FONDAZIONE ITALIANA LINFOMI

IMMUNOTERAPIA E FARMACI BIOLOGICI NEL TRATTAMENTO DEI LINFOMI E LLC: ATTUALITÀ E FUTURO

NGS nella valutazione della MRD nelle patologie linfoproliferative

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Disclosures of Veronica Scaglia

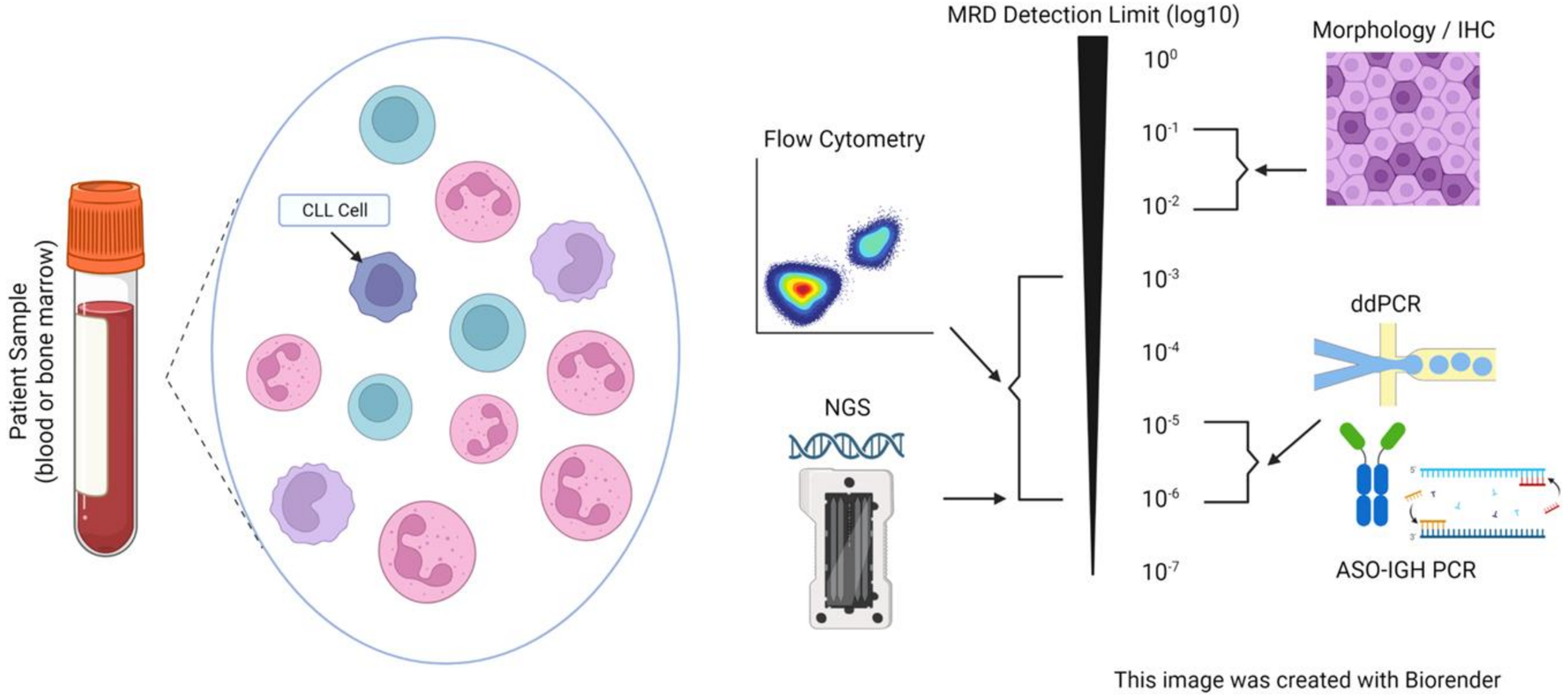
Company name	Research support	Employee	Consultant	Stockholder	Speakers bureau	Advisory board	Other

MRD in CLL

- Il Raggiungimento di uno stato di MRD negativa (uMRD) si è dimostrato un potente fattore prognostico, correlato a più lunga progressione libera da malattia e sopravvivenza globale.

- Negli ultimi anni le tecniche di NGS si sono affermate come valido strumento per la valutazione della MRD.

MRD in CLL: tecnologie utilizzate



Joanna M. Rhodes, Carlos A. Lopez, Jacqueline C. Barrientos; MRD-directed therapy in CLL: ready for prime time?. *Hematology Am Soc Hematol Educ Program* 2023; 2023 (1): 413–420.

