09.

SCHOOL OF NGS

un evento promosso da

Nuove frontiere del Next Generation Sequencing nella diagnostica oncologica ed ematologica

04 NOVEMBRE 2022
HOTEL CONTINENTAL, NAPOLI

L'NGS nella CLL: il laboratorio incontra il clinico

Federico Chiurazzi, A.O.U. Federico II Napoli
Ida Pisano, Santa Errichiello,
Laboratorio di Ematologia Oncologica e Citogenomica -CEINGE Biotecnologie Avanzate

LEUCEMIA LINFATICA CRONICA

- La LLC è un disordine linfoproliferativo cronico che coinvolge i linfociti B CD5-positivi e che rientra tra le neoplasie a cellule B-mature della classificazione WHO (Muller-Hermelink HK et al, 2008). E' più frequente nei maschi che nelle femmine (1,5-2,0/1), ed ha un'incidenza nei paesi occidentali, riferita a 100.000 abitanti, compresa tra 2-6 casi/anno, mentre è rara in Giappone e nei paesi orientali, ove l'incidenza è <1 caso/100.000 abitanti (Redaelli A et al, 2004).

- L'età media alla diagnosi è attorno ai 70 anni, e l'incidenza aumenta da 1 caso/anno/100.000 abitanti nella fascia 40-50 anni a 20 casi nella fascia 70-80 anni. Oltre il 40% delle LLC è diagnosticata ad un'età >75 anni, mentre meno del 10% è diagnosticata prima dei 50 anni (Brenner H et al, 2008).

Chronic lymphocytic leukaemia: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up

B. Eichhorst, M. Hallek & M. Dreyling
On behalf of the ESMO Guidelines Working Group*
Annals of Oncology 21 (Supplement 5): v162–v164, 2010

The fitness and comorbidity of patients should be evaluated for the choice of the treatment. Improved survival has recently been demonstrated following first-line immunochemotherapy with FCR in physically fit patients with CLL [II, A].

Therefore in this patient group (physically active, no major health problems, normal renal function) FCR is now the standard first-line therapy.

In patients with relevant comorbidity chlorambucil [II, B] remains the standard therapy. Alternatives are dose-reduced purine analogue-based therapies (FC, PCR) [III, B] or bendamustine [II, B].

Patients showing a chromosomal defect del(17p) frequently do not respond to conventional chemotherapy with fludarabine or FC. Even after FCR therapy, progression free survival of these patients remains short. Therefore, these patients with sufficient fitness should be offered allogeneic stem cell transplantation within clinical trials [III, B].

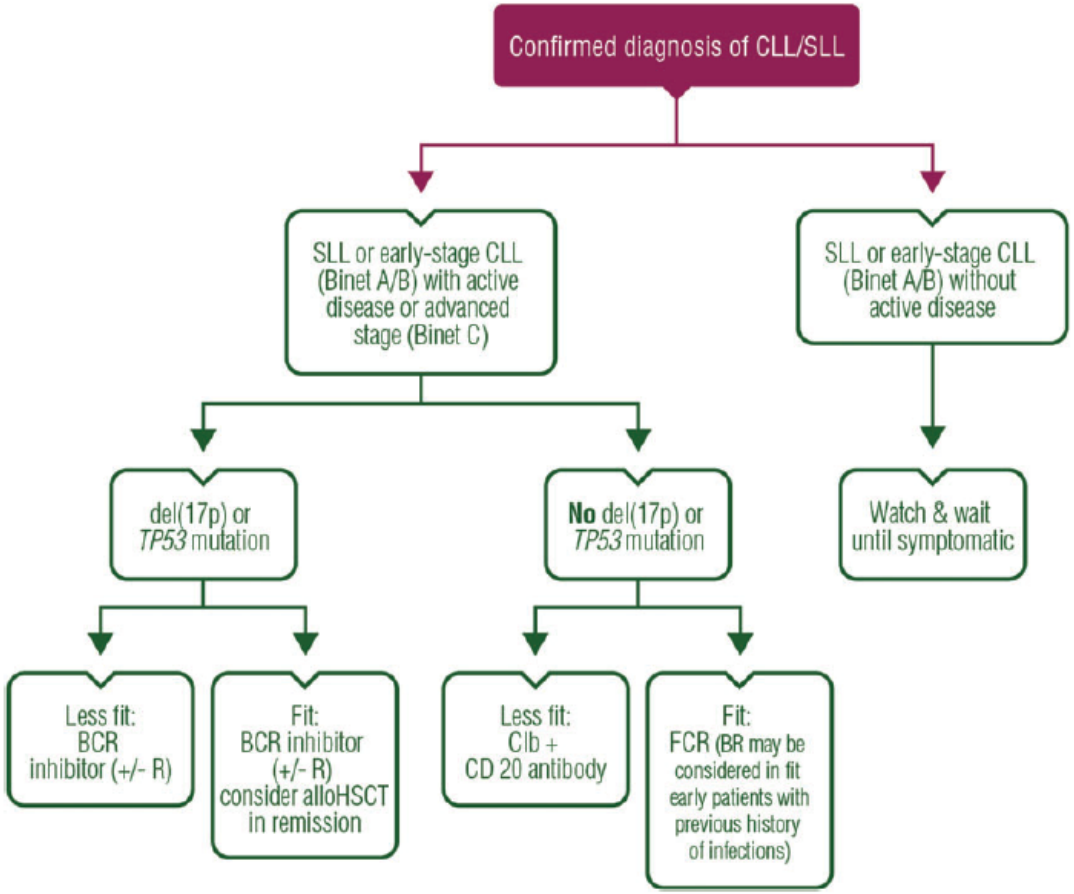


Figure 1. Front-line treatment. CLL, chronic lymphocytic leukaemia; SLL, small lymphocytic leukaemia; BCR, B-cell receptor; R, rituximab; alloHSCT, allogeneic haematopoietic stem cell transplantation; FCR, fludarabine, cyclophosphamide and rituximab; BR, bendamustine plus rituximab; Clb, chlorambucil.

