNX1* is one of the genes most commonly disrupted by balanced translocations in AML (*Okuda T. et al. 1996).* Up to 39 different translocation partners generating fusion genes have been described (*de Braekeleer E. et al. 2009).* In translocations involving *RUNX1*, the fusion proteins retain the N-terminal domain but lack the C-terminal regulatory domain. One exception is the *RUNX1-ETV6* fusion gene where the 3'sequence of *RUNX1* fuses to the 5'sequence of *ETV6* retaining the RHD; thus, fusion proteins lose the transcription activation potential but are still capable of competing with RUNX1 wildtype protein for DNA binding through a dominant negative effect against RUNX1 wild type protein (*de Braekeleer E. et al. 2009).* In addition to balanced translocations, intragenic *RUNX1* mutations have been identified in AML with an incidence ranging from 5-10 % among different groups (*Tang J.L. et al. 2009, Dicker F. et al. 2010, Gaidzik V.I. et al. 2011, Schnittger S. et al. 2011, Mendler J.H. et al. 2012, Greif P. et al. 2012*).

1.2.3 *RUNX1* mutations in other myeloid neoplasm

***RUNX1* mutations in myelodysplastic syndrome (MDS)**

In MDS, *RUNX1* is one of the most frequently mutated genes with an incidence of 10-20 % with a high frequency in therapy related MDS (t-MDS). *RUNX1* mutations have been associated with more advanced disease stages, rapid progression to AML irrespective of the International Prognostic Scoring System and decreased OS *(Bejar R. et al. 2011, Haferlach T. et al. 2014, Papaemmanuil E. et al. 2013, Tang J.L. et al. 2009, Dicker F. et al. 2010).*

Balanced translocations involving the *RUNX1* gene are very rare in MDS and are almost exclusively found in AML. In contrast, point mutations are found in both AML and MDS. An important observation regarding the role of *RUNX1* mutations in MDS/AML results from the studies on atomic bomb survivors. A high incidence of somatic point mutations (N=6/13, 46 %) in the *RUNX1* gene was identified among MDS patients who survived atomic bomb in Hiroshima indicating a high susceptibility to radiation of the *RUNX1* gene. Half of the patients had a late MDS onset and were exposed to low dose of radiation (below 50cGy). Similar findings were reported in patients with MDS living in the vicinity of the

Semipalatinsk Nuclear Testing Site. Of 18 exposed patients with MDS/AML, 7 harbored *RUNX1* point mutations comparing to none in 13 patients with MDS/AML without exposure. These findings support the correlation of *RUNX1* point mutations and radiation exposure in a dose-dependent manner. In contrast to acute leukemias resulting from exposure to chemo- and / or radiotherapy where double strand DNA breaks occur, the low dose radiation induces point mutations but further genetic alterations are needed to develop MDS. This resembles the course of FDP with an adult onset of AML indicating that *RUNX1* germline mutations alone are not sufficient for inducing the leukemic phenotype (*Harada H. et al. 2003, Zharlyganova D. et al. 2008).* Frequent co-occurring mutations are mutations in genes involved in RNA splicing (*SRSF*2), cohesion complex (*STAG*2) and chromatin modifiers (*ASXL1* and *BCOR*) *(Haferlach T. et al. 2014, Papaemmanuil E. et al. 2013).*

***RUNX1* mutations in chronic myelomonocytic leukemia (CMML)**

For CMML data are restricted to two studies. In the study by Meggendorfer and colleagues *(Meggendorfer M. et al. 2012*) *RUNX1* mutations were identified in 61 of 274 (22 %) patients. In the second study *(Kuo M.C. et al. 2009)* the authors reported *RUNX1* mutations in 32 of 87 patients (37 %). Both analyses were performed in unselected patient cohorts and a trend towards progression to AML was demonstrated for mutations affecting the C-terminus *(Kuo M.C. et al. 2009).*

***RUNX1* mutations in myeloproliferative neoplasms (MPN)**

RUNX1 mutations could be detected during transformation to leukemic phase but not during the chronic phase of myeloproliferative neoplasms. One study (*Ding Y. et al. 2009)* identified that 28 % of patients with MPN had *RUNX1* mutations (5 of 18). Another study (*Zhao L.J. et al. 2012)* on blast crisis of chronic myeloid leukemia identified *RUNX1* point mutations in the RHD in 11 of 85 (12.9 %) patients. Taken together, these findings suggest that *RUNX1* mutations promote leukemic transformation in a subset of patients with MPN.

1.2.4 *RUNX1* mutations in preleukemic conditions

In the last years, more knowledge could be gained in identifying genetic syndromes causing bone marrow diseases and the upcoming WHO Classification dedicates a new section to

"*Familial neoplasms with Germline Predisposition*". More than 30 pedigrees with Familial Platelet Disorders/ Acute myeloid leukemia (FPD/AML) have been described within the last years. The disease is inherited in an autosomal dominant manner displaying a great genotypic and phenotypic variability *(Nickels E.M. et al. 2013).* Affected individuals harbor a heterozygous *RUNX1* mutation. The mutations are predominantly located at the N-terminus within the conserved RHD with few cases harboring mutations in the C-terminus. There was no specific type of *RUNX1* mutations; frameshift, missense or nonsense mutations as well as intragenic deletions were described. In all cases they disrupt the DNA binding capacity of the RUNX1 protein *(Be'ri-Dexheimer M. et al. 2008, Liew E. et al. 2011).* Notably, only 20-50 % of patients with *RUNX1* germline mutations and FPD progress into full-blown AML. The underlying mechanism of leukemogenesis is still to be discovered; due to the fact that not all affected individuals develop overt leukemia, it is hypothesized that *RUNX1* germline haploinsufficiency *per se* is not enough to induce leukemia and that a second genetic event is needed, for example the acquisition of a *de novo* mutation in the non-mutated allele, or the acquisition of cooperating somatic mutation *(Nickels E.M. et el. 2013, Preudhomme C. et al. 2009).*

Interestingly, also cases of T-acute lymphoblastic leukemia following FPD have been described *(Owen C.J. et al. 2008, Preudhomme C. et al. 2009).*

Another congenital disease recently shown to harbor a high frequency of somatic *RUNX1* mutations is severe congenital neutropenia with a 20 % risk of evolving into MDS or AML. Using Next-Generation Sequencing (NGS) and single-cell analysis, Skokowa J. et al. identified *RUNX1* mutations in 64 % of the patients with congenital neutropenia who developed MDS or AML; in addition, a strong cooperation of *RUNX1* mutations with mutations in the hematopoietic cytokine receptor *(CSFR3)* could be detected unraveling a novel pathway of leukemogenesis *(Skokowa J. et al. 2014).*

In Fanconi anemia it was demonstrated *(Quentin S. et al. 2011)* that disease progression from Fanconi anemia to MDS or acute leukemia is accompanied by secondary acquired genetic abnormalities such as -7/7q- or *RUNX1* abnormalities. The cumulative incidence of MDS or AML in Fanconi anemia patients by the age of 40 years ranges between 30-40 %. Using Comparative Genomic Hybridization (CGH), Single Nucleotide Polymorphism (SNP) array and Fluorescence in Situ Hybridization (FISH) analysis with *RUNX1* break-apart probes, a

total of 20 % of patients with Fanconi anemia and severe MDS or AML harbored *RUNX1* mutations in form of cryptic balanced translocations [i.e. t(1;21)(p36;q22); *PRDM16-RUNX1*], unbalanced translocations, deletions or insertions. In this situation, *RUNX1* mutations seem to be secondary genetic events that contribute to disease progression. No other AML-associated mutations such as *TP*53, *TET2*, *CBL*, *NPM1* and *CEBPA* were found.

1.3 Aims

In the last years, several groups have reported on the frequency and clinical significance of *RUNX1* mutations in AML patients (*Tang J.L. et al. 2009, Dicker F. et al. 2010, Gaidzik V.I. et al. 2011, Schnittger S. et al. 2011, Mendler J.H. et al. 2012, Greif P. et al. 2012).* One major drawback of most studies was patient selection, that is, only subsets of AML such as cytogenetically-normal AML, *de novo* AML, or younger adult patients, were included in the analysis, thereby providing biased results.

The aim of this work was to study the frequency and clinical impact of *RUNX1* mutations in a large, unselected cohort of adult patients with AML. Specific aims were: 1) to characterize the role of *RUNX1* mutation in adult AML patients focusing on the interaction with other molecular and cytogenetic markers 2) to evaluate its impact on treatment response and outcome and 3) to investigate distinct combined genotypes with regard to their specific clinical characteristics. All patients were enrolled in multicenter treatment trials of intensive therapy performed by the German-Austrian AML Study Group (AMLSG).

2. PATIENTS, MATERIALS AND METHODS

2.1 Patients and Samples

Diagnostic bone marrow (BM) or peripheral blood (PB) samples from 2439 AML patients (18 to 84 years of age) were analyzed. Patients were enrolled on four consecutive AMLSG multicenter treatment trials: AML HD98A (NCT00146120) (n=804) *(Schlenk R.F. et al. 2010)*, AMLSG 07-04 (NCT00151242) (n=885) (*Schlenk R.F. et al. 2011*); AML HD98B (n=307) (*Schlenk R.F. et al. 2006*) and AMLSG 06-04 (NCT00151255) (n=443) (*Tassara M. et al. 2014*). Patients with acute promyelocytic leukemia (APL) were treated in the APL HD95 trial (*Schlenk R.F. et al. 2005*). The characterization of *RUNX1* mutations in a subset of 945 patients with AML has been previously published (n=651, AML HD98A and n=294, AML 07-04, *Gaidzik V.I. et al. 2011*). All patients gave informed consent for treatment and genetic analysis according to the Declaration of Helsinki. In 16 cases we were able to analyze paired BM samples from diagnosis and relapse. In 10 cases, germline material (DNA obtained from buccal swabs or from PB in CR) was studied for the presence of *RUNX1* germline mutations. In addition, PB samples from 29 healthy volunteers were analyzed for the presence of *RUNX1* polymorphisms. The resulted sequences of exons 1 to 8 were aligned against the reference sequence for *RUNX1* [GenBank accession number X79549.1, http://www.ncbi.nlm.nih.gov/gene/861]. Finally, all *RUNX1* sequence variations were aligned to different SNP databases [http://www.ncbi.nlm.nih.gov/sites/snp, http://genome.ucsc.edu/cgi-bin/hg_Gateway, http://www.ensembl.org] to detect known polymorphisms. The protein encoded by the mutated DNA sequence was identified with the ORF-Finder [http://www.ncbi.nlm.nih.gov/gorf/gorf.html]. The deduced amino acid sequence is then searched against the amino acid sequence of the wild type protein generated using the X79549.1 accession number

AML diagnosis was based on morphology and flow cytometry with a specific antigen panel. Cytogenetic and molecular genetic analysis [*RUNX1-RUNX1T1*, *MYH11-CBFß*, *PML-RARA* and mutations of following genes: *RUNX1*, *NPM1*, *FLT3* (ITD and TKD), *CEBPA* *ASXL1*, *IDH1*, *IDH2* (*IDH2*^{R140}, *IDH2*^{R172}), *KMT2A* (PTD), *DNMT3A*] on diagnosis samples was performed for all patients. Classification of AML was made using the current WHO 2008 Classification. According to their *RUNX1* mutation status, patients were divided into

2 subgroups: *RUNX1* wildtype (wt) and *RUNX1* mutated (mut) and analyzed for concurrent mutations, associated chromosomal abnormalities, clinical characteristics and outcome.

2.2 Cytogenetic analysis

Conventional cytogenetic analysis was performed using Giemsa-banding (G-banding) as described in the following. For each patient PB and BM aspirate were collected in heparinized tubes (Na Heparin 1:10). The cells were counted in the Sysmex Cell Counter and the cell concentration was adjusted to 0,5 - 3 x 10^7 /ml. 1 ml was added in a sterile glass bottle supplied with growth factors and culture medium and incubated at 37 °C and 5 % CO2 for 24 h, 48 h and 72 h. Prior to harvest, 100 µl ethidium bromide (EB) was added to the cultures in order to induce chemical elongation of the DNA. Subsequently, 50 µl colcemid was added to the cultures 90 minutes before harvesting to induce cell cycle synchronization. A larger percentage of cells are thus blocked in metaphase at the time of harvesting. The next step was hypotonic treatment with potassium chloride (KCl). Cell cultures were filled in 12 ml tubes and centrifuged for 8 min at 1200 rpm at room temperature; the cell pellet was suspended in 9 ml 0.075 M KCl with the first 1,5 ml by drop-wise addition. The tubes were placed for exactly 16 min in bain-marie at 37°C; the suspension of swollen mitotic cells was centrifuged at 1200 rpm for 8 min and fixative was added drop-wise and filled up to 10 ml and stored for at least 20 min (up to 1-2 h); the cell suspension was again centrifuged at 1200 rpm for 8 min; the washing process was repeated 2-3 times until the suspension remained clear by removing erythrocytes and free hemoglobin. Fixed pellets were stored in the refrigerator at 2°-8°C until slides were prepared.

Preparation of the slides for G-banding

The cell pellets suspended in fixative were washed again with fresh prepared fixative solution (methanol: glacial acetic acid 3:1) for at least 3 times before dropping. The slides were humidified with water vapor and the cell suspension dropped from 20-30 cm distance directly to the glass surface. Afterwards, the slides were again washed with fixative and dried at room temperature for up to 10-15 min. The quality of the metaphases was checked in the phase contrast microscope. Adequate slides were aged overnight in a drying chamber at 37°C. Heat-aged slides were introduced in trypsin for 2 seconds by swinging the slides in the fresh prepared solution. Next, slides were rapidly moved into 37°C preheated PBS for 5

to 6 seconds. After that the slides were moved in PBS at room temperature and swinged for another 10-15 seconds. Finally, the slides were Giemsa stained for exactly 4 minutes and then successively washed with Buffer and Aqua dest for 6 to 8 swings in each cuvette. In the end, the stained slides were dried at room temperature. Metaphases were visualized on an Axioplan microscope with green light filter. Giemsa-banded chromosomes show an alternating dark and light band pattern along the chromosome length that is unique and reproducible for each chromosome. One band covers 5-10 Mb. Giemsa dark bands (positive bands) are AT-rich, gene poor and late replicating. By contrast, Giemsa light bands are CG-rich, gene rich and early replicating. The band resolution varies between 200-400 bands per haploid karyotype. For analysis the karyotyping system Ikaros from Metasystem was used. Karyotype description was performed according to the International System for Human Cytogenetic Nomenclature 2013.

2.3. Identification of *RUNX1* mutations

2.3.1 Mononuclear cell isolation

Mononuclear cells from PB and BM were isolated using density gradient centrifugation. Samples with a high cell count were initially diluted with RPMI 1600 at 1:2-1:3. BM or PB were layered over the density medium in a ratio of 1:2 and centrifuged at 2800 rpm for 20 min at room temperature (without brake). Then, the mononuclear cell layer was transferred in falcon using a sterile pipette and washed twice with 50 ml RPMI 1640 and centrifuged at 1200 rpm for 10 min at room temperature (with brake). Based on the final cell number, cells were stored at -80°C as 5 x 10^5 to 5 x 10^7 cell pellets.

2.3.2 DNA/RNA extraction

Materials

- AllPrep DNA Mini Spin Columns
- Collection Tubes (1,5 ml)
- Collection Tubes (2 ml)
- Buffer RLT Plus
- Buffer AW1 (concentrate)

- Buffer AW2 (concentrate)
- Buffer EB

DNA/RNA extraction was performed using the AllPrep DNA/RNA Mini Kit that allows simultaneous purification of genomic DNA and total RNA from the same sample. Cells were lysed and homogenized in 600 µl RLT Puffer Plus. In this way DNase and RNases are removed together with other proteins and RNA and DNA strands are stabilized. The cell lysate is loaded on a QIA shredder column and centrifuged for 2 minutes at 13000 rpm. Finally, the homogenized lysate was loaded on an All Prep DNA spin column so that DNA binds to the column and again centrifuged for 1 minute at 13000 rpm. The DNA bounded to the All Prep columns was purified with 500 µl AW1 buffer and centrifuged at 13000 rpm for 1 min removing protein excess. The washing process was repeated with 500 µl AW 2-Puffer and 2 min centrifugation, removing the remaining salts. Then, DNA was dried on a Savant DNA Speed Vac 110 and re-dissolved with 50-100 µl Tris-EDTA buffer and again centrifuged at 13000 rpm for 1 min. The extracted DNA was stored at -20°C and 80°C.

2.3.3 Amplification of the *RUNX1* gene

A polymerase chain reaction (PCR) was used for the amplification of DNA *in vitro*. The whole coding region of the *RUNX1* gene comprising exons 1-8 was analyzed using PCR. The primers were designed to cover also the exon-intron junction regions in order to capture possible mutations in the splicing sites. To ensure quality control, a negative template control (NTC) – without genomic DNA- was pipetted for each exon. In case of detection of a band in the gel electrophoresis, the reaction was repeated.

