BHS course: Laboratory hematology

Molecular hematology

Apr. PhD Marie-Berthe Maes

Labo klinische biologie - Hematologie

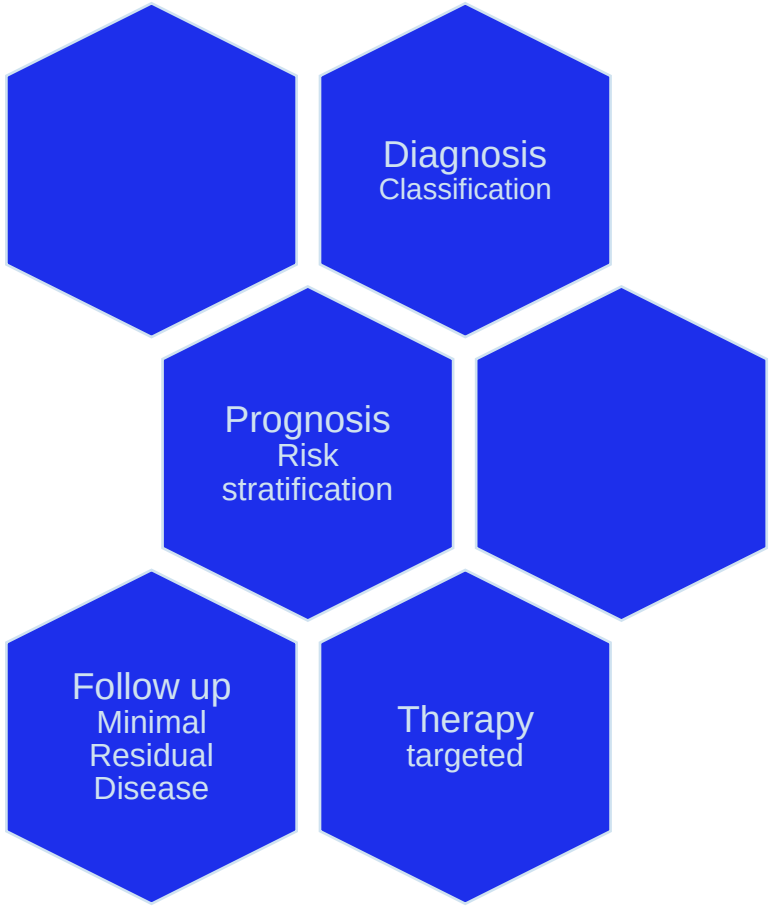
Universitair Ziekenhuis Antwerpen

10/2023

Content

Introduction

Molecular testing in hematology



Molecular genetic testing should screen for all the genetic abnormalities that define disease (**diagnosis and classification**), and risk categories (**prognosis**) or that are needed for targeted treatment modalities (**therapy**).

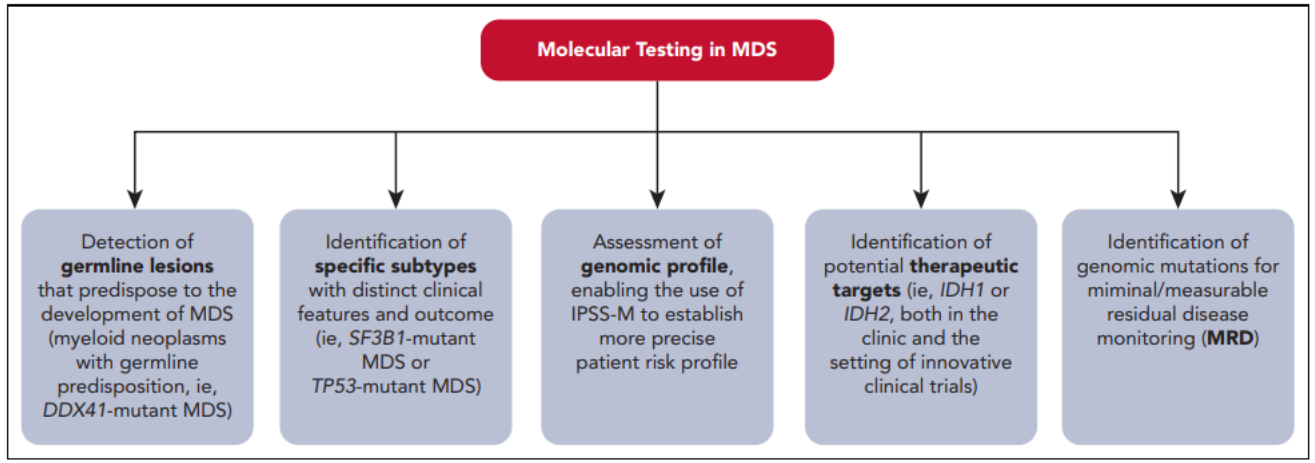


Figure 1. How molecular profiling can inform clinical decision making in MDS. IPSS-M, Molecular International Prognostic Scoring System; MDS, myelodysplastic syndrome; MRD, minimal/measurable residual disease. Professional illustration by Patrick Lane, ScEYEnce Studios.

Introduction

Molecular testing in hematology: Classification

Leukemia

www.nature.com/leu

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The 5th edition of the World Health Organization Classification of Haematolymphoid Tumours: Myeloid and Histiocytic/ Dendritic Neoplasms

Joseph D. Khoury[1✉], Eric Solary[2✉], Oussama Abla[3], Yassmine Akkari[4], Rita Alaggio[5], Jane F. Apperley[6], Rafael Bejar[7], Emilio Berti[8], Lambert Busque[9], John K. C. Chan[10], Weina Chen[11], Xueyan Chen[12], Wee-Joo Chng[13], John K. Choi[14], Isabel Colmenero[15], Sarah E. Coupland[16], Nicholas C. P. Cross[17], Daphne De Jong[18], M. Tarek Elghetany[19], Emiko Takahashi[20], Jean-Francois Emile[21], Judith Ferry[22], Linda Fogelstrand[23], Michaela Fontenay[24], Ulrich Germing[25], Sumeet Gujral[26], Torsten Haferlach[27], Claire Harrison[28], Jennelle C. Hodge[29], Shimin Hu[1], Joop H. Jansen[30], Rashmi Kanagal-Shamanna[1], Hagop M. Kantarjian[31], Christian P. Kratz[32], Xiao-Qiu Li[33], Megan S. Lim[34], Keith Loeb[35], Sanam Loghavi[1], Andrea Marcogliese[19], Soheil Meshinchi[36], Phillip Michaels[37], Kikkeri N. Naresh[35], Yasodha Natkunam[38], Reza Nejati[39], German Ott[40], Eric Padron[41], Keyur P. Patel[1], Nikhil Patkar[42], Jennifer Picarsic[43], Uwe Platzbecker[44], Irene Roberts[45], Anna Schuh[46], William Sewell[47], Reiner Siebert[48], Prashant Tembhare[42], Jeffrey Tyner[49], Srdan Verstovsek[31], Wei Wang[1], Brent Wood[50], Wenbin Xiao[51], Cecilia Yeung[35] and Andreas Hochhaus[52✉]

www.nature.com/leu Leukemia

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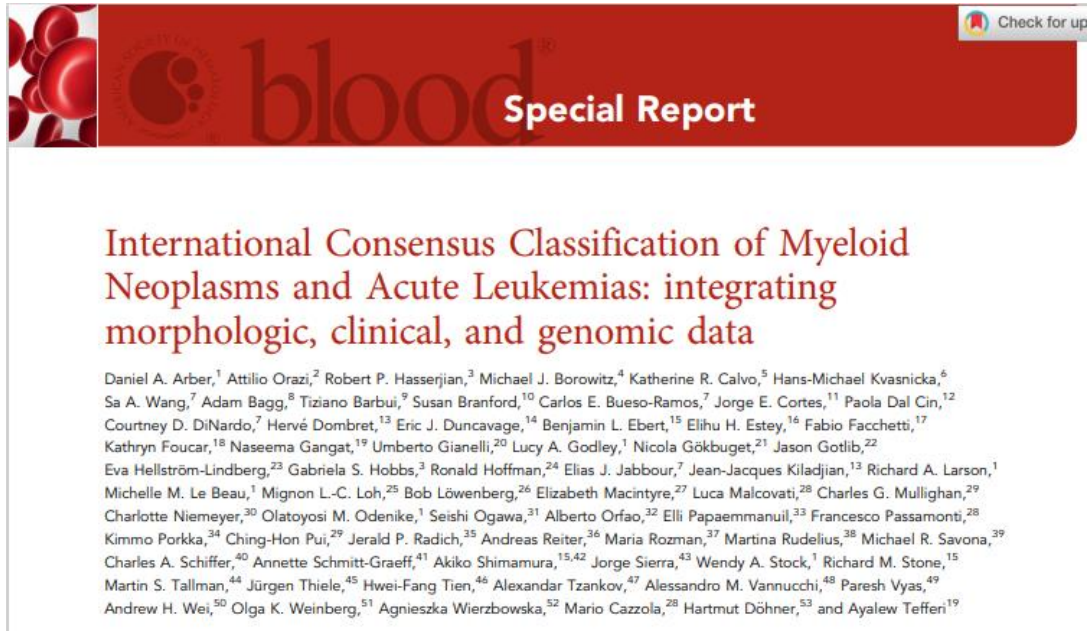
LYMPHOMA

The 5th edition of the World Health Organization Classification of Haematolymphoid Tumours: Lymphoid Neoplasms

Rita Alaggio[1], Catalina Amador[2], Ioannis Anagnostopoulos[3], Ayoma D. Attygalle[4], Iguaracyra Barreto de Oliveira Araujo[5], Emilio Berti[6], Govind Bhagat[7], Anita Maria Borges[8], Daniel Boyer[9], Mariarita Calaminici[10], Amy Chadburn[11], John K. C. Chan[12], Wah Cheuk[12], Wee-Joo Chng[13], John K. Choi[14], Shih-Sung Chuang[15], Sarah E. Coupland[16], Magdalena Czader[17], Sandeep S. Dave[18], Daphne de Jong[19], Ming-Qing Du[20✉], Kojo S. Elenitoba-Johnson[21], Judith Ferry[22✉], Julia Geyer[11], Dita Gratzinger[23], Joan Guitart[24], Sumeet Gujral[25], Marian Harris[26], Christine J. Harrison[27], Sylvia Hartmann[28], Andreas Hochhaus[29], Patty M. Jansen[30], Kennosuke Karube[31], Werner Kempf[32], Joseph Khoury[33], Hiroshi Kimura[34], Wolfram Klapper[35], Alexandra E. Kovach[36], Shaji Kumar[37], Alexander J. Lazar[38], Stefano Lazzi[39], Lorenzo Leoncini[39], Nelson Leung[40], Vasiliki Leventaki[41], Xiao-Qiu Li[42], Megan S. Lim[21], Wei-Ping Liu[43], Abner Louissaint Jr.[22], Andrea Marcogliese[44], L. Jeffrey Medeiros[33], Michael Michal[45], Roberto N. Miranda[33], Christina Mitteldorf[46], Santiago Montes-Moreno[47], William Morice[48], Valentina Nardi[22], Kikkeri N. Naresh[49], Yasodha Natkunam[23], Siok-Bian Ng[50], Ilske Oschlies[35], German Ott[51✉], Marie Parrens[52], Melissa Pulitzer[53], S. Vincent Rajkumar[54], Andrew C. Rawstron[55], Karen Rech[48], Andreas Rosenwald[3], Jonathan Said[56], Clémentine Sarkozy[57], Shahin Sayed[58], Caner Saygin[59], Anna Schuh[60], William Sewell[61], Reiner Siebert[62✉], Aliyah R. Sohani[22], Reuben Tooze[63], Alexandra Traverse-Glehen[64], Francisco Vega[33], Beatrice Vergier[65], Ashutosh D. Wechalekar[66], Brent Wood[36], Luc Xerri[67] and Wenbin Xiao[53]



Introduction

Molecular testing in AML: Diagnosis and classification

WHO 2022

Acute myeloid leukaemia

Introduction

Acute myeloid leukaemia with defining genetic abnormalities

- Acute promyelocytic leukaemia with PML::RARA fusion
- Acute myeloid leukaemia with RUNX1::RUNX1T1 fusion
- Acute myeloid leukaemia with CBFB::MYH11 fusion
- Acute myeloid leukaemia with DEK::NUP214 fusion
- Acute myeloid leukaemia with RBM15::MRTFA fusion
- Acute myeloid leukaemia with BCR::ABL1 fusion
- Acute myeloid leukaemia with KMT2A rearrangement
- Acute myeloid leukaemia with MECOM rearrangement
- Acute myeloid leukaemia with NUP98 rearrangement
- Acute myeloid leukaemia with NPM1 mutation
- Acute myeloid leukaemia with CEBPA mutation
- Acute myeloid leukaemia, myelodysplasia-related
- Acute myeloid leukaemia with other defined genetic alterations

Acute myeloid leukaemia, defined by differentiation

- Acute myeloid leukaemia with minimal differentiation
- Acute myeloid leukaemia without maturation
- Acute myeloid leukaemia with maturation
- Acute basophilic leukaemia
- Acute myelomonocytic leukaemia
- Acute monocytic leukaemia
- Acute erythroid leukaemia
- Acute megakaryoblastic leukaemia

Myeloid sarcoma

- Myeloid sarcoma

85%

Translocations / fusion genes

Gene mutations

Table 8. Cytogenetic and molecular abnormalities defining acute myeloid leukaemia, myelodysplasia-related.