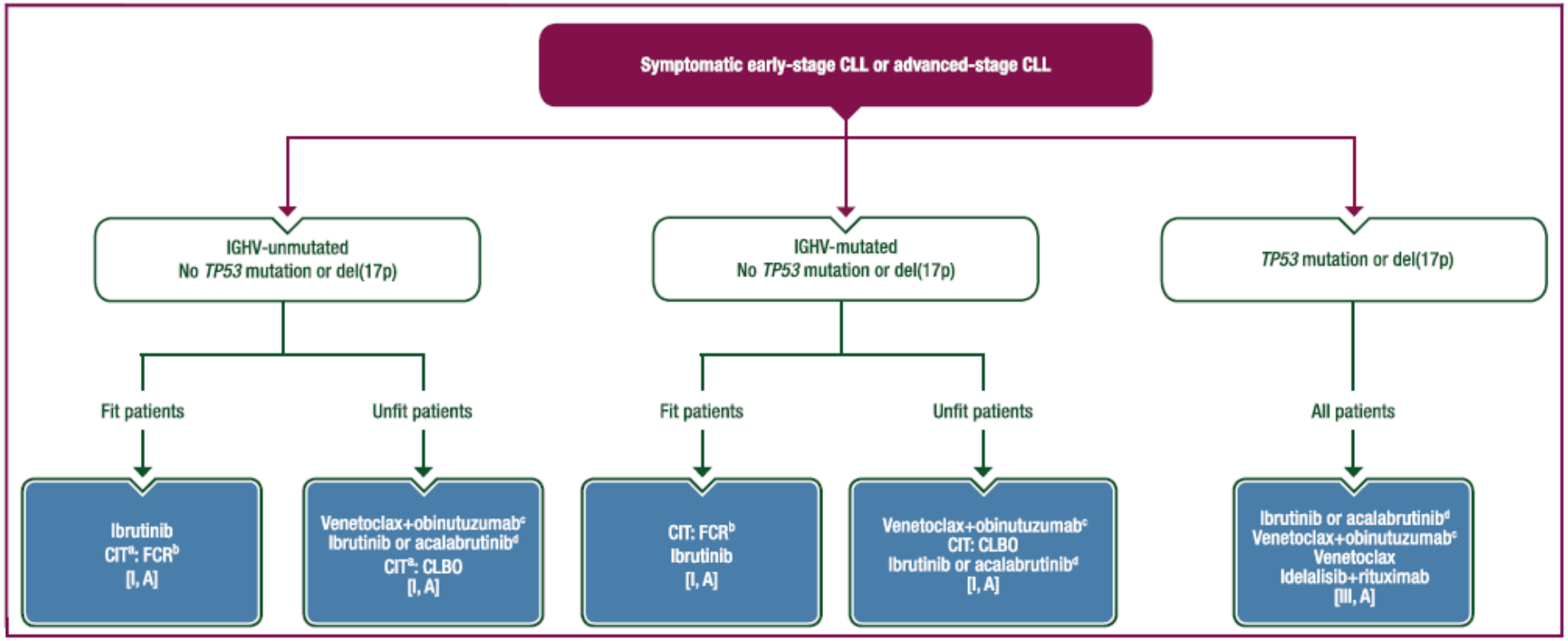


Figure 1. Front-line therapy.

The order of the recommended treatments for each subgroup is based on expert opinion considering time-limited as more valuable therapy, if there is equal evidence for two different treatment options.

BR, bendamustine plus rituximab; CIT, chemoimmunotherapy; CLBO, chlorambucil plus obinutuzumab; CLL, chronic lymphocytic leukaemia; FCR, fludarabine, cyclophosphamide and rituximab; IGHV, immunoglobulin heavy chain variable.

[a] CIT as alternative treatment, only if reasons against treatment with targeted therapies or non-availability.

[b] BR might be considered alternatively in patients above the age of 65 years.

[c] If available.

[d] If approved and available.