Primer sequences used for PCR amplification *(Gaidzik V.I. et al. 2011)*

	Forward Primer	Reverse Primer
Exon 1	**M13**-TGAGGCTGAAACAGTGACCTG	GAGAGGAATTCAAACTA
Exon 2	AACCACGTGCATAAGGAACAG	**M13**-CAGCGTTTACCATAGGTGCA
Exon 3	**M13**-GAGCTGCTTGCTGAAGATCC	GGGTCGGTCTTCCTAGCTTG
Exon 4	**M13**-CATTGCTATTCCTCTGCAACC	GATGGAATCCCTAGAAACTCGG
Exon 5	**M13**-GTAACTTGTGCTGAAGGGCTG	GGACCATGTCTCAGATTCCTTG
Exon 6	CCCAAATTCAGCTGGCATATC	**M13**-CACACACCTTCCCAGACCAAC
Exon 7	AAACCCTGGTACATAGGCCAC	**M13**-GCATGAAGGAGTTGGCAGAA
Exon 8	**M13**-TCCGCTCCGTTCTCTTGC	CTCCTGTTCGCCGACAAGC

PCR amplification protocol (for one PCR reaction)

- 1 µl genomic DNA (100 ng/µl)
- 1 µl forward primer (10 µmol/I; Thermo Fischer Scientific, Ulm)
- 1 µl reverse primer (10 µmol/I; Thermo Fischer Scientific, Ulm)
- 0.25 µl dNTP (10 µmol/l: dATP, dCTP, dGTP, dTTP; Roche Diagnostics, Mannheim)
- 0.25 µl Hotstar Taq Plus Polymerase (Qiagen, Hilden)
- 0.6 µl DMSO (Sigma Aldrich, München)
- 2.5 µl 10x PCR buffer
- 18,4 µl Aqua dest (Braun, Berlin)

Amplification was performed on a 9800 Fast Thermal Cycler (Applied Biosystems, Darmstadt, Germany).

PCR conditions:

- Initial denaturation: 97°C, 2 min x1
  - Denaturation: 97°C, 2 min
  - Annealing: 63°C, 1 min
  - Extension: 72°C, 1 min

  } X 35 cycles
- Final cycle: 72°C, 5 min x1
- End: ∞ at 4°C.

2.3.4 Visualization of PCR products on agarose gel electrophoresis

Materials

TAE-Buffer 1x 0.04 mol/I Tris-Acetat, 0.001 mmol/I EDTA, pH 8.0 Store at room temperature
Agarose Store at room temperature
Tracklt 100 bp Ladder Contains 16 DNA fragments of different lengths: 15 range from 100 to 1500bp and one fragment with 2072 bp length Store at 4°C

Preparation of the agarose gel

For the generation of 2 % agarose gel, 6 g of LE agarose (Seakem, Lonza, Rockland USA) were mixed with 300 ml TAE buffer and then microwaved for 4 min at 900 W to melt. The content is cooled down at 60 °C and poured into a gel tray with the well comb in place. Then, the newly poured gel is set on room temperature for about 30 min to solidify and the well comb removed.

Then, the casting tray is transferred into the gel box (Electrophoresis Power Supply EPS 100, Amersham Pharmacia Biotech Freiburg) and covered with TAE buffer. Previous to loading on the agarose gel, 5 µl of each PCR product is mixed on a microtiter plate with loading buffer [OrangeG (Sigma Aldrich München) Glycerol (Sigma Aldrich München), and Aquadest (Braun, Berlin)].

A molecular weight ladder (100 bp, Invitrogen) was loaded in the first lane of the gel and the 8 samples in the additional wells of the gel. After successful loading, the gel was run at 130-135 V and 400 mA for about 45 min. Finally, the gel was placed in a container filled with Ethidium Bromide (EtBr) (1 µg/ml) so that PCR products are stained. DNA fragments were visualized on the transilluminator using a UV light device and images were captured using the Bio Imaging System (Syngene, Frederick USA).

2.3.5 Purifications of the PCR products

Only amplified products that yield an expected band with gel electrophoreses were purified. Purification of the PCR products was performed using the QIAquick PCR purification Kit (Qiagen, Hilden). In this way DNA binds to a silicone gel membrane in the QIAquick column and the flow-through with the remaining PCR products (enzymes, primers, nucleotides, salts) is removed. Subsequently, DNA elution happens in a basic milieu.

Materials

- QIAquick Spin Columns
- Sodium phosphate buffer (PB-buffer)
- Dilution Buffer (EB-buffer) 10 mM Tris-HCl pH 8.5.
- Collection Tubes a 2 ml

Initial 200 µl of PB buffer was mixed with each sample at pH<7.5. A QIA quick column was placed into a 2 ml Eppendorf tube. The sample was placed carefully in the column and the tube is centrifuged for 1 min at 13000 rpm. The flow-through is discarded and the column containing the DNA is placed in another tube. To elute the DNA from the column, 40 µl elution buffer (EB buffer) are added and rest for 1 minute. Then, the column is centrifuged again at 13000 rpm for 1 min, the elute containing purified DNA was transferred in another tube and stored at -20°C.

2.3.6 Cycle Sequencing Reactions (CSR)

In contrast to common PCR, the reaction mix contains a second type of nucleotides called dideoxynucleotides (ddNTP) that are stained with a fluorescent dye. Every time a ddNTP is incorporated in the complementary DNA strand, the synthesis stops, so the amplification follows only one direction, forward or reverse ddNTP are incorporated randomly in the complementary strand. At the end of the reaction, the tube contains collections of double strained DNA which length differ only by one nucleotide.

Reaction mix for amplification of exons 1 to 8

- 2 µl purified DNA
- 1 µl M13-tailored primer (10 µmol/I; Thermo Fischer Scientific, Schwerte)
- 1,7 µl Big Dye Terminator Puffer (10 µmol/I; Applied Biosystems, Darmstadt)
- 2 µl Big Dye Terminator v1.1
  (Contains: the 4 ddNTP: 2´3´ddATP green, 2`3`ddCTP marked red, 2´3´ddGTP marked blue, 2´3´ddTTP marked black, the 4 dNTP: dATP, dCTP, dGTP, dUTP, Ampli Tag Polymerase, $MgCl_2$ and Tris-HCL Puffer, pH 9,0] (Cycle Sequencing RR-100; Applied Biosystems, Darmstadt).
- 8,3 µl HPLC Water (Aqua ad injectabila, Braun, Berlin)
- Store at 4°C

CSR conditions

Amplification was performed using the Gene Amp® PCR System 2700 (Applied Bioscience, Darmstadt).

- Initial denaturation: 97°C, 2 min x1
  - Denaturation: 94°C, 2 min
  - Annealing: 55°C, 1 min
  - Extension: 60°C, 3 min

  } X 35 cycles
- Final cycle: 72°C, 5 min x1
- End: ∞ at 4°C.

All PCR products were amplified using the same M13 Primer. This was possible because for each exon amplification an identical M13-identifying sequence had been tagged during PCR. After binding of the M13 primer to the identification sequence, elongation was possible to begin. CSR products were stored at 4°C under UV protection.

Primer	**5`-3`Sequence**
M13	GTAAAACGACGGCCAGT

Purification of CSR products

Materials

- DyeEx Spin Columns (Single Columns)
- Colelction Tubes a 2 ml

- DyeEX 96 Plates (Purification Plates)
- Collection Plates, 48 well

Fluorescently labeled reaction products must be purified from residual unincorporated dye terminators that may impair accurate sequencing. For that purpose a DyeEx2.0-Spin-Kit (Qiagen, Hilden) or DyeEx 06 plate was used. Using gel filtration technology in a convenient microspin format allows cleanup of sequencing reactions in a very short time.

Purification with DyeEX2.0-Spin-Kit. Small D. molecular weight products are captured in the pores while larger DNA fragments pass through the gel and are captured in the tubes. The DyeEx Spin Columns were centrifuged at 3000 rpm for 3 min. After discarding the overflow the columns contained only the gel matrix. The columns were placed in collection tubes. The CSR products were added in the center of the columns and centrifuged at 3000 rpm for 3 min. The flow through was stored at -20°C.

Purification with DyeEx 96 plate. Prior to load the products on the gel containing columns, the DyeEx 96 plate is centrifuged for 6.5 min at 2350 rpm. Afterwards, the products are loaded on the gel and centrifuged again at 6.5 min at 2350 rpm. The resulted flow-through in the collection plates contains only the labeled fragments necessary for sequencing while the unincorporated fragments are retained within the pores of the gel. Products are stored at 20°C protected from light.

2.3.7 Product sequencing and identification of mutations

Materials

- Genetic Analyzer Buffer (10x) with EDTA (Applied Biosystems, Darmstadt)
- POP-6: Performance Optimized Polymer 6 % (Applied Biosystems, Darmstadt)
- HPLC-Watter Chrosolv-Watter for chromatography (Merck, Darmstadt)
- Capillary: 310, 3130 XL Genetic Analyzer 47 cm x 10 µm Capillaries (Applied Biosystems, Darmstadt)
- Heat plate: Aluminiumbplate25 QBT2 (Grant Instruments Ltd., Cambridge, GB)
- Computer: Power Macintosh 7500/100 (Apple Computer, Ismaning)
- Software: ABI PRISM DNA Sequencing Analysis Software Version 3.4 (Applied Biosystems, Darmstadt)

Sequencing relies on Sanger method using capillary electrophoresis. The PCR products (prior denaturation at 95°C, 2 min) are injected electrokinetically into capillaries filled with polymer (injection time 20 sec, 50°C). High voltage is applied so that the fluorescent DNA fragments are separated by size and are detected by an Argon-laser/camera system. Each of the 4 ddNTP emits a color light of a different wavelength that is recorded as a colored band on a simulated gel image. The computer interprets the row data and outputs an electropherogram with colored peaks, each peak corresponds to a specific letter (nucleotide) in the target sequence. The resulted sequences of exons 1 to 8 were aligned against the

reference sequence for *RUNX1* [GenBank accession number X79549.1, http://ncbi.nlm.nih.gov/gene/861]. Finally, all *RUNX1* sequence variations were aligned to different SNP databases [http://ncbi.nlm.nih.gov/sites/snp, http://genome.ucsc.edu/cgi-bin/hg Gateway, http://ensembl.org] to detect known polymorphisms. The protein encoded by the mutated DNA sequence was identified with the ORF Finder [http://ncbi.nlm.nih.gov/gorf/gorf.html]. The deduced amino acid sequence is then searched against the amino acid sequence of the wild type protein generated using the X79549.1 accession number.

2.4 Reagents

Cytogenetic analysis (G-banding)	
Acetic acid	Fa. Applichem, Darmstadt, Germany
Aqua dest	Fa. Fresenius, Bad Homburg, Germany
Colcemid solution	Fa. Gibco, Darmstadt, Germany
Erythropoietin	Fa. Calbiochem, Darmstadt, Germany
Ethidium bromide	Fa. Sigma Aldrich, St. Louis, USA
FCS	Fa. Biochrom, Berlin, Germany
G-CSF	Fa. Miltenyi Biotech, Bergisch Gladbach, Germany
Giemsa stain	Fa. Merck, Darmstadt, Germany
IL1 alpha	Fa. Miltenyi Biotech, Bergisch Gladbach, Germany
IL3	Fa. Miltenyi Biotech, Bergisch Gladbach, Germany
Kaliumhydrogenphosphate	Fa. Merck, Darmstadt, Germany
KCl	Fa. Merck, Darmstadt, Germany
L-Glutamine	Fa. Biochrom, Berlin, Germany
Methanol	Fa. Sigma Aldrich, St. Louis, USA
Sodiumhydroxid	Fa. Applichem, Darmstadt, Germany
PBS	Fa. Biochrom, Berlin, Germany
Penicillin/Streptomycin	Fa. Biochrom, Berlin, Germany
RPMI Media 1640	Fa. Biochrom, Berlin, Germany
SCF	Fa. Miltenyi Biotech, Bergisch Gladbach, Germany
Trypsin	Fa. Gibco, Darmstadt, Germany
Identification of *RUNX1* mutations	

Agarose	Fa. Carl Roth, Karlsruhe, Germany
Acetic acid	Fa. Carl Roth, Karlsruhe, Germany
AllPrep DNA/RNA mini Kit	Fa. Qiagen, Hilden, Germany
Aqua ad injectabila	Fa. Braun, Melsungen, Germany
ß-Mercaptoethanol	Fa. Sigma Aldrich, St. Louis, USA
Big Dye Terminator	Fa. Applied Biosystems, Weiterstadt, Germany
Blue juice gel loading buffer	Fa. Invitrogen, Groningen, Germany
DNAzol-Reagent	Fa. Gibco, Darmstadt, Germany
ddNTP (ddATP, ddTTP, ddGPT, ddCPT)	Fa. Roche Diagnostics, Mannheim, Germany
DyeEx 96Kit	Fa. Qiagen, Hilden, Germany
EDTA	Fa. Merck, Darmstadt, Germany
Ethanol (70 %, 100 %)	Fa. Sigma Aldrich, St. Louis, USA
Ethidium bromide	Fa. Sigma Aldrich, St. Louis, USA
Falcon 15 ml	Fa. Becton, New Jersey, USA
FTA Classic Card	Fa. Whatman, Maidstone, GB
FTA Purification Reagent	Fa. Whatman, Maidstone, GB
HOT Star Taq DNA Polymerase Kit	Fa. Qiagen, Hilden, Germany
HPLC-Water	Fa. Merck, Darmstadt, Germany
MicroAmp Optical 8-Cap Strip	Fa. Applied Biosystems, Weiterstadt, Germany
MicroAmp Optical 8-Tube Strip (0,2 ml)	Fa. Applied Biosystems, Weiterstadt, Germany
MicroAmp Optical 96-Well Reaction Plate	Fa. Applied Biosystems, Weiterstadt, Germany
MicroAmp Reaction Tube with Cap (0,2 ml)	Fa. Applied Biosystems, Weiterstadt, Germany
Sodium Acetate	Fa. Merck, Darmstadt, Germany
Sodium Heparin	Fa. Braun, Melsungen, Germany
QIAquick PCR Purification Kit	Fa. Qiagen, Hilden, Germany
QIAshredder	Fa. Qiagen, Hilden, Germany
RNase A	Fa. Roche Diagnostics, Mannheim, Germany
RNase-free DNA Set	Fa. Qiagen, Hilden, Germany
Safe Lock Tubes (0,5 ml, 1,5 ml, 2,0 ml)	Fa. Eppendorf , Hamburg, Germany
Sterile foam tipped applicators	Fa. Whatman, Maidstone, GB
Sterile Omni Swabs	Fa. Whatman, Maidstone, GB
Superase Inhibitor	Fa. Applied Biosystems, Weiterstadt, Germany
TE Buffer	Fa. Sigma Aldrich, St. Louis, USA
Tracklt 100 bp DNA Ladder	Fa. Invitrogen, Groningen, Germany
Tris EDTA Buffer	Fa. Sigma Aldrich, St. Louisa, USA

2.5 Statistical analysis

The main focus of the thesis was to characterize the role of *RUNX1* mutations in AML by analyzing its interaction with other molecular and cytogenetic markers, to describe its impact on outcome and to investigate distinct combined genotypes. Statistical analyses were performed in the Clinical Trial Unit of the Department of Internal Medicine III Ulm. All available data from patients enrolled in the above mentioned multicenter treatment were used and supervised by Prof. Dr. R.F. Schlenk To study the organization of *RUNX1* mutations within the category of AML with recurrent genetic abnormalities and the association with additional gene mutations, constraint-sorting analyses of gene signatures were performed. Constraint sorting is based on the greedy algorithm for the minimal Set Covering Problem. Tests were performed at the Institute of Neuroinformatics of the University of Ulm and were supervised by Prof. Dr. rer. nat. Dipl.-Ing. H. Kestler. The definition of CR, EFS, RFS, and OS, as well as cytogenetic categorization into favorable-, intermediate-, and adverse-risk groups followed recommended criteria (*Döhner H. et al. 2010, Cheson B.D. et al. 2003).* Pairwise comparisons between patient characteristics (covariates) were performed by using the Mann-Whitney test for continuous variables and by using Fisher's exact test for categorical variables. The median follow-up for survival was calculated according to the method of Korn (*Korn E.L. 1986).* The Kaplan-Meier and Simon Makuch methods were used to estimate the distribution of EFS, RFS and OS (*Kaplan E. 1958 and Simon R., Makuch R.W. 1984).* Estimation of confidence intervals (CI's) for the survival curves was based on Greenwood's formula for the standard error estimation. A logistic regression model was used to analyze associations between baseline characteristics and the achievement of CR. A Cox model was used to identify prognostic variables (*Therneau T.M. 2000).* Exploratory variables in the regression analyses included age, sex, hemoglobin level, logarithm of white blood cell (WBC), type of AML (*de novo*, secondary AML, therapy-related AML), percentage of PB and BM blasts, cytogenetic risk group, and mutational status of *RUNX1*, *NPM1*, *FLT3* (ITD and TKD), *CEBPA* (*CEBPA*^{dm}), *ASXL1*, *IDH1*, *IDH2* (*IDH2*^{R140}, *IDH2*^{R172}), *KMT2A* (PTD), and *DNMT3A*. Missing data for covariates were estimated by using 50 multiple imputations in chained equations that incorporated predictive mean matching (*Harrell F. 2001*). All statistical analyses were performed with the statistical software environment R version 2.14.0, using the R packages rms version 3.3-1, survival version 2.36-8, and cmprsk version 2.2-2.33

3. RESULTS

3.1 Frequency and types of *RUNX1* mutations

Overall, 280 *RUNX1* mutations were found in 245 of 2439 (10 %) patients. Mutations were located as follows: exon 3, n=48, exon 4, n=81, exon 5, n=42, exon 6, n=23, exon 8, n=63. In total, 139 (49.6 %) mutations were found in the RHD (exon 3-5) and 95 (40 %) in the TAD (exon 6-8). There were 146 frameshift (FS), 96 missense (MS) and 38 nonsense (NS) mutations. In 33 patients, two *RUNX1* mutations were found. No impact on outcome was identified for any of the three types of mutations (OS, *P*=0.91, RFS, *P*=0.99, EFS, *P*=0.26). No hot spot mutations were detected.