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Metodologie a confronto

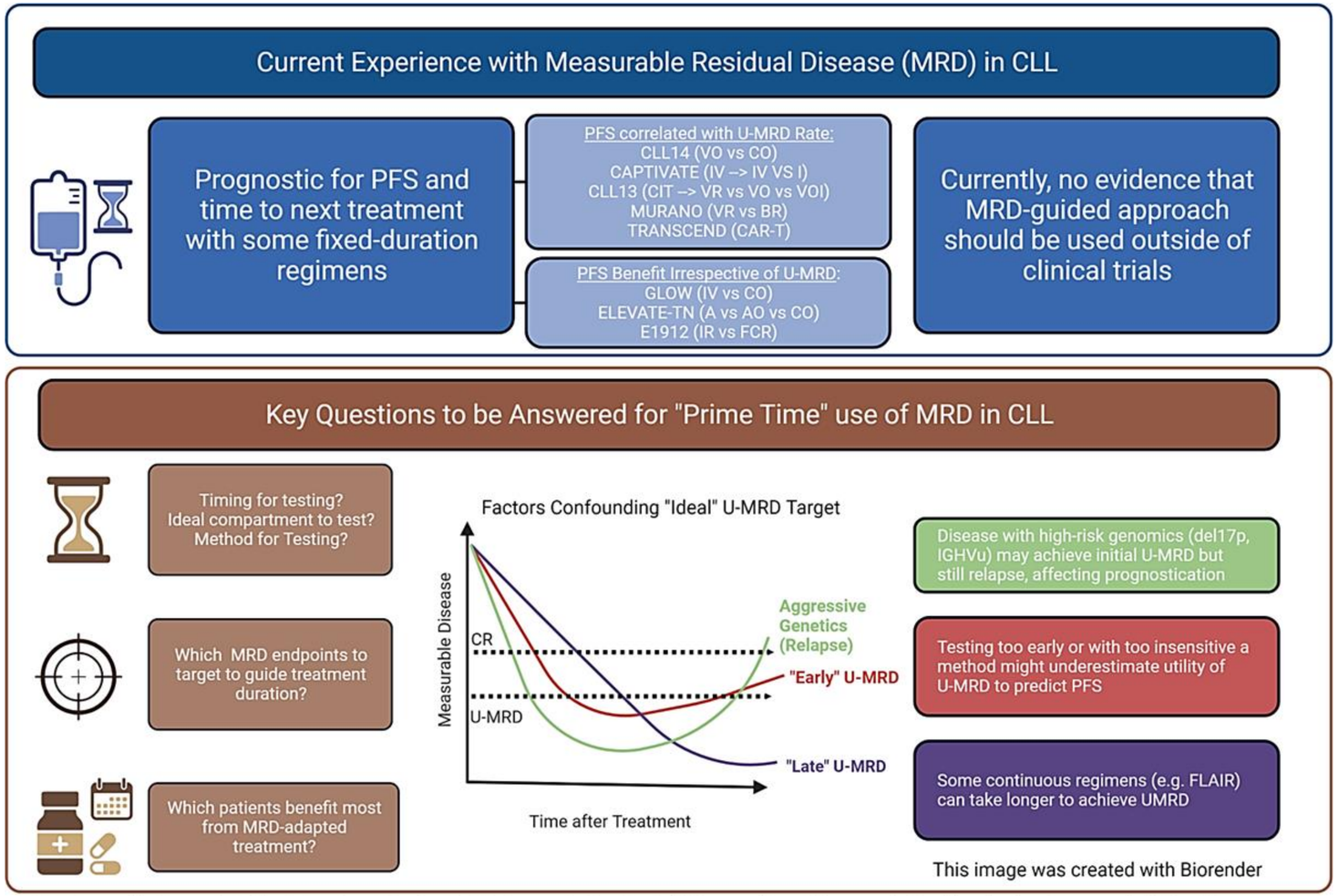
Methodology	Strengths	Weaknesses
Multiparameter Flow Cytometry	Fast; Absolute Quantification; Information at a cellular level; Wide availability	Variable antigen expression could lead to false negative results; High grade of expertise needed; Medium sensitivity with less than 8-colours
Allele-Specific Oligonucleotide PCR	High sensitivity	Time-consuming in the design of patient-specific primers; Requirement for optimal DNA quality and quantity
Digital PCR	Absolute quantification; High sensitivity; Avoids PCR inhibitors due to compartmentalization of target sequences	Lack of standardization No possibility to find new variants Allele-specific design
Next-Generation Sequencing or High-Throughput Sequencing	High Sensitivity ($>10^{-6}$); Patient-specific primers not necessary; Versatility	Lack of standardization; High degree of bioinformatics expertise; Expensive

Sánchez R, Ayala R, Martínez-López J. MinimalResidualDiseaseMonitoring with Next-Generation Sequencing Methodologies in HematologicalMalignancies. Int J Mol Sci. 2019 Jun 10;20(11):2832.