Defining cytogenetic abnormalities
Complex karyotype (≥3 abnormalities)
5q deletion or loss of 5q due to unbalanced translocation
Monosomy 7, 7q deletion, or loss of 7q due to unbalanced translocation
11q deletion
12p deletion or loss of 12p due to unbalanced translocation
Monosomy 13 or 13q deletion
17p deletion or loss of 17p due to unbalanced translocation
Isochromosome 17q
idic(X)(q13)
Defining somatic mutations
ASXL1
BCOR
EZH2
SF3B1
SRSF2
STAG2
U2AF1
ZRSR2

Introduction

Molecular testing in AML: Diagnosis and classification

ICC 2022

Table 25. Classification of AML with percentage of blasts required for diagnosis

Acute promyelocytic leukemia (APL) with t(15;17)(q24.1;q21.2)/*PML::RARA* ≥ 10%
APL with other *RARA* rearrangements* ≥ 10%
AML with t(8;21)(q22;q22.1)/*RUNX1::RUNX1T1* ≥ 10%
AML with inv(16)(p13.1q22) or t(16;16)(p13.1;q22)/*CBFB::MYH11* ≥ 10%
AML with t(9;11)(p21.3;q23.3)/*MLLT3::KMT2A* ≥ 10%
AML with other *KMT2A* rearrangements† ≥ 10%
AML with t(6;9)(p22.3;q34.1)/*DEK::NUP214* ≥ 10%
AML with inv(3)(q21.3q26.2) or t(3;3)(q21.3;q26.2)/*GATA2; MECOM(EVI1)* ≥ 10%
AML with other *MECOM* rearrangements‡ ≥ 10%
AML with other rare recurring translocations (see supplemental Table 5) ≥ 10%
AML with t(9;22)(q34.1;q11.2)/*BCR::ABL1*§ ≥ 20%
AML with mutated *NPM1* ≥ 10%
AML with in-frame bZIP *CEBPA* mutations ≥ 10%
AML and MDS/AML with mutated *TP53*† 10-19% (MDS/AML) and ≥ 20% (AML)
AML and MDS/AML with myelodysplasia-related gene mutations 10-19% (MDS/AML) and ≥ 20% (AML) Defined by mutations in *ASXL1, BCOR, EZH2, RUNX1, SF3B1, SRSF2, STAG2, U2AF1,* or *ZRSR2*
AML with myelodysplasia-related cytogenetic abnormalities 10-19% (MDS/AML) and ≥ 20% (AML) Defined by detecting a complex karyotype (≥ 3 unrelated clonal chromosomal abnormalities in the absence of other class-defining recurring genetic abnormalities), del(5q)/t(5q)/add(5q), −7/del(7q), +8, del(12p)/t(12p)/add(12p), i(17q), −17/add(17p) or del(17p), del(20q), and/or idic(X)(q13) clonal abnormalities
AML not otherwise specified (NOS) 10-19% (MDS/AML) and ≥ 20% (AML)
Myeloid sarcoma

Translocations / fusion genes

Gene mutations

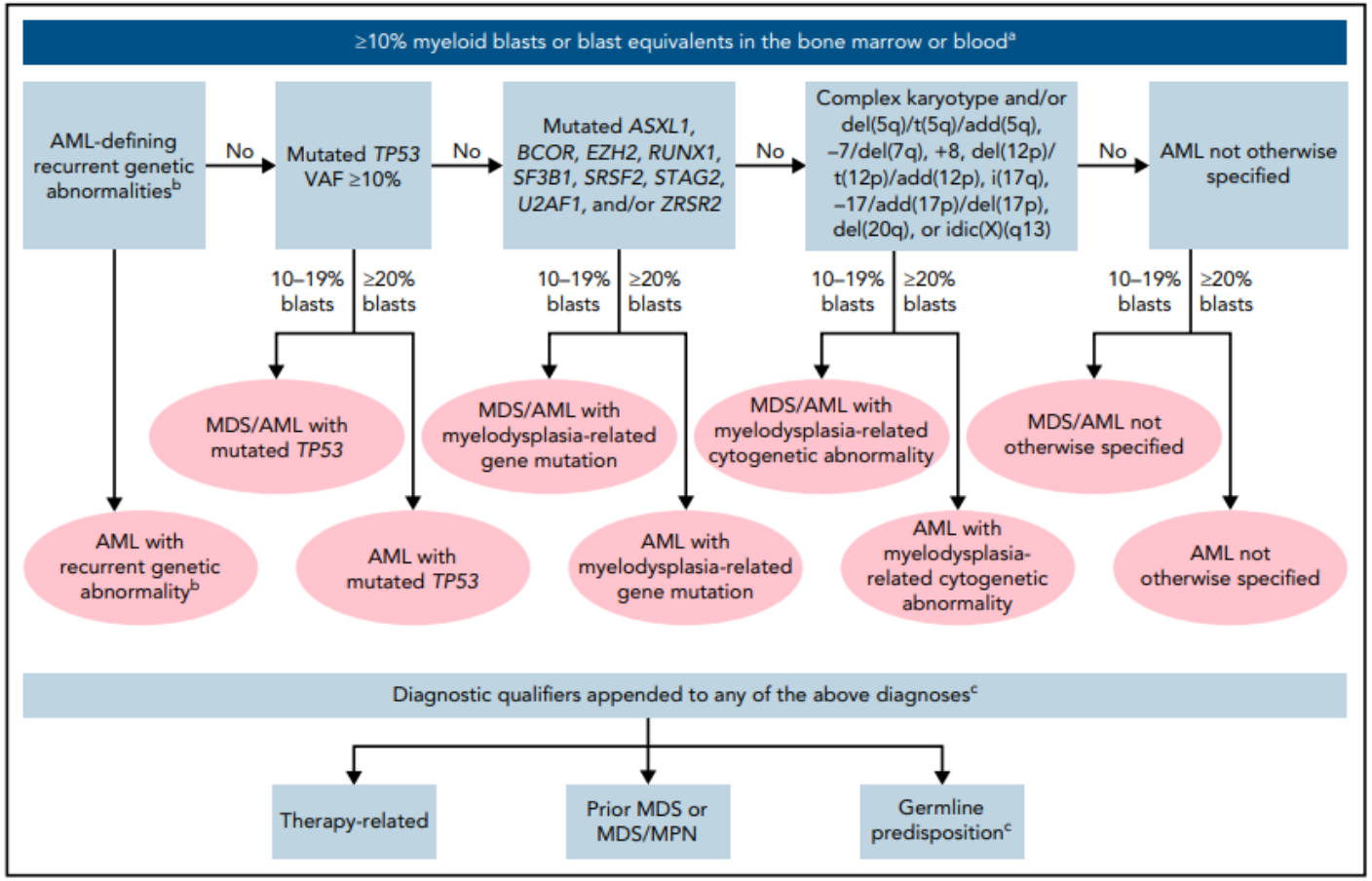


Figure 1. Hierarchical classification of the International Consensus Classification of AML. The classification is hierarchical (ie, AML with recurrent genetic

Introduction

Molecular testing in AML: Prognosis and risk stratification

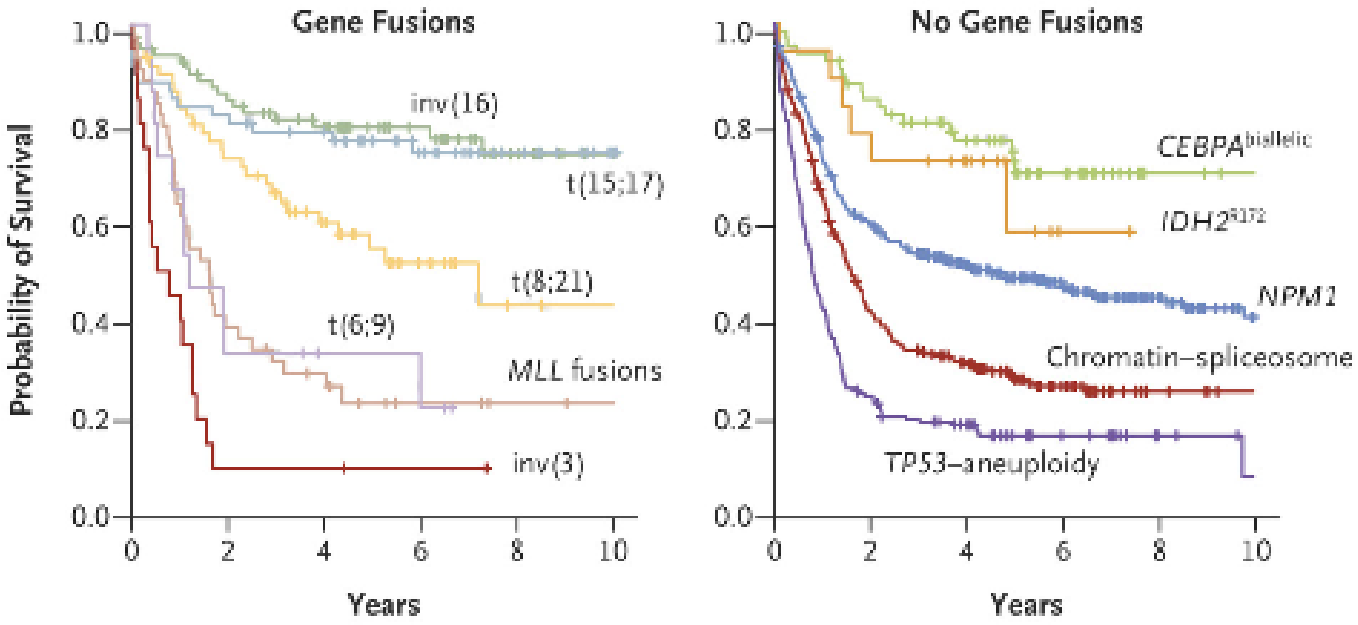
ELN 2022

Table 6. 2022 ELN risk classification by genetics at initial diagnosis*

Risk category†	Genetic abnormality
Favorable	• t(8;21)(q22;q22.1)/*RUNX1::RUNX1T1*†,‡ • inv(16)(p13.1q22) or t(16;16)(p13.1;q22)/*CBFB::MYH11*†,‡ • Mutated *NPM1*†,§ without *FLT3*-ITD • bZIP in-frame mutated *CEBPA*‖
Intermediate	• Mutated *NPM1*†,§ with *FLT3*-ITD • Wild-type *NPM1* with *FLT3*-ITD (without adverse-risk genetic lesions) • t(9;11)(p21.3;q23.3)/*MLLT3::KMT2A*†,¶ • Cytogenetic and/or molecular abnormalities not classified as favorable or adverse
Adverse	• t(6;9)(p23.3;q34.1)/*DEK::NUP214* • t(v;11q23.3)/*KMT2A*-rearranged# • t(9;22)(q34.1;q11.2)/*BCR::ABL1* • t(8;16)(p11.2;p13.3)/*KAT6A::CREBBP* • inv(3)(q21.3q26.2) or t(3;3)(q21.3;q26.2)/*GATA2, MECOM(EVI1)* • t(3q26.2;v)/*MECOM(EVI1)*-rearranged • −5 or del(5q); −7; −17/abn(17p) • Complex karyotype,** monosomal karyotype†† • Mutated *ASXL1, BCOR, EZH2, RUNX1, SF3B1, SRSF2, STAG2, U2AF1, and/or ZRSR2*‡‡ • Mutated *TP53*[a]