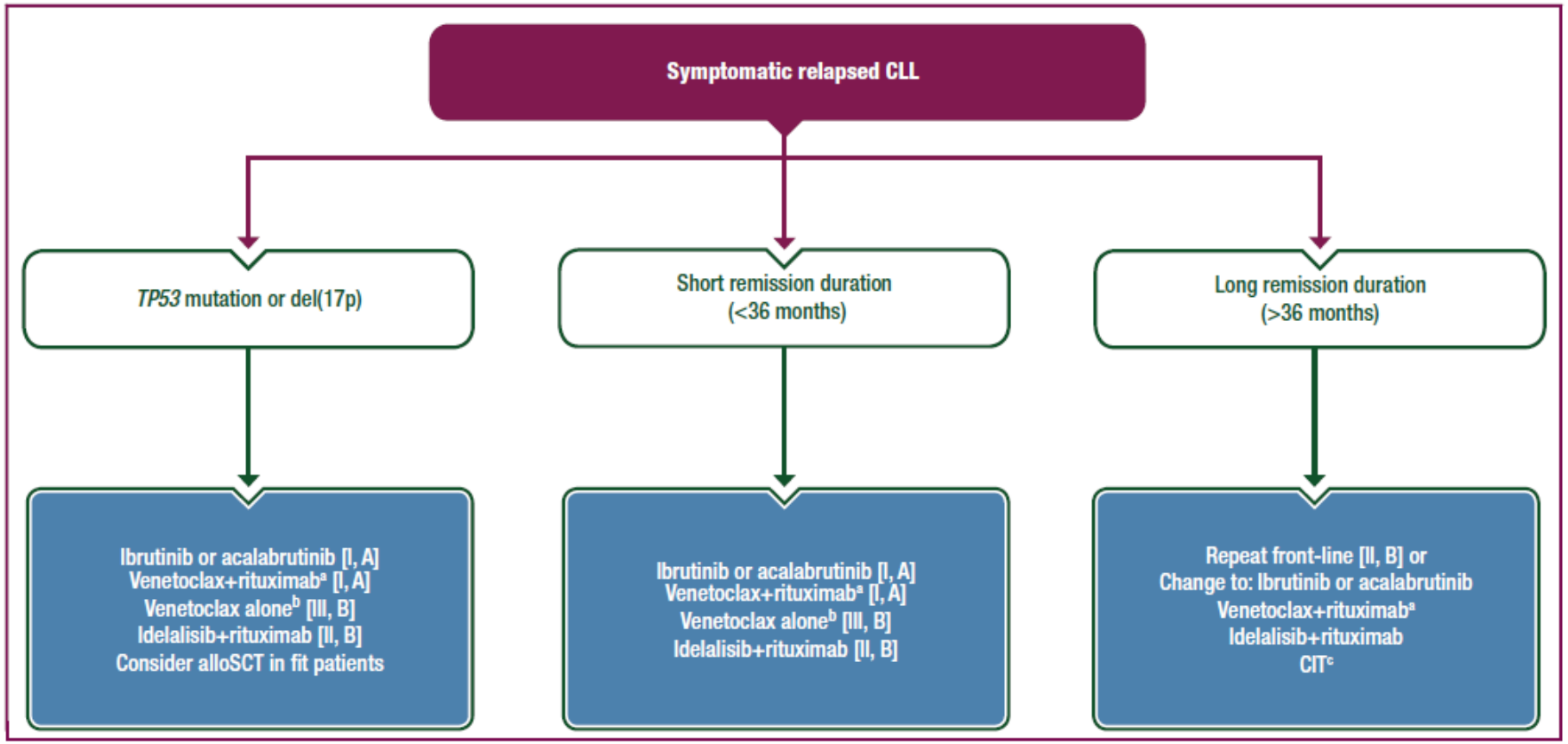


Figure 2. Relapse therapy.
alloSCT, allogeneic stem cell transplantation; BCRi, B-cell receptor inhibitor; CIT, chemoimmunotherapy; CLL, chronic lymphocytic leukaemia; FCR, fludarabine, cyclophosphamide and rituximab; R, rituximab.
[a] After prior ibrutinib, preferred therapy.
[b] After prior CIT and BCRi.
[c] Repetition of FCR not recommended.

Phase 2
R/R CLL*
(N=54)

→ Ibrutinib + Venetoclax

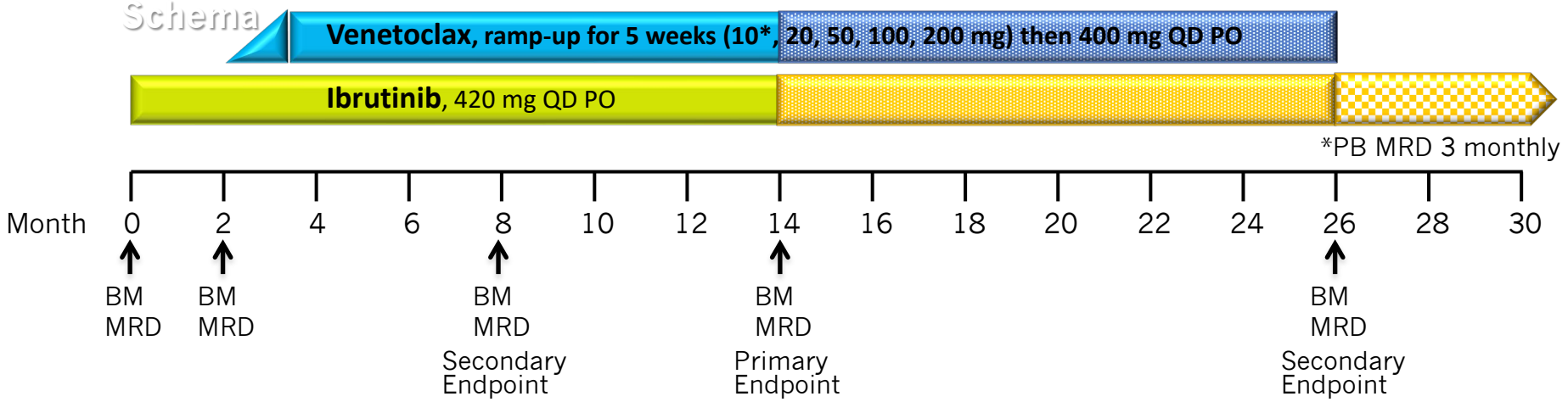
*No previous BTKi or venetoclax

Primary Endpoint:
MRD eradication (<0.01% CLL cells) in BM after 12 months ibrutinib + venetoclax

Secondary Endpoints: *ORR, PFS, OS, AEs*

Treatment

Schema



- Venetoclax and ibrutinib stopped at 14 months if 8-month BM is MRD(neg)
- Venetoclax and ibrutinib stopped at 26 months if 14-month BM is MRD(neg)
- Ibrutinib alone continues if 26-month BM is MRD(pos)

