



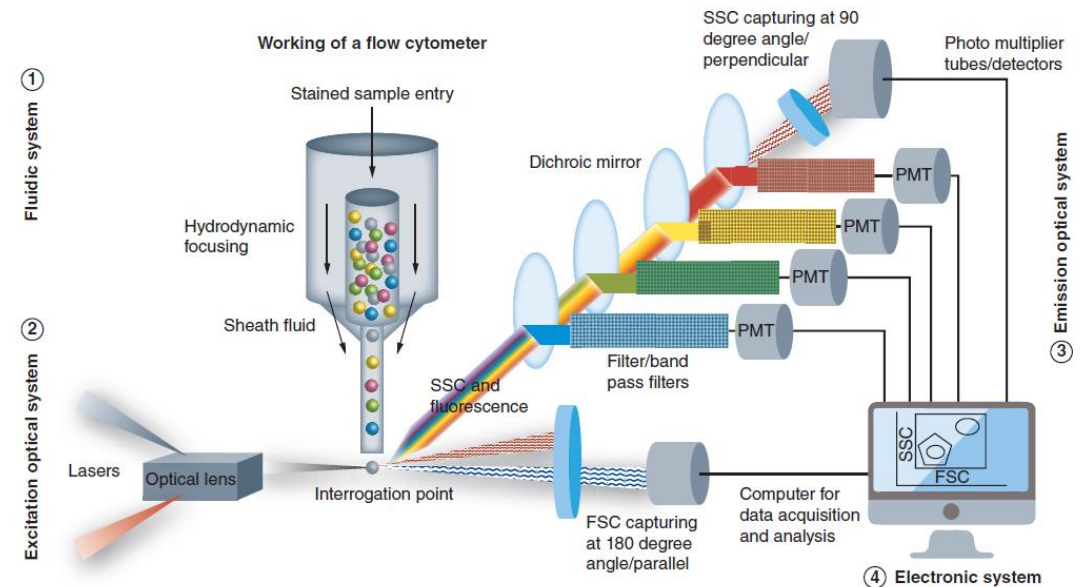
Imunofenotipizacija akutnih leukemija

Antonija Babić, dr.med.
Specijalist laboratorijske imunologije

Odjel za laboratorijsku imunologiju
Klinički zavod za laboratorijsku dijagnostiku
KBC Zagreb

Protočna citometrija

- brza multiparametrijska analiza fizičkih i imunokemijskih karakteristika stanica
- 3 međusobno povezana sustava: protočni, optički i elektronski
- stanice iz stanične suspenzije pojedinačno laminarnim protokom kroz sustav uske kapilare dolaze do snopa laserskog svjetla
- raspršena svjetlost omogućava mjerenje fizičkih osobina stanica - veličine (FSC - *engl. forward scatter*) i zrnatosti (SSC, *engl. side scatter*), a emitirana fluorescencija se usmjerava kroz sustav dihroičnih ogledala i optičkih filtera, te bilježi na PMT detektorima
- svjetlosne signale elektronski sustav pretvara u digitalne signale koji se prenose u elektroničko računalo i služe za analizu



Manohar, S. M., Shah, P., & Nair, A. (2021). Flow Cytometry: Principles, Applications and Recent Advances. *Bioanalysis*, 13(3), 181–198. <https://doi.org/10.4155/bio-2020-0267>

Priprema i analiza

- Svježi uzorci stanica
 - periferna krv, koštana srž, pleuralni izljev, BAL, punktati limfnog čvora, likvor
- Obilježavanje stanica
 - uklanjanje eritrocita liziranjem
 - inkubacija sa specifičnim protutijelima na koja je vezan pojedini fluorokrom
 - ispiranje i resuspendiranje u odgovarajućem puferu
- Propuštanje kroz citometar
- Analiza točkastih prikaza uz pomoć programa za analizu



Akutne leukemije

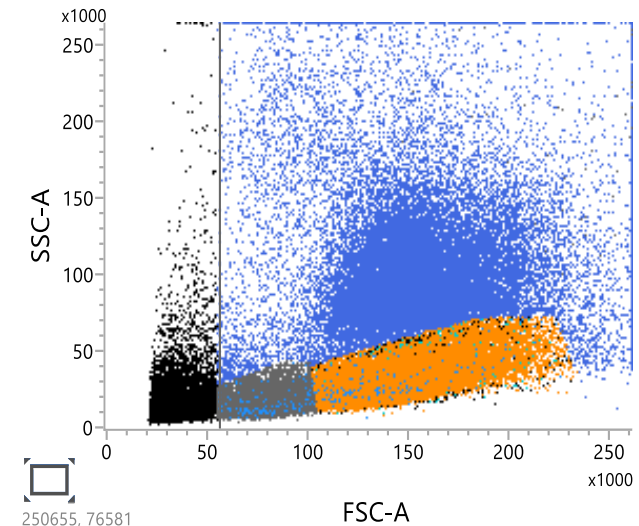
Table 1. WHO classification of AML and ALL.