MRD-directed therapy in CLL: stato dell'arte

- Studi condotti durante trattamento con BTKis non hanno messo in evidenza PFS e OS correlata allo stato MRD
- Successivamente, nel trattamento con BCL-2 inibitori (+obinotuzumab/rituximab), la valutazione dello stato MRD si è reso fondamentale nella pratica clinica.
- Studi come CLL14 hanno dimostrato una percentuale significativa di pazienti che ottengono uMRD dopo trattamento.
- Gruppo di lavoro CLL-AVEN su caratteristiche biologiche e outcome in pazienti affetti da CLL trattati con target agents (valutazione MRD in NGS at EOCT, EOT, Follow-up ogni 6 mesi)

MRD-directed therapy in CLL: stato dell'arte



MRD: Measurable Disease; CLL: Chronic Lymphocytic Leukemia; PFS: Progression-Free Survival; VO: venetoclax + obinutuzumab; CO: chlorambucil + obinutuzumab; IV: Ibrutinib + Venetoclax; I: Ibrutinib; CIT: Chemoimmunotherapy; VR: Venetoclax + Rituximab; VOI: venetoclax + obinutuzumab + ibrutinib; BR: Bendamustine + rituximab; CAR-T: Chimeric antigen receptor T-cell Therapy; CR: Complete Remission; U-MRD: undetectable MRD; A: acalabrutinib; AO: acalabrutinib + obinutuzumab; IGHVu: IGHV unmutated

Joanna M. Rhodes, Carlos A. Lopez, Jacqueline C. Barrientos; MRD-directed therapy in CLL: ready for prime time?. *Hematology Am Soc Hematol Educ Program* 2023; 2023 (1): 413–420.

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NCCN Guidelines Version 3.2025
Chronic Lymphocytic Leukemia/Small Lymphocytic Lymphoma

Table 1: Undetectable MRD Rates for BCL2i-Containing Regimens

Disease Setting	Trial	Regimen	No. of Patients	Patient Characteristics	Median Follow-up	Undetectable MRD ($\leq 10^{-4}$, uMRD4)	Method used for MRD detection
Previously Untreated CLL/SLL	CLL14[28] (Phase III)	**Venetoclax + obinutuzumab (VenO)**	216	≥65 years (CIRS >6; CrCl <70 mL/min)	65 months	EOT+2: 75% (blood)	ASO-PCR; MRD-flow; NGS
		Chlorambucil + obinutuzumab	216			EOT+2: 33% (blood)	
	CAPTIVATE[32] (Phase II; Time-limited cohort)	**Venetoclax + ibrutinib**	159	≤70 years; ECOG PS 0–1	28 months	EOT+3: 77% (blood); 60% (BM)	MRD-flow (8-color flow cytometry)
	CAPTIVATE[33] (Phase II; MRD cohort)	**Venetoclax + ibrutinib** (3 cycles of lead-in ibrutinib followed by 12 cycles of ibrutinib + venetoclax)	164	≤70 years; ECOG PS 0–1 (Prerandomization)	31 months	75% (blood); 68% (BM)	
		Venetoclax + ibrutinib	32	≤70 years; ECOG PS 0–1 (Randomization; uMRD not confirmed)		69% (blood); 66% (BM)	
		Ibrutinib	31			45% (blood); 42% (BM)	
	GLOW (Phase III)[27]	**Venetoclax + ibrutinib**	106	≥65 years or <65 years who also had CIRS >6 or CrCl <70 mL/min	34 months	EOT+3: 55% (blood); 52% (BM)	NGS
		Chlorambucil + obinutuzumab	105			EOT+3: 39% (blood); 17% (BM)	
	GAIA/CLL13[29] (Phase III)	**Venetoclax + ibrutinib + obinutuzumab**	231	≤65 years or >65 years [without del(17p) or *TP53* mutation]	39 months	15 months: 92% (blood); 78% (BM)	MRD-flow (4-color flow cytometry)
		VenO	229			15 months: 87% (blood); 73% (BM)	
		Venetoclax + rituximab (VenR)	237			15 months: 57% (blood); 43% (BM)	
		Chemoimmunotherapy (FCR ≤65 years; BR >65 years)	229			15 months: 52% (blood); 37% (BM)	
	AMPLIFY[31]	**Venetoclax + acalabrutinib**	291	Median age: 61 years [without del(17p) or *TP53* mutation])	41 months	EOT: 45% (blood); EOT+3: 38% (blood)	NGS; MRD-flow
		VenO + acalabrutinib	286			EOT: 95% (blood); EOT+3: 94% (blood)	
		FCR or Bendamustine + rituximab (BR)	290			EOT: 73% (blood); EOT+3: 78% (blood)	
Relapsed or Refractory CLL/SLL	MURANO (Phase III)[82]	**VenR**	194	≥18 years; ECOG PS 0–1; adequate bone marrow, liver, and kidney function	36 months	62% (blood)	ASO-PCR and/or MRD-flow (4-color flow cytometry)
		BR	195			13% (blood)	
	CLARITY[83] (Phase II)	Ibrutinib + venetoclax	53	Median age: 64 years ECOG PS 0–2	21 months	53% (blood); 36% (BM)	MRD-flow