Based on samples availability, germline screening was possible in 10 patients, n=8 with *NPM1*^{mut}/*RUNX1*^{mut} genotype, n=1 with *CEBPA*^{dm}/*RUNX1*^{mut} genotype and n=1 with t(8;21)(q22;q22)/*RUNX1*^{mut}. Only in one patient with *NPM1*^{mut}/*RUNX1*^{mut} genotype a *RUNX1* mutation (c.G991A.p.M267I) was identified both in bone marrow sample from diagnosis and in peripheral blood sample at the time of complete remission and *NPM1* MRD negativity, making this mutation a candidate for a germline mutation.

3.2 Association of mutations with clinical characteristics

RUNX1 mutations were significantly associated with increasing age (P>0.001), male gender (*P*=0.02), secondary AML evolving from MDS (*P*<0.001), higher platelet count (*P*=0.007), lower LDH serum levels (*P*<0.0001) and with FAB M0 morphology (*P*=0.004). **(Table 7).**

3.3 Distribution of molecular and cytogenetic data

***Cytogenetics*.** Cytogenetic data were available in 2231/2439 (91.5 %) cases. *RUNX1* mutations were identified in 245/2439 (10 %) patients. The distribution of *RUNX1* mutations among the ELN genetic categories was as follows: favorable, n=8 (3.5 %), intermediate I, n=107 (47.6 %), intermediate II, n=69 (30 %), adverse, n=41 (18.2 %). Thus, most patients

clustered in the intermediate risk group 176/245 (77.6 %), with 107/245 (47.6 %) having a normal karyotype.

Within the favorable ELN genetic category we identified 1 case with t(8;21)(q22;q22) and 1 case with inv(16)(p13.1q22). The remaining 6 cases had a normal karyotype and concurrent *NPM1* and *RUNX1* mutations. The following recurrent genetic abnormalities could be identified in the intermediate II and adverse risk groups: 1 patient had a t(9;11)(p22;q23) (intermediate II), 1 patient had a t(11;19)(q23;p13) (adverse) and 2 patients had inv(3)(q21q26.2) (adverse). No *RUNX1* mutation was identified among cases with t(15;17)(q22;q12) or t(6;9)(p23;q34). With regard to specific cytogenetic abnormalities, *RUNX1* mutations were significantly associated with the presence of chromosome 7 abnormalities (-7/7q-) (*P*=0.04) and trisomy 13 (*P*=0.0001). Among cases with *RUNX1* mutations 12 cases were therapy-related AML. Within these patients subset, no balanced translocations involving *RUNX1* were detected. In 2 patients a normal karyotype was found, 9 patients had a complex karyotype and 1 patient had a t(9;11)(p22;q23) translocation. The latency period after cytotoxic exposure varied from less than one year to 10 years. **(Table 6)** ***Molecular genetics***. *RUNX1* mutations inversely correlated with *NPM1* mutations (*P*<0.0001), biallelic *CEBPA* mutations (*P*=0.02) and the recurrent genetic abnormalities t(15;17)(q22;q12); *PML-RARA*, inv(16)(p13.1q22); *CBFß-MYH11* and t(8;21)(q22;q22); *RUNX1-RUNX1T1*. There was a significant association with mutations in the epigenetic modifiers *ASXL1* (*P*<0.0001), *KMT2A*-PTD (*P*<0.0001) and *IDH2* (*P*=0.02). Secondary AML with *RUNX1* mutation had more frequently concurrent *ASXL1* mutations when compared to de novo AML with *RUNX1* mutation (52.6 % vs 15 %, *P*<0.0001). **(Table 7).**

Table 6. Correlation of *RUNX*1 mutation status of the entire cohort with different cytogenetic abnormalities and ELN risk categories. According to their mutation status, patients were divided in 2 subgroups, with (n=245) and without *RUNX1* (n=2194) mutations

***RUNX1*^{mut} (n=245, %)**	***RUNX1*^{wt} (n=2194, %)**	***P***
Cytogenetic abnormality		
t(15;17)(q22;q22)	0	77, 3.8 %	0.0003
t(8;21)(q22;q22)	1, 0.4 %	102, 5.1 %	0.0003
inv(16)(p13.1q22)/ t(16;16)(p13.1;q22)	1, 0.4 %	124, 6.2 %	<0.0001
t(9;11)(p22;q23)	1, 0.4 %	33, 1.65 %	0.25

Table 6 to be continued on page 35

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t(11q23)var	2, 0.9 %	29, 1,45 %	0.76
-7/7q-	13, 5.75 %	135, 6.73 %	0.04
+8	19, 8.4 %	202, 10 %	0.5
9q-	6, 2.65 %	66, 3.3 %	0.84
+11	3, 1.33 %	36, 1.8 %	0.8
+13	11, 4.9 %	28, 1.4 %	0.001
+21	8, 3.5 %	55, 2.75 %	0.5
-17/abnl(17p)	9, 4 %	114, 5.7 %	0.36
CK	22, 9.7 %	248, 12.4 %	0.28
MK	21, 9.3 %	211, 10,5 %	0.65
NK	113, 50 %	974, 48.6 %	0.73
Others	27, 11 %	123, 5.6 %	0.002
Missing	19, 7.7 %	189, 8.6 %	
ELN 2010 Classification			
Favorable	8, 3.4 %	580, 29 %	<0.0001
Intermediate I	107, 47,4 %	619, 31 %	<0.0001
Intermediate II	69, 30.7 %	403, 20 %	<0.0001
Adverse risk	41, 18.2 %	401, 20 %	0.38
Missing	20, 8 %	191, 9 %	

Abbreviations: ELN, European LeukemiaNet| **CK**, complex karyotype| **MK**, monosomal karyotype| **NK**, normal karyotype| ***P***, p-value| **"+"** and **"-"**, gain or loss of a whole chromosome or a chromosome region |**p** and **q**, short and long arms of a chromosome.

Table 7. Correlation of *RUNX*1 mutation status of the entire cohort with clinical and biological features, FAB classification and molecular markers. According to their mutation status, patients were divided in 2 subgroups, with (n=245) and without *RUNX1* (n=2194) mutations

	***RUNX1*^{mut} (n=245, %)**	***RUNX1*^{wt} (n=2194, %)**	***P***
Age (years)			<0.0001
Median (Range)	59.2 (19.2 to 79)	53.6 (16.3 to 84.5)	
Male sex	147, 60 %	1137, 51.8 %	0.01
AML history			
de novo	194, 79.5 %	1920, 88.5 %	<0.0001
Secondary	38, 15.6 %	119, 5.5 %	<0.0001

Table 7 to be continued on page 35

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Therapy-related	12, 4.9 %	131, 6 %	0.57
WBC count, x10^9/I			0.77
Median (Range)	13.6 (0.3 to 533)	13 (0.1 to 440)	
Missing	3, 1.2 %	45, 2 %	
Plt count, x10^9/I			0.007
Median (Range)	67 (4 to 575)	53 (2 to 933)	
Missing	3, 1.2 %	46, 2.2 %	
Hemoglobin, g/dL			0.56
Median (Range)	9.2 (2.7 to 14.6)	9.1 (2.5 to 17.6)	
Missing	3, 1.2 %	48, 2.2 %	
PB blasts, %			0.74
Median (Range)	34.5 (0 to 100)	35 (0 to 100)	
Missing	17, 7 %	201, 9 %	
BM blasts, %			0.74
Median (Range)	75 (2.9 to 100)	75 (0 to 100)	
Missing	12, 5 %	211, 9.5 %	
LDH, U/I			<0.0001
Median (Range)	322 (110 to 5406)	418 (40 to 15098)	
Missing	8, 3.2 %	72, 3.2 %	
FAB Classification			
M0	11, 13.7 %	49, 5.1 %	0.04
M1	14, 17.5 %	164, 17.2 %	0.36
M2	19, 23.7 %	259, 26.2 %	0.08
M3	0	95, 10 %	<0.0001
M4	22, 27.5 %	241, 25.3 %	0.38
M5	12, 15 %	114, 12 %	1.0
M6	2, 2.5 %	27, 2.8 %	0.76
M7	0	13, 1.4 %	0.63
Missing	165, 67 %	1241, 56 %	
NPM1			<0.0001
Wildtype	230, 94,7 %	1509, 70 %	
Mutated	13, 5.3 %	651, 30 %	
Missing	2, 1 %	34, 1.5 %	

Table 7 to be continued on page 36

Continuation of Table 7 from page 37

***FLT3*-ITD**			0.05
Wildtype	220, 96 %	1929, 92.7 %	
Mutated	9, 4 %	153, 7.3 %	
Missing	16, 6.5 %	112, 5 %	
CEBPA			0.01
Wildtype	225, 95 %	1890, 92.4 %	
Mutated			
• Monoallelic	10, 4.2 %	70, 3.4 %	
• Biallelic	2, 0.8 %	86, 4.2 %	
Missing	8, 3.2 %	148, 6.7 %	
***KMT2A*-PTD**			<0.0001
Absent	126, 82,9 %	1487, 95.6 %	
Present	26, 17 %	68, 4.4 %	
Missing	93	639	
IDH1			0.3
Wildtype	222, 91 %	2008, 93 %	
Mutated	21, 8.6 %	149, 7 %	
Missing	2, 1 %	37, 1.7 %	
IDH2			0.02
Wildtype	205, 84.5 %	1926, 89.3 %	
Mutated	38, 15.6 %	230, 10.7 %	
Missing	2, 1 %	38, 1.8 %	
***DNMT*3A**			0.66
Wildtype	193, 82 %	1663, 80.3 %	
Mutated	43, 18.2 %	409, 19.7 %	
Missing	9, 3.6 %	122, 5.5 %	
ASXL1			<0.0001
Wildtype	193, 79.4 %	2016, 94 %	
Mutated	50, 20.6 %	129, 6 %	
Missing	2, 1 %	49, 2.2 %	

Abbreviations: **AML**, acute myeloid leukemia| **PB**, peripheral blood| **BM** bone marrow| **FAB**, French-American-British| **WBC**, white blood cells| **Plt**, Platelets| **LDH**, Lactat dehydrogenase| ***NPM1***, Nucleophosmin | ***FLT3*-ITD**, FMS-like tyrosine kinase 3 internal tandem duplication| ***CEBPA*** CCAAT/enhancer-binding protein α |***KMT2A*-PTD**, Lysine [K]-specific methyltransferase 2A-partial tandem duplication | ***IDH* 1,2,** Isocytrate dehydrogenase 1,2 |***DNMT3A***, *DNA (cytosine-5)-methyltransferase 3A* |***ASXL1***, Additional sex combs like 1 gene | **M1-7**, FAB morphologic classification

Constraint sorting of gene signature. *RUNX1* mutations were almost entirely mutually exclusive of the recurrent genetic abnormalities defined in the current WHO Classification (2008) meaning, they form a distinct cluster within this category of AML. *RUNX1* mutations rarely co-occurred with *NPM1* mutations, n=13, biallelic *CEBPA* mutations, n=2, t(8;21), n=1, inv(16), n=1, t(9;11), n=1 and inv(3), n=3. **(Figure 2)**

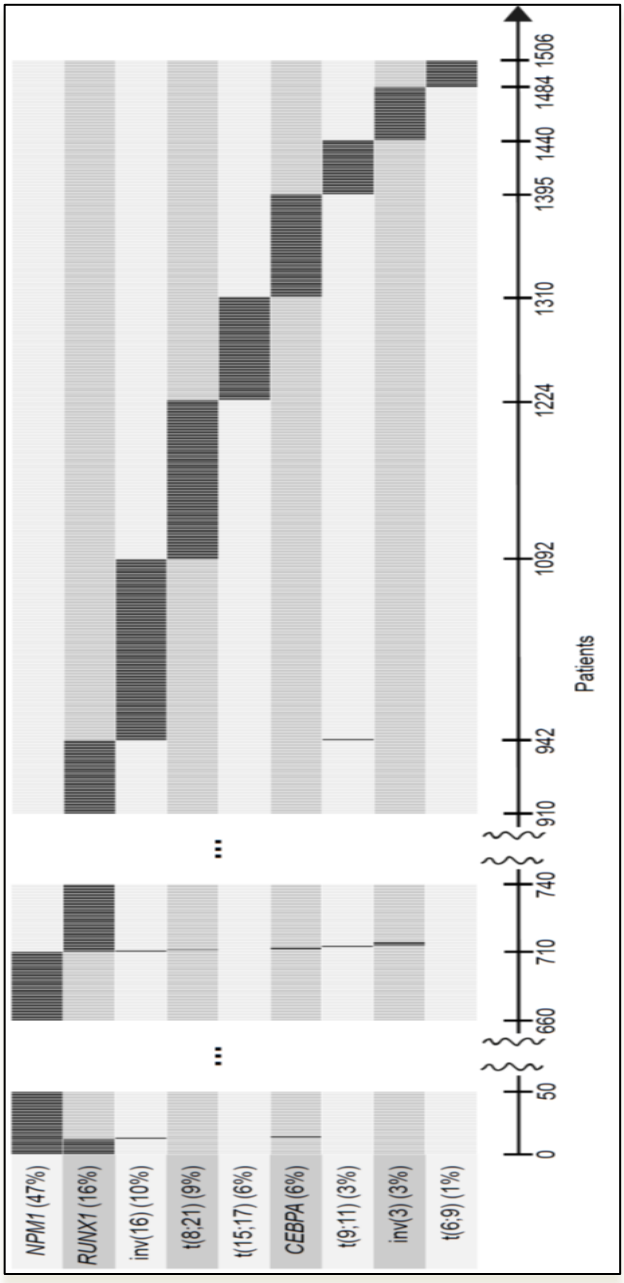


Figure 2. Organization of *RUNX1* mutations within the WHO category "AML with recurrent genetic abnormalities". Vertical lines represent individual patients (n=1506 patients included in the analysis). The specific abnormalities within this category and their incidence (%) are listed in the left boxes. To allow an overview on all abnormalities, fractions of patients (with *NPM1* mutation between numbers 50 and 660 and patients with *RUNX1* mutation between numbers 740 and 910) were omitted showing no overlap with the abnormalities. Only *CEBPA* biallelic mutations were included in this analysis. The recurrent genetic abnormalities form distinct clusters indicating the mutual exclusivity of the genetic abnormalities within this AML category. **Abbreviations:** ***NPM1***, Nucleophosmin 1| ***RUNX1***, Runt-related transcription factor 1 | ***CEBPA***, CCAAT/enhancer-binding protein α.

3.4 Response to induction therapy

Clinical correlation analysis was possible for 2404 patients (information missing in 35 cases). The CR rate was significantly lower in patients with *RUNX1* mutations than in patients without mutation (48.4 % vs 68 %, *P*=0.0001) **(Table 8)**, this effect was irrespective of age or additional chromosomal abnormalities. The lower CR rate was mainly attributable to a higher rate of resistant disease (40.6 % vs 23.4 %, *P*=0.03).

Table 8. Univariable analysis showing different clinical endpoints (CR rate, ED and RD) after double induction therapy for patients with (n=245) and without (n=2194) *RUNX1* mutations for the entire cohort and separately for patients younger and older than 60 years according to their *RUNX1* mutation status.

Clinical endpoint	*RUNX1*^{mut}, n=245, %	*RUNX1*^{wt}, n=2194, %	*P*
Entire cohort			
CR	118, 48.4 %	1470, 67 %	<0.0001
ED	27, 11 %	185, 8.5 %	
RD	99, 40.6 %	505, 23 %	
Missing	1, 0.4 %	34, 1.5 %	
Patients ≤ 60 years			
CR	81, 61 %	1153, 74.5 %	0.003
ED	13, 10 %	120, 8 %	
RD	38, 29 %	275, 17.5 %	
Missing	0	9, 0.4 %	
Patients >60 years			
CR	37, 32,7 %	317, 50 %	0.0008
ED	14, 12.4 %	65, 10 %	
RD	61, 54 %	230, 36 %	
Missing	1, 0,9 %	25, 4 %	

Abbreviations: CR, complete remission| **ED**, early death| **RD** residual disease | ***P***, p-value

Among patients with *RUNX1* mutation, in particular those with secondary AML had a poor response to double induction therapy as illustrated in **Table 9**.

Table 9. Univariable analysis of different clinical endpoints (CR rate, ED and RD) after double induction therapy for all patients with *RUNX1* mutations (n=245) according to history of AML (secondary vs *de novo* AML)

Clinical endpoint	Secondary AML, n=38, %	*de novo* AML, n=194, %	*P*
CR	6, 15.8 %	105, 54.4 %	<0.0001
ED	5, 13.2 %	20, 10.4 %	0.6
RD	27, 71 %	68, 35.2 %	<0.0001
Missing	0	1, <0.0004 %	

Abbreviations: AML, acute myeloid leukemia| **CR**, complete remission| **ED**, early death| **RD**, resistant disease| ***P***, p-value.