Cell Lineage	Classification	Subtype
Lymphoid	B-cell ALL with certain genetic abnormalities	<ul style="list-style-type: none"> • B-cell ALL with hypodiploidy • B-cell ALL with hyperdiploidy • B-cell ALL with t(9;22) (Philadelphia chromosome, BCR-ABL1 fusion) • B-cell ALL with translocation involving chromosome 11 • B-cell ALL with t(12;21) • B-cell ALL with t(1;19) • B-cell ALL with t(5;14) • B-cell ALL with iAMP21 * • B-cell ALL with BCR-ABL1-like ALL * • B-cell ALL, not otherwise specified
	T-cell ALL	<ul style="list-style-type: none"> • Early T-cell precursor lymphoblastic leukaemia *
Myeloid	AML with defining genetic abnormalities	<ul style="list-style-type: none"> • Acute promyelocytic leukaemia with PML::RARA fusion • AML with RUNX1::RUNX1T1 fusion • AML with CBFβ::MYH11 fusion • AML with DEK::NUP214 fusion • AML with RBM15::MRTEA fusion • AML with BCR::ABL1 fusion • AML with KMT2A rearrangement • AML with MECOM rearrangement • AML with NUP98 rearrangement • AML with NPM1 mutation • AML with CEBPA mutation • AML, myelodysplasia-related • AML with other defined genetic alterations
	AML, defined by differentiation	<ul style="list-style-type: none"> • AML with minimal differentiation • AML without maturation • AML with maturation • Acute basophilic leukaemia • Acute myelomonocytic leukaemia • Acute monocytic leukaemia • Acute erythroid leukaemia • Acute megakaryoblastic leukaemia
	Acute leukaemia of ambiguous lineage (ALAL) and mixed-phenotype acute leukaemia (MPAL)	ALAL/MPAL with defining genetic abnormalities
	ALAL, immunophenotypically defined	<ul style="list-style-type: none"> • Mixed-phenotype acute leukaemia, B/myeloid • Mixed-phenotype acute leukaemia, T/myeloid • Mixed-phenotype acute leukaemia, rare types • Acute leukaemia of ambiguous lineage, not otherwise specified • Acute undifferentiated leukaemia

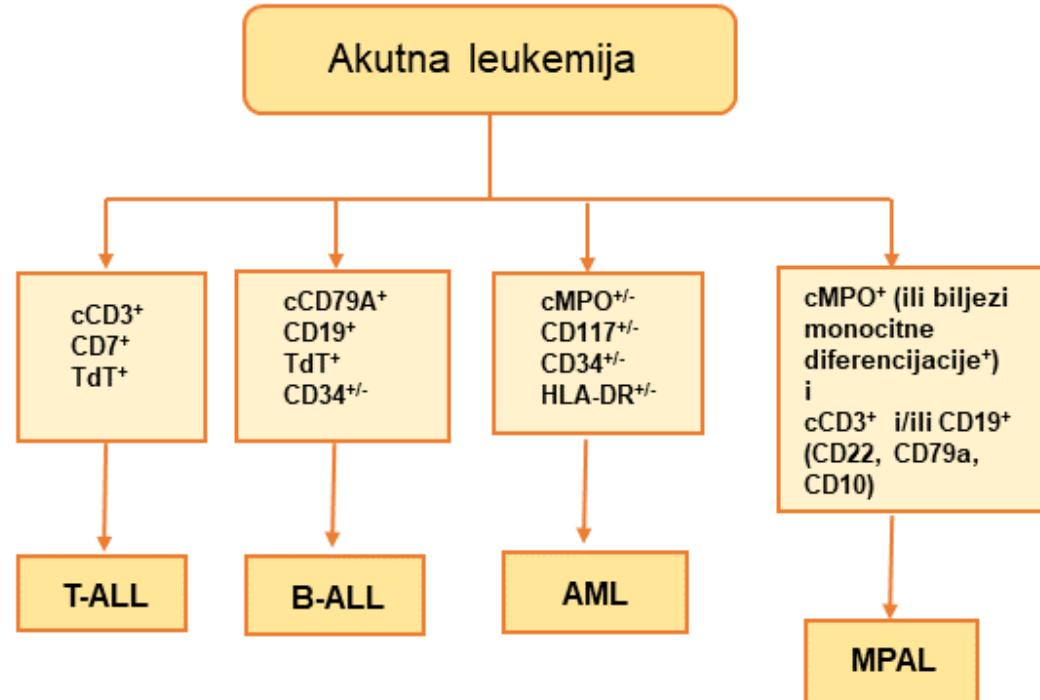
Wemyss C, Jones E, Stentz R, Carding SR. Acute Myeloid Leukaemia and Acute Lymphoblastic Leukaemia Classification and Metabolic Characteristics for Informing and Advancing Treatment. *Cancers (Basel)*. 2024 Dec 11;16(24):4136. doi: 10.3390/cancers16244136. PMID: 39766036; PMCID: PMC11675077.