MRD-directed therapy in CLL: ruolo dell' NGS

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NCCN Guidelines Version 3.2025
Chronic Lymphocytic Leukemia/Small Lymphocytic Lymphoma

NCCN Guidelines Index
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Discussion

RESPONSE DEFINITIONS AFTER TREATMENT FOR CLL/SLL[a]

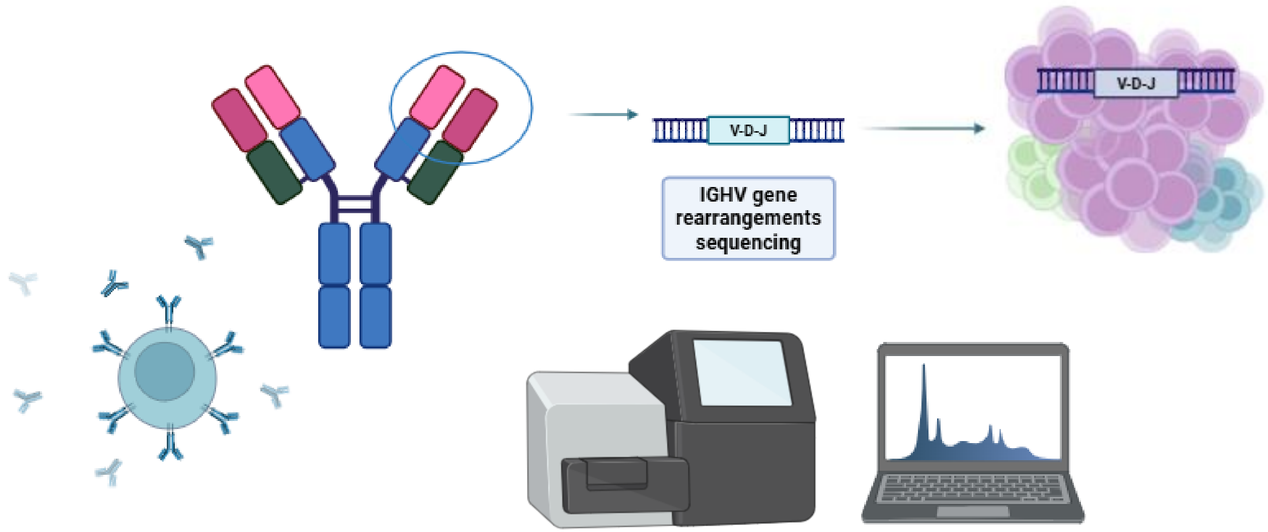
Minimal Residual Disease (MRD) Assessment:

- **Evidence from clinical trials suggests that undetectable MRD in the peripheral blood after the end of time-limited treatment is an important predictor of efficacy.[e,f,g,h,i]**
- **Allele-specific oligonucleotide polymerase chain reaction (ASO-PCR) and six-color flow cytometry (MRD flow) are the two validated methods used for the detection of MRD at the level of 10^{-4} to 10^{-5}.[j,k] Next-generation sequencing (NGS)-based assays have been shown to be more sensitive, thus allowing for the detection of MRD at the level of 10^{-6}.[l,m,n]**
- **MRD evaluation should be performed using an assay with a sensitivity of 10^{-4} according to the standardized European Research Initiative on CLL (ERIC) method or standardized NGS method.**