In multivariate analysis, *RUNX1* mutations were an independent poor prognostic factor for achievement of CR in the entire cohort (odds ratio [OR] 0.70; 95 % CI, 0.51-0.96; *P*=0.03). This effect was even more pronounced in the older patients (OR, 0.48; 95 %CI, 0.28-0.81; *P*=0.006).

3.5 Survival analysis

The median follow-up time for survival for the entire cohort (n= 2439 patients) was 5.8 years (95 % CI, 5.57-5.98). In univariate analysis, *RUNX1* mutations were significantly associated with inferior 5-year EFS (24 % vs 9 %, *P*<0.0001), RFS (36 % vs 22 %, *P*=0.01) and OS (37 % vs 22 %, *P*<0.0001) **(Figures 3, 4 and 5).**

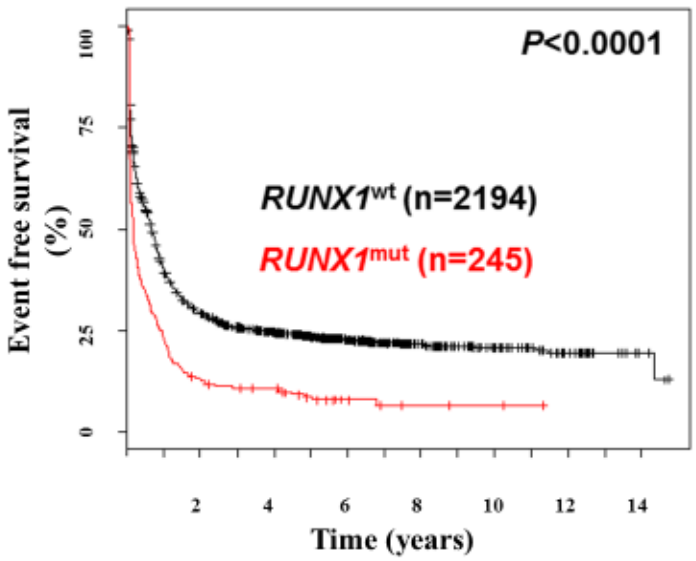


Figure 3. Univariable outcome analysis showing event free survival (EFS) according to *RUNX1* mutation status for the entire cohort. Patients with *RUNX1* mutations (n=245) are marked red and patients without *RUNX1* mutations are marked black (n=2194). There was a significant impact of *RUNX1* mutation on EFS, patients with *RUNX1* mutations having a 5-year EFS of 9 % comparing to 24 % for patients without *RUNX1* mutations, $P<0.0001$.

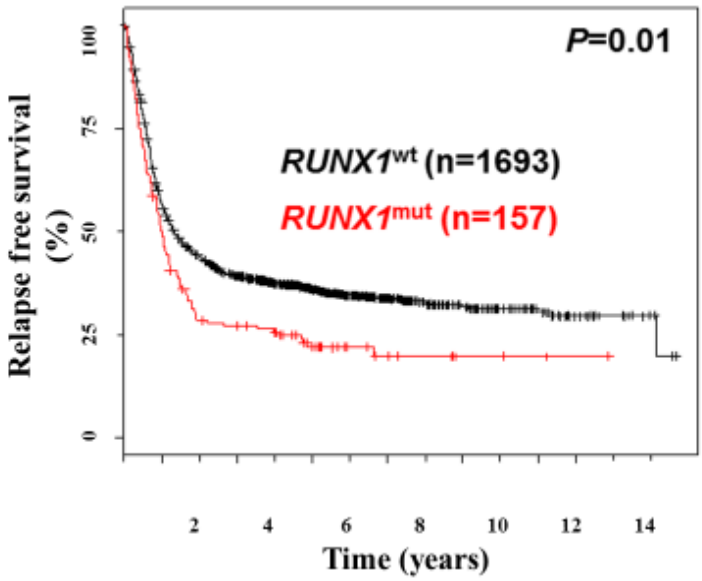


Figure 4. Univariable outcome analysis showing relapse free survival (RFS) according to *RUNX1* mutation status for the entire cohort. Patients with *RUNX1* mutations (n=157) are marked red and patients without *RUNX1* mutations (n=1693) are marked black. There was a significant impact of *RUNX1* mutation on RFS, patients with *RUNX1* mutations having a 5-year RFS of 22 % comparing to 36 % in patients without *RUNX1* mutations, $P=0.01$.

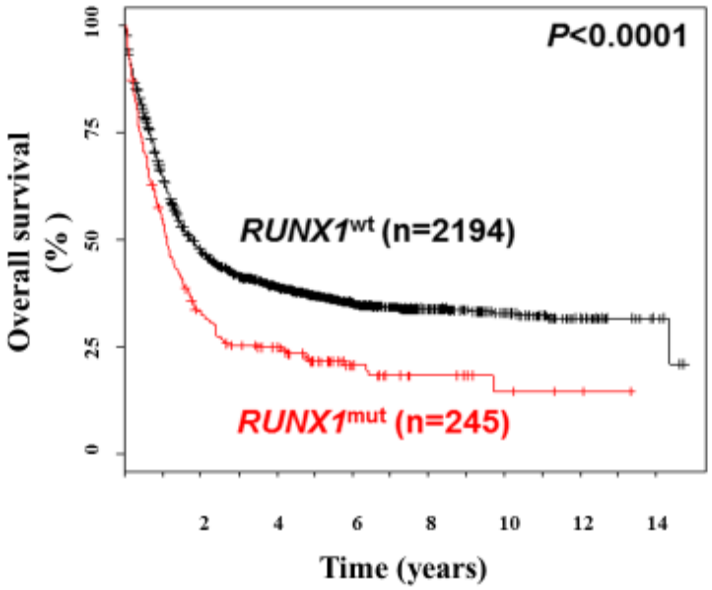


Figure 5. Univariable outcome analysis showing overall survival according to *RUNX1* mutation status for the entire cohort. Patients with *RUNX1* mutations (n=245) are marked red and patients without *RUNX1* mutations (n=2194) are marked black. There was a significant impact of *RUNX1* mutation on OS, patients with *RUNX1* mutations having a 5-year OS of 22 % comparing to 37% in patients without *RUNX1* mutations, $P<0.0001$.

The effect was similar for younger patients in whom the presence of *RUNX1* mutations negatively impacted 5-year OS (43 % vs 33 %, $P=0.002$), EFS (30 % vs 12 %, $P<0.00001$) and RFS (42 % vs 26 %, $P=0.0007$). In patients over 60 years of age, *RUNX1* mutations were associated with inferior 5-year EFS (8 % vs 4 %, $P=0.0009$) and in trend with poor OS (15 % vs 8 %, $P=0.09$) but there was no effect on RFS (15 % vs 14 %, $P=0.43$) (**Tables 10A** and **10B**).

Table 10A. Univariable outcome analysis for younger patients (n=1689, ≤ 60 years) according to *RUNX1* mutation status showing event free survival, relapse free survival and overall survival.

Event free survival					
***P*<0.001**	N	Events	Median (months)	95 % CI	5-year survival %
RUNX1^{wt}	1557	1103	10.1	9.1-11.3	30 (28-32)
RUNX1^{mut}	132	116	3.1	1.4-6.8	12 (8-20)
Relapse free survival					
***P*=0.007**	N	Events	Median (months)	95 % CI	5-year survival %
RUNX1^{wt}	1336	791	23.4	18.8-28.8	42 (39-45)
RUNX1^{mut}	106	76	14.1	10.8-21.3	26 (19-37)
Overall survival					
***P*=0.002**	N	Events	Median (months)	95 % CI	5-year survival %
RUNX1^{wt}	1557	855	38.6	30.3-50.2	46 (43-48)
RUNX1^{mut}	132	90	18.0	13.6-25.3	33 (26-43)

Table 10B. Univariable outcome analysis for elderly patients (n=750, > 60 years) according to *RUNX1* mutation status showing event free survival, relapse free survival and overall survival.

Event free survival					
***P*=0.009**	N	Events	Median (months)	95 % CI	5-year survival %
RUNX1^{wt}	637	572	3.2	2.5-4.6	8 (6-11)
RUNX1^{mut}	113	108	1.7	1.3-2.2	4 (2-10)
Relapse free survival					
***P*=0.43**	N	Events	Median (months)	95 % CI	5-year survival %
RUNX1^{wt}	357	300	9.8	8.9-11	15 (12-19)
RUNX1^{mut}	51	44	9.9	6.4-13	14 (7-27)
Overall survival					
***P*=0.09**	N	Events	Median (months)	95 % CI	5-year survival %
RUNX1^{wt}	637	525	11.1	9.9-12.5	15 (13-19)
RUNX1^{mut}	113	101	9.6	6.3-12.8	8 (4-15)

In an exploratory manner we further looked if combined genotypes influenced outcome. The following genotypes including co-occurring mutations in at least 15 % of cases were included in the analyses: *RUNX1*^{mut}/*FLT3*-ITD^{pos}, *RUNX1*^{mut}/*DNMT3A*^{mut}, *RUNX1*^{mut}/*ASXL1*^{mut}, *RUNX1*^{mut}/*KMT2A*-PTD^{pos} and *RUNX1*^{mut}/*IDH2*^{mut}. The worst outcome was conferred by the genotype *RUNX1*^{mut}/*ASXL1*^{mut} when compared with the *RUNX1*^{mut}/*ASXL1*^{wt} genotypes (OS, *P*=0.004 and RFS, *P*=0.05). (**Figures 6 and 7**). By contrast, patients with the genotype *RUNX1*^{mut}/*IDH2*^{mut} had a better outcome (median OS of 1.67 years vs 1.06 years, *P*=0.04, median RFS 2.61 years vs 0.93 years, *P*=0.02) (**Figures 8 and 9**). For all the other combined genotypes, no significant impact on survival was found. (*RUNX1*^{mut}/*KMT2A*-PTD^{pos} vs *RUNX1*^{mut}/*KMT2A*-PTD^{wt}, OS, *P*=0.38, RFS, *P*=0.97; *RUNX1*^{mut}/*FLT3*-ITD^{pos} vs *RUNX1*^{mut}/*FLT3*-ITD^{wt} , OS, *P*=0.14, RFS, *P*=0,24; *RUNX1*^{mut}/*DNMT3A*^{mut} vs *RUNX1*^{mut}/*DNMT3A*^{wt}, OS, *P*=0,54, RFS=0.5)

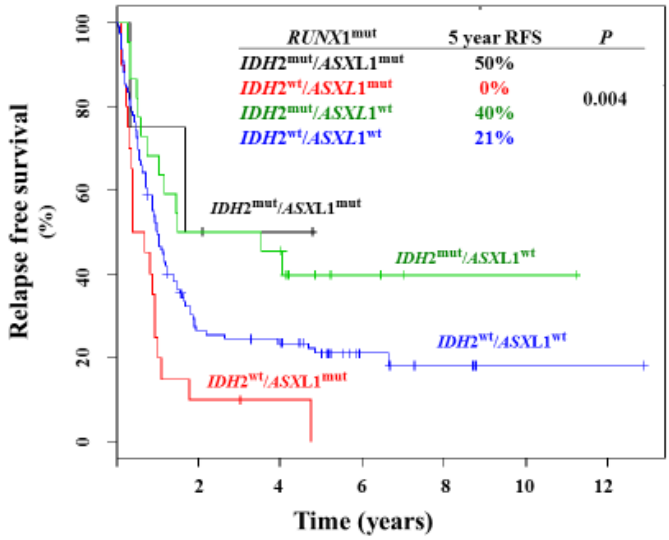


Figure 7. Relapse free survival in patients with *RUNX1* mutations according to combined genotypes: black curve shows patients with *RUNX1* mutations and *IDH1*^{mut}/*ASXL1*^{mut} genotype, the red curve shows patients with *RUNX1* mutations and *IDH2*^{wt}/*ASXL1*^{mut} genotype, in blue are shown patients with *IDH2*^{mut}/*ASXL1*^{wt} genotype and the green curve shows patients with *RUNX1*^{mut} and *IDH1*^{wt}/*ASXL1*^{wt} genotype. The poorest outcome was identified for the *RUNX1*^{mut}/*IDH2*^{wt}/*ASXL1*^{mut} genotype with no patient reaching 5 year RFS. |RFS, relapse free survival

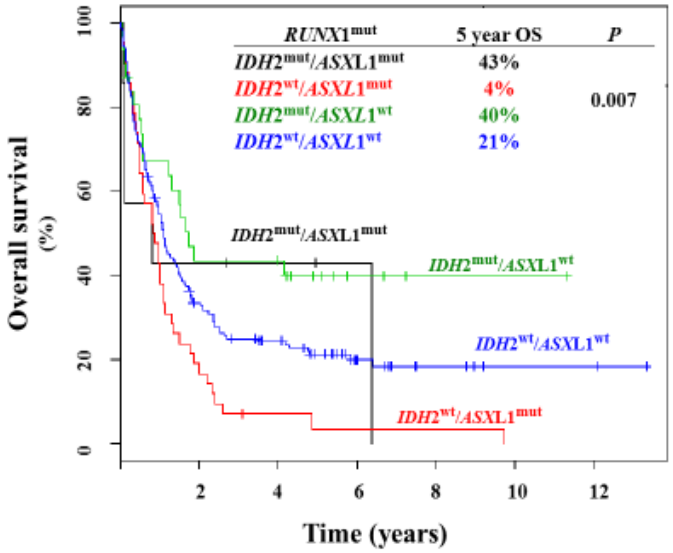


Figure 8. Overall survival in patients with *RUNX1* mutations according to combined genotypes: black curve shows patients with RUNX1 mutations and *IDH1*^{mut}/*ASXL1*^{mut} genotype, the red curve shows patients with *RUNX1* mutations and *IDH2*^{wt}/*ASXL1*^{mut} genotype, in blue are shown patients with *IDH2*^{mut}/*ASXL1*^{wt} genotype and the green curve shows patients with *RUNX1*^{mut} and *IDH1*^{wt}/*ASXL1*^{wt} genotype. Patients with *IDH2*^{wt}/*ASXL1*^{mut} have a very poor outcome with only 4% survival rate at 5 years. |OS, overall survival

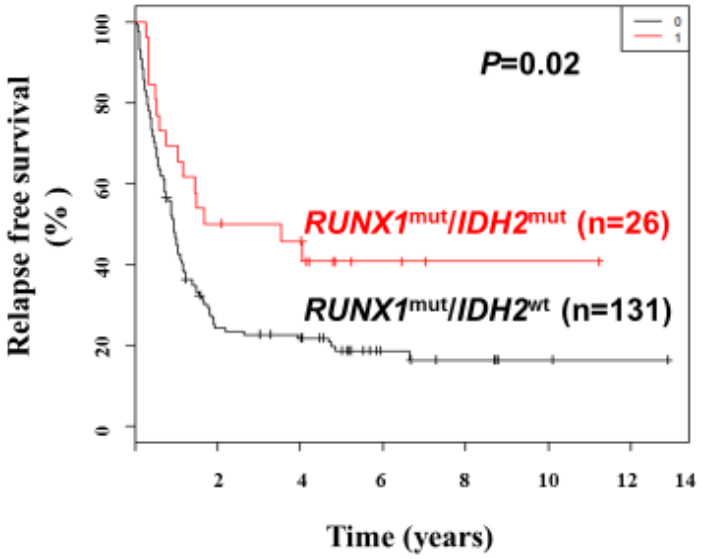


Figure 9. Relapse free survival in patients (RFS) with *RUNX1* mutations according to combined genotypes: black curve shows patients with *RUNX1* mutations and *IDH1*^{wt} genotype and the red curve shows patients with concomitant *RUNX1* and *IDH2* mutations. There is a significant better RFS for patients with the *RUNX1*^{mut}/*IDH2*^{mut} genotype compared to *RUNX1*^{mut}/*IDH2*^{wt} genotype, 2.61 years vs 0.93 years, *P*=0.02.

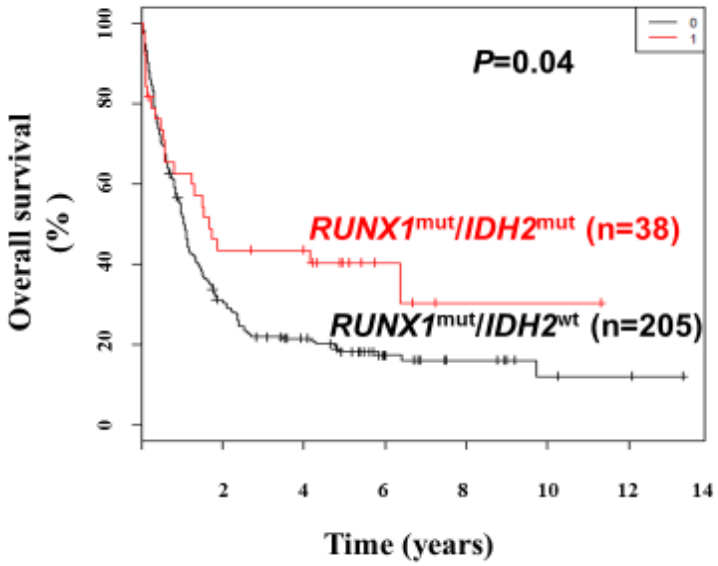


Figure 10. Overall survival (OS) in patients with *RUNX1* mutations according to combined genotypes: black curve shows patients with *RUNX1* mutations and *IDH1*^{wt} genotype and the red curve shows patients with concomitant *RUNX1* and *IDH2* mutations. There is a significant better OS for patients with the *RUNX1*^{mut}/*IDH2*^{mut} genotype compared to *RUNX1*^{mut}/*IDH2*^{wt} genotype, 1.67 years vs 1.06 years, *P*=0.04.