Glavna uloga imunofenotipizacije

- razlikovanje malignih od normalnih stanica
- određivanje loze stanica
- određivanje diferencijacijskog stupnja malignih stanica
- određivanje aberantnih osobina malignih populacija stanica
- probir na citogenetske i genetske promjene
- detekcija biljega nužnih za primjenu specifične imunoterapije
- praćenje mjerljive ostatne bolesti



Imunofenotipski probir

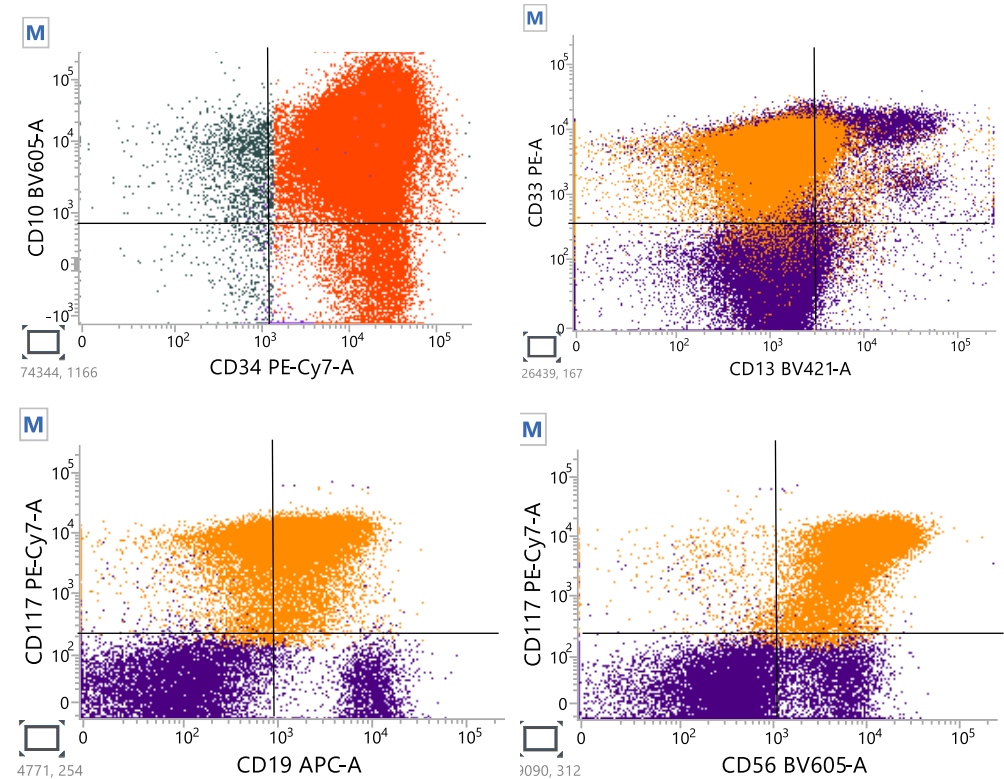


- sve negativno osim:

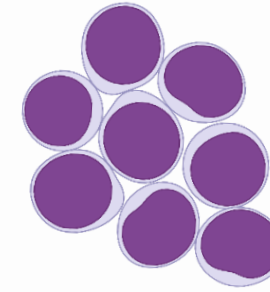
- biljega nezrelosti (CD34+TdT+HLA-DR+) – **AUL** (*akutna nediferencirana leukemija*)
- CD56, CD123, CD4, HLA-DR (**BPDCN** – *neoplazma blastičnih plazmacitoidnih dendritičkih stanica*)

Imunofenotipske aberacije

- pojačani ili sniženi izražaj pojedinih biljega u odnosu na normalnu koštanu srž (npr. CD34 i CD10 u prekursorskoj B-ALL)
- potpuni gubitak biljega normalno prisutnih na određenim nezrelim stanicama (npr. gubitak biljega CD13 i/ili CD33 na mijeloblastima)
- asinkroni izražaj biljega zrelosti na nezrelim stanicama (npr. CD15 na mijeloblastima)
- „cross lineage“ izražaj biljega (npr. CD13 i CD33 na limfoblastima, CD19, CD7 i CD56 na mijeloblastima)



Akutna limfoblastična leukemija (ALL)



Cell Lineage	Classification	Subtype
Lymphoid	B-cell ALL with certain genetic abnormalities	<ul style="list-style-type: none"> • B-cell ALL with hypodiploidy • B-cell ALL with hyperdiploidy • B-cell ALL with t(9;22) (Philadelphia chromosome, BCR-ABL1 fusion) • B-cell ALL with translocation involving chromosome 11 • B-cell ALL with t(12;21) • B-cell ALL with t(1;19) • B-cell ALL with t(5;14) • B-cell ALL with iAMP21 * • B-cell ALL with BCR-ABL1-like ALL * • B-cell ALL, not otherwise specified
	T-cell ALL	<ul style="list-style-type: none"> • Early T-cell precursor lymphoblastic leukaemia