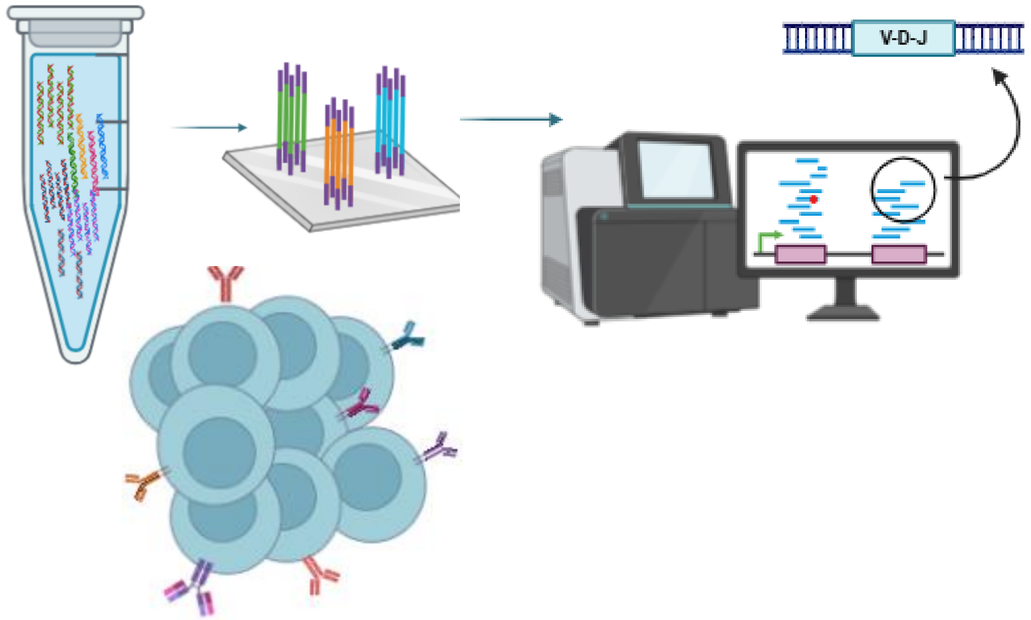
- MRD su campioni BM o PB
- L'analisi NGS richiede un campione pre-trattamento
- La valutazione della MRD può essere utile nella pratica clinica per fornire informazioni dettagliate sulla PFS dopo il trattamento, ma non è ad oggi raccomandata nelle decisioni terapeutiche.

MRD-directed therapy in CLL: workflow NGS

Esordio:

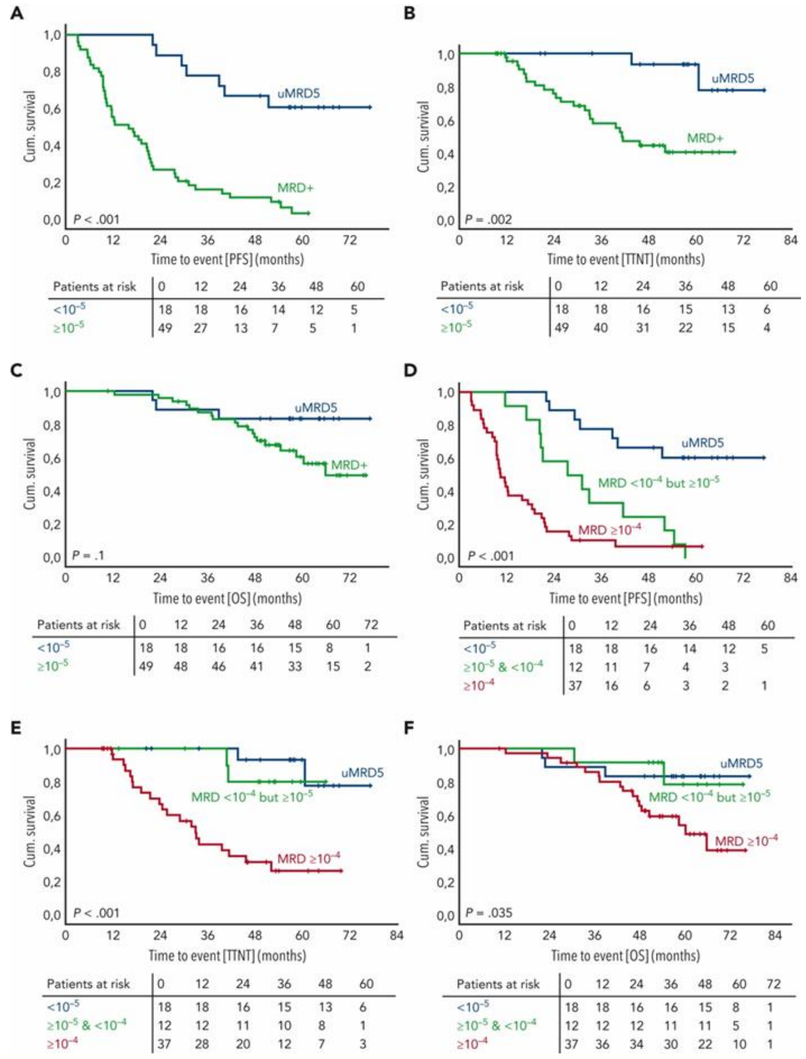


Rivalutazione MRD:



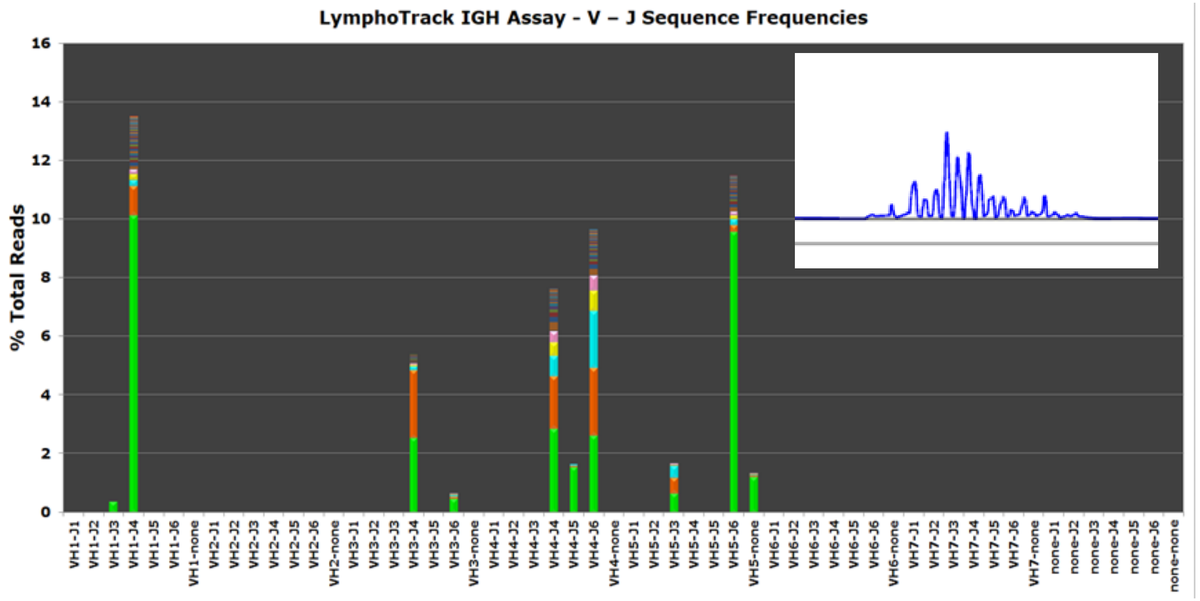
Vantaggi della tecnologia NGS: sensibilità

- uMRD <10^{-5} è associata a maggiore PFS e intervallo al successivo trattamento.
- 10^{-4}>MRD≥10^{-5} presentano PFS inferiore rispetto a uMRD<10^{-5}, ma superiore al gruppo con MRD>10^{-4}.
- I risultati supportano l'utilità clinica del test NGS IGHV leader-based.



Paul J. Hengeveld et al.; Detecting measurable residual disease beyond 10^{-4} by an IGHV leader-based NGS approach improves prognostic stratification in CLL. *Blood* 2023; 141 (5): 519–528

Vantaggi della tecnologia NGS: caratterizzazione cloni multipli



Total coun 201 387

Rank	Sequence	Length	Merge cou	V-gene	J-gene	% total rea	Cumulative	Mutation rate to partial V-gene (%	In-frame (Y/N	No Stop codon (Y/
1	GATCCTCTTTTTG	478	14030	IGHV1-3_01	IGHJ4_02	13.51	13.51	0.68	Y	Y
2	CTCGCCCTCCTCC	472	12780	IGHV5-51_01	IGHJ6_02	12.30	25.81	0.00	Y	Y
3	TCCTGGTGGCAG	461	4461	IGHV4-4_02	IGHJ4_02	4.29	30.10	7.77	Y	Y
4	TCCTGCTGGTGG	471	3912	IGHV4-39_01	IGHJ6_02	3.76	33.87	0.67	Y	Y
5	TCCTGCTGGTGG	468	3601	IGHV4-39_01	IGHJ4_02	3.47	37.34	2.68	Y	Y
6	TCCTCCTGGTGG	455	3572	IGHV4-4_02	IGHJ6_02	3.44	40.77	1.01	Y	Y
7	GGTTTTCCTTGTT	475	3114	IGHV3-74_01	IGHJ4_02	2.99	43.77	2.03	Y	Y
8	GGTTTTCCTCGTT	482	2875	IGHV3-33_01	IGHJ4_02	2.76	46.53	1.35	Y	Y
9	TCCTCCTGGTGG	450	2360	IGHV4-34_01	IGHJ6_02	2.27	48.80	0.00	Y	Y
10	TCCTCCTGGTGG	448	1771	IGHV4-34_01	IGHJ5_02	1.70	50.51	0.00	N	N

Vantaggi della tecnologia NGS: analisi dei subcloni

- LLC è considerata come patologia monoclonale, meno del 5% dei pazienti presenta riarrangiamenti multipli (Plevova et. al,2014)

- Grazie alla tecnologia NGS si possono identificare più facilmente diverse sequenze clonali all'interno del campione di esordio.