In multivariable analysis, *RUNX1* mutation was an independent prognostic marker for inferior EFS (hazard ratio [HR] 1.22, *P*=0.01) but not for RFS (HR 1.03, *P*=0.75) and OS (HR 1.10, *P*=0.26) **(Table 11)**. In younger patients, *RUNX1* mutations only had a negative impact on EFS (HR 1.09, *P*=0.48). In patients over 60 years, *RUNX1* mutation had no independent prognostic value (EFS, HR 1.22, *P*=0.08, OS HR 1.08, *P*=0.53, RFS, HR 0.88, *P*=0.4).

Table 11. Multivariable analysis for the entire cohort (excluding acute promyelocytic leukemia), stratified analysis according to age showing the endpoints event free survival, relapse free survival and overall survival.

Variables	HR	95 % CI	*P*
Endpoint: Event free survival			
RUNX1 mutation	1.22	1.04-1.42	0.01
Age (10 years difference)	1.17	1.10-1.24	<0.0001
Gender (female)	0.87	0.78-0.96	0.004
s-AML	1.21	1.00-1.46	0.05
t-AML	1.12	0.92-1.37	0.27
*FLT*3-ITD	1.32	1.13-1.54	0.0004
*FLT*3-TKD	0.93	0.75-1.15	0.51
NPM1 mutation	0.66	0.58-0.76	0.0001
DNMT3A mutation	1.13	0.99-1.29	0.07
ASXL1 mutation	1.04	0.87-1.24	0.68
BM blasts	1	1-1	0.51
WBC (log10)	1.25	1.13-1.37	<0.0001
LDH (log10)	1.1	0.92-1.33	0.3
ELN Intermediate I	1.77	1.51-2.07	<0.0001
ELN Intermediate II	1.81	1.54-2.14	<0.0001
Adverse	3.17	2.70-3.73	<0.0001
Endpoint: Relapse free survival			
RUNX1 mutation	1.03	0.84-1.27	0.75
Age (10 years difference)	1.18	1.10-1.26	<0.0001
Gender (female)	0.96	0.85-1.1	0.54
s-AML	1.05	0.8-1.4	0.71
t-AML	1.49	1.16-1.91	0.002

Table 11 be continued on page 49

Continuation of Table 11 from page 48

*FLT*3-ITD	1.49	1.23-1.80	<0.0001
*FLT*3-TKD	0.86	0.66-1-11	0.24
NPM1 mutation	0.75	0.63-0.9	0.0007
DNMT3A mutation	1.11	0.95-1.3	0.2
ASXL1 mutation	1.18	0.93-1.50	0.2
BM blasts	1	1-1	0.7
WBC (log10)	1.23	1.1-1.4	0.7
LDH (log10)	1.3	1-1.63	<0.001
ELN Intermediate I	1.5	1.25-1.8	<0.0001
ELN Intermediate II	1.42	1.2-1.73	0.0004
ELN adverse	2.2	1.77-2.64	<0.0001
Endpoint: Overall survival			
RUNX1 mutation	1.1	0.93-1.30	0.26
Age (10 years difference)	1.4	1.3-1.5	<0.0001
Gender (female)	0.94	0.84-1	0.3
s-AML	1.23	1-1.6	0.02
t-AML	1.2	0.96-1.5	0.11
FLT3-ITD	1.53	1.3-1.8	<0.0001
FLT3-TKD	1	0.82-1.32	0.72
NPM1 mutation	0.9	0.75-1	0.1
DNMT3A mutation	1	0.9-1.2	0.5
ASXL1 mutation	1.14	0.95-1.4	0.2
BM blasts	1	1-1	1
WBC (log10)	1.21	1.10-1.35	0.0002
LDH (log10)	1.4	1.13-1.7	0.002
ELN Intermediate I	1.7	1.44-2	<0.0001
ELN Intermediate II	1.8	1.5-2.2	<0.0001
Adverse	3.4	2.9-4	1

Abbreviations: AML, acute myeloid leukemia| **t-AML**, therapy-related AML| **s-AML**, secondary AML evolving from myelodysplastic syndrome| **EFS**, event free survival| **RFS**, relapse free survival| **OS**, overall survival| **HR**, hazard ratio| CI confidence interval| ***P***, p-value| **TKD** tyrosine kinase domain| **ELN**, European LeukemiaNet| **BM**, bone marrow| **WBC**, white blood cells| **LDH**, serum lactate dehydrogenase|***FLT3*** ITD FMS-like tyrosine kinase 3 internal tandem duplication| ***NPM1*** Nucleophosmin 1| ***DNMT3A*** *DNA (cytosine-5)-methyltransferase 3A*| ***ASXL1***, Additional sex combs like 1 gene**.**

The role of allogeneic hematopoietic stem cell transplantation (alloHSCT). In younger patients the role of alloHSCT was estimated using Simon-Makuch survival curves. Of 81 patients younger than 60 years with *RUNX1* mutation achieving a CR, 36 were transplanted in first CR. A benefit for prolonged RFS was obtained for patients who were transplanted in first CR (*P*=0.01) but no significant difference in OS for those patients (*P*=0.53) **(Figures 9** and **10)**

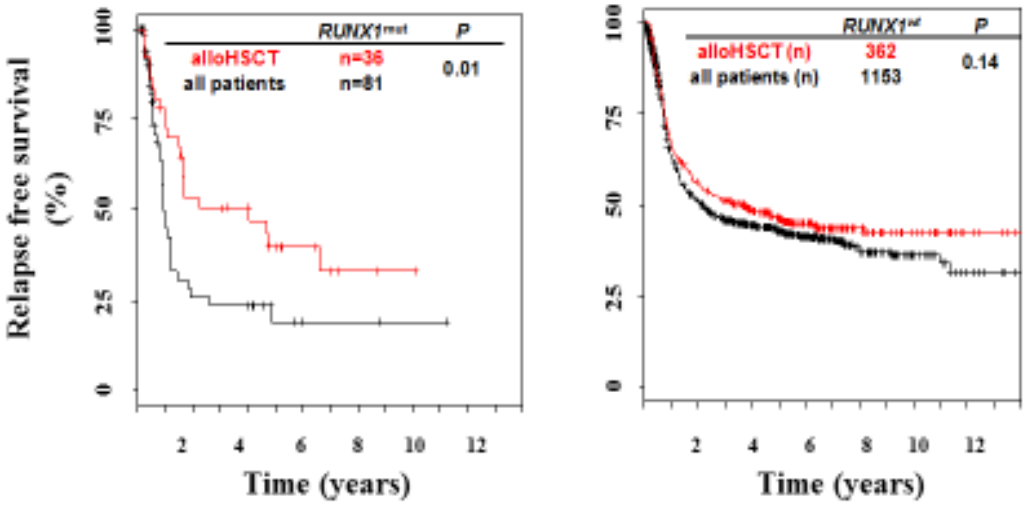


Figure 9. Simon Makuch survival estimates showing relapse free survival in younger patients (≤ 60 years) with or without *RUNX1* mutations who underwent an alloHSCT in first CR. There was a benefit in terms of prolonged RFS for patients with *RUNX1* mutation who received an alloHSCT, *P*.<0.01(left figure).|**alloHSCT**, hematopoietic stem cell transplantation

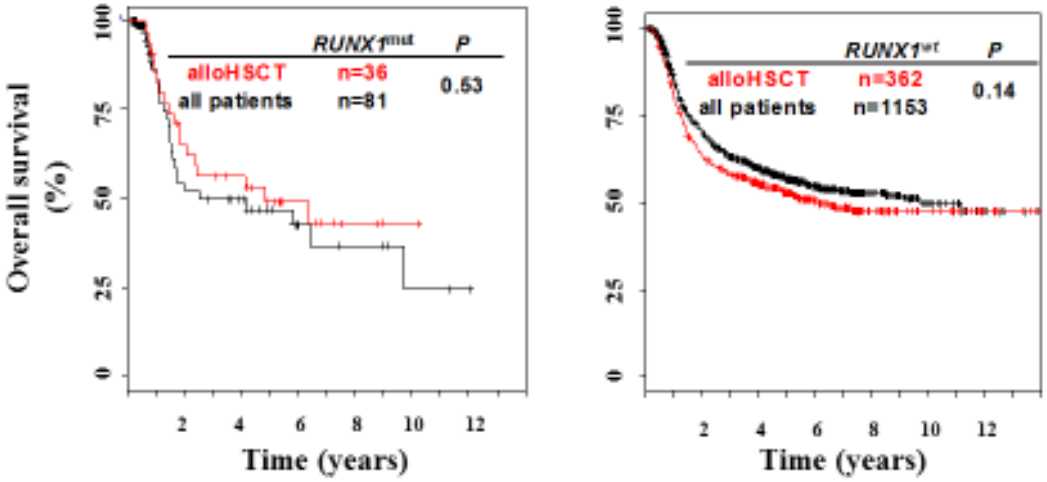


Figure 10. Simon Makuch survival estimates showing overall survival in younger patients (≤ 60 years) with or without *RUNX1* mutations who underwent an alloHSCT in first CR. There was no improvement in OS for patients who underwent an alloHSCT regardless of their *RUNX1* mutation status.| **alloHSCT,** allogeneic hematopoietic stem cell transplantation.

3.6 Stability of *RUNX1* mutations

RUNX1 mutation status at the time of diagnosis and relapse could be analyzed in 16 out of 88 relapsed cases. The mutation was lost in 3 of 16 cases. In 2 cases, 2 mutations were present at diagnosis with loss of one mutation at the time of relapse. (**Table 12**)

Table 12. Representation of the *RUNX1* mutation status at diagnosis and relapse for 16 patients with available samples.

N	Study	Diagnosis		Relapse	
		Exon of mutation	Mutation	Exon of mutation	Mutation
1	07-04	3	c.377_383del;p.A63LfsX7	3	c.377_383del;p.A63LfsX7
2	07-04	3 & 4	c.482delC;p.L98SfsX24;c.513delinsTCCC;p.S141SfsX	3 & 4	c.482delC;p.L98SfsX24;c.513delinsTCCC;p.S141SfsX
3	07-04	8	c.1544_1545insAACCAAAGCGACG;p.V452EfsX	8	c.1544_1545insAACCAAAGCGACG;p.V452EfsX
4	**07-04**	**8**	**c.1226_1227insC;p.R346PfsX**	**No mutation**	**No mutation**
5	**HD98A**	**5**	**c.C791T;p.R201X**	**No mutation**	**No mutation**
6	HD98A	8	c.1226_1227insC;p.R346PfsX	8	c.1226_1227insC;p.R346PfsX
7	HD98A	3 & 4	c.530_534del;p.I114FfsX22;T148_A149fsX10	3 & 4	c.530_534del;p.I114FfsX22;T148_A149fsX10
8	**07-04**	**3**	**c.466_467insTGTGCGCACC;p.D93CfsX48**	**No mutation**	**No mutation**
9	HD98A	4	c.G683C;p.G165R	4	c.G683C;p.G165R
10	HD98A	7	c.1127_1128del;p.L313LfsX	7	c.1127_1128del;p.L313LfsX
11	06-04	8	c.1538_1539insA;p.S450KfsX	8	c.1538_1539insA;p.S450KfsX
12	06-04	4	c.665_666insTTA;p.N159I	4	c.665_666insTTA;p.N159I
13	06-04	3 & 7	c.C528T;p.P113L;c.1148_1155delinsTCCCCCCGGCGG;p.320SfsX	3 & 7	c.C528T;p.P113L;c.1148_1155delinsTCCCCCCGGCGG;p.320SfsX
14	06-04	5 & 7	c.717_718insG;p.T176TfsX37;c.1031_1032insAG;p.Y281X	5 & 7	c.717_718insG;p.T176TfsX37;c.1031_1032insAG;p.Y281X
15	06-04	4	c.C612A;p.S141X	4	c.C612A;p.S141X
16	06-04	4	c.T678A;p.F163Y	4	c.T678A;p.F163Y

Abbreviations: N, patient number| **del**, deletion| **ins**, insertion| **fs**, frameshift| **c,** cDNA| **A**=Adenine**, C**=Cytosine**, G**=Guanine**; T**=Thymine| **p,** protein, **A-Y**, amino acids symbols

4. DISCUSSION

This work presents a comprehensive analysis on *RUNX1* mutations in 2439 intensively treated adults patients with AML. A broad spectrum of molecular markers were available allowing to investigate the pattern of cooperating mutations as well as clinical and genetic characteristics and outcome.

Overall, *RUNX1* mutations were identified in 10 % (245 of 2439) of cases, 24.2 % in secondary AML and 9.2 % in *de novo* AML. The incidence of *RUNX1* mutations increased with age. In our study the median age of patients with *RUNX1* mutations was 59.2. Based on the latest SEER data, the median age at diagnosis for AML patients is 67 years, thus *RUNX1* emerges as one of the most frequently mutated genes in AML.

Our results are in accordance with previously published data. In the study by Mendler *(Mendler J.H. et al. 2012), RUNX1* mutations were found more frequently in patients over 60 years of age (16 % vs 8 %) but only patients with CN-AML were included in that study. In the Taiwanese study published by Tang (*Tang J.L. et al. 2009)* on 470 patients with *de novo* AML, the incidence of *RUNX1* mutations was 13 % as compared with 8 % incidence of *RUNX1* mutations in *de novo* AML in the present study. The difference may be caused by the selection of the study population with only *de novo* non-APL included or with ethnic differences. In another study in *de novo* AML with normal karyotype or non-complex karyotype (*Schnittger S. et al. 2011*), the incidence of *RUNX1* mutations was 32.7 %.

With regard to morphology, we identified a strong association of *RUNX1* mutations with undifferentiated AML (FAB M0). This feature was previously reported *(Preudhomme C. et al. 2000)* and confirmed in subsequent studies and also by the positive association with a more immature immunophenotype (*Tang J.L. et al. 2009, Schnittger S. et al. 2011).* The authors identified a positive association with CD34 and HLA DR expression and an inverse correlation with CD33, CD15, CD56 and CD19. Notably, also the type of *RUNX1* involvement in the genetic change influences the leukemic phenotype as patients with point mutations have a more undifferentiated phenotype, whereas patients with balanced translocations like t(8;21)(q22;q22) or t(12;21)(p13;q22) present with AML with FAB M2 morphology or with lymphoblastic leukemia FAB L1 or L2 (*Vardiman J.W. et al. 2008).* Regarding other clinical characteristics, *RUNX1* mutations occurred more frequently in males, presented with higher platelet counts and lower LDH serum levels. These findings are not uniformly reported across published studies. Tang et al. reported lower LDH levels

in patients with *RUNX1* mutations in *de novo* AML, whereas Greif et al. reported higher LDH levels for *RUNX1* mutated patients with CN-AML. Regarding the WBC in our study, no difference was found between patients with or without *RUNX1* mutations. By contrast, Mendler et al. and Greif et al. reported lower WBC and blasts in the peripheral blood and Schnittger S. et al. reported lower platelets counts in association with *RUNX1* mutations. These differences can result from differences in the analyzed cohorts but the lower WBC count can also reflect the association with a previous history of MDS (*Xu X.Q. et al. 2014).* *RUNX1* mutations were significantly associated with secondary AML evolving from MDS, a finding that is in accordance with earlier reports on smaller patient cohorts (*Harada H. et al. 2004)* and is not surprising considering that *RUNX1* mutations are detected in 5-10 % of advanced MDS (*Bejar R. et al. 2012, Papaemmanuil E. et al. 2013, Haferlach T. et al. 2014).* Of the cases with *RUNX1* mutations and t-AML in our patient cohort, none harbored balanced translocations involving *RUNX1*, otherwise one of the most frequently disrupted gene by translocations in t-AML (*Roulston D. et al. 1998).* This finding points to different pathways in which *RUNX1* mutations can be involved in leukemogenesis.

The most frequent cytogenetic abnormalities identified in patients with *RUNX1* mutated AML in our study were abnormalities of chromosome 7 (-7/7q-) and trisomy 13. Other previously reported abnormalities (*Tang J.L. et al.* 2009, *Gaidzik V.I. et al. 2012*) such as trisomy 8 and trisomy 21 were not significantly associated with *RUNX1* mutations. Regarding trisomy 13, Döhner et al. first reported the abnormality in association with an undifferentiated phenotype in 1990. In that study, a series of 8 cases with isolated trisomy or tetrasomy 13 were reported, all with an undifferentiated phenotype and classified as AML (5 cases), biphenotypic leukemia (1 case), ALL (1 case) and undifferentiated leukemia (1 case). Diagnosis was based on histology, immunphenotyping and conventional cytogenetics. Since then, other groups reported the distinct undifferentiated phenotype in association with trisomy 13 and *RUNX1* mutations (*Silva F.F. et al. 2007, Dicker F. et al. 2007 and Schnittger S. et al. 2011*). In a recent publication on 34 AML patients with trisomy 13 by Herold et al *RUNX1* mutations were identified in 75 % of the patients and the presence of trisomy 13 conferred an inferior survival within the ELN Intermediate II risk group. Based on these findings, one may hypothesize that there are genes mapped on chromosome 13 that cooperate with *RUNX1* mutations in leukemogenesis.