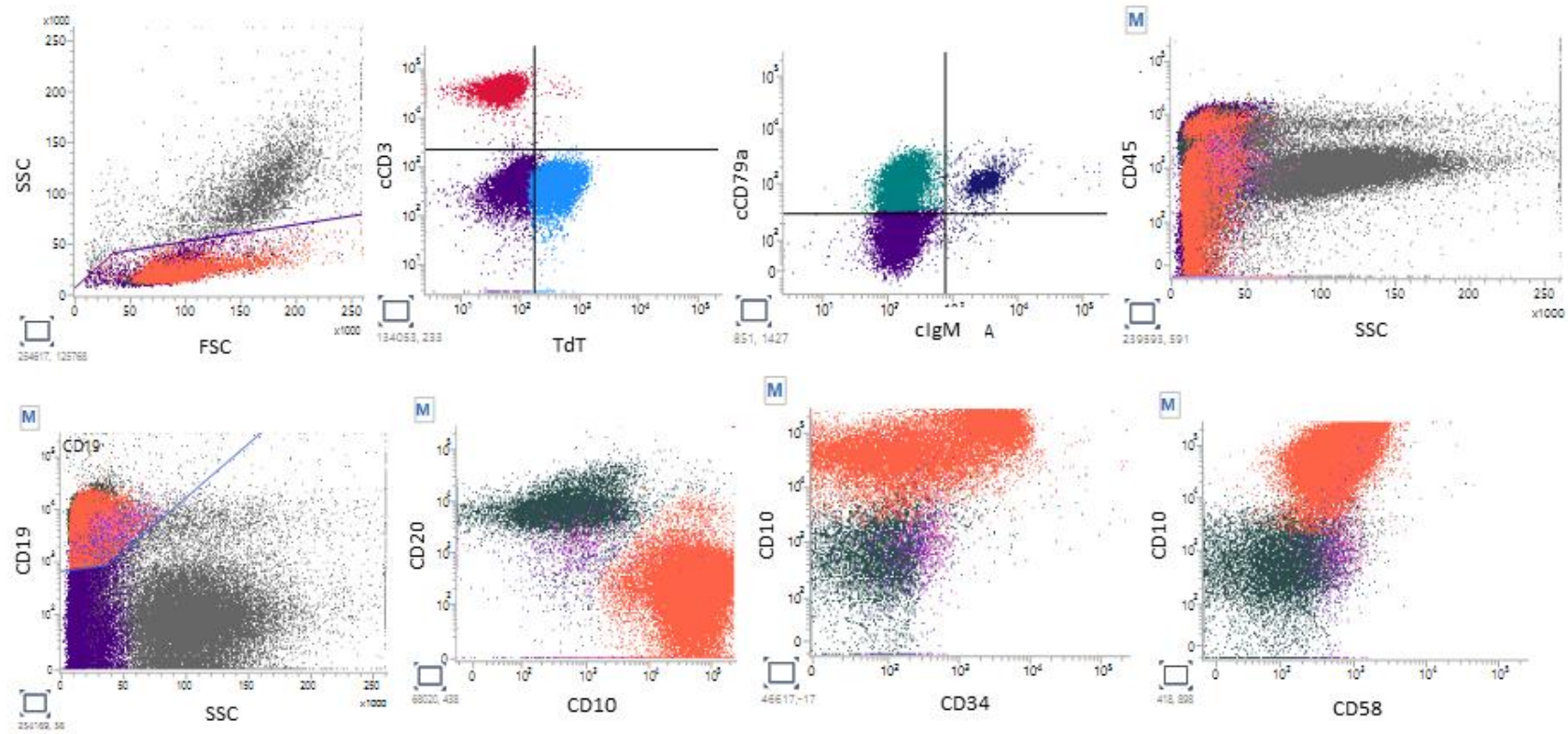
EGIL klasifikacija ALL-a				
	B-loza		T-loza	
I	Pro-B	TdT+, C19+, cCD79a+, CD22+, CD10- , clgM-, mlgM-	Pro-T	TdT+, cCD3+ , mCD3-, CD7+ , CD2-, CD5-, CD1a-, CD4-, CD8-
II	Common	TdT+, CD19+, cCD79a+, CD22+, CD10+ , clgM-, mlgM-	Pre-T	TdT+, cCD3+, mCD3-, CD7+ CD2+/-, CD5+/-, CD1a- , CD4+/-, CD8+/-
III	Pre-B	TdT+, CD19+, cCD79a+, CD22+, CD10+, clgM+ , mlgM-	Kortikalni T	TdT+/-, cCD3+, mCD3+/-, CD7+ CD2+, CD5+, CD1a+ , CD4+CD8+
IV	Zrela B	TdT-, C19+, cCD79a+, CD22+, CD10+, clgM+, mlgM+	Zrela T	TdT-, cCD3+, mCD3+ , CD7+ CD2+, CD5+, CD1a-, CD4+/CD8+

- **leukemija ranih T-prekursora** (engl. *early T precursors*, **ETP-ALL**) - jedan ili više biljega nezrelosti i/ili mijeloidne loze (CD11b, CD13, CD33, CD34, CD65, CD117 i/ili HLA-DR), uz CD5^{slabo+/-} i CD8⁻