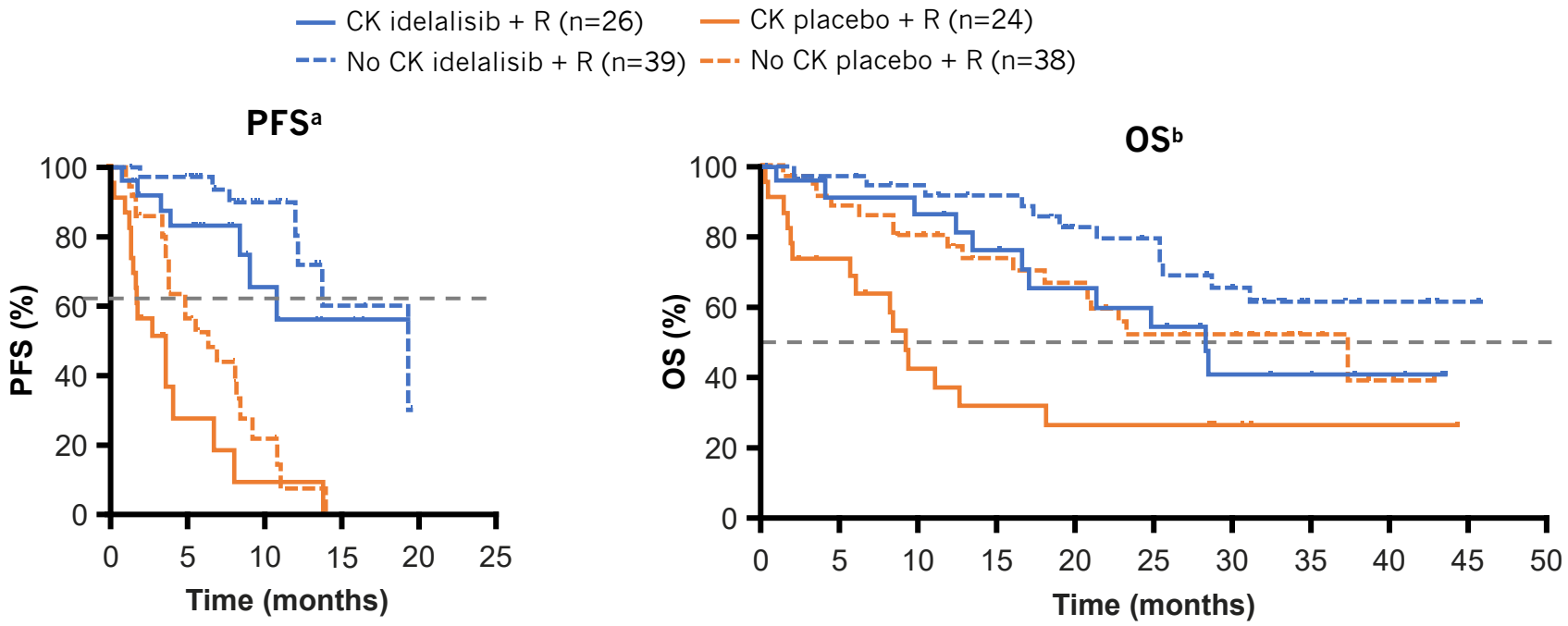
*First 3 patients only.

AE=Adverse Event. BM=Bone Marrow. CT=Computed Tomography. CLL=Chronic Lymphocytic Leukemia. MRD=Minimal Residual Disease. neg=negative. ORR=Overall Response Rate. OS=Overall Survival. PFS=Progression Free Survival. PO=By Mouth (Orally). pos=positive. QD=Daily. R/R=Relapsed/Refractory.

Hillmen P, et al. Oral #428. 59th ASH Annual Meeting and Exposition; December 8-12, 2017; Atlanta, GA.

CK does not significantly influence PFS and OS outcomes in idelalisib + rituximab-treated patients

Study 116/117: Idelalisib + R vs. placebo + R treatment in patients with R/R CLL



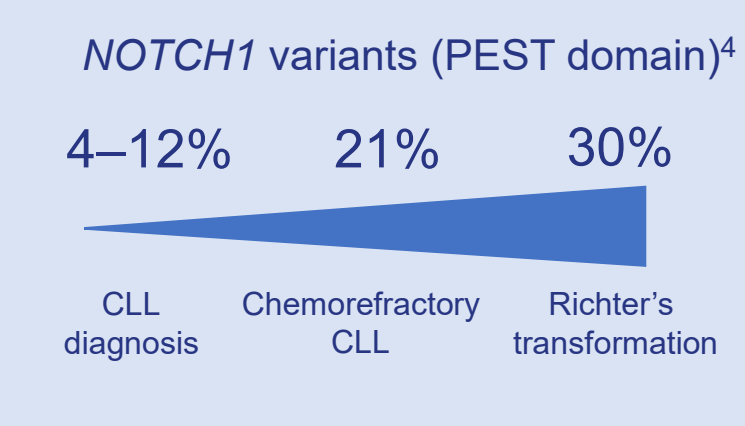
No significant adverse effect of CK on OS in idelalisib-treated patients
(HR 1.97; 95% CI=0.87, 4.48; p=0.10)

[a] Data cut-off: 15 October 2014; Study 116 only
[b] Data cut-off: 2 May 2016, Studies 116 + 117; median (range) follow-up: 25.0 months (0.3–45.6) for idelalisib + rituximab and 15.9 months (0.2–44.3) for placebo + rituximab

Kreuzer KA, *et al.* iwCLL 2017 (Abstract 410; poster).

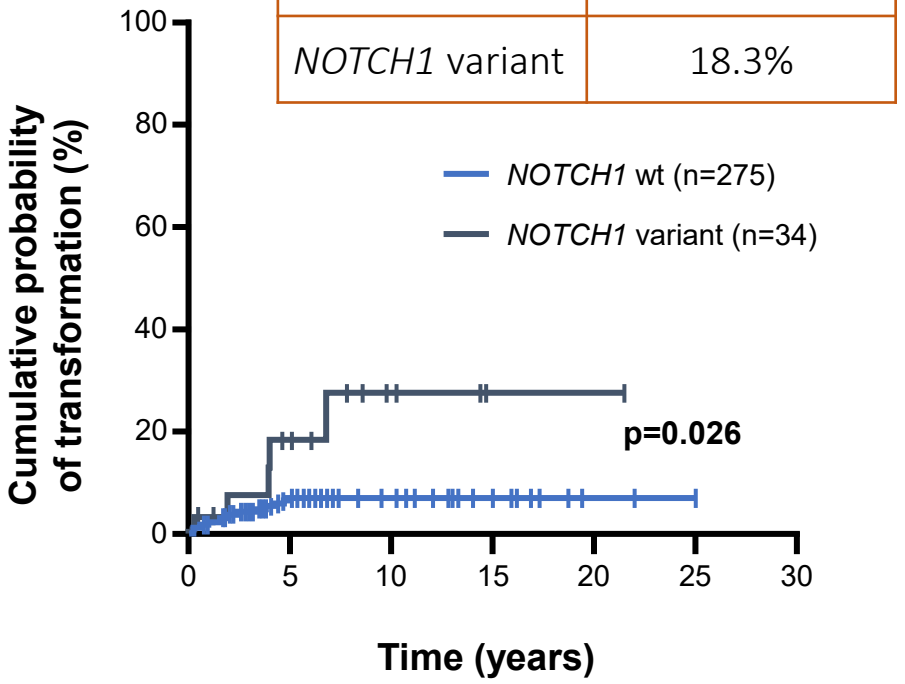
NOTCH1 variants are found in patients with CLL and are associated with Richter's transformation