Regarding the association of *RUNX1* mutations with other genetic abnormalities, we found a negative association with the abnormalities listed in the current WHO category "AML with recurrent genetic abnormalities". *RUNX1* mutations inversely correlated with the presence

of *NPM1* mutation and biallelic *CEBPA* mutations. We identified only 13 cases with concurrent *NPM1* and *RUNX1* mutation and 2 cases with biallelic *CEBPA* mutation and *RUNX1* mutation. These results are similar to those reported by other groups. Mendler J.H. et al. reported 4 patients with concurrent *RUNX1* and *NPM1* mutation out of 472 AML cases with normal karyotype or sole trisomy 8 and Fasan A. et al. reported concurrent mutations in 11 out of 2722 AML cases in the intermediate risk group. Similar findings were also reported by the Cancer Genome Atlas Research Network (*Ley T.J. et al. 2013)* identifying a mutual exclusivity of *RUNX1* mutations and the transcription factor fusions *PML-RARA, MYH11-CBFß, RUNX1-RUNX1T1* and with *NPM1* and *CEBPA* mutations

Genes that were frequently co-mutated in patients with *RUNX1* mutations, were genes encoding epigenetic modifiers such as *ASXL1*, *IDH2* and *KMT2A*. In exploratory analyses we investigated the clinical significance of such secondary genotypes. In particular patients with the *RUNX1*^{mut}/*ASXL1*^{mut} genotype had more advanced age at diagnosis and more frequently presented with a history of previous myelodysplastic syndrome than patients with *RUNX1*^{mut}/*ASXL1*^{wt} genotype; this finding is in line with the high incidence of *ASXL1* mutation in MDS (*Gelsi-Boyer V. et al. 2012).* An interesting finding was the high proliferative nature conferred by this genotype (median WBC 21G/I for *RUNX1*^{mut}/*ASXL1*^{mut} genotype vs 11G/I for *RUNX1*^{mut}/*ASXL1*^{wt} genotype, P=0.04). As already shown in several studies, the presence of *ASXL1* mutation in MDS/AML confers a proliferative phenotype and predicts for rapid disease progression (*Bejar R. et al. 2012, Thol F. et al. 2012, Gelsi-Boyer V. et al. 2012).*

Inherited *RUNX1* mutations are associated with familial platelet disorder and approximately 20-50 % of cases progress to AML/MDS. In our cohort testing for *RUNX1* germline mutations was possible in 10 patients (8 with *NPM1*^{mut}/*RUNX1*^{mut} genotype, 1 patient with *CEBPA*^{dm}/*RUNX1*^{mut} genotype and 1 patient with t(8;21)(q22;q22)/*RUNX1*^{mut}). Only in one patient with *NPM1*^{mut}/*RUNX1*^{mut} genotype a *RUNX1* mutation (c.G991A.p.M267I) was identified both in bone marrow sample from diagnosis and in peripheral blood sample at the time of complete hematologic remission. MRD analysis for *NPM1* mutation was also negative. For this case the germline origin of the mutation cannot be excluded. No data on family history for this patient was available.

In a report by Mendler J.H. et al., *RUNX1* mutations were detected in the germline material (buccal swabs) in 3 of 4 patients with co-occurring *NPM1* mutation. This finding could not be confirmed in a subsequent study by Fasan A. et al. and is also not supported by our results.

In the present study, we identified *RUNX1* mutations as stable marker during the course of the disease meaning that the same mutation identified at diagnosis was present at relapse. We were able to analyze 16 cases with paired samples at diagnosis and relapse and identified 13 of 16 cases maintaining at least one mutation present at diagnosis. In the study by Tang J.L. et al. matched paired samples from diagnosis and relapse were available in 6 patients, 2 had the same mutation at diagnosis and relapse, 2 lost the mutation at relapse and the remaining 2 patients had initially 2 mutations and one was lost at the time of relapse. This feature of mutation can be further used to establish a molecular marker for minimal residual disease monitoring. For that purpose, Kohlmann A. et al. addressed the stability of *RUNX1* mutations in a study analyzing 57 paired AML samples at diagnosis and relapse. In 47/57 (82.5 %) cases, the same *RUNX1* mutation was detected and in one case the mutation was lost at relapse. In 9 cases a novel *RUNX1* mutation was acquired. New *RUNX1* mutations at the time of relapse were not restricted to the initial affected gene region.

Using next-generation sequencing (NGS), a cut-off level for MRD was defined that allowed patient stratification in "good" (mutation burden <3.6 %) and "bad" responders (mutation burden ≥3.6 %) with significant impact on OS (57 months vs 32 months, P=0.002). These first data suggest that *RUNX1* can be used as a suitable marker for MRD monitoring, however, these data need to be validated in larger and prospective cohorts.

Regarding the site of mutations, in our study mutations located in the RHD were more frequent than mutations in the TAD (49.6 % vs 40 %) and missense mutations prevailed in the RAD while frameshift were more frequent in the TAD. These findings are in line with the results published by Tang J.L. et al. and Schnittger S. et al. We did not find any impact on response to therapy or outcome according to whether mutation were in the RHD or TAD, but mutations located in the RHD were associated with older age (61.5 years vs 56 years) and prevailed in the ELN Intermediate II risk group. The lack of difference regarding other clinical and biological characteristics between mutations in the functional domains despite biochemical differences suggests a common mechanism by which they contribute to leukemogenesis. Harada H. et al. and Harada Y. et al. postulated that *RUNX1* mutants lead to loss of the normal transactivation potential and suggested 3 possible mechanism of action: through haploinsufficiency for tumor suppression, dominant negative effect on normal *RUNX1* function or cooperating mutations.

Regarding the impact on response to therapy, the presence of *RUNX1* mutations was associated with a poor response to double induction therapy and this was mainly attributed to the high rate of resistant disease in patients with *RUNX1* mutations (40.6 %). This negative

effect on response was even more pronounced in the subgroup of patients with *RUNX1* mutations and secondary AML (71 %). The poor response to induction therapy was uniformly reported across studies and was identified in all age groups and also the group of CN-AML. Tang J.L. et al. identified a lower CR rate among patients with *de novo* AML with *RUNX1* mutations with CR rates of 56 % vs 77.5 % in patients with *RUNX1* wildtype status. In the cohorts published by Mendler J.H. et al. and Greif P. et al. patients with *RUNX1* mutations achieved lower CR rates after standard induction therapy compared to patients with wild type status (47 % and 30 % vs 77 % and 73 %).

In survival analysis, we identified *RUNX1* mutations as a negative prognostic factor for EFS, RFS and OS only in univariate analysis. In multivariate analysis, the negative impact was retained for EFS but not for RFS or OS. Previous data reported a negative impact for *RUNX1* mutations on survival but the study populations were restricted to *de novo* AML *(Tang J.L. et al. 2009)* or CN-AML *(Mendler J.H. et al. 2012).* In the present study, patients covering all ELN genetic risk groups, elderly patients, as well as patients with secondary AML were included in the survival analysis and we identified a significant association of *RUNX1* mutations with both secondary AML and more advanced age, already established unfavorable prognostic markers. In this context it is difficult to attribute the dismal outcome to only one risk factor.

Given the high resistance rate to induction therapy and inferior outcome for patients with *RUNX1* mutations, the value of alloHSCT in first remission was evaluated in 36 of 81 patients younger than 60 years transplanted in first CR. AlloHSCT had a positive impact on RFS, but there was no significant benefit with regard to OS, likely due to the fact that patients with *RUNX1* mutations could be successfully salvaged in second-line treatment. This suggests that alloHSCT may be a reasonable treatment option for fit patients in first CR who have a matched-related donor. In the Taiwanese study (*Tang J.L. et al. 2009*) alloHSCT improved both RFS and OS but only *de novo* AML patients were analyzed and the number of patients was small. (only 11 of the 96 transplanted patients had a *RUNX1* mutation).

Finally, we performed explorative analysis of secondary genotypes. As already published by Paschka P. et al., the $RUNX1^{mut}/ASXL1^{mut}$ genotype emerged as particularly unfavorable in terms of achieving CR and long term outcome when compared with other $RUNX1^{mut}$ genotypes. For younger patients the role of alloHSCT needs further evaluation; pharmacologic inhibition of the H3K27 demethylation as a therapeutic target given the loss of H3K27me3 induced by *ASXL1* mutations (*Kruidenier L. et al. 2012)* may be a further approach for these patients. On the other side, the $RUNX1^{mut}/IDH2^{mut}$ genotype had a

positive impact on outcome compared to the *RUNX1*^{mut}/*IDH2*^{wt} genotype. This finding may be of interest considering treatment option with an IDH2 inhibitor. Consistent with previous data (*Schnittger S. et al. 2011*), the combined genotype *RUNX1*^{mut}/*KMT2A*-PTD did not predict for shorter RFS or OS in comparison to *RUNX1*^{mut}/*KMT2A*^{wt} genotype.

In summary, some biologically and clinically relevant characteristics of AML with *RUNX1* mutations could be described in the present study:

1. *RUNX1* mutations are among the most frequent genetic lesions in AML.
2. *RUNX1* mutations are mutually exclusive of the other abnormalities included in the current WHO category "AML with recurrent genetic abnormalities".
3. *RUNX1* mutations exhibit a characteristic pattern of concurrent genetic lesions.
4. *RUNX1* mutations are associated with specific presenting clinical and pathologic features.
5. *RUNX1* mutations are associated with a high resistance rate to standard induction therapy and inferior outcome urging for new therapeutic approaches.

Still, the precise role of *RUNX1* mutation as a disease defining genetic alteration needs further evaluation. More sensitive methods unraveled new mutations - with mutations in the cohesion family genes and spliceosome genes being recently reported (*Thota S. et al. 2014)* as significantly associated with *RUNX1* mutations - and will help to better understand the role of *RUNX1* in leukemogenesis.

5. CONCLUSION

Acute myeloid leukemia (AML) is characterized by a great cytogenetic and molecular genetic diversity. Past years research has shed more light on the molecular background of the disease and identified new mutations and pathways, for example *NPM1* and *FLT3* mutations that allowed a more precise prognostic stratification beyond cytogenetics and opened the way to targeted therapies.

This study aimed to characterize the role of *RUNX1* mutations in AML focusing on the distribution of *RUNX1* mutations among different WHO categories of AML, identifying cytogenetic and molecular markers associated with *RUNX1* mutations and evaluating their clinical impact.

To this aim, a total of 2439 adult AML patients enrolled treatment trials of the German-Austrian Study Group (AMLSG) were analyzed for *RUNX1* mutations using Sanger sequencing. Overall, 280 *RUNX1* mutations were detected in 245 (10 %) of 2439 AML patients. The majority of mutations clustered in exon 4 (n=81, 28.9 %) and exon 8 (n=63, 22.5 %). Approximately half of the *RUNX1* mutations (49.6 %) were located in the Runt homology domain (RHD) and 40 % in the transactivation domain (TAD). Frameshift mutations prevailed in the TAD and missense mutation in the RHD.

Regarding clinical characteristics, compared to patients with *RUNX1* wildtype patients, *RUNX1* mutated patients presented with more advanced age (59.2 years *vs* 53.6 years), had more immature FAB morphology (FAB M0) and secondary AML evolving from myelodysplastic syndrome. In the current category of "AML with recurrent genetic abnormalities" of the WHO 2008 Classification, *RUNX1* mutations were largely mutually exclusive of all recurrent genetic abnormalities. We identified a significant association between *RUNX1* mutation and abnormalities of chromosome 7 (-7/7q-) and trisomy 13. Molecular markers significantly associated with *RUNX1* mutations were mutations in the epigenetic modifiers *ASXL1*, *IDH2* and *KMT2A*. Among combined genotypes, the *RUNX1*^{mut}/*ASXL1*^{mut} genotype conferred a particularly poor prognosis.

A significant finding was the high rate of resistant disease after double induction (41 %) for the *RUNX1* mutated patients. In univariable analysis, *RUNX1* mutations were a negative prognostic factor for all survival endpoints, whereas in multivariable analysis only event-free survival retained significance. For young patients eligible for allogeneic hematopoietic cell transplantation, a benefit in terms of prolonged relapse-free survival was found

suggesting that allogeneic transplantation may be a therapeutic option for selected patients with *RUNX1* mutations.

The results of the present study identified *RUNX1* as one of the most frequently mutated genes in AML. AML with *RUNX1* mutations have distinct clinical and biological characteristics and may be considered as a genetic lesion defining a novel disease entity. Given the association with older age and the high rate of resistant disease future work will focus on identifying pathways by which *RUNX1* mutations contribute to leukemic transformation with the aim of developing new targeted therapies and improve outcome.

6. REFERENCES

1. **Alexandrow LB**, Nik-Zainal S, Wedge DC, Aparicio SA, Behjati S, Biankin AV, Bignell GR, Bolli N, Borg A, Børresen-Dale AL, Boyault S, Burkhardt B, Butler AP, Caldas C, Davies HR, Desmedt C, Eils R, Eyfjörd JE, Foekens JA, Greaves M, Hosoda F, Hutter B, Ilicic T, Imbeaud S, Imielinski M, Jäger N, Jones DT, Jones D, Knappskog S, Kool M, Lakhani SR, López-Otín C, Martin S, Munshi NC, Nakamura H, Northcott PA, Pajic M, Papaemmanuil E, Paradiso A, Pearson JV, Puente XS, Raine K, Ramakrishna M, Richardson AL, Richter J, Rosenstiel P, Schlesner M, Schumacher TN, Span PN, Teague JW, Totoki Y, Tutt AN, Valdés-Mas R, van Buuren MM, van 't Veer L, Vincent-Salomon A, Waddell N, Yates LR; Australian Pancreatic Cancer Genome Initiative; ICGC Breast Cancer Consortium; ICGC MMML-Seq Consortium; ICGC Ped Brain, Zucman-Rossi J, Futreal PA, McDermott U, Lichter P, Meyerson M, Grimmond SM, Siebert R, Campo E, Shibata T, Pfister SM, Campbell PJ, Stratton MR: Signatures of mutational process in human cancer. Nature 500: 415-21 (2012)
2. **Bejar R**, Stevenson KE, Abdel-Wahab O, Galili N, Nilsson B, Garcia-Manero G, Kantarjian H, Raza A, Levine RL, Neuberg D, Ebert BL: Clinical effect of point mutations in myelodysplastic syndromes. N Engl J Med 364: 2496-506 (2011)
3. **Bejar R**, Stevenson KE, Caughey BA, Abdel-Wahab O, Steensma DP, Galili N, aza A, Kantarjian H, Levine RL, Neuberg D, Garcia-Manero G, Ebert BL: Validation of a prognostic model and the impact of mutations in patients with lower-risk myelodysplastic syndromes. J Clin Oncol 30: 3376-82 (2012)
4. **Béri-Dexheimer M**, Latger-Cannard V, Philippe C, Bonnet C, Chambon P, Roth V, Grégoire MJ, Bordigoni P, Lecompte T, Leheup B, Jonveaux P: Clinical phenotype of germline RUNX1 haploinsufficiency: from point mutations to large genomic deletions. Eur J Hum Genet 16: 1014-18 (2008)
5. **Cheson BD,** Bennett JM, Kopecky KJ, Büchner T, Willman CL, Estey EH, Schiffer CA, Doehner H, Tallman MS, Lister TA, Lo-Coco F, Willemze R, Biondi A, Hiddemann W, Larson RA, Löwenberg B, Sanz MA, Head DR, Ohno R, Bloomfield CD: International Working Group for Diagnosis, Standardization of Response Criteria, Treatment Outcomes, and Reporting Standards for Therapeutic Trials in Acute Myeloid Leukemia. Standardization of Response Criteria, Treatment Outcomes, and Reporting Standards for Therapeutic Trials in Acute Myeloid Leukemia. J Clin Oncol 21: 4642-49 (2003)

6. Corbacioglu A, Scholl C, Schlenk R.F. RF, Eiwen K, Du J, Bullinger L, Fröhling S. S, Reimer P, Rummel M, Derigs HG, Nachbaur D, Krauter J, Ganser A, Döhner H, Döhner K: Prognostic impact of minimal residual disease in CBFß-MYH11-positive acute myeloid leukemia. J Clin Oncol 28: 3724-3729 (2010)

7. Cornelissen JJ, Gratwohl A, Schlenk R.F. RF, Sierra J, Bornhäuser M, Juliusson G.G, Råcil Z, Rowe JM, Russell N, Mohty M, Löwenberg B, Socié G, Niederwieser D, Ossenkoppele GJ:The European LeukemiaNet AML Working Party consensus statement on allogeneic HSCT for patients with AML in remission: an integrated-risk adapted approach. Nat Rev Clin Oncol 9: 579-90 (2012)

8. Dang L, White DW, Gross S, Bennett BD, Bittinger MA, Driggers EM, Fantin VR, Jang HG, Jin S, Keenan MC, Marks KM, Prins RM, Ward PS, Yen KE, Liau LM, Rabinowitz JD, CantLey T.J. LC, Thompson CB, Vander Heiden MG, Su SM: Cancer-associated IDH1 mutations produce 2-hydroxyglutarate. Nature 462: 739-44 (2009)

9. De Braekeleer E, Férec C, De Braekeleer M: RUNX1 translocations in malignant hemopathies. Anticancer Res 29: 1031-1037 (2009)

10. Ding Y, Harada Y, Imagawa J, Kimura A, Harada H: AML1/RUNX1 point mutation possibly promotes leukemic transformation in myeloproliferative neoplasms. Blood 114: 5201-5 (2009)