Translocations fusion genes

Gene mutations



Panel shows Kaplan–Meier curves for overall survival among patients in the 11 genomically defined subgroups. Papaemmanuil NEJM 2016

Introduction

Molecular testing in AML: Minimal Residual Disease (MRD)

MRD assesment in AML

- Establish a deeper remission status (monitoring respons to therapy)
- Refine postremission relapse risk assessment (prognosis)
- Identify impending relapse

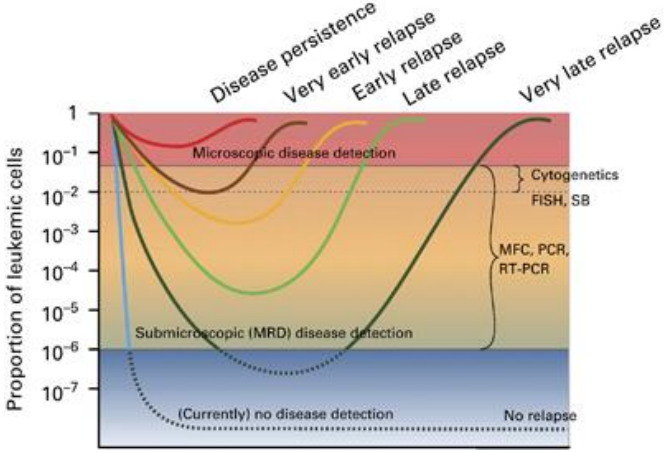


Table 7. Methods for detection of MRD in AML

	Method	Target	Sensitivity	Applicable in % of AML	Turn-around time (days)	Limitations/ problems
Established	Multi-parameter flow cytometry (MFC)	Leukemia-associated immunophenotype (LAIP) or different from normal (DfN)	10^{-3} to 10^{-4}	85-90	2	Less sensitive, more subjective analysis
Established	Real-time quantitative PCR (RT-qPCR)	Robust data: *NPM1*, *CBFB::MYH11*, *RUNX1::RUNX1T1* Less validated: *KMT2A::MLLT3*, *DEK::NUP214*, *BCR::ABL1*, *WT1*	10^{-4} to 10^{-5}	40-50*	3-5	Limited applicability
Exploratory	Next-generation sequencing (NGS)†,‡	Potentially any somatic mutation†	10^{-2} to 10^{-4}	~100	5-10	Less sensitive, costly, technically challenging
Exploratory	Digital PCR (dPCR)	Specific targeted mutations	10^{-3} to 10^{-4}	~70	3-5	Specific assay necessary for every mutation, limited sensitivity

Döhner et al Blood 2022

*Less frequent in elderly patients with AML.

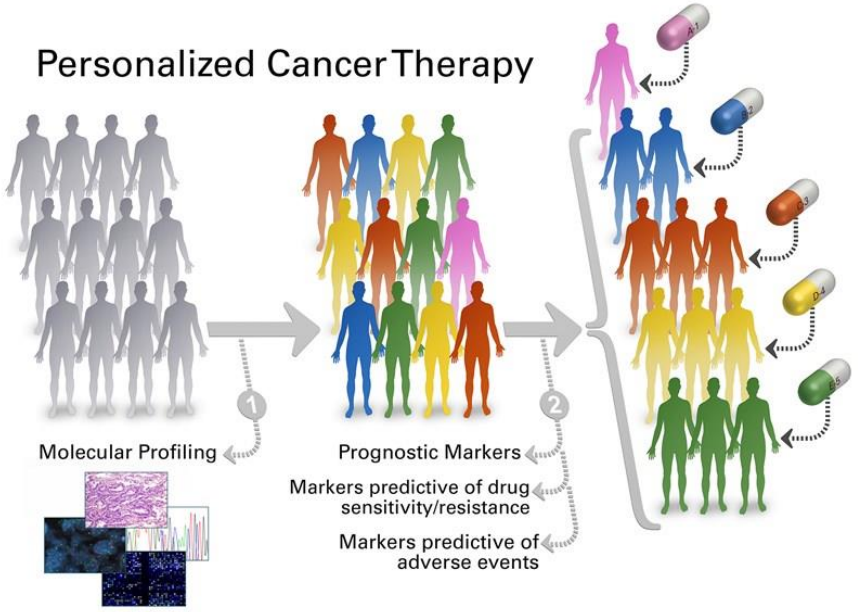
†The NGS-MRD threshold has not been defined for individual mutations; NGS-MRD positivity is provisionally defined as ≥ 0.1% variant allele frequency, excluding mutations related to clonal hematopoiesis and germline mutations.

‡Common gene mutations consistent with pre-malignant clonal hematopoiesis such as *DNMT3A*, *TET2*, and *AXSL1* excluded; further study is required to determine which mutations are truly indicative of residual AML and not clonal hematopoiesis.

e.g. RT-qPCR of mutated NPM1, WT1 expression, PML::RARA, RUNX1::RUNX1T1, BCR::ABL1 gene fusions, …

Introduction

Molecular testing in AML: Targeted therapy



Genetic analyses	Results preferably available within
Cytogenetics§	• 5-7 d
Screening for gene mutations required for establishing the diagnosis and to identify actionable therapeutic targets#	
• *FLT3*,¶ *IDH1*, *IDH2*	• 3-5 d
• *NPM1*	• 3-5 d
• *CEBPA*,# *DDX41*, *TP53*; *ASXL1*, *BCOR*, *EZH2*, *RUNX1*, *SF3B1*, *SRSF2*, *STAG2*, *U2AF1*, *ZRSR2*	• 1st cycle
Screening for gene rearrangements**	
• *PML*::*RARA*, *CBFB*::*MYH11*, *RUNX1*::*RUNX1T1*, *KMT2A* rearrangements, *BCR*::*ABL1*, other fusion genes (if available)	• 3-5 d

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FLT3 mutated AML	Standard CT + **FLT3-inhibitor** (eg midostaurin)
IDH1/2 mutated AML	Standard CT + **IDH1/2-inhibitor** (eg ivosidenib, enasidenib)
PML-RARA fusion gene AML	**ATRA/ATO**
CBF-fusion gene AML	Frontline CT + **gemtuzumab ozogamicin**
NPM1 mutated AML	Standard CT
Genetically defined **MR**-AML	Standard CT vs **CPX-351** vs Ven-HMA?
TP53 mutated AML	**Experimental** therapy (eg magrolimab, eprenetapopt)

Content

Molecular testing in AML

Laboratory diagnosis of AML

By bone marrow aspiration and biopsy using

morphologic		Cytology, anatomopathologie 
immunophenotypic		Flowcytometry 
cytogenetic/molecular analysis		Molecular testing, genetics 

➡ Multidisciplinary approach: integrated conclusion WHO2022 + ICC2022

Genetic analyses	Results preferably available within
Cytogenetics§	• 5-7 d
Screening for gene mutations required for establishing the diagnosis and to identify actionable therapeutic targets#	
• *FLT3*,¶ *IDH1*, *IDH2*	• 3-5 d
• *NPM1*	• 3-5 d
• *CEBPA*,# *DDX41*, *TP53*; *ASXL1*, *BCOR*, *EZH2*, *RUNX1*, *SF3B1*, *SRSF2*, *STAG2*, *U2AF1*, *ZRSR2*	• 1st cycle
Screening for gene rearrangements**	
• *PML::RARA*, *CBFB::MYH11*, *RUNX1::RUNX1T1*, *KMT2A* rearrangements, *BCR::ABL1*, other fusion genes (if available)	• 3-5 d

Döhner et al Blood 2022

DNA
Stable
Variability in breakpoints
1 copy/cell: less sensitive

RNA
Not stable (sample 4°C, RBC lysis <72h)
Less variability in fusion genes
> copies/cell: sensitive

Molecular testing in hematology

Different techniques

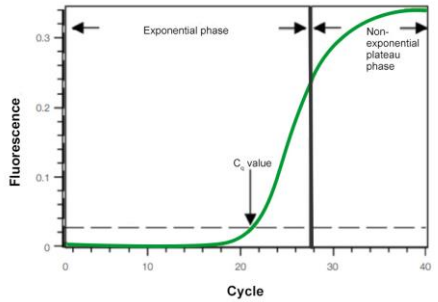
Molecular testing is defined as any testing that reveals the changes in the nucleotide in a DNA and RNA sequence.

PCR

Translocations (RNA)

Gene mutations (DNA)

- Conventional PCR
- RT-PCR
- Quantitative PCR
- Allele-specific PCR
- Multiplex PCR
- …



Sequencing

Translocations (RNA)

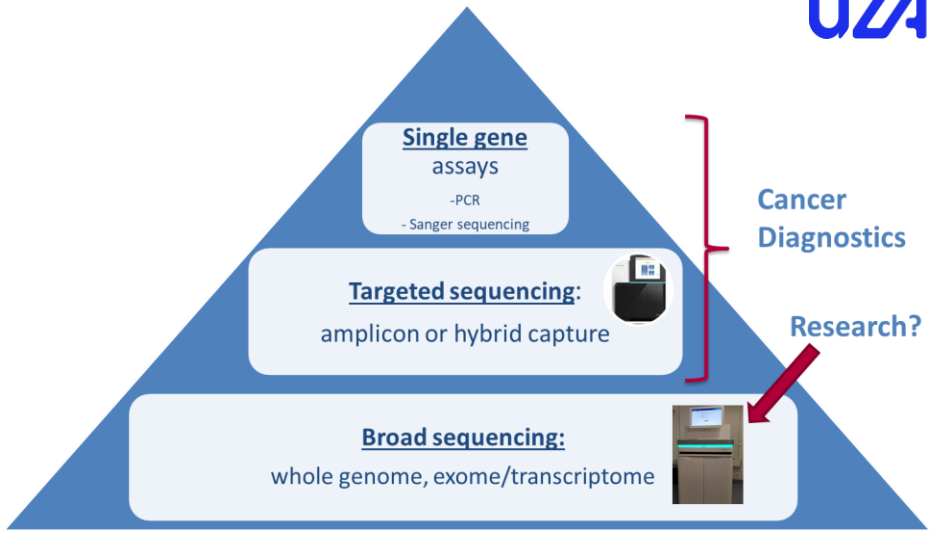
Gene mutations (DNA)