Probir na citogenetske i molekularne promjene

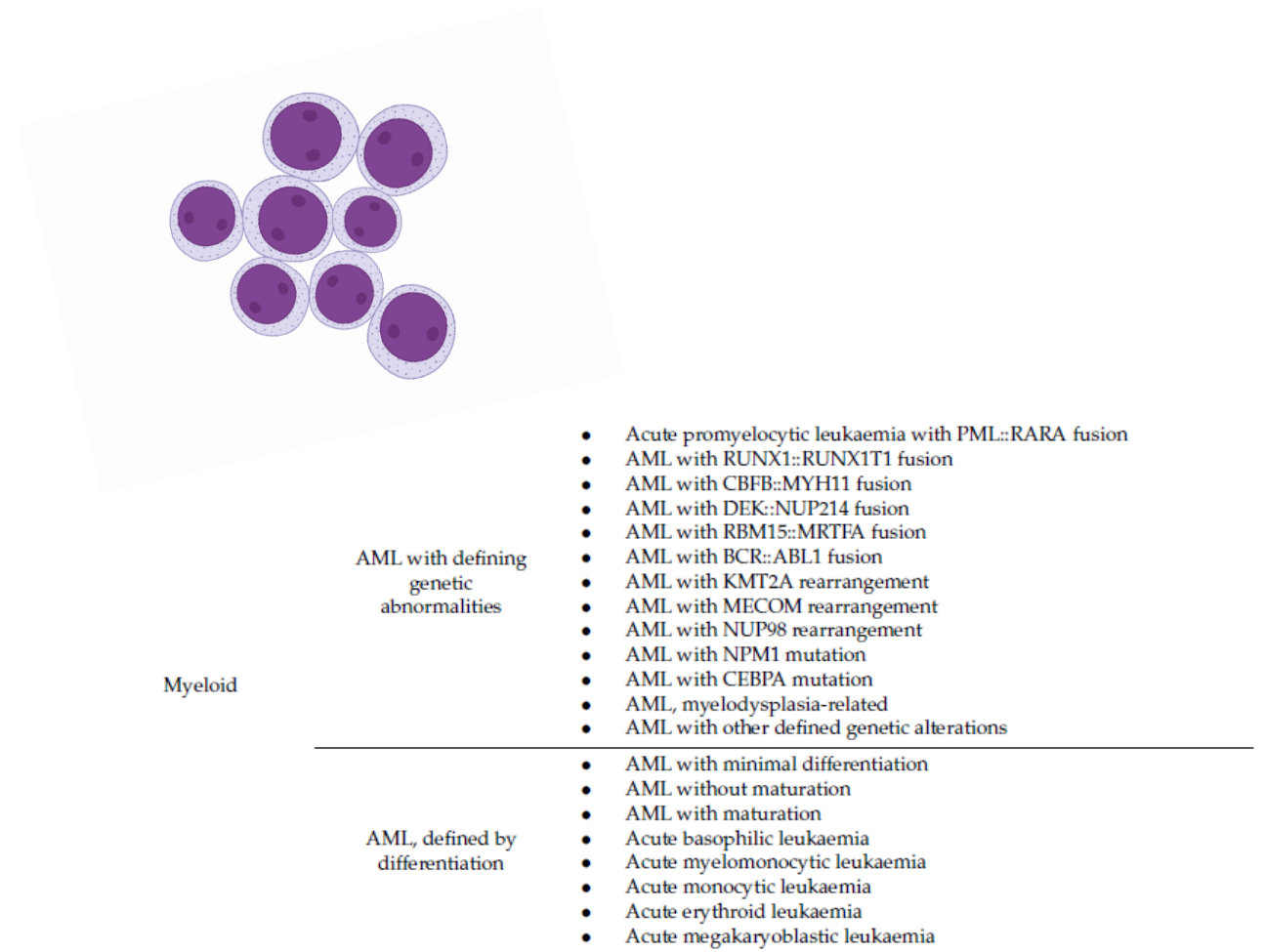
Marker	Prekursorska B-ALL			
	11q23	t(9;22)	t(12;21)	t(1;19)
CD19	+	+/++	+	+
CD10	-	+	++	+
CD20	-/+ dim	-/+	-/p+	-/+
clgM	-	-	-/p+	+
CD45	+	+	-/+	+
CD15	+	-	-	-
CD34	+	++	-/+	-
CD13	-/+dim	+dim	+dim	-