11. Döhner H, DC Arthur, ED Ball, RE Sobol, FR Davey, D Lawrence, L Gordon, SR Patil, RB Surana, JR Testa: Trisomy 13: a new recurring chromosome abnormality in acute leukemia. Blood 76: 1614-21 (1990)

12. Döhner H, Estey EH, Amadori S, Appelbaum FR, Büchner T, Burnett AK, Dombret H, Fneaux P, Grimwade D.D, Larson RA, Lo-Coco F, Naoe T, Niederwieser D, Ossenkoppele GJ, Sanz MA, Sierra J, Tallman MS, Löwenberg B, Bloomfield CD: Diagnosis and management of acute myeloid leukemia in adults: recommendations from an international expert panel, on behalf of the European LeukemiaNet. Blood 115: 453-74 (2015)

13. Döhner H, Gaidzik VI: Impact of genetic features on treatment decisions in AML: Hematology Am Soc Hematol Educ Program 36-42 (2013)

14. Döhner H, Weisdorf DJ, Bloomfield CD**:** Acute myeloid leukemia. N Engl J Med 373: 1136-1152 (2015)

15. Döhner K, Döhner H: Molecular characterization of acute myeloid leukemia. Haematologica 93: 976-82 (2008)

16. **Döhner K,** Paschka P: Intermediate-risk acute myeloid leukemia therapy: current and future. Hematology Am Soc Hematol Educ Program 34-43 (2014)
17. **El Hajj H**, Dassouki Z, Berthier C, Raffoux E, Ades L, Legrand O, Hleihel R, Sahin U, Tawil N, Salameh A, Zibara K, Darwiche N, Mohty M, Dombret H, Fenaux P, de Thé H, Bazarbachi A: Retinoic acid and arsenic trioxide trigger degradation of mutated NPM1, resulting in apoptosis of AML cells. Blood 125: 3447-54 (2015)
18. **Estey E**, Döhner H: Acute myeloid leukemia. Lancet 368: 1894-907 (2006)
19. **Falini B**, Mecucci C, Tiacci E, Alcalay M, Rosati R, Pasqualucci L, La Starza R, Diverio D, Colombo E, Santucci A, Bigerna B, Pacini R, Pucciarini A, Liso A, Vignetti M, Fazi P, Meani N, Pettirossi V, Saglio G, Mandelli F, Lo-Coco F, Pelicci PG, Martelli MF; GIMEMA Acute Leukemia Working Party: Cytoplasmic nucleophosmin in acute myelogenous leukemia with a normal karyotype**.** N Engl J Med 20: 352: 254-66 (2005)
20. **Fröhling S**, Schlenk R.F. RF, Stolze I, Bihlmayr J, Benner A, Kreitmeier S, Tobis K, Döhner H, Döhner K: CEBPA mutations in younger adults with acute myeloid leukemia and normal cytogenetics: prognostic relevance and analysis of cooperating mutations. J Clin Oncol 22: 624-633 (2004)
21. **Gaidzik VI**, Bullinger L, Schlenk R.F. RF, Zimmermann AS, Röck J, Paschka P, Corbacioglu A, Krauter J, Schlegelberger B, Ganser A, Späth D, Kündgen A, Schmidt-Wolf IG, Götze K, Nachbaur D, Pfreundschuh M, Horst HA, Döhner H., Döhner K: RUNX1 mutations in acute myeloid leukemia: results from a comprehensive genetic and clinical analysis from the AML study group. J Clin Oncol 29: 1364-72 (2011)
22. **Gaidzik VI**, Paschka P, Späth D, Habdank M, Köhne CH, Germing U, von Lilienfeld-Toal M, Held G, Horst HA, Haase D, Bentz M, Götze K, Döhner H., Schlenk R.F. RF, Bullinger L, Döhner K: TET2 mutations in acute myeloid leukemia (AML): results from a comprehensive genetic and clinical analysis of the AML study group. J Clin Oncol 30: 1350-7 (2012)
23. **Gaidzik VI**, Schlenk R.F. RF, Paschka P, Stölzle A, Späth D, Kuendgen A, von Lilienfeld-Toal M, Brugger W, Derigs HG, Kremers S, Greil R, Raghavachar A, Ringhoffer M, Salih HR, Wattad M, Kirchen HG, Runde V, Heil G, Petzer AL, Girschikofsky M, Heuser M, Kayser S. S, Goehring G, Teleanu MV, Schlegelberger B, Ganser A, Krauter J, Bullinger L, Döhner H., Döhner K: Clinical impact of DNMT3A mutations in younger adult patients with acute myeloid leukemia: results of the AML Study Group (AMLSG). Blood 121: 4769-77 (2013)

24. Gelsi-Boyer V, Brecqueville M, Devillier R, Murati A, Mozziconacci MJ, Birnbaum D: Mutations in ASXL1 are associated with poor prognosis across the spectrum of malignant myeloid diseases. J Hematol & Oncol 5:12 (2012)

25. Genovese G, Kähler AK, Handsaker RE, Lindbergh J, Rose S, Bakhoum S, Chambert K, Mick E, Neale B, Fromer M, Purcell S, Svantesson O, Landen M, Höglund M, Lehmann S, Gabriel S, Moran J, Lander E, Sullivan P, Sklar P, Grönberg H, Hultman C, McCaroll S: Clonal hematopoiesis and blood-cancer risk inferred from blood DNA sequence. N Engl J Med 371: 2477-87 (2014)

26. Gilliland DG: Hematologic malignancies. Curr Opin Hematol 8: 189-91 (2001)

27. Gorin NC, Labopin M, Frassoni F, Milpied N, Attal M, Blaise D, Meloni G, Iori AP, Michallet M, Willemze R, Deconninck E, Harousseau JL, Polge E, Rocha V: Identical outcome after autologous or allogeneic genoidentical hematopoietic stem-cell transplantation in first remission of acute myelocytic leukemia carrying inversion 16 or t(8;21): a retrospective study from the European Cooperative Group for Blood and Marrow Transplantation. J Clin Oncol 26: 3183-8 (2008)

28. Goyama S, Schibler J, Cunningham L, Zhang Y, Rao Y, Nishimoto N, Nakagawa M, Olsson A, Wunderlich M, Link KA, Mizukawa B, Grimes HL, Kurokawa M, Liu PP, Huang G, Mulloy JC: Transcription factor RUNX1 promotes survival of acute myeloid leukemia cells. J Clin Invest 123: 3876-88 (2013)

29. Greif PA, Konstandin NP, Metzeler KH, Herold T, Pasalic Z, Ksienzyk B, Dufour A, Schneider F, Schneider S, Kakadia PM, Braess J, Sauerland MC, Berdel WE, Büchner T, Woermann BJ, Hiddemann W, Spiekermann K, Bohlander SK: RUNX1 mutations in cytogenetically normal acute myeloid leukemia are associated with a poor prognosis and up-regulation of lymphoid genes. Haematologica 97: 1909-15 (2012)

30. Grimwade D, Freemann SD: Defining minimal residual disease in acute myeloid leukemia: which platforms are ready for "prime time"?. Blood 124: 3345-3355 (2014)

31. Grimwade D: The clinical significance of cytogenetic abnormalities in acute myeloid leukaemia. Best Pract Res Clin Haematol. 14: 497-529 (2001)

32. Gröschel S, Sanders MA, Hoogenboezem R, de Wit E, Bouwman BA, va der Velden VH, Havermans M, Avellino R, van Lom K, Rombouts EJ, van Duin M, Döhner K, Beverloo HB, Bradner JE, Döhner H., Löwenberg B, Valk PJ, Bindels EM, de Laat W, Delwel R: A single oncogeneic enhancer rearrangement causes concomitant EVI1 and GATA2 deregulation in leukemia. Cell 157: 369-81 (2014)

33. **Haferlach T**, Nagata Y, Grossmann V, Okuno Y, Bacher U, Nagae G, Schnittger S, Sanada M, Kon A, Alpermann T, Yoshida K, Roller A, Nadarajah N, Shiraishi Y, Shiozawa Y, Chiba K, Tanaka H, Koeffler HP, Klein HU, Dugas M, Aburatani H, Kohlmann A, Miyano S, Haferlach C, Kern W, Ogawa S: Landscape of genetic lesions in 944 patients with myelodysplastic syndromes. Leukemia 28: 241-7 (2014)
34. **Harada H**, Harada Y, Niimi H, Kyo T, Kimura A, Inaba T: High incidence of somatic mutations in the AML1/RUNX1 gene in myelodysplastic syndrome and low blast percentage myeloid leukemia with myelodysplasia. Blood 103: 2316-24 (2004)
35. **Harada H**, Harada Y, Tanaka H, Kimura A, Inaba T: Implications of somatic mutations in the AML1 gene in radiation-associated and therapy-related myelodysplastic syndrome/acute myeloid leukemia. Blood 101: 673-80 (2003)
36. **Harada Y**, Harada H: Molecular pathways mediating MDS / AML with focus on AML1/RUNX1 point mutations. J Cell Physiol 220: 16–20 (2009)
37. **Harrell FE**: Regression modeling strategies: with applications to linear models, logistic regression, and survival analysis. Springer, Berlin, Heidelberg, New York, P. 147-176 (2001)
38. **Ichikawa M**, Yoshimi A, Nakagawa M, Nishimoto N, Watanabe-Okochi N, Kurokawa M:. A role for RUNX1 in hematopoiesis and myeloid leukemia. Int J Hematol 97: 726-34 (2013)
39. **Ito Y**, Bae SC, Chuang LS: The RUNX family: developmental regulators in cancer Nat Rev Cancer 15: 81-95 (2015)
40. **Jaiswal S,** Fontanilla P, Flannic J, Manning A, Graumann P, Mar B, Coleman L, Mermel C, Burtt N, Chaves A, Higgins J, Moltchanov V, Kuo F, Kluk M, Henderson B, Kinnunen L, Koistinen H, Ladenvall C, Getz G, Correa A, Banahan B, Gabriel S, Kathiresan S, Stringham H, McCarthy M, Boehnke M, Tuomilehto J, Haiman C, Groop L, Atzmon G, Wilson J, Neuberg D, Altshuler D, Ebert B: Age related clonal hematopoiesis associated with adverse outcome. N Engl J Med 371: 2488-2498 (2014)
41. **Janin M**, Mylonas E, Saada V, Micol JB, Renneville A, Quivoron C, Koscielny S, Scourzic L, Forget S, Pautas C, Caillot D, Preudhomme C, Dombret H, Berthon C, Barouki R, Rabier D, Auger N, Griscelli F, Chachaty E, Leclercq E, Courtier MH, Bennaceur-Griscelli A, Solary E, Bernard OA, Penard-Lacronique V, Ottolenghi C, de Botton S: Serum 2-hydroxyglutarate production in IDH1- and IDH2-mutated de novo acute myeloid leukemia: a study by the Acute Leukemia French Association group. J Clin Oncol 32: 297-305 (2014)

42. Juliusson G, Antunovic P, Derolf Å, Lehmann S, Möllgard L, Stockelberg D, Tidefelt U, Wahlin A, Höglund M: Age and acute myeloid leukemia: real world data on decision to treat and outcomes from the Swedish Acute Leukemia Registry. Blood 113: 4179-4187 (2009)

43. Kao HW, Liang DC, Wu JH, Kuo MC, Wang PN, Yang CP, Shih YS, Lin TH, Huang YH, Shih LY: Gene mutation patterns in patients with minimally differentiated acute myeloid leukemia. Neoplasia 16: 481-8 (2014)

44. Kaplan EL, Meier P: Nonparametric estimation from incomplete observations. J Am Stat Assoc 53: 457-81 (1958)

45. Kohlmann A, Nadarajah N, Alpermann T, Grossmann V, Schindela S, Dicker F, Roller A, Kern W, Haferlach C, Schnittger S, Haferlach T: Monitoring of residual disease by next-generation deep-sequencing of RUNX1 mutations can identify acute myeloid leukemia patients with resistant disease. Leukemia 28:129-37 (2014)

46. Korn EL: Censoring distributions as a measure of follow-up in survival analysis. Stat Med. 5: 255-60 (1986)

47. Krönke J, Schlenk R.F. RF, Jensen KO, Tschürtz F, Corbacioglu A, Gaidzik VI, Paschka P, Onken S, Eiwen K, Habdank M, Späth D, Lübbert M, Wattad M, Kindler T, Salih HR, Held G, Nachbaur D, von Lilienfeld-Toal M, Germing U, Haase D, Mergenthaler HG, Krauter J, Ganser A, Göhring G, Schlegelberger B, Döhner H., Döhner K: Monitoring of minimal residual disease in NPM1-mutated acute myeloid leukemia: a study from the German-Austrian acute myeloid leukemia study group. J Clin Oncol 29: 2709-16 (2011)

48. Kruidenier L, Chung CW, Cheng Z, Liddle J, Che K, Joberty G, Bantscheff M, Bountra C, Bridges A, Diallo H, Eberhard D, Hutchinson S, Jones E, Katso R, Leveridge M, Mander PK, Mosey J, Ramirez-Molina C, Rowland P, Schofield CJ, Sheppard RJ, Smith JE, Swales C, Tanner R, Thomas P, Tumber A, Drewes G, Oppermann U, Patel DJ, Lee K, Wilson DM: A selective jumonji H3K27 demethylase inhibitor modulates the proinflammatory macrophage response. Nature 488: 404–408 (2012)

49. Kuo MC, Liang DC, Huang CF, Shih YS, Wu JH, Lin TL, Shih LY: RUNX1 mutations are frequent in chronic myelomonocytic leukemia and mutations at the C-terminal region might predict acute myeloid leukemia transformation. Leukemia 23: 1426-31 (2009)

50. Levis M, Small D: FLT3 tyrosine kinase inhibitors. Int J Hematol 82 100-7 (2005)

51. Ley TJ, Ding L, Walter MJ, McLellan MD, Lamprecht T, Larson DE, Kandoth C, Payton JE, Baty J, Welch J, Harris CC, Lichti CF, Townsend RR, Fulton RS, Dooling

DJ, Koboldt DC, Schmidt H, Zhang Q, Osborne JR, Lin L, O'Laughlin M, McMichael JF, Delehaunty KD, McGrath SD, Fulton LA, Magrini VJ, Vickery TL, Hundal J, Cook LL, Conyers JJ, Swift GW, Reed JP, Alldredge PA, Wylie T, Walker J, Kalicki J, Watson MA, Heath S, Shannon WD, Varghese N, Nagarajan R, Westervelt P, Tomasson MH, Link DC, Graubert TA, DiPersio JF, Mardis ER, Wilson RK:. DNMT3A mutations in acute myeloid leukemia. N Engl J Med 363: 2424-33 (2010)

52. **Liakhovitskaia A**, Rybtsov S, Smith T, Batsivari A, Rybtsova N, Rode C, de Bruijn M, Buchholz F, Gordon-Keylock S, Zhao S, Medvinsky A: Runx1 is required for progression of CD41+ embryonic precursors into HSCs but not prior to this. Development 141: 3319-23 (2014)
53. **Lie-A-Ling M**, Marinopoulou E, Li Y, Patel R, Stefanska M, Bonifer C, Miller C, Kouskoff V, Lacaud G: RUNX1 positively regulates a cell adhesion and migration program in murine hemogenic endothelium prior to blood emergence. Blood 124: e11-20 (2014)
54. **Liew E**, Owen C: Familial myelodysplastic syndromes: a review of the literature. Haematologica 96: 1536-42 (2011)
55. **Mangan JK,** Speck NA: RUNX1 mutations in clonal myeloid disorders: from conventional cytogenetics to next generation sequencing, a story 40 years in the making. Crit Rev Oncog 16: 77-91 (2011)
56. **Matheny CJ**, Speck ME, Cushing PR, Zhou Y, Corpora T, Regan M, Newman M, Roudaia L, Speck CL, Gu TL, Griffey SM, Bushweller JH, Speck NA: Disease mutations in RUNX1 and RUNX2 create nonfunctional, dominant-negative, or hypomorphic alleles. EMBO 26: 1163-75 (2007)
57. **Meggendorfer M**, Roller A, Haferlach T, Eder C, Dicker F, Grossmann V, Kohlmann A, Alpermann T, Yoshida K, Ogawa S, Koeffler HP, Kern W, Haferlach C, Schnittger S: SRSF2 mutations in 275 cases with chronic myelomonocytic leukemia (CMML). Blood 120: 3080-8 (2012)
58. **Mendler JH**, Maharry K, Radmacher MD, Mrózek K, Becker H, Metzeler KH, Schwind S, Whitman SP, Khalife J, Kohlschmidt J, Nicolet D, Powell BL, Carter TH, Wetzler M, Moore JO, Kolitz JE, Baer MR, Carroll AJ, Larson RA, Caligiuri MA, Marcucci G, Bloomfield CD: RUNX1 mutations are associated with poor outcome in younger and older patients with cytogenetically normal acute myeloid leukemia and with distinct gene and MicroRNA expression signatures. J Clin Oncol 30: 3109-18 (2012)

59. Metzeler KH, Maharry K, Radmacher MD, Mrózek K, Margeson D, Becker H, Curfman J, Holland KB, Schwind S, Whitman SP, Wu YZ, Blum W, Powell BL, Carter TH, Wetzler M, Moore JO, Kolitz JE, Baer MR, Carroll AJ, Larson RA, Caligiuri MA, Marcucci G, Bloomfield CD: TET2 mutations improve the new European LeukemiaNet risk classification of acute myeloid leukemia: a Cancer and Leukemia Group B study. J Clin Oncol 29: 1373-81 (2011)