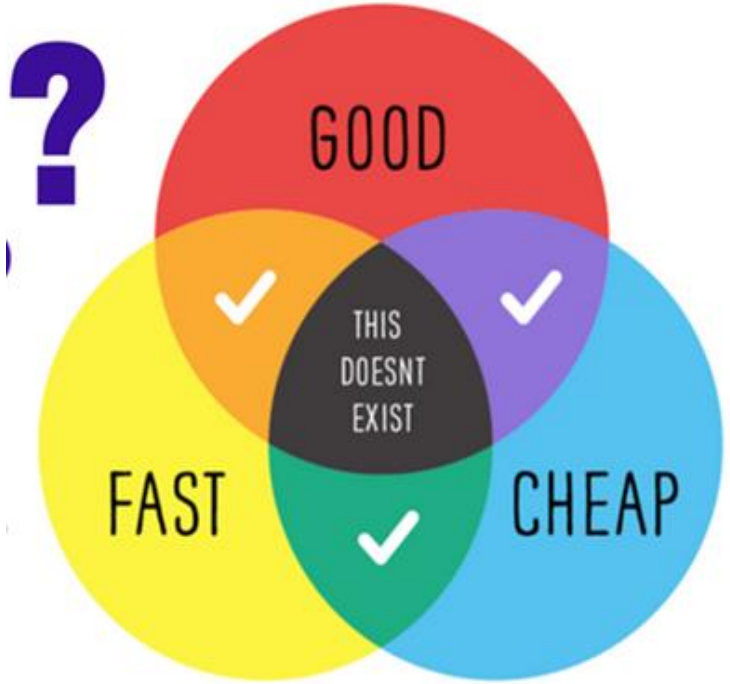
- Sanger Sequencing
- Next generation Sequencing
  - Targeted next generation sequencing
  - Whole genome sequencing
  - Whole exome sequencing
  - RNA sequencing
- …

Molecular testing in hematology

Different Techniques: Choice of technique

- Target?
  - Detection of fusiontranscripts / DNA mutations?
  - Throughput? Single / Multiple genes / hotspot?
- Purpose?
  - TAT: Diagnosis/Therapy?
  - Sensitivity (detection limit): MRD?
- Many commercial kits on the market
  - IVDR?
  - Labor intensivity?
  - Price?
- Reimbursement from RIZIV/Inami?
- Guidelines (Belgium/European)
  - minimum requirements
  - rarely with a specific method or technology





Content

Molecular testing in AML

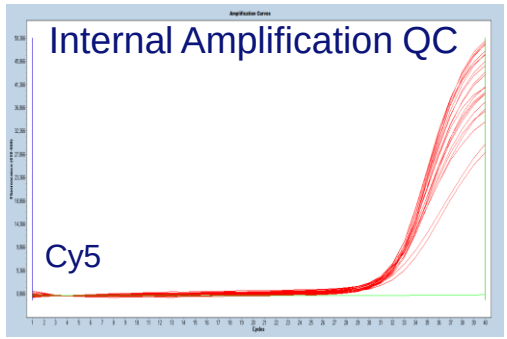
Translocations: detection of fusion genes

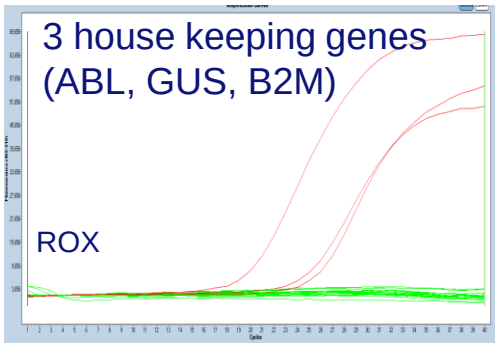
PCR

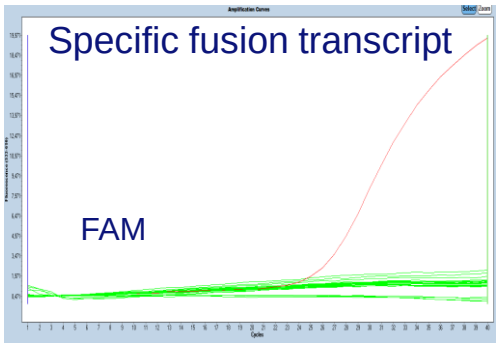
Multiplex screening Real Time-PCR

Hemavision®

- 28 different chromosomal rearrangements/translocations
- (Semi)-Qualitative
- Diagnosis and classification of acute leukemia (AML, ALL)
- CE/IVD kit







Hemavision®

Tube	Translocation	Fusion Gene	Fw primer - Rev primer	Flourochrome	
1	t(15;17)(q24;q21)	PML-RARA (bcr2, V)	PML ex5-RARA ex5	FAM	CY5
	Inv(16)(p13;q22)	CBFB-MYH11	CBFB ex3-MYH11 ex30	ROX	CY5
2	Inv(16)(p13;q22)	CBFB-MYH11	CBFB ex4-MYH11 ex34	FAM	CY5
	t(8;21)(q22;q22)	RUNX1-RUNX1T1	RUNX1 ex6-RUNX1T1 ex9	ROX	CY5
3	t(15;17)(q24;q21)	PML-RARA (bcr1, L)	PML ex6a-RARA ex5	FAM	CY5
	t(9;11)(p22;q23)	MLL-MLLT3	MLL ex7-MLLT3 ex7	ROX	CY5
4	t(15;17)(q24;q21)	PML-RARA (bcr3, S)	PML ex3-RARA ex5	FAM	CY5
	t(9;11)(p22;q23)	MLL-MLLT3	MLL ex8-MLLT3 ex11	ROX	CY5
5	t(11;19)(q23;p13.3)	MLL-ELL	MLL ex7-ELL ex3	FAM	CY5
	t(16;21)(p11;q22)	FUS-ERG	FUS ex6-ERG ex14	ROX	CY5
6	t(12;22)(p13;q11-12)	ETV6-MN1	ETV6 ex2-MN1 ex2	FAM	CY5
	t(6;9)(p23;q34)	DEK-NUP214	DEK ex9-NUP214 ex19	ROX	CY5
7	Reference gene	GUS	GUS ex11-GUS ex12	FAM	CY5
8	Reference gene	B2M	B2M ex2-B2M ex4	FAM	CY5
9	t(1;11)(p32;q23)	MLL-EPS15	MLL ex8+9-EPS15 ex3	FAM	CY5
	t(6;11)(q27;q23)	MLL-MLLT4	MLL ex8+9-MLLT4 ex2	ROX	CY5
10	t(1;19)(q23;p13)	TCF3-PBX1	TCF3 ex16-PBX1 ex3	FAM	CY5
	t(12;21)(p13;q22)	ETV6-RUNX1	ETV6 ex5-RUNX1 ex4b	ROX	CY5
11	t(11;19)(q23;p13.3)	MLL-MLLT1	MLL ex8+9-MLLT1 ex2	FAM	CY5
	t(4;11)(q21;q23)	MLL-AFF1	MLL ex8+9-AFF1 ex10	ROX	CY5
12	t(17;19)(q22;p13)	TCF3-HLF	TCF3 ex14-HLF ex4	FAM	CY5
	del(1)(p32)	STIL-TAL1	STIL ex1-TAL1 ex1	ROX	CY5
13	t(9;22)(q34;q11)	BCR-ABL1 (m-bcr, P190)	BCR ex1-ABL1 ex4	FAM	CY5
	t(9;9)(q34;q34)	SET-NUP214	SET ex9-NUP214 ex19	ROX	CY5
14	t(11;19)(q23;p13.3)	MLL-MLLT1	MLL ex7-MLLT1 ex9	FAM	CY5
	t(9;22)(q34;q11)	BCR-ABL1 (M-bcr, P210)	BCR ex12-ABL1 ex4	ROX	CY5
15	t(9;22)(q34;q11)	BCR-ABL1 (µ-bcr, P230)	BCR ex19-ABL1 ex4	FAM	CY5
	t(11;17)(q23;q21)	ZBTB16-RARA	ZBTB16 ex4-RARA ex5	ROX	CY5
16	Reference gene	ABL1	ABL1 ex3-ABL1 ex4	FAM	CY5
17	t(9;12)(q34;p13)	ETV6-ABL1	ETV6 ex2+5-ABL1 ex4	FAM	CY5
	t(5;12)(q33;p13)	ETV6-PDGFRB	ETV6 ex2+5-PDGFRB ex12	ROX	CY5
18	t(10;11)(p12;q23)	MLL-MLLT10	MLL ex8+9-MLLT10 ex20	FAM	CY5
	t(1;11)(q21;q23)	MLL-MLLT11	MLL ex8+9-MLLT11 ex2	ROX	CY5
19	t(X;11)(q13;q23)	MLL-FOXO4	MLL ex7-FOXO4 ex2	FAM	CY5
	t(11;17)(q23;q21)	MLL-MLLT6	MLL ex7-MLLT6 ex12	ROX	CY5
20	t(3;21)(q26;q22)	RUNX1-MECOM	RUNX1 ex6-MECOM ex2	FAM	CY5
	t(10;11)(p12;q23)	MLL-MLLT10	MLL ex7-MLLT10 ex9	ROX	CY5
21	t(5;17)(q35;q21)	NPM1-RARA	NPM1 ex4-RARA ex5	FAM	CY5
	t(3;5)(q25.1;q35)	NPM1-MLF1	NPM1 ex4-MLF1 ex4	ROX	CY5
22	t(10;11)(p12;q23)	MLL-MLLT10	MLL ex7-MLLT10 ex13	FAM	CY5
	t(3;21)(q26;q22)	RUNX1-MECOM	RUNX1 ex6-MECOM ex9	ROX	CY5
23	t(10;11)(p12;q23)	MLL-MLLT10	MLL ex8-MLLT10 ex12	ROX	CY5
24	-	-	-	-	-

Molecular testing in AML

Translocations: detection of fusion genes

PCR

Multiplex **screening** RT-PCR

Specific Real Time-PCR

- Quanitative, sensit 10^{-5}
- Specific
- *Urgent diagnosis APL: PML::RARA (<48h)*
- *MRD FU AML (PML::RARA, RUNX1::RUNX1T1, CBFB::MYH11, …)*
- *MRD pediatric ALL*
- *FU Chronic Myeloid Leukemia: BCR-ABL1*

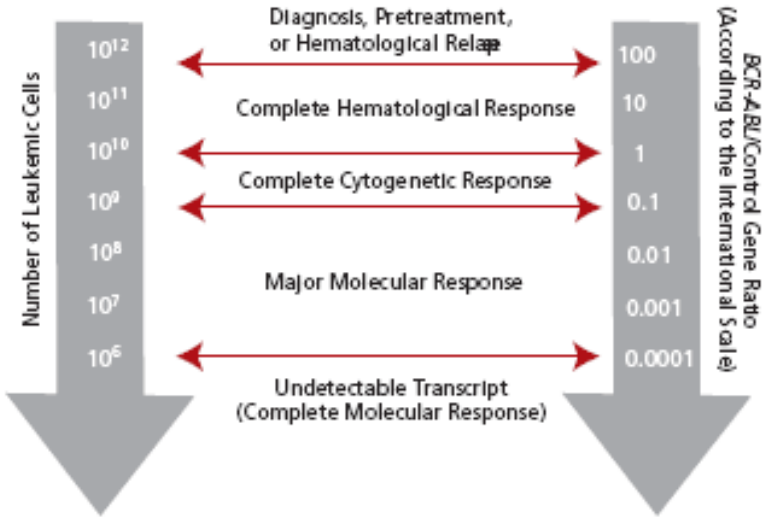
Molecular testing in AML

Translocations: detection of fusion genes

Specific real time PCR

- Diagnosis and classification
- Follow up: MRD monitoring
  - Quantitative
  - Sensitive (10^{-4} - 10^{-6})
  - Reproducible
  - Standardized

Figure 1. The *BCR-ABL* Transcript Percentage Parallels the Number of Leukemic Cells



Adapted with permission from Baccarani M et al. Blood. 2006;108:1809-1820.[10]

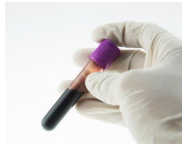
- Standardization MRD EAC-protocol (Gabert et al 2003):

  Taqman based quantitative RT-PCR for fusion gene transcripts *RUNX1::RUNX1T1, CBFB::MYH11, PML::RARA, …*
- ELN MRD consensus (Schuurhuis et al 2018)
- ELN MRD updated consensus (Heuser et al. 2021)