- *NOTCH1* mutations are found in ~7% of CLL cases at diagnosis[1]
- *NOTCH1* mutations lead to oncogenic pathway activation[2] and downregulation of CD20[3]



Risk of Richter's transformation in patients with *NOTCH1* variants[5]

	5-year risk of RT
NOTCH1 wt	6.8%
NOTCH1 variant	18.3%

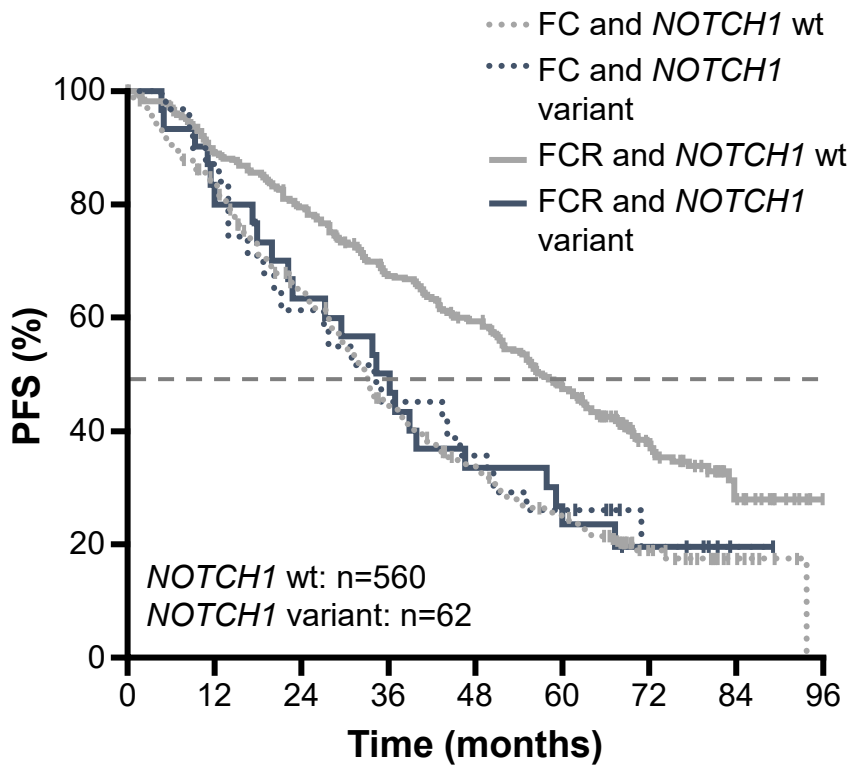


RT: Richter's transformation; wt: wild type

1. Willander K, *et al. BMC Cancer* 2013; 13:274. 2. Stilgenbauer S, *et al. Blood* 2014; 123:3247–3254. 3. Pozzo F, *et al. Leukemia* 2016; 30:182–189. 4. Sutton LA & Rosenquist R. *Haematologica* 2015; 100:7–16. 5. Rossi D, *et al. Blood* 2012; 119:521–529.

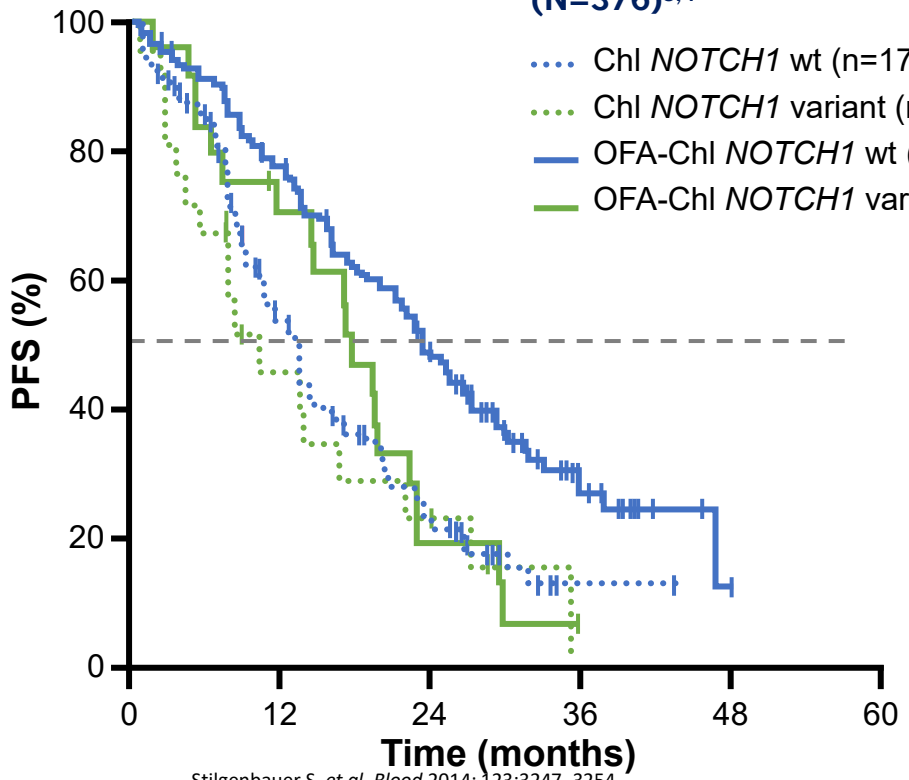
Patients with *NOTCH1* variants treated with FC or Chl do not benefit from addition of anti-CD20 therapy

CLL8: Phase III, randomised, open-label trial of FCR as a first-line treatment for patients with CLL (N=817)[1,2]



Rituximab failed to improve PFS in patients with *NOTCH1* variants

COMPLEMENT-1: Phase III, randomised, open-label trial of Chl vs. Chl-OFA as first-line treatment in patients with CLL (N=376)[3,4]



1. Stilgenbauer S, *et al. Blood* 2014; 123:3247–3254.
2. https://www.clinicaltrials.gov/ct2/show/NCT00281918 (accessed Feb 2018).
3. Tausch E, *et al. Blood* 2013;122:527.

- Chl: chlorambucil; OFA: ofatumumab

BTK^{C481S}-Mediated Resistance to Ibrutinib in Chronic Lymphocytic Leukemia

Jennifer A. Woyach, Amy S. Ruppert, Daphne Guinn, Amy Lehman, James S. Blachly, Arletta Lozanski, Nyla A. Heerema, Weiqiang Zhao, Joshua Coleman, Daniel Jones, Lynne Abruzzo, Amber Gordon, Rose Mantel, Lisa L. Smith, Samantha McWhorter, Melanie Davis, Tzyy-Jye Doong, Fan Ny, Margaret Lucas, Weihong Chase, Jeffrey A. Jones, Joseph M. Flynn, Kami Maddocks, Kerry Rogers, Samantha Jaglowski, Leslie A. Andritsos, Farrukh T. Awan, Kristie A. Blum, Michael R. Grever, Gerard Lozanski, Amy J. Johnson, and John C. Byrd

www.oncotarget.com Oncotarget, 2018, Vol. 9, (No. 76), pp: 34357-34378

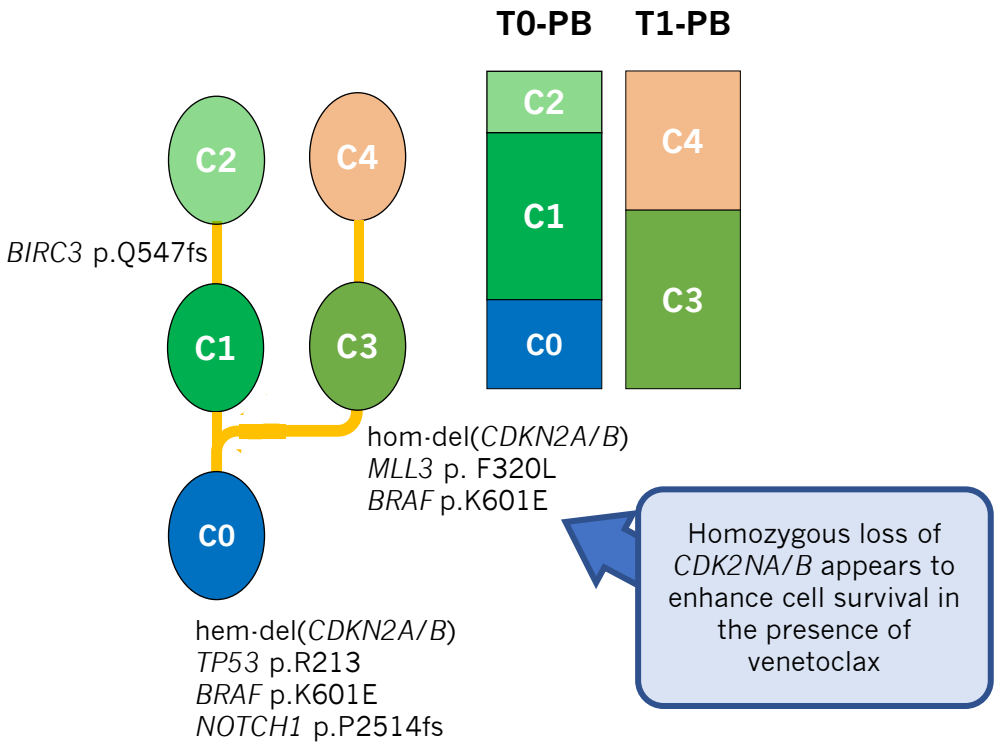
Research Paper

Functional characterization of phospholipase C-γ_2 mutant protein causing both somatic ibrutinib resistance and a germline monogenic autoinflammatory disorder

Claudia Walliser[1], Martin Wist[1], Elisabeth Hermkes[1], Yuan Zhou[1], Anja Schade[1], Jennifer Haas[1], Julia Deinzer[1], Laurent Désiré[2], Shawn S.C. Li[3], Stephan Stilgenbauer[4], Joshua D. Milner[5] and Peter Gierschik[1]

CDKN2A/B and BTG1 mutations may play a role in venetoclax resistance

Clonal evolution profiling in a *TP53*-deficient CLL patient with stable disease[1]



BTG1 loss of function is associated with many solid and haematological malignancies[2]

- *BTG1* is an important molecular mechanism contributing to the cytotoxic effects of BCL-2-mediated treatments
- Loss of function mutations in *BTG1* may abrogate the function of BCL-2 inhibitors such as venetoclax

- Recurrent genomic changes that evolved during venetoclax treatment were homozygous deletions affecting *CDKN2A/B* ($p16^{Ink4a}$/$p14^{Arf}$) in 3 patients and *BTG1* missense mutations in 2 cases (N=8)[1]

1. Herling CD, *et al. Nat Commun* 2018; 9:727.
2. Nahta R, *et al. Mol Cancer Ther* 2006; 5:1593–1601.

- Donna caucasica, anni 57
- APR: 2012 osteonecrosi della testa del femore, in tale occasione primo riscontro di leucocitosi con inversione della formula leucocitaria.
- Negli anni progressivo lento incremento della linfocitosi
- 15/10/2017: primo accesso al nostro ambulatorio: Hb 13,4 gr/dl MCV 90 GB 77.710/mmc N 8% L 88%
- 18/12/2018: progressione di malattia; inizia immunochemioterapia secondo lo schema FCR (Fludarabina, Ciclofosfamide, Rituximab). Pratica 4 cicli a cadenza mensile che interrompe per citopenia; al termine remissione completa citofluorimetrica.
- 11/07/2022: ripresa di malattia

Risultati dell'indagine molecolare:

Valutazione stato mutazionale riarrangiamento Ig

- IG-unmutated: prognosi sfavorevole

(Rosenquist R, et al Immunoglobulin gene sequence analysis in chronic lymphocytic leukemia Updated ERIC Recommendations Leukemia 2017)

Valutazione stato mutazionale TP53

- TP53 mutated

Risultati dell'indagine FISH:

- Delezione del gene ATM nel 6,7% dei nuclei analizzati
- Delezione biallelica del gene DLEU1 nel 39% dei nuclei analizzati

11/07/2022: inizia terapia con Ibrutinib cp 420 mg/die

Emocromo 12/10/2022: Hb 12,0 gr/dl GB 246.200/mmc Pt 203.000/mmc

Possibili opzioni terapeutiche

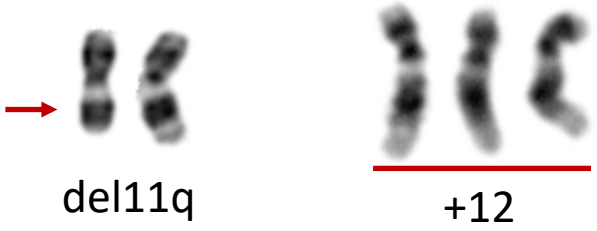
- ***Prima linea***:
  - Ibrutinib (terapia continuativa)
  - Acalabrutinib (terapia continuativa)
  - Venetoclax + Obinutuzumab (terapia a termine)
  - Immunochemioterapia (terapia a termine)
  - Chlorambucil + Obinutuzumab (terapia a termine)
  - Idelalisib + Rituximab (terapia continuativa)

Possibili opzioni terapeutiche

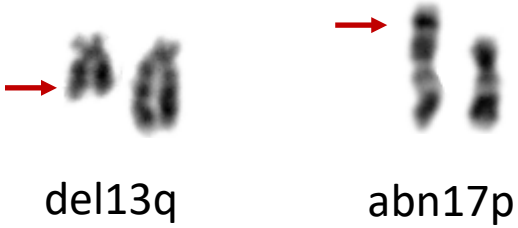
- ***Seconda linea***:
  - Ibrutinib (terapia continuativa)
  - Acalabrutinib (terapia continuativa)
  - Venetoclax (terapia continuativa)
  - Venetoclax + Rituximab (terapia a termine)
  - Idelalisib + Rituximab (terapia continuativa)
  - Immunochemioterapia (terapia a termine)
  - Allotrapianto

ANOMALIE CROMOSOMICHE RICORRENTI NELLA CLL

CARIOTIPO (CBA)

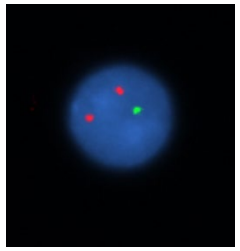


del11q +12

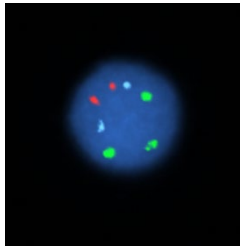


del13q abn17p

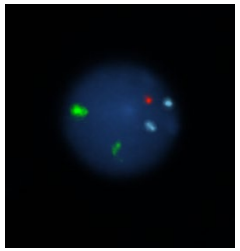
FLUORESCENT IN SITU HYBRIDIZATION (FISH)



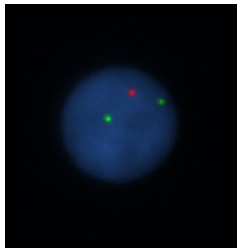
- TP53 (17p13) x2
- ATM (11q22.3) x1



- CEN12 x3
- DLEU1 (13q14) x2
- LAMP (13q34) x2



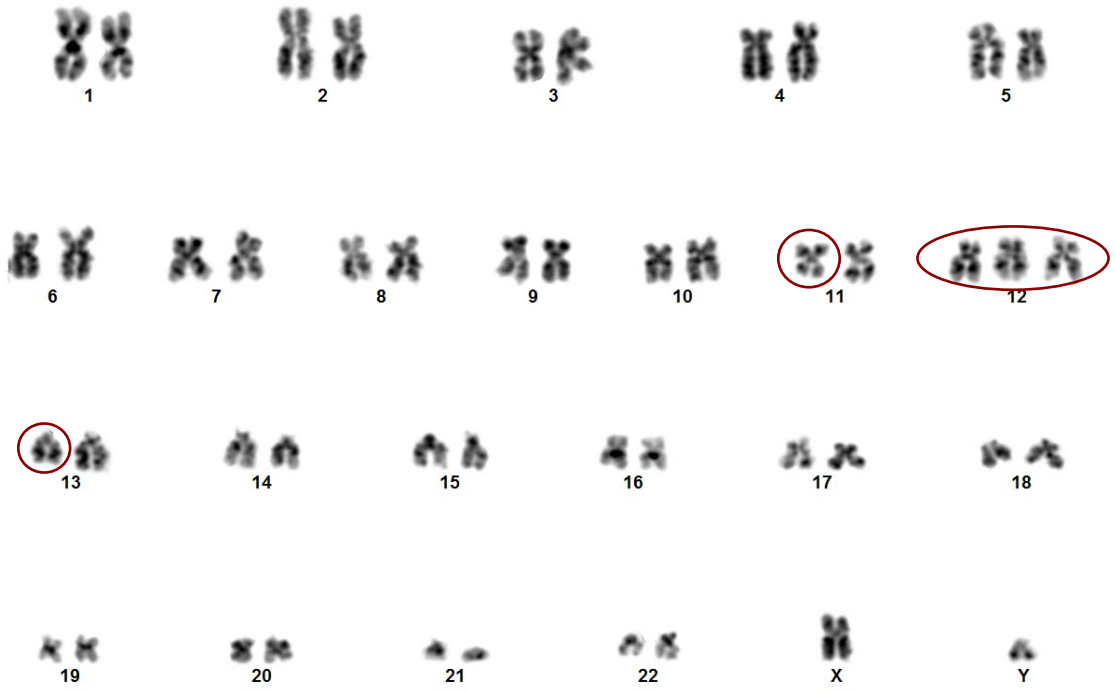
- CEN12 x2
- DLEU1 (13q14) x1
- LAMP (13q34) x2



- TP53 (17p13) x1
- ATM (11q22.3) x2

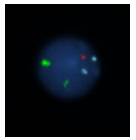
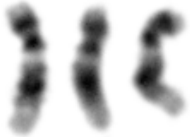
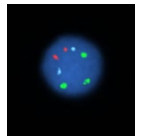
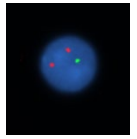
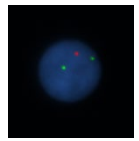
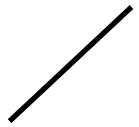
CARIOTIPO COMPLESSO (CK) NELLA CLL

presenza di un clone caratterizzato da tre o più anomalie cromosomiche



47,XY,del(11)(q14q24),+12,del(13)(q12q21)

INTERPRETAZIONE DEI DATI CITOGENETICI NEL CONTESTO CLINICO DELLA CLL

ABN	CBA	FISH	PI
del13q			+
+12			+/-
del11q			-
abn/del17p			-
CK	≥3 abn		-

CARATTERISTICHE DEL CAMPIONE

TIPOLOGIA

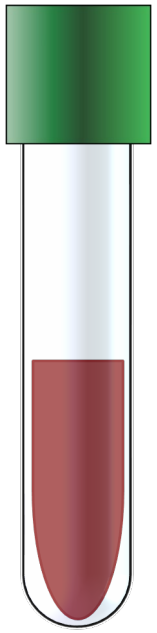
Sangue periferico

ANTICOAGULANTE

Eparina

TEMPERATURA

Non refrigerare



TEMPO

Entro 24h

VOLUME

0,5-5ml

CELLULARITA’

$1\sim2x10^7$

CARATTERISTICHE DELLE COLTURE

HemaSphere

EHA EUROPEAN HEMATOLOGY ASSOCIATION

Guideline Article - Expert opinion
Open Access

Cytogenetics in Chronic Lymphocytic Leukemia: ERIC Perspectives and Recommendations

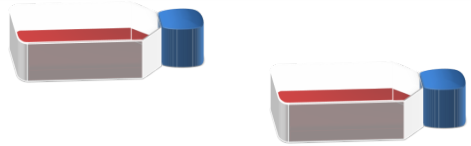
Panagiotis Baliakas[1,2], Blanca Espinet[3,4], Clemens Mellink[5], Marie Jarosova[6,7], Anastasia Athanasiadou[8], Paolo Ghia[9], Arnon P. Kater[10], David Oscier[11], Claudia Haferlach[12], Kostas Stamatopoulos[13,14], on behalf of ERIC, the European Research Initiative on CLL

Correspondence: Blanca Espinet (bespinet@parcdesalutmar.cat); Panagiotis Baliakas (panagiotis.baliakas@igp.uu.se).

ABSTRACT

Mounting evidence underscores the clinical value of cytogenetic analysis in chronic lymphocytic leukemia (CLL), particularly as it allows the identification of complex karyotype, that has recently emerged as a prognostic and potentially predictive biomarker. That said, explicit recommendations regarding the methodology and clinical interpretation of either chromosome banding analysis (CBA) or chromosome microarray analysis (CMA) are still lacking. We herein present the consensus of the Cytogenetic Steering Scientific Committee of ERIC, the European Research Initiative on CLL, regarding methodological issues as well as clinical interpretation of CBA/CMA and discuss their relevance in CLL. ERIC considers CBA standardized and feasible for CLL on the condition that standards are met, extending from the use of novel mitogens to the accurate interpretation of the findings. On the other hand, CMA, is also standardized, however, robust data on its clinical utility are still scarce. In conclusion, cytogenetic analysis is not yet mature enough to guide treatment choices in CLL. That notwithstanding, ERIC encourages the wide application of CBA, and potentially also CMA, in clinical trials in order to obtain robust evidence regarding the predictive value of specific cytogenetic profiles towards refining risk stratification and improving the management of patients with CLL.

NUMERO



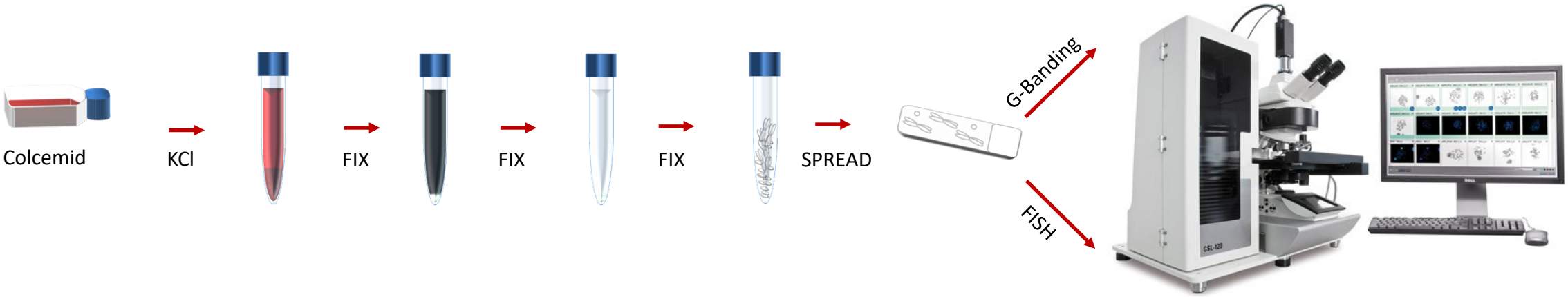
DENSITA’

1~2x10^{6}cells/ml

DURATA