60. Mitelman F, Johansson B, Mertens F: The impact of translocations and gene fusions on cancer causation. Nat Rev Cancer 7: 233-45 (2009)

61. Mrózek K, Heerema NA, Bloomfield CD: Cytogenetics in acute leukemia. Blood Rev. 18: 115-36 (2004)

62. Nickels EM, Soodalter J, Churpek JE, Godley LA: Recognizing familial myeloid leukemia in adults. Ther Adv Hematol 4: 254-69 (2013)

63. Okuda T, van Deursen J, Hiebert SW, Grosveld G, Downing JR**:** AML1, the target of multiple chromosomal translocations in human leukemia, is essential for normal fetal liver hematopoiesis. Cell 84: 321-30 (1996)

64. Papaemmanuil E, Gerstung M, Malcovati L, Tauro S, Gundem G, Van Loo P, Yoon CJ, Ellis P, Wedge DC, Pellagatti A, Shlien A, Groves MJ, Forbes SA, Raine K, Hinton J, Mudie LJ, McLaren S, Hardy C, Latimer C, Della Porta MG, O'Meara S, Ambaglio I, Galli A, Butler AP, Walldin G, Teague JW, Quek L, Sternberg A, Gambacorti-Passerini C, Cross NC, Green AR, Boultwood J, Vyas P, Hellstrom-Lindberg E, Bowen D, Cazzola M, Stratton MR, Campbell PJ: Chronic Myeloid Disorders Working Group of the International Cancer Genome Consortium: Clinical and biological implications of driver mutations in myelodysplastic syndromes. Blood 122: 3616-27 (2013)

65. Paschka P, Du J, Schlenk R.F. RF, Gaidzik VI, Bullinger L, Corbacioglu A, Späth D, Kayser S, Schlegelberger B, Krauter J, Ganser A, Köhne CH, Held G, von Lilienfeld-Toal M, Kirchen H, Rummel M, Götze K, Horst HA, Ringhoffer M, Lübbert M, Wattad M, Salih HR, Kündgen A, Döhner H., Döhner K: Secondary genetic lesions in acute myeloid leukemia with inv(16) or t(16;16): a study of the German-Austrian AML Study Group (AMLSG). Blood 121: 170-7 (2013)

66. Paschka P, Schlenk R.F. RF, Gaidzik VI, Herzig JK, Aulitzky T, Bullinger L, Späth D, Teleanu V, Kündgen A, Köhne CH, Brossart P, Held G, Horst HA, Ringhoffer M, Götze K, Nachbaur D, Kindler T1, Heuser M1, Thol F, Ganser A, Döhner H., Döhner K: ASXL1 mutations in younger adult patients with acute myeloid leukemia: a study by the

German-Austrian Acute Myeloid Leukemia Study Group. Haematologica 100: 324-30 (2015)

67. **Patel JP**, Gönen M, Figueroa ME, Fernandez H, Sun Z, Racevskis J, Van Vlierberghe P, Dolgalev I, Thomas S, Aminova O, Huberman K, Cheng J, Viale A, Socci ND, Heguy A, Cherry A, Vance G, Higgins RR, Ketterling RP, Gallagher RE, Litzow M, van den Brink MR, Lazarus HM, Rowe JM, Luger S, Ferrando A, Paietta E, Tallman MS, Melnick A, Abdel-Wahab O, Levine RL: Prognostic relevance of integrated genetic profiling in acute myeloid leukemia. N Engl J Med 366: 1079-89 (2012)
68. **Pileri SA**, Ascani S, Cox MC, Campidelli C, Bacci F, Piccioli M, Piccaluga PP, Agostinelli C, Asioli S, Novero D, Bisceglia M, Ponzoni M, Gentile A, Rinaldi P, Franco V, Vincelli D, Pileri S.A. A Jr, Gasbarra R, Falini B, Zinzani PL, Baccarani M: Myeloid sarcoma: clinico-pathologic, phenotypic and cytogenetic analysis of 92 adult patients. Leukemia 21: 340-50 (2007)
69. **Preudhomme C**, Warot-Loze D, Roumier C, Grardel-Duflos N, Garand R, Lai JL, Dastugue N, Macintyre E, Denis C, Bauters F, Kerckaert JP, Cosson A, Fenaux P: High incidence of biallelic point mutations in the Runt domain of the AML1/PEBP2 alpha B gene in Mo acute myeloid leukemia and in myeloid malignancies with acquired trisomy 21. Blood 96: 2862-2869 (2000)
70. **Preudhomme C**, Renneville A, Bourdon V, Philippe N, Roche-Lestienne C, Boissel N, Dhedin N, André JM, Cornillet-Lefebvre P, Baruchel A, Mozziconacci MJ, Sobol H: High frequency of RUNX1 biallelic alteration in acute myeloid leukemia secondary to familial platelet disorder. Blood 113: 5583-5587 (2009)
71. **Roulston D**, Espinosa R , Nucifora G, Larson RA, Le Beau MM, RowLey T.J. JD: CBFA2 (AML1) translocations with novel partner chromosomes in myeloid leukemias: association with prior therapy. Blood 92: 2879-85 (1998)
72. **Schlenk RF,** Germing U, Hartmann F, Glasmacher A, Fischer JT, del Valle y Fuentes F, Götze K, Pralle H, Nerl C, Salwender H, Grimminger W, Petzer A, Hensel M, Brenner A, Zick L, Döhner K, Fröhling S. S, Döhner H: High dose cytarabine and mitoxantrone in consolidation therapy for acute promyelocytic leukemia. Leukemia 19: 978-983 (2005)
73. **Schlenk RF**, Döhner K, Krauter J, Fröhling S. S, Corbacioglu A, Bukkinger L, Habdank M, Späth D, Morgan M, Benner A, Schlegelberger B, Heil G, Ganser A, Döhner H: Mutations and treatment outcome in cytogenetically normal acute myeloid leukemia. N Engl J Med 358: 1909-18 (2008)

74. Schlenk RF, Döhner K, Mack S, Stoppel M, Király F, Götze K, Hartmann F, Horst HA, Koller E, Petzer A, Grimminger W, Kobbe G, Glasmacher A, Salwender H, Kirchen H, Haase D, Kremers S, Matzdorff A, Benner A, Döhner H: Prospective evaluation of allogeneic hematopoietic stem-cell transplantation from matched related and matched unrelated donors in younger adults with high-risk acute myeloid leukemia: German-Austrian trial AMLHD98A. J Clin Oncol 28: 4642-4648 (2010)

75. Schlenk RF, Taskesen E, van Norden Y, Krauter J, Ganser A, Bullinger L, Gaidzik VI, Paschka P, Corbacioglu A, Göhring G, Kündgen A, Held G, Götze K, Vellenga E, Kuball J, Schanz U, Passweg J, Pabst T, Maertens J, Ossenkoppele GJ, Delwel R, Döhner H., Cornelissen JJ, Döhner K, Löwenberg B: The value of allogeneic and autologous hematopoietic stem cell transplantation in prognostically favorable acute myeloid leukemia with double mutant CEBPA. Blood 122: 1576-1582 (2013)

76. Schlenk RF, Döhner H: Genomic application in the clinic: use in treatment paradigm of acute myeloid leukemia. Hematology Am Soc Hematol Educ Program: 324-330 (2013)

77. Schlenk RF: Post-remission therapy for acute myeloid leukemia. Haematologica 99 1663-70 (2014)

78. Schmit JM, Turner DJ, Hromas RA, Wingard JR, Brown RA, Li Y, Li MM, Slayton WB, Cogle CR: Two novel RUNX1 mutations in a patient with congenital thrombocytopenia that evolved into a high grade myelodysplastic syndrome. Leuk Res Rep 4: 24–27 (2015)

79. Schnittger S, Dicker F, Kern W, Wendland N, Sundermann J, Alpermann T, Haferlach C, Haferlach T: RUNX1 mutations are frequent in de novo AML with noncomplex karyotype and confer an unfavorable prognosis. Blood 117: 2348-57 (2011)

80. Shaffer LG, McGowan-Jordan J, Schmid M**,** (editors). S Karger, ISCN (2013). An International System for Human Cytogenetic Nomenclature. Basel (2013)

81. Silva FP, Lind A, Brouwer-Mandema G, Valk PJ, Giphart-Gassler M: Trisomy 13 correlates with RUNX1 mutation and increased FLT3 expression in AML-M0 patients. Haematologica 92: 1123-6 (2007)

82. Simon R, Makuch RW: A non-parametric graphical representation of the relationship between survival and the occurrence of an event: application to responders versus non-responders bias. Statistics in Medicine 3:35-44 (1984)

83. Skokowa J, Steinemann D, Katsman-Kuipers JE, Zeidler C, Klimenkova O, Klimiankou M, Unalan M, Kandabarau S, Makaryan V, Beekman R, Behrens K, Stocking C, Obenauer J, Schnittger S, Kohlmann A, Valkhof MG, Hoogenboezem R, Göhring G,

Reinhardt D, Schlegelberger B, Stanulla M, Vandenberghe P, Donadieu J, Zwaan CM, Touw IP, van den Heuvel-Eibrink MM, Dale DC, Welte K: Cooperativity of RUNX1 and CSF3R mutations in severe congenital neutropenia: a unique pathway in myeloid leukemogenesis. Blood 123: 2229-37 (2014)

84. Steensma DP, Gibbons RJ, Mesa RA, Tefferi A, Higgs DR: Somatic point mutations in RUNX1/CBFA2/AML1 are common in high-risk myelodysplastic syndrome, but not in myelofibrosis with myeloid metaplasia. Eur J Haematol 74: 47-53 (2005)

85. Stein EM, Altman JK, Collins R, DeAngelo DJ, Fathi AT, Flinn I, Frankel A, Levine RL, Medeiros BC, Patel M, Daniel A, Pollyea DA, Roboz GJ, Stone RM, Swords RT, Tallman MS, Agresta S, Fan B, Yang H, Yen K, de Botton S: AG-221, an Oral, Selective, First-in-Class, Potent Inhibitor of the IDH2 Mutant Metabolic Enzyme, Induces Durable Remissions in a Phase I Study in Patients with IDH2 Mutation Positive Advanced Hematologic Malignancies. Blood 124: Abstract 115 (2014)

86. Stone RM, Mandrekar S, Sanford BL, Geyer S, Bloomfield CD, Dohner K, Thiede C, Marcucci G, Lo-Coco F, Klisovic RB, Wei A, Sierra J, Sanz M, Brandwein JM, de Witte T, Niederwieser D, Appelbaum FR, Medeiros BC, Tallman MS, Krauter J, Schlenk R.F. RF, Ganser A, Serve H, Ehninger G, Amadori S, Larson RA and Dohner H: The Multi-Kinase Inhibitor Midostaurin Prolongs Survival Compared with Placebo in Combination with Daunorubicin Cytarabine Induction, High-Dose Consolidation and As Maintenance Therapy in Newly Diagnosed Acute Myeloid Leukemia Patients Age 18-60 with *FLT3* Mutations: An International Prospective Randomized Controlled Double-Blind Trial (CALGB 10603/RATIFY [Alliance]). Abstract 6, ASH Meeting (2015)

87. Surveillance Epidemiology and End Results, National Cancer Institute (SEER) 18, 2008-2012

88. Tang JL, Hou HA, Chen CY, Liu CY, Chou WC, Tseng MH, Huang CF, Lee FY, Liu MC, Yao M, Huang SY, Ko BS, Hsu SC, Wu SJ, Tsay W, Chen YC, Lin LI, Tien HF: AML1/RUNX1 mutations in 470 adult patients with de novo acute myeloid leukemia: prognostic implication and interaction with other gene alterations. Blood 114: 5352-61 (2009)

89. Taskesen E, Bullinger L, Corbacioglu A, Sanders MA, Erpelinck CA, Wouters BJ, van der Poel-van de Luytgaarde SC, Damm F, Krauter J, Ganser A, Schlenk R.F. RF, Löwenberg B, Delwel R, Döhner H., Valk PJ, Döhner K: Prognostic impact, concurrent genetic mutations, and gene expression features of AML with CEBPA mutations in a

cohort of 1182 cytogenetically normal AML patients: further evidence for CEBPA double mutant AML as a distinctive disease entity. Blood 17: 2469-75 (2011)

90. Tassara M, Döhner K, Brossart P, Held G, Götze K, Horst HA, Ringhoffer M, Köhne CH, Kremers S, Raghavachar A, Wulf G, Kirchen H, Nachbaur D, Derigs HG, Wattad M, Koller E, Brugger W, Matzdorff A, Greil R, Heil G, Paschka P, Gaidzik VI, Göttlicher M, Döhner H., Schlenk R.F. RF: Valproic acid in combination with all-trans retinoic acid and intensive therapy for acute myeloid leukemia in older patients. Blood 123: 4027-36 (2014)

91. Tawana K, Wang J, Renneville A, Bödör C, Hills R, Loveday C, Savic A, Van Delft FW, Treleaven J, Georgiades P, Uglow E, Asou N, Uike N1, Debeljak M1, Jazbec J, Ancliff P, Gale R, Thomas X, Mialou V, Döhner K, Bullinger L, Mueller B, Pabst T, Stelljes M, Schlegelberger B, Wozniak E, Iqbal S, Okosun J, Araf S, Frank AK, Lauridsen FB, Porse B, Nerlov C, Owen C, Dokal I, Gribben J, Smith M, Preudhomme C, Chelala C, Cavenagh J, Fitzgibbon J: Disease evolution and outcomes in familial AML with germline CEBPA mutations. Blood 126: 1214-23 (2015)

92. Thein MS, Ershle WB, Jemal Y, Yates JW, Baer MR: Outcome of older patients with acute myeloid leukemia: an analysis of SEER data over 3 decades. Cancer 119: 2720-27 (2013)

93. Therneau TM, Grambusch PM: Modeling survival data: extending the Cox model. New York, NY: Springer Verlag (2000)

94. Thol F, Friesen I, Damm F, Yun H, Weissinger EM, Krauter J, Wagner K, Chaturvedi A, Sharma A, Wichmann M, Göhring G, Schumann C, Bug G, Ottmann O, Hofmann WK, Schlegelberger B, Heuser M, Ganser A: Prognostic significance of ASXL1 mutations in patients with myelodysplastic syndromes. J Clin Oncol. 29: 2499-506 (2011)

95. Vardiman JW, Thiele J, Arber DA: The 2008 revision of the World Health Organization (WHO) classification of myeloid neoplasms and acute leukemia: rationale and important changes. Blood 114: 937-51 (2009)

96. Wander SA, Levis MJ, Fathi AT: The evolving role of FLT3 inhibitors in acute myeloid leukemia: quizartinib and beyond**.** Ther Adv Hematol 5: 65-77 (2014)

97. Wang ES. Treating acute myeloid leukemia in older adults. Hematology Am Soc Hematol Educ Program 14-20 (2014)

98. Wetzler M, Mrózek K, Kohlschmidt J, Dombret H, Döhner H., Pilorge S, Krug U, Carroll AJ, Larson RA, Marcucci G, Hiddemann W, Büchner T, Bloomfield CD:

Intensive induction is effective in selected octogenarian acute myeloid leukemia patients: prognostic significance of karyotype and selected molecular markers used in the European LeukemiaNet classification. Haematologica 99: 308-13 (2014)

99. Wouters BJ, Löwenberg B, Erpelinck-Verschueren CA, van Putten WL, Valk PJ, Delwel R: Double CEBPA mutations, but not single CEBPA mutations, define a subgroup of acute myeloid leukemia with a distinctive gene expression profile that is uniquely associated with a favorable outcome. Blood 13: 3088-91 (2009)

100. Xu XQ, Wang JM, Gao L, Qiu HY, Chen L, Jia L, Hu XX, Yang JM, Ni X, Chen J, Lü SQ, Zhang WP, Song XM: Characteristics of acute myeloid leukemia with myelodysplasia-related changes: A retrospective analysis in a cohort of Chinese patients. Am J Hematol 89: 874-81 (2014)

101. Zhang DE, Zhang P, Wang ND, Hetherington CJ, Darlington GJ, Tenen DG: Absence of granulocyte colony-stimulating factor signaling and neutrophil development in CCAAT enhancer binding protein alpha-deficient mice. Proc Natl Acad Sci U S A 94: 569-574 (1997)

102. Zhao LJ, Wang YY, Li G, Ma LY, Xiong SM, Weng XQ, Zhang WN, Wu B, Chen Z, Chen SJ: Functional features of RUNX1 mutants in acute transformation of chronic myeloid leukemia and their contribution to inducing murine full-blown leukemia. Blood 119: 2873-82 (2012)

103. Zharlyganova D, Harada H, Harada Y, Shinkarev S, Zhumadilov Z, Zhunusova A, Tchaizhunusova NJ, Apsalikov KN, Kemaikin V, Zhumadilov K, Kawano N, Kimura A, Hoshi M: High frequency of AML1/RUNX1 point mutations in radiation-associated myelodysplastic syndrome around Semipalatinsk nuclear test site. J Radiat Res 49: 549-55 (2008)

104. Zuckerman T, Ganzel C, Tallman MS, Rowe JM: How I treat hematologic emergencies in adults with acute leukemia. Blood 120: 1993-2002 (2012)

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