Molecular testing in AML

Translocations: detection of fusion genes

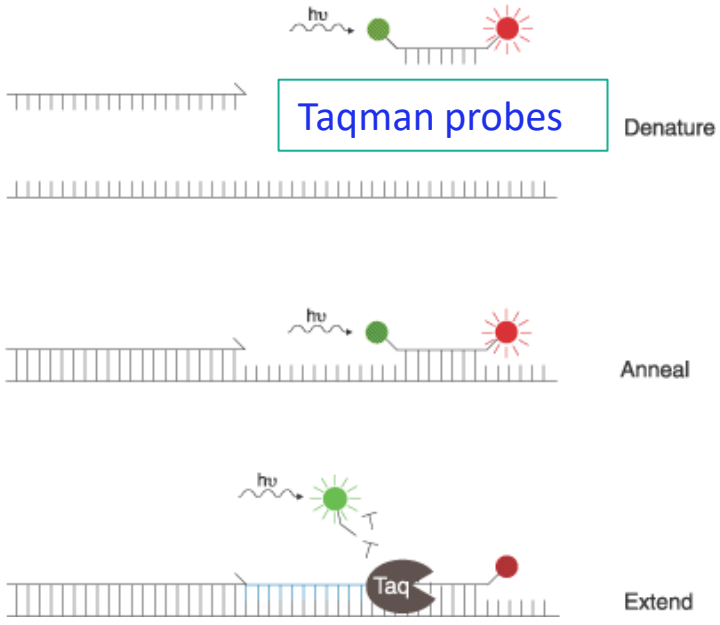
Specific real time PCR

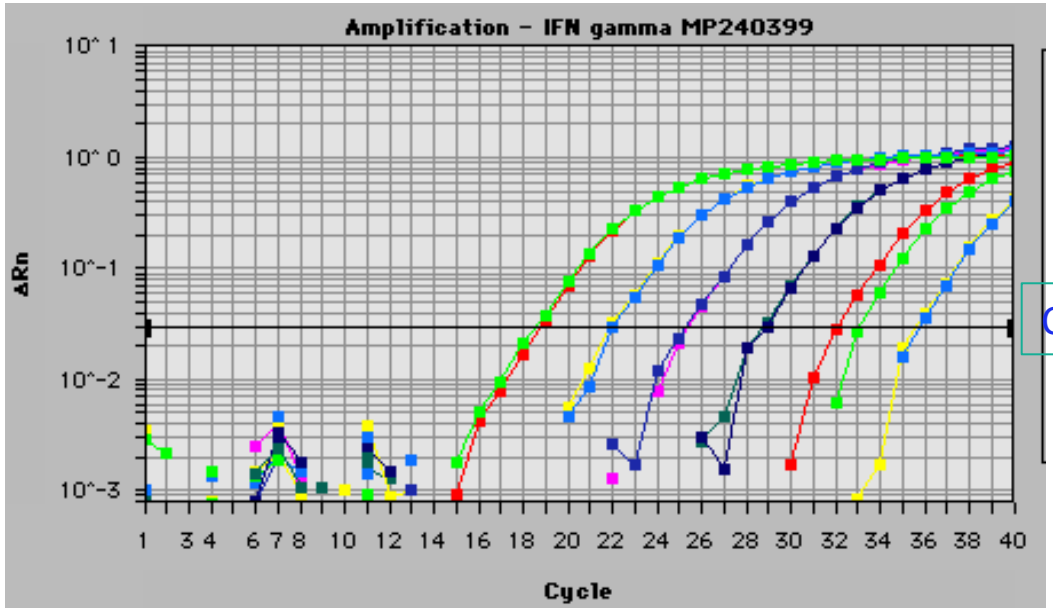


Reverse transcriptase

Taqman probes

RNA isolation → cDNA → RT-PCR





Cycle threshold (Ct)

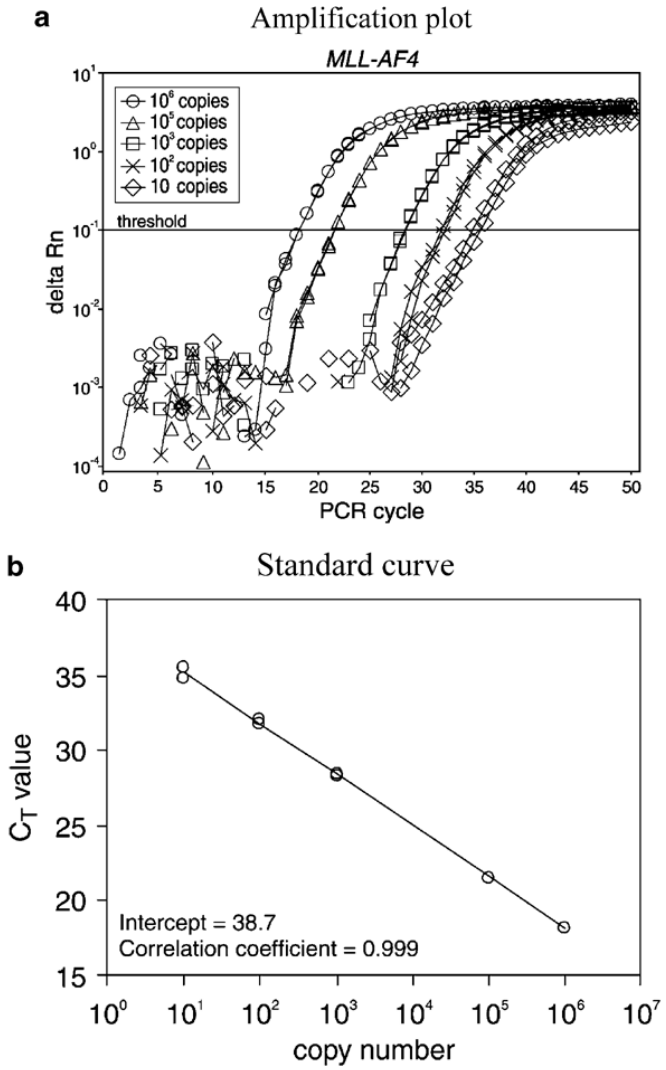
Molecular testing in AML

Translocations: detection of fusion genes

Specific real time PCR

Standardization

- Quantification using standard curves
- Each analysis performed in duplicate/triplicate
- Controls included (negative, positive, nontarget)
- Results compared to housekeeping gene
  - ABL, B2M, GUS
  - Gene expression quantitation
  - ➢ Normalised Copy Number (NCN/100 ABL)
  - Sample to sample quality variations
  - Sensitivity (# copies housekeeping genes)
  - ➢ “sample-specific LOD”

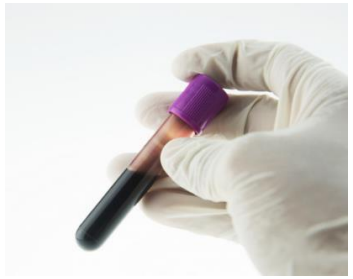


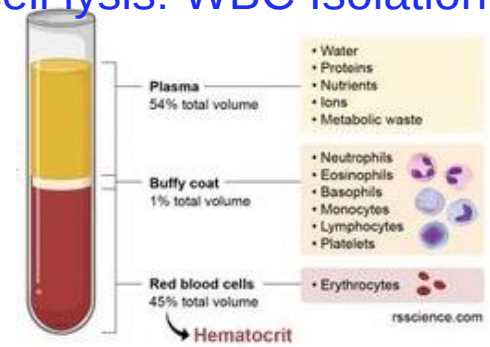
Molecular testing in AML

Translocations: detection of fusion genes

Real time PCR

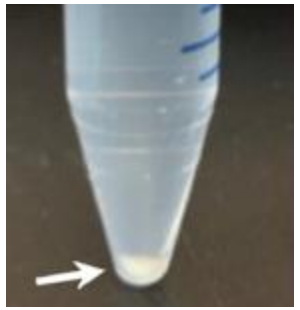
Red blood cell lysis: WBC Isolation





1-2h

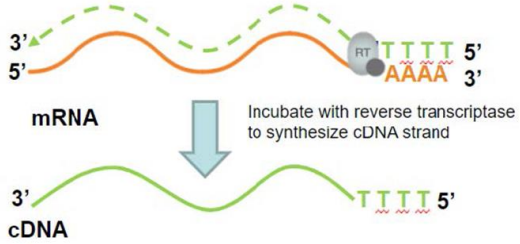
10×10^6 cells



RNA extraction

2h

Reverse transcription

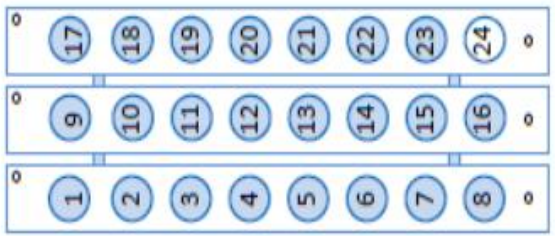


1h

HEMAVISION

Primers and Probes

(Target 1 (FAM) + Target 2 (ROX) + IAC (Cy5))



2-3h

PCR

Molecular testing in AML

Translocations: detection of fusion genes

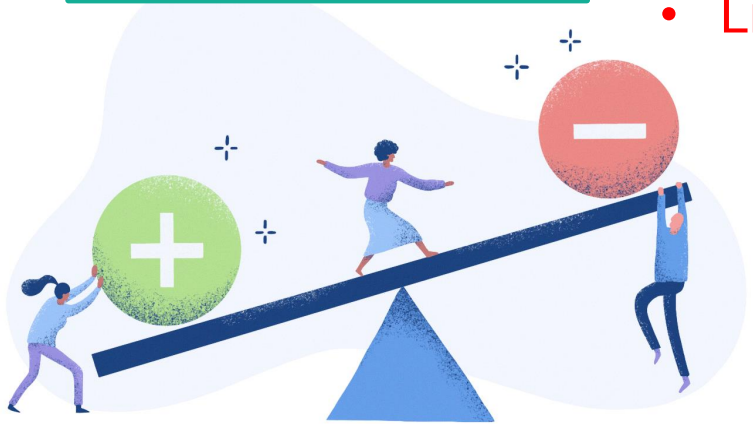
Specific RT-PCR

- Quantitative, Very sensitive
- Fast (1 working day)
- Not too labor intensive
- Affordable

Multiplex screening RT-PCR

- Many relevant translocations in one test
- Fast (1 working day)
- Not too labor intensive
- Simple method (PCR)
- CE/IVD