„Common” B-ALL - primjer

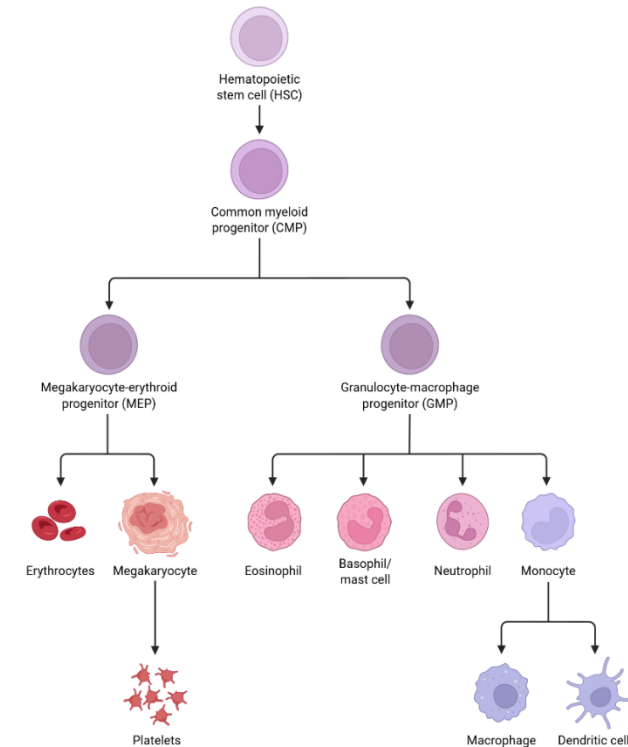


Akutna mijeloična leukemija

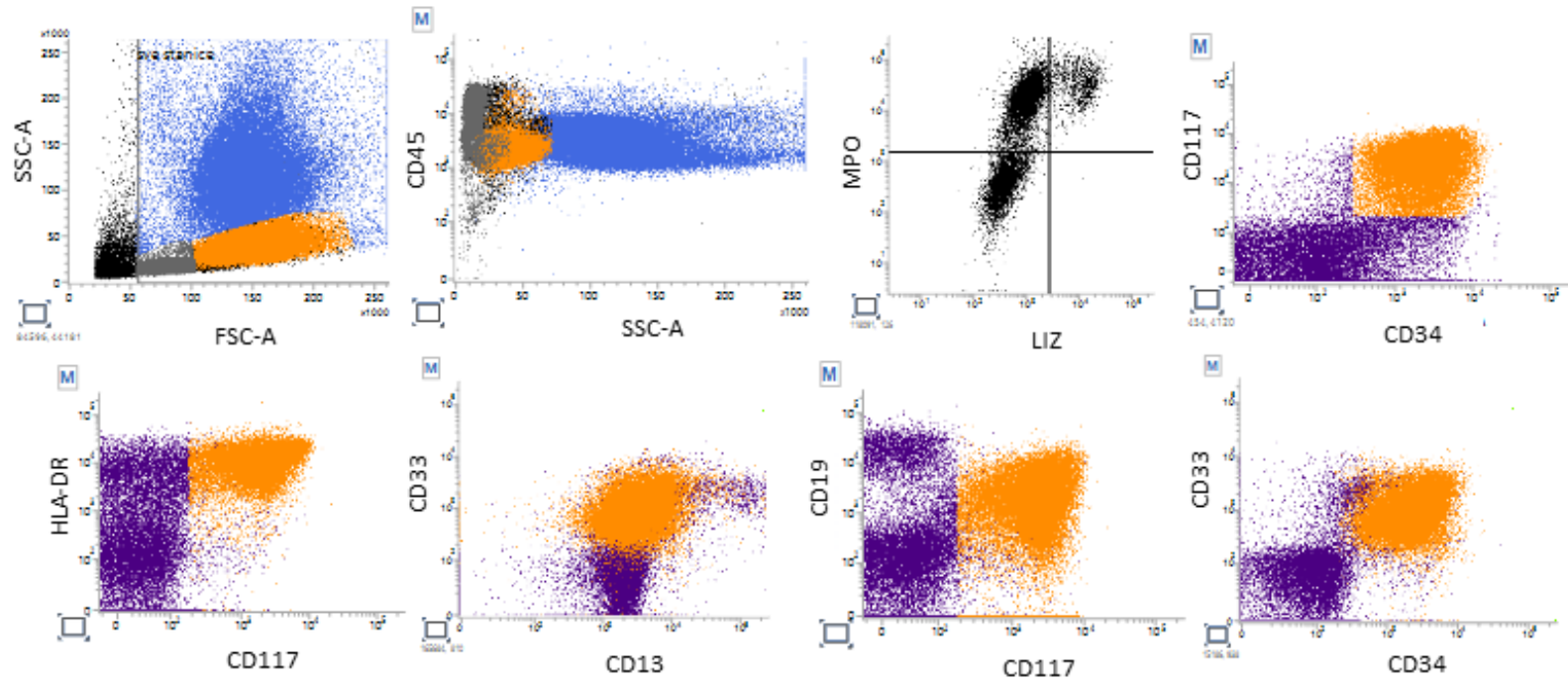
- Definirana prema stupnju diferencijacije i prema citogenetskim i molekularnim promjenama
- Nema imunofenotipske podjele
- Uloga imunofenotipa:
 - Brza potvrda dijagnoze AML-a
 - Probir na potencijalne citogenetske i molekularne promjene
 - Isključenje dijagnoze MPAL
 - Definiranje diferencijacijskog stupnja blasta i aberacija na njima u odnosu na izražaj pojedinih CD biljega
 - Prisutnost izražaja pojedinih biljega bitna za liječenje ciljanom terapijom (gemtuzumab)



Tip blasta	Imunofenotip
Mijeloblasti	CD34⁺, CD117⁺, CD13⁺, CD33⁺, HLA-DR⁺, CD45⁺, MPO^{+/-}, CD45RA⁺, TdT^{+/-}, CD7^{-/+}, CD56^{-/+}, CD71^{+/-}, CD133⁺
Promijelociti	CD34⁻, CD117⁺, CD13⁺, CD33⁺⁺, CD11c⁻, HLA-DR⁻, MPO⁺, CD45⁺, TdT⁻, CD56^{-/+}, CD64^{+(slabo)/-}, CD7⁻
Monoblasti	CD34^{-(rijetko+)}, CD117^{-/+}, CD13⁺, CD33⁺⁺, MPO⁻, HLA-DR⁺, CD45⁺⁺, CD14^{+/-}, CD64⁺⁺, CD11b^{+/-}, CD11c⁺, CD4⁺, CD56^{+/-}, CD71^{+/-}, CD123^{+/-}
Eritroblasti	CD34⁻, CD117^{-/+}, CD13/CD33⁻, CD36⁺, HLA-DR⁻, MPO⁻, CD45⁻, TdT⁻, CD71⁺⁺, GlyA^{+/-}
Megakarioblasti	CD34^{-/+}, CD117^{-/+}, MPO⁻, CD13/CD33^{+/-}, HLA-DR⁻, CD45^{+/-}, TdT⁻, CD41⁺, CD42⁺, CD61⁺
BPDCN	CD4⁺, CD13⁻, CD33^{-/+}, CD56⁺⁺, CD123⁺⁺, CD11b⁻, CD11c⁻, HLA-DR⁺, CD45⁺, TdT^{+/-}, MPO⁻, CD64⁻
Nediferencirani blasti	CD34⁺, CD117⁻, CD13⁻, CD33⁻, CD38⁺, HLA-DR⁺, CD45⁺, TdT^{+/-}, CD7⁻, CD19⁻, CD3⁻, MPO⁻, CD14⁻, CD64⁻, CD11b⁻, CD11c⁻, CD56⁻