- Attualmente manca una standardizzazione in merito al follow-up MRD dei campioni con riarrangiamenti clonali multipli.

Controlli di qualità

UK NEQAS
Leucocyte Immunophenotyping

Sheffield Teaching Hospitals NHS
NHS Foundation Trust

Measurable Residual Disease for Lymphoid Neoplasms by Molecular Methods (Not Accredited)

- Disponibile controllo qualità UK NEQAS dal 2023 (Not Accredited)
- Composto da 2 campioni MRD rilevabile (soglie decrescenti) e 1 campione MRD non rilevabile.
- Nell' anno 2024 è stata evidenziata la necessità di standardizzare il LoD e la soglia di positività MRD. Raccomandano il raggiungimento di sensibilità 10^{-4} – 10^{-5} per i saggi in NGS.

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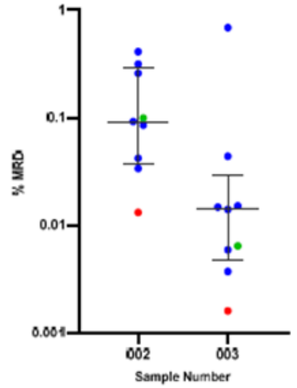

Figure 1: Scatter plot to show all participants % MRD results for samples 002 and 003 for participants calculating % MRD using the following approach: (clonal sequences detected / total number of IG sequences analysed) x100. Long horizontal black line = median. Short horizontal lines = interquartile range. Clone A = Blue, clone B = Green, unknown clone = Red.

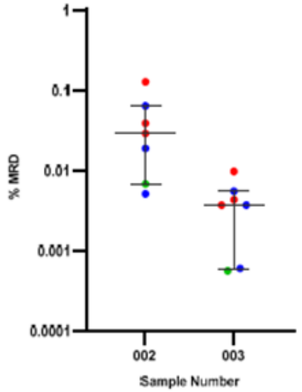

Figure 2: Scatter plot to show all participants % MRD results for samples 002 and 003 for participants calculating % MRD using the following approach: number of estimated clonal cell equivalents per million total nucleated cells detected x100. Long horizontal black line = median. Short horizontal lines = interquartile range. Clone A = Blue, clone B = Green, unknown clone = Red.

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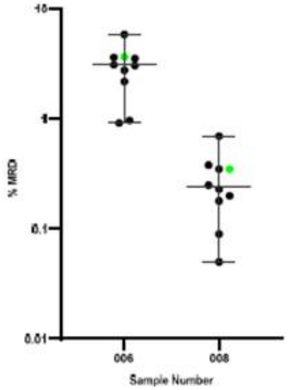


Figure 1: Scatter plot to show all participants % MRD results for samples 006 and 008 for participants calculating % MRD using the following approach: (clonal sequences detected / total number of IG sequences analysed) x100. Long horizontal black line = median. Short horizontal lines = range. Clone A = black, clone B = blue, clone C = red, clone D = green (see table 5).

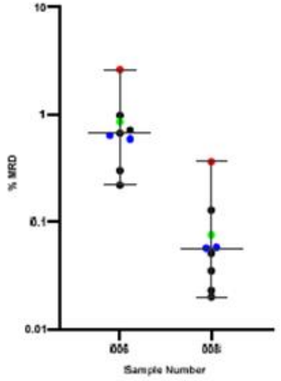
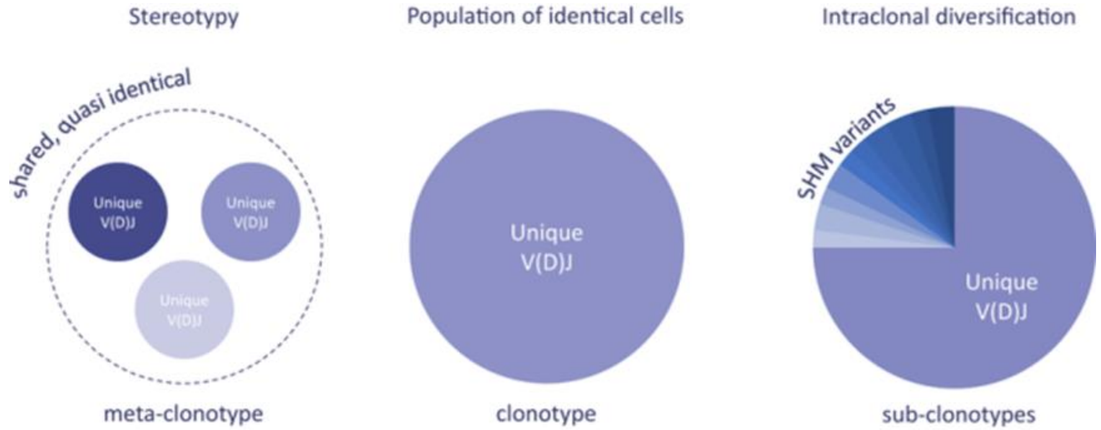