MRD monitoring



Classification / Diagnosis

Specific RT-PCR

- Limited to 1 specific alteration

Multiplex screening RT-PCR

- Semi-quantitative
- Expensive (~250 €)
- Limited to the list of targets

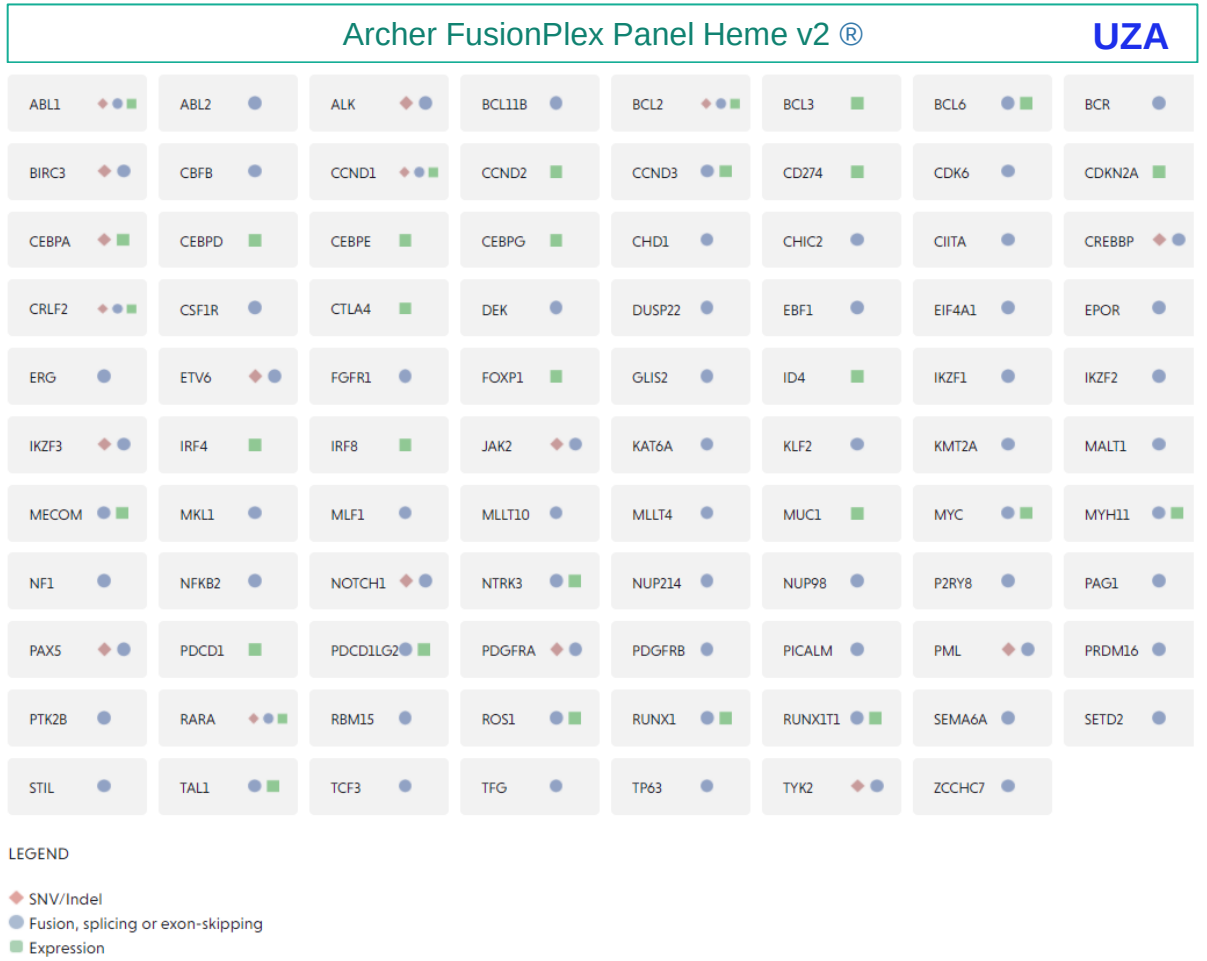
Molecular testing in AML

Translocations: detection of fusion genes

NGS: RNA sequencing

- Qualitative
- Targeted; but not necessary to know the fusion partner

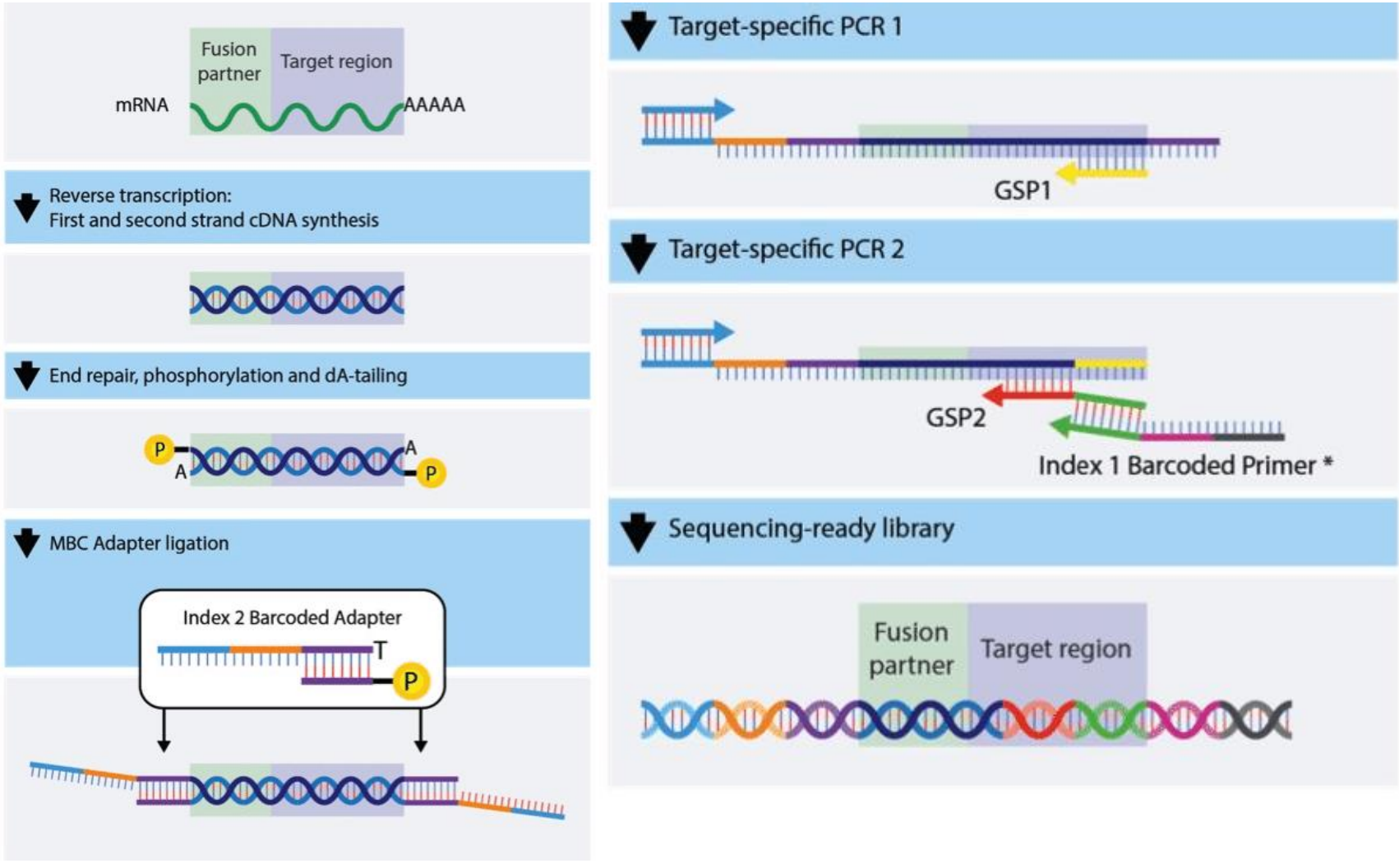
  Much bigger panels than with RT-PCR
- Diagnosis and classification of acute leukemia (AML, ALL)
- Many commercial kits available



Molecular testing in AML

Translocations: detection of fusion genes

NGS: RNA sequencing



Molecular testing in AML

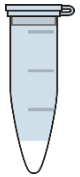
Translocations: detection of fusion genes

NGS: RNA sequencing

RBC Lysis

½ - 1 day

RNA Extraction



Bone marrow (Blood)

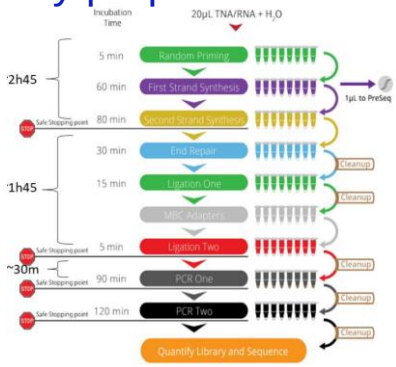
WBC pellet

Qiacube

1,5 days

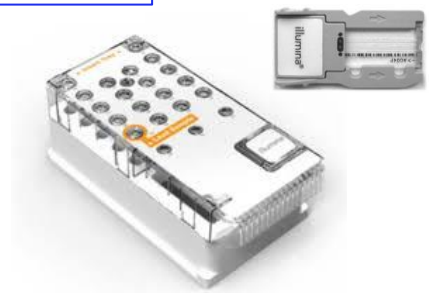
NGS Library prep

50ng



illumina

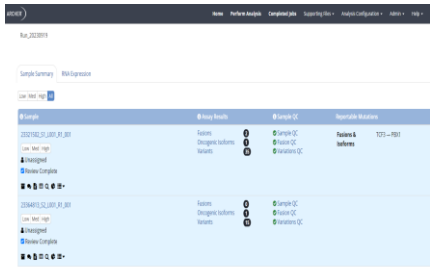
1-2 days



Miseq Sequencer

Sequencing

1 day



Archer Software

Fastq files

Data Analysis

Molecular testing in AML

Translocations: detection of fusion genes

NGS: RNA sequencing

- Large panel of fusion transcripts
- No need to know the fusion partner
- Easy analysis
- Potential to expand to mutation and/or Expression analysis

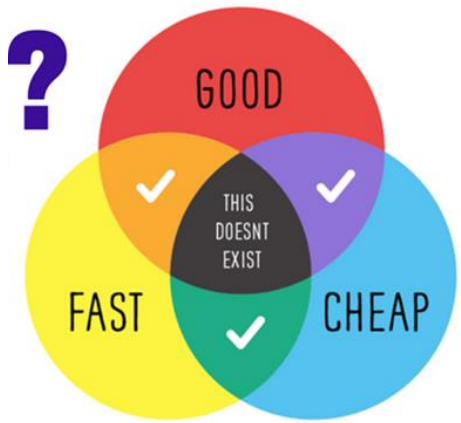
NGS: RNA sequencing

- Long TAT (1 week)
- Labor intensive
- Expensive (~600 €)

Molecular testing in AML

Translocations: detection of fusion genes

Conclusion



	Quantitative RT-PCR	Translocation screening (RT-PCR)	RNA sequencing (NGS)
Sensitivity	+++	++	+
Quantitative	+++	+	+/-
Throughput	-	+	++
Labor Intensive	No	No	Yes
TAT	1-2 days	1-2 days	1 week
Cost	<100 €	~250 €	~600 €

Content

Molecular testing in AML

Gene mutations

Next Generation Sequencing (NGS)

- Screening
- Targeted (panel), Multiparametric
- Semi-quantitative, sensitivity~5%
- Diagnosis and classification of AML
- Prognosis and risk stratification in AML
- Many commercial panels available

- ComPermed guidelines and workflows (2023)

TEST Variant analysis*: ASXL1, BCOR, CEBPA, DDX41, DNMT3A, EZH2, FLT3, IDH1/2, KIT, NPM1, RUNX1, SF3B1, SRSF2, STAG2, TET2, TP53, U2AF1, WT1, ZRSR2

Overzicht van onderzochte genen/exonen van het Myeloid NGS haloplex HS panel. UZA

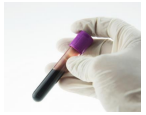
Referentiegenoom GRCh37(hg19)