AML -primjer



Probir na citogenetske i molekularne promjene

Marker	AML			
	t(8;21)	t(15;17)	Inv(16)	NPM1 ^{mut}
MPO	+ /+++	+ /+++	++	+
CD4	-	-	+	+/-
CD11B	-	-	+	+/-
CD13	+	+	+	+
CD33	+	+	+	+
CD14	-	-	+	+/-
CD15	+het	-	+	
CD34	+	-/+	p+	2/3 neg
CD117	+	+	p+	+
HLA-DR	+	-	+	1/3 neg
CD2	-	-/+dim	-/+	-
CD19	+	-	-	-
CD56	+ /+++	-/+	-	-

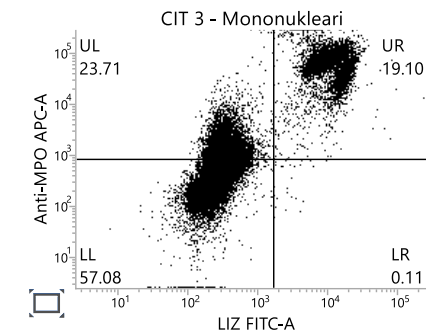
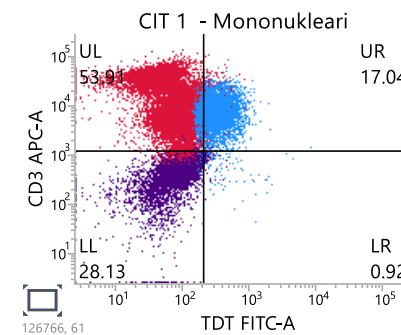
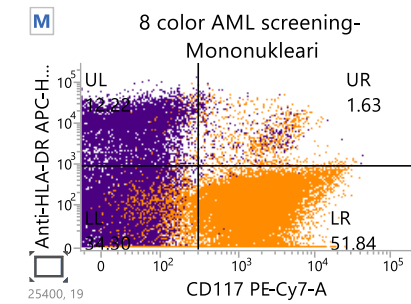
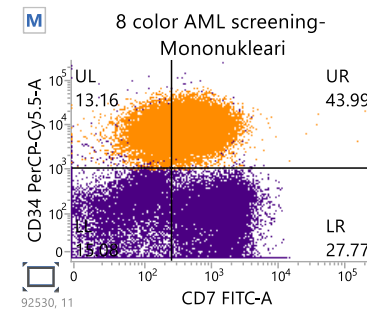


“APL like”

Akutne leukemije nejasnog podrijetla

MPAL kriteriji prema SZO	
Mijeloidna loza	MPO ⁺ ili biljezi monocitne diferencijacije (barem 2 pozitivna od CD11c, CD14, CD64, lizozim, NSE)
T-loza	Jak izražaj cCD3 ili mCD3
B-loza	Jak izražaj CD19 i barem još jedan dodatni biljeg B-loze (cCD79a, CD22, CD10) ili slabi izražaj CD19 i barem još 2 dodatna biljega B-loze (cCD79a, CD22, CD10)

Acute leukaemia of ambiguous lineage (ALAL) and mixed-phenotype acute leukaemia (MPAL)	ALAL/MPAL with defining genetic abnormalities	<ul style="list-style-type: none"> Mixed-phenotype acute leukaemia with BCR::ABL1 fusion Mixed-phenotype acute leukaemia with KMT2A rearrangement Acute leukaemia of ambiguous lineage with other defined genetic alterations Mixed-phenotype acute leukaemia with ZNF384 rearrangement Acute leukaemia of ambiguous lineage with BCL11B rearrangement
	ALAL, immunophenotypically defined	<ul style="list-style-type: none"> Mixed-phenotype acute leukaemia, B/myeloid Mixed-phenotype acute leukaemia, T/myeloid Mixed-phenotype acute leukaemia, rare types Acute leukaemia of ambiguous lineage, not otherwise specified Acute undifferentiated leukaemia



Praćenje mjerljive rezidualne bolesti (MRD)

- prisutnost ostatnih leukemijskih stanica nakon primjenjene terapije ispod granice detekcije konvencionalnih morfoloških metoda
- indikator prognoze i predviđanja rizika od relapsa
- metode - FISH, protočna citometrija, PCR i NGS

Maximum sensitivity (no. cancer cells per no. nucleated cells)	Percentage	Sensitivity threshold
1 in 20	5%	
1 in 1,000	0.1%	10^{-3}
1 in 10,000	0.01%	10^{-4}
1 in 100,000	0.001%	10^{-5}
1 in 1,000,000	0.0001%	10^{-6}