Figure 2: Scatter plot to show all participants % MRD results for samples 006 and 008 for participants calculating % MRD using the following approach: number of estimated clonal cell equivalents per million total nucleated cells detected x100. Long horizontal black line = median. Short horizontal lines = range. Clone A = black, clone B = blue, clone C = red, clone D = green (see table 5).

Prospettive future della tecnologia NGS

Diversità intraclonale

- L'accumulo di SHM dovuto alla continua stimolazione antigenica porta alla diversificazione delle sequenze BcR rilevabili nel repertorio immunitario sequenziato.
- Lo studio del repertorio può dare informazioni utili in merito all'evoluzione della malattia e alle stimolazioni antigeniche in corso.
- Attualmente determinabile tramite programmi bioinformatici di creazione alberi filogenetici delle famiglie dei geni IgH (ClonalTREE, GCTree, GLaMST, IgTree, Mtree et al.)

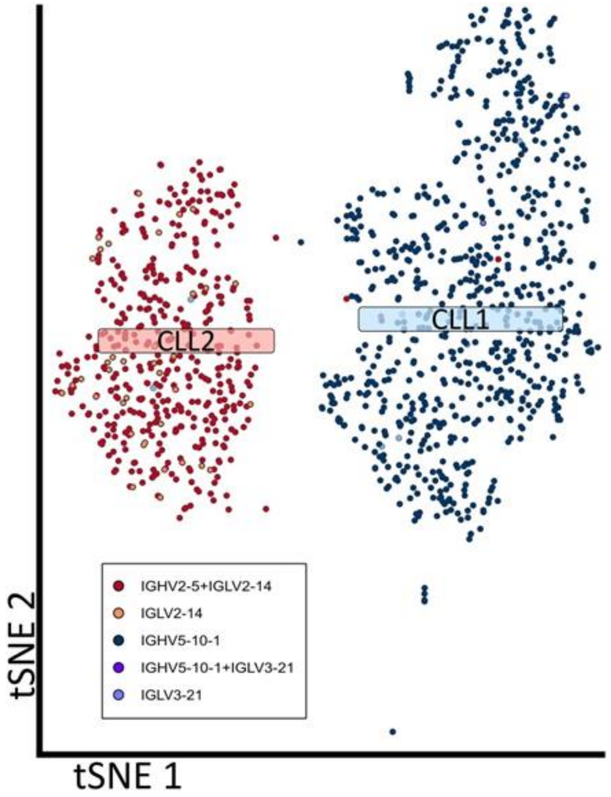


Clonotype definitions for immunogenetic studies: proposals from the EuroClonality NGS Working Group
June 2023
Leukemia 37(8):1-3

Prospettive future della tecnologia NGS

Single-cell analysis

- Permette di identificare l'evoluzione e l'etrogeneità clonale ed eventuali nuovi target terapeutici.
- Nella CLL permetterebbe una migliore distinzione tra pool di cellule biclonali o bialleliche (Dampmann et al.,2024)



" T-distributed stochastic neighbor embedding (tSNE) displaying IG gene heavy (HV) and light chain (LV) usage of the CLL cell populations as determined by single-cell RNA analysis. Cells using IGHV5/IGLV3-21 were assigned to cluster CLL1, cells using IGHV2/IGLV2-14 to cluster CLL2. Cells are colored in lighter shades (orange, lilac, light blue) if only one heavy or one light chain was found in these cells".

Dampmann, M., Kibler, A., von Tresckow, J., Reinhardt, H. C., Küppers, R., & Budeus, B. (2024). Single-cell analysis of a bi-clonal chronic lymphocytic leukemia reveals two clones with distinct gene expression pattern. *Leukemia & Lymphoma*, *66*(4), 744–752.

Conclusioni

- La valutazione MRD con tecnologia NGS rappresenta oggi uno strumento promettente per la valutazione della risposta al trattamento a durata fissa nella LLC.
- Si necessita standardizzazione in merito al LoD e agli endpoint di valutazione della MRD.
- In futuro la metodica si presenta come un valido strumento per espandere le conoscenze in merito alla cinetica del repertorio immunitario nella LLC.

Grazie per l'attenzione