Gen	RefSeq	Exon(en)	Type
ANKRD26	NM_014915.2	Alle	Tumor Suppressor
ASXL1	NM_015338.5	13 (laatste exon)	Tumor Suppressor
BCOR	NM_017745.5	Alle	Tumor Suppressor
CALR	NM_004343.3	9	Oncogen
CBL	NM_005188.3	8-9	Oncogen/ Tumor Suppressor
CEBPA	NM_004364.3	1	Tumor Suppressor
CSF3R	NM_156039.3	14,17	Oncogen
DDX41	NM_016222.4	Alle	Tumor Suppressor
DNMT3A	NM_175629.2	4,8-23	Tumor Suppressor
ETNK1	NM_018638.4	3	Oncogen
ETV6	NM_001987.4	Alle	Tumor Suppressor
EZH2	NM_004456.4	Alle	Tumor Suppressor/ Oncogen
FLT3	NM_004119.2	14-15, 20	Oncogen
GATA2	NM_032638.5	Alle	Oncogen
IDH1	NM_005896.3	4	Oncogen/ Tumor Suppressor
IDH2	NM_002168.3	4	Oncogen
JAK2	NM_004972.3	12,14	Oncogen
KIT	NM_000222.2	2,8-11,13,14,17	Oncogen
KRAS	NM_004985.4	Alle	Oncogen
MPL	NM_005373.2	10	Oncogen
NF1	NM_001042492.3	Alle	Tumor Suppressor
NPM1	NM_002520.6	11	Oncogen
NRAS	NM_002524.3	2-3	Oncogen
PTPN11	NM_002834.4	3, 13	Oncogen
RUNX1	NM_001754.4	Alle	Tumor Suppressor
SETBP1	NM_015559.3	4	Oncogen
SF3B1	NM_012433.3	13-17	Oncogen
SRSF2	NM_003016.4	1	Oncogen
STAG2	NM_001042749.2	Alle	Tumor Suppressor
TET2	NM_001127208.2	3, 9-11	Tumor Suppressor
TP53	NM_000546.5	Alle	Tumor Suppressor
U2AF1	NM_006758.2	2,6-7	Oncogen
WT1	NM_024426.4	7-9	Tumor Suppressor/ Oncogen
ZRSR2	NM_005089.3	Alle	Tumor Suppressor

Molecular testing in AML

Gene mutations

Next Generation Sequencing (NGS)



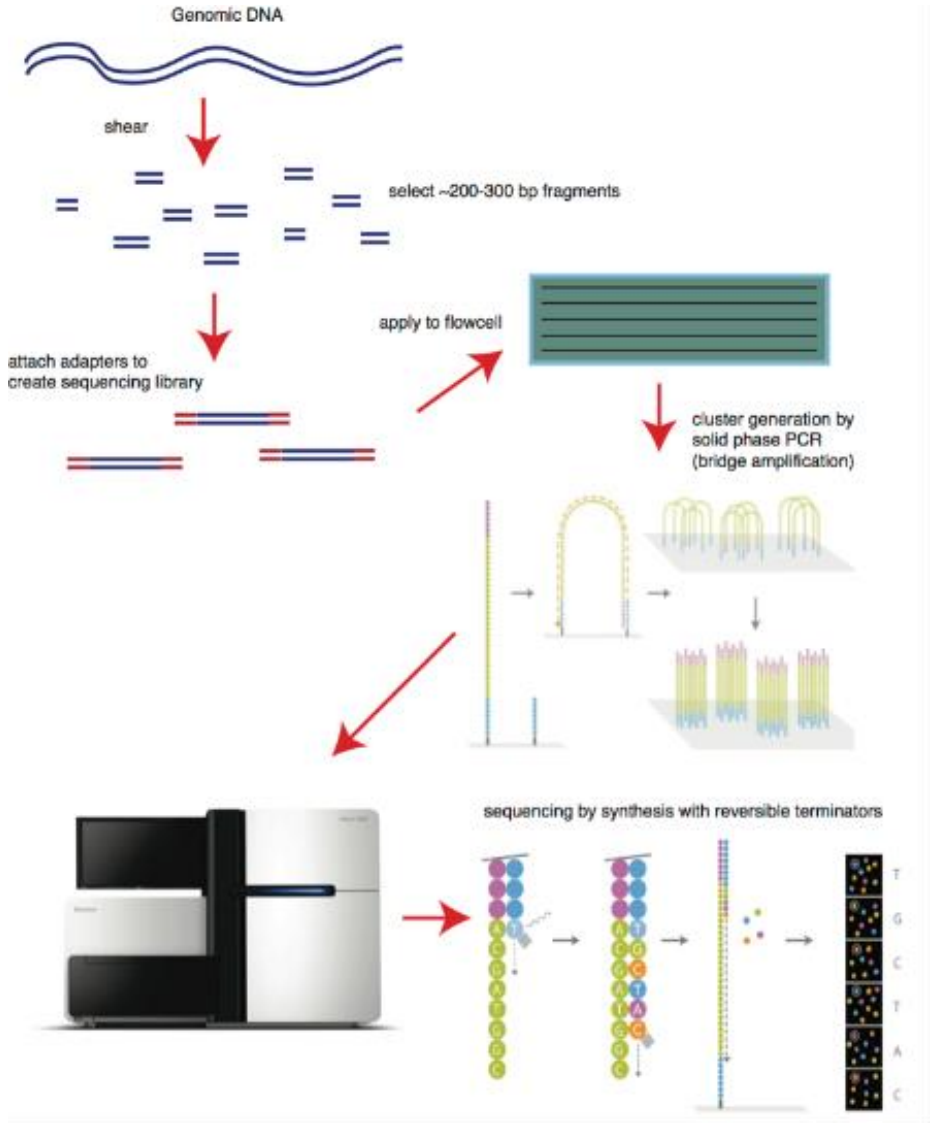
DNA isolation

DNA enrichment / library preparation

Sequencing run

Analysis data: Variant interpretation + clinical report

min 1 week



Molecular testing in AML

Gene mutations

NGS: Variant interpretation and clinical report standardization”

Variant calling and annotation

Biological Interpretation

Pathogenic

on report

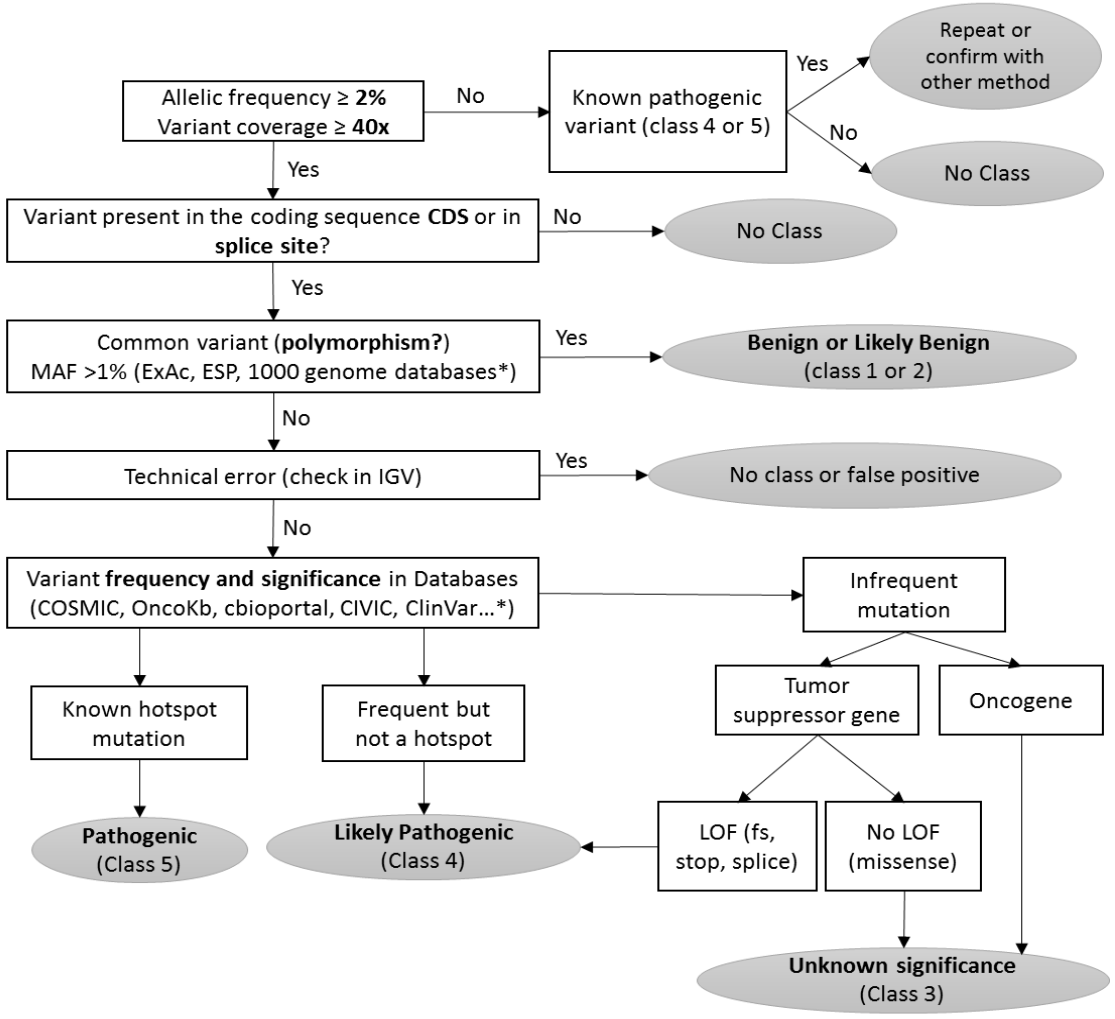
Probably pathogenic

Variant Unknown Significance (VUS)

Probably benign

Benign

Clinical Interpretation



Molecular testing in AML

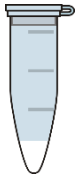
Gene mutations

NGS

½ day

RBC Lysis

Bone marrow (Blood)



WBC pellet

Genomic DNA Extraction

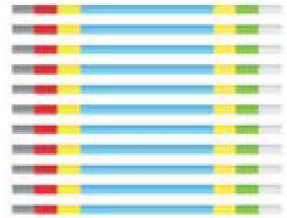
Qiacube

1,5 days

NGS Library prep

50ng

Agilent Technologies



HaloPlex HS Target Enrichment System

illumina

2 days

Miseq Sequencer

Sequencing

1-2 days

Fastq files

SeqNext + Pindel

Data Analysis

Molecular testing in AML

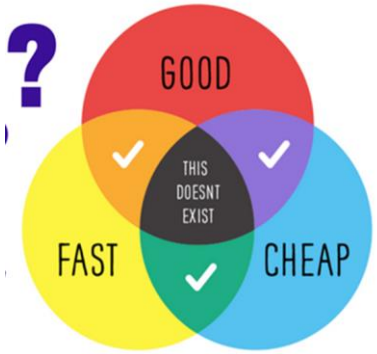
Gene mutations

NGS

- High throughput
- All relevant genes in one test
- Flexible
- Sensitivity: 2-5% VAF

NGS

- Labor intensive
- Sensitivity: 2-5% VAF
- Long TAT (>1 week)
- Expensive (300-500 €)



Molecular testing in AML

Gene mutations

Next Generation Sequencing (NGS)

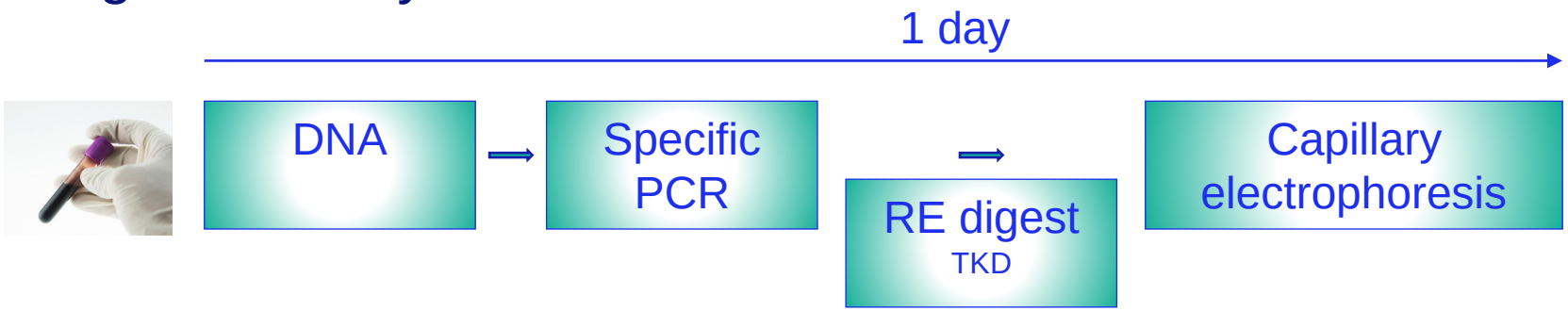
Specific PCR + fragment analysis (PCR)