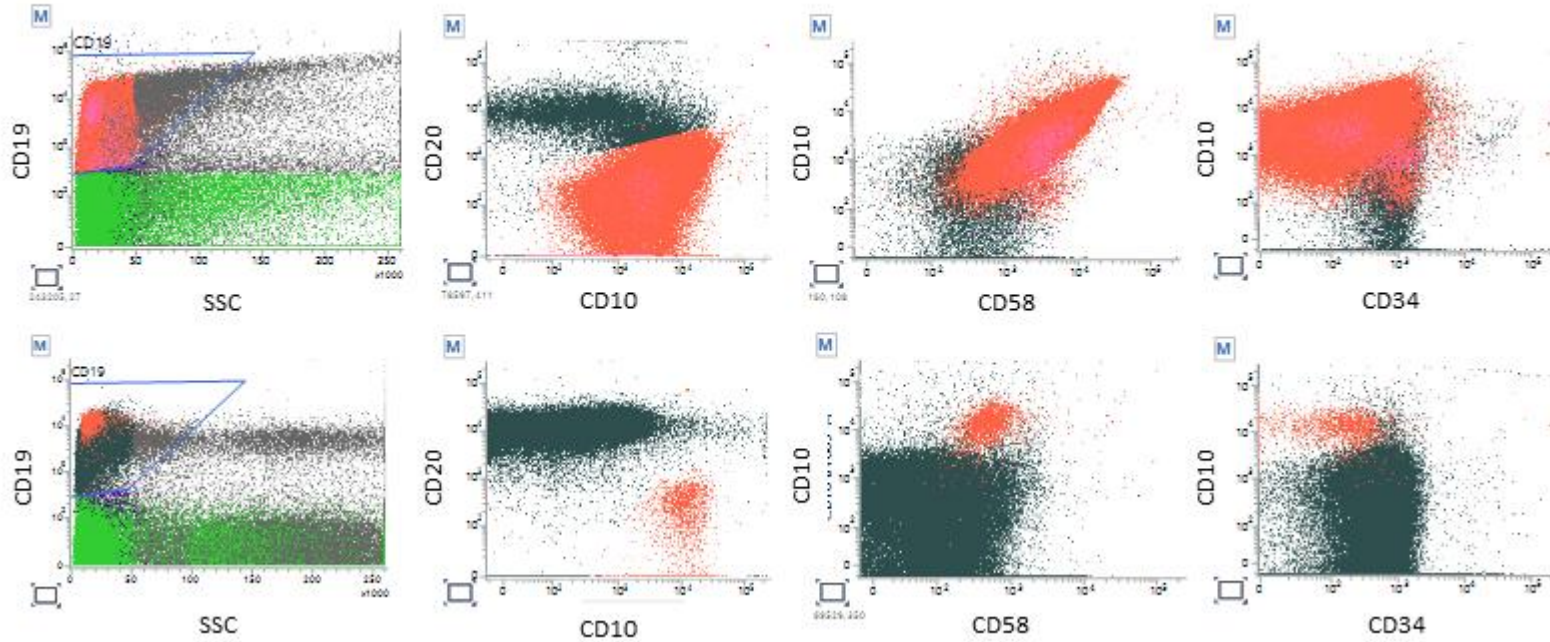
https://www.lls.org/sites/default/files/2021-05/FSHP5_MRD_Factsheet

Mjerenje MRD-a protočnom citometrijom

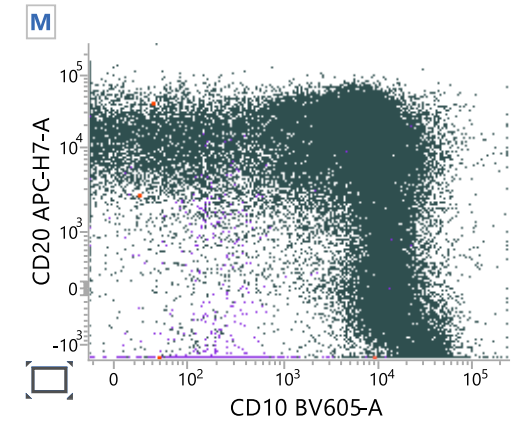
- relativno brza i osjetljiva metoda
- potrebno prikupljanje vrlo velikog broja stanica za analizu ($>10^6$)
- značajno iskustvo operatera za detektiranje aberacija u izražaju pojedinih biljega
- promjene u imunofenotipu tijekom liječenja
- nedostatak standardizacije (u AML-u)
- bitno informirati laboratorij ukoliko je pacijent primao ciljanu terapiju monoklonskim protutijelima usmjerenim na određene stanične biljege (npr. CD19 ili CD20) - negativizacija biljega koji se koriste za praćenje

- Dva pristupa:
 - **LAIP** (eng. *leukemia associated immunophenotype*) – praćenje leukemijskog imunofenotipa prisutnog pri dijagnozi – rizik lažno negativnih nalaza
 - **DfN** (eng. *different from normal*) – praćenje promjena u imunofenotipu (aberracija) u odnosu na normalnu koštanu srž – rizik lažno pozitivnih nalaza

De novo



MRD



Imunofenotipizacija akutnih leukemija

- temeljni dio dijagnostike zajedno sa citomorfološkim, citogenetskim i molekularnim metodama
 - omogućuje precizno određivanje loze i stupnja diferencijacije stanica
 - prepoznavanje aberantnih obrazaca izražaja pojedinih biljega
 - pravovremeno usmjeravanje na specifične citogenetske i molekularne analize
 - odabir odgovarajuće ciljane terapije
 - procjenu dubine remisije i rizika relapsa praćenjem mjerljive rezidualne bolesti
- 