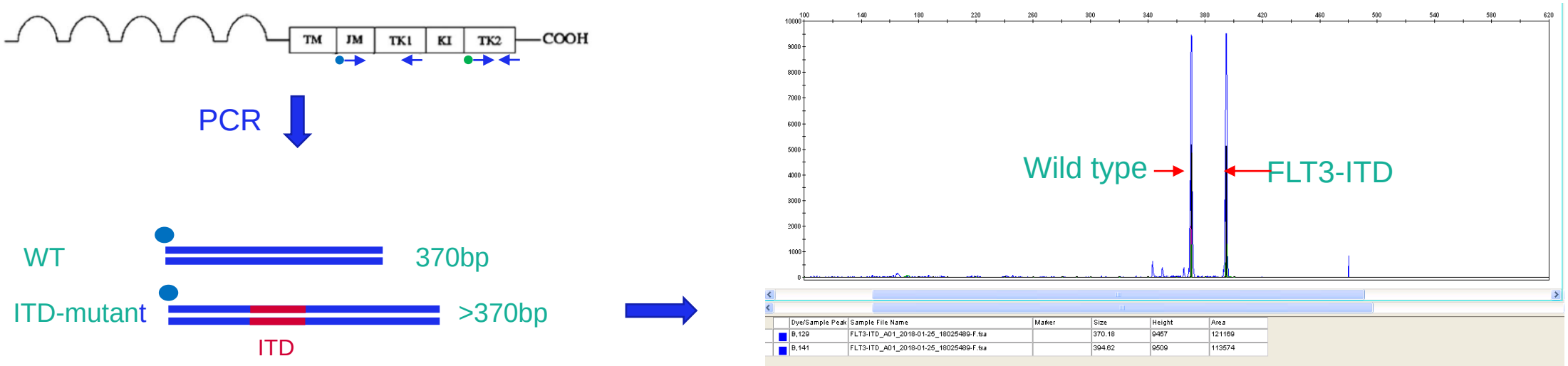
- (Semi)-qualitative
- Sensitivity ~10%
- Specific (1 test per mutation type)
- Easily standardized
- *Only used in special situations:*
  - *Semi-urgent for therapy: FLT3-ITD/TKD*
  - *More sensitive technique: CEBPa, large FLT3-ITD (some NGS panels bad coverage)*

Molecular testing in AML

Gene mutations

PCR + fragment analysis





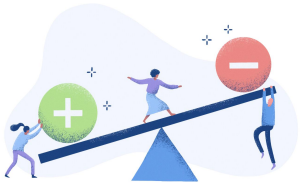
Fragments analysis
(Capillary electrophoresis)

Murphy et al 2003

Molecular testing in AML

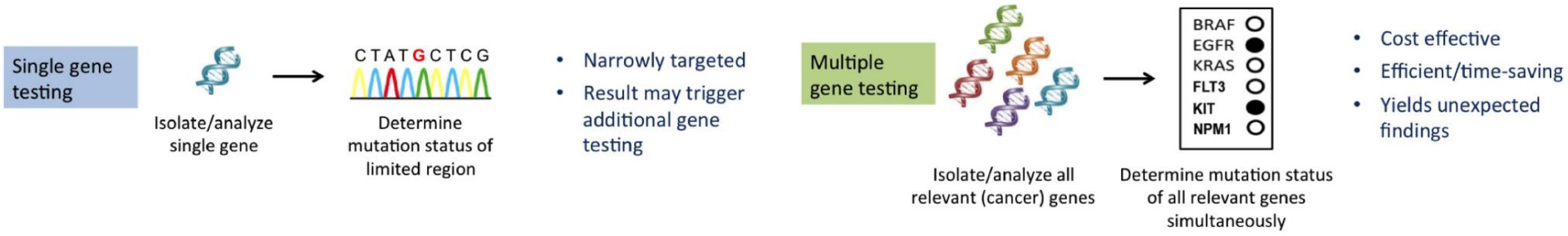
Gene mutations

FLT3 molecular testing



Fragmentanalysis	NGS
Specific (1 test per mutation type)	Multigene myeloid mutation panel
Easily standardised	Labor intensive, highly specialized interpretive skills
TAT 3-5d	TAT 2-3w
~ 100 euro (in house)	~ 400 euro
	False negative results

Targeted therapy (FLT3-inhibitor)
TAT <8days



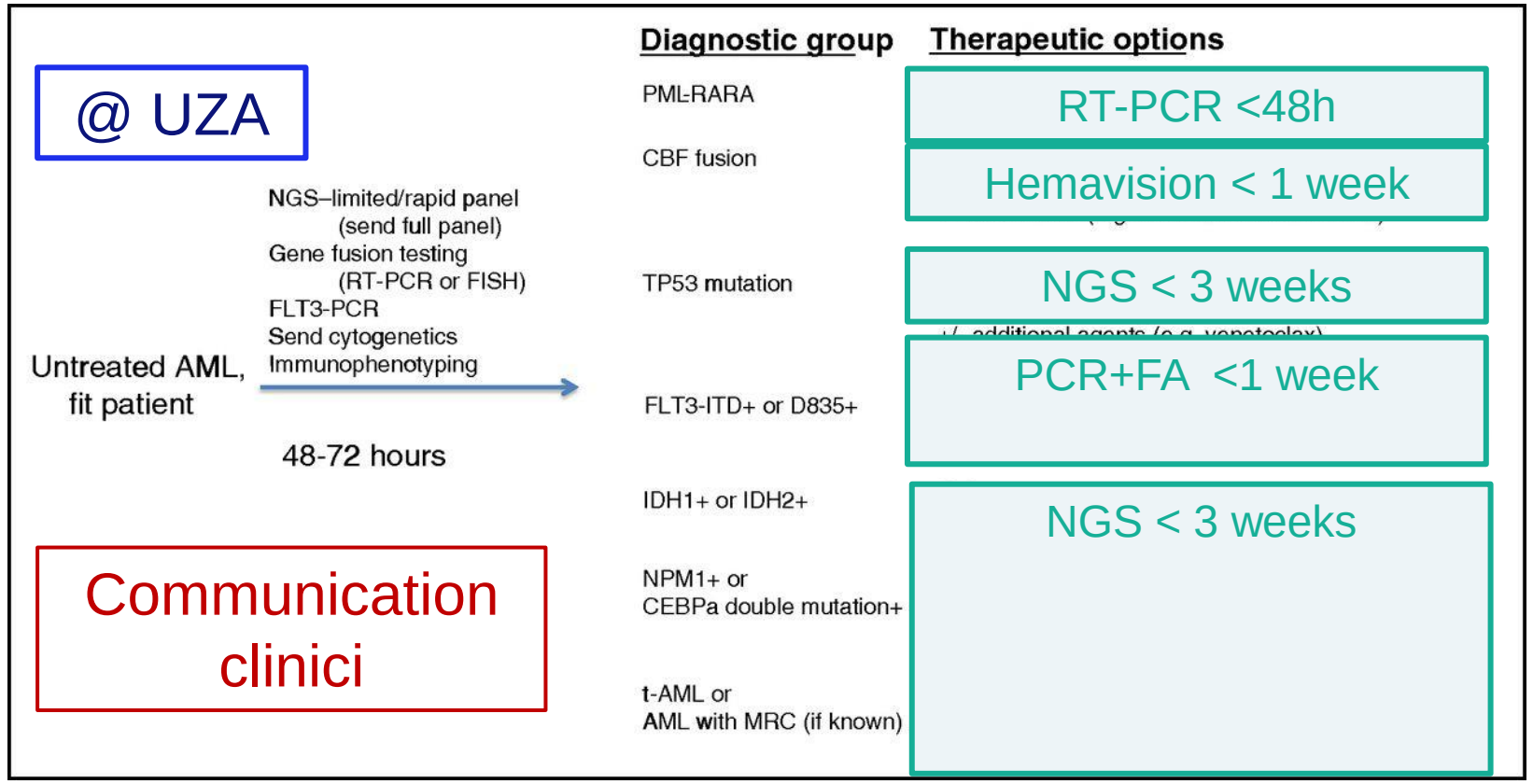
Molecular testing in AML

Optimal approach to ensure patients receive precision medicine diagnostics in an expeditious manner?

Genetic analyses	Results preferably available within
Cytogenetics§	• 5-7 d
Screening for gene mutations required for establishing the diagnosis and to identify actionable therapeutic targets#	
• *FLT3,¶ IDH1, IDH2*	• 3-5 d
• *NPM1*	• 3-5 d
• *CEBPA,# DDX41, TP53; ASXL1, BCOR, EZH2, RUNX1, SF3B1, SRSF2, STAG2, U2AF1, ZRSR2*	• 1st cycle
Screening for gene rearrangements**	
• *PML::RARA, CBFB::MYH11, RUNX1::RUNX1T1, KMT2A* rearrangements, *BCR::ABL1*, other fusion genes (if available)	• 3-5 d

Döhner et al Blood 2022

FLT3 mutated AML	Standard CT + **FLT3-inhibitor**
IDH1/2 mutated AML	Standard CT + **IDH1/2-inhibitor**
CBF-fusion gene AML	Frontline CT + **GO**
NPM1 mutated AML	Standard CT
Genetically defined **MR**-AML	Standard CT vs **CPX-351** vs Ven-HMA?
TP53 mutated AML	**Experimental** therapy



ATRA, all-trans retinoic acid; ATO, arsenic trioxide; CBF, core-binding factor; FISH, fluorescent in situ hybridization; GO, gemtuzumab ozogamicin; HMA, hypomethylating agent; MRC, myelodysplasia-related change; RT-PCR, reverse transcriptase-polymerase chain reaction; t-AML, therapy-related AML; 713, cytarabine by continuous infusion (7 doses) plus anthracycline (3 doses).

Perl et al Blood Advances 2017

Molecular testing in AML

Case report: Man 61y pancytopenia

Cytologie/immunofenotyping: Acute Myeloid Leukemia

Fusion genes (Hemavision)

° Hemavision	negatief
° RUNX1-RUNX1T1t(8;21) (q22;q22)	nt gedet
° CBFB-MYH11 inv(16)(p13;q22)	nt gedet
° PML-RARA t(15;17) (q22;q21)	nt gedet
° MLLT3-KMT2A t(9;11)(p22;q23)	nt gedet
° DEK-NUP214 t(6;9)(p23;q34)	nt gedet
° BCR-ABL1 t(9;22)(q34;q11.2)	nt gedet
° KMT2A herschikt t(v;11q23)	nt gedet

Gene mutations (PCR+fragmentanalysis)

° FLT3/int.tandem duplicatie	gedetec.
° FLT3/puntmutatie D835	nt gedet

WT1 overexpression

° WT1 beenmerg (Otsuka)	↑	**15436**	copies/µg	<1300

Gene mutations (NGS)

° **Pathogene varianten**	2
° Variant 1	FLT3 c.1786_1787insGGGCGAAAGAGTACTTCTACGTTGATTTCA _Glu596insGlyAlaLysGluTyrPheTyrValAspPheArg) - 33%
° Variant 2	SRSF2 c.284C>T; p.(Pro95Leu) - 19%
° **Vermoedelijk pathog. variant**	1
° Variant 1	RUNX1 c.496C>T; p.(Arg166Ter) - 38%

AML with myelodysplasia related gene mutations (mutated SRSF2 and RUNX1)

FLT3 mutated: sensitive for FLT3-inhibitors
- standard CT + midostaurin

Associated with adverse prognosis
- Allo-SCT

WT1 overexpression
- MRD monitoring

References

- The 5th edition of the WHO classification of haematolymphoid tumours: myeloid and histiocytic/dendritic neoplasms. *Khoury et al. Leukemia 2022*
- The 5th edition of the WHO classification of haematolymphoid tumours: Lymphoid neoplasms. *Alaggio et al. Leukemia 2022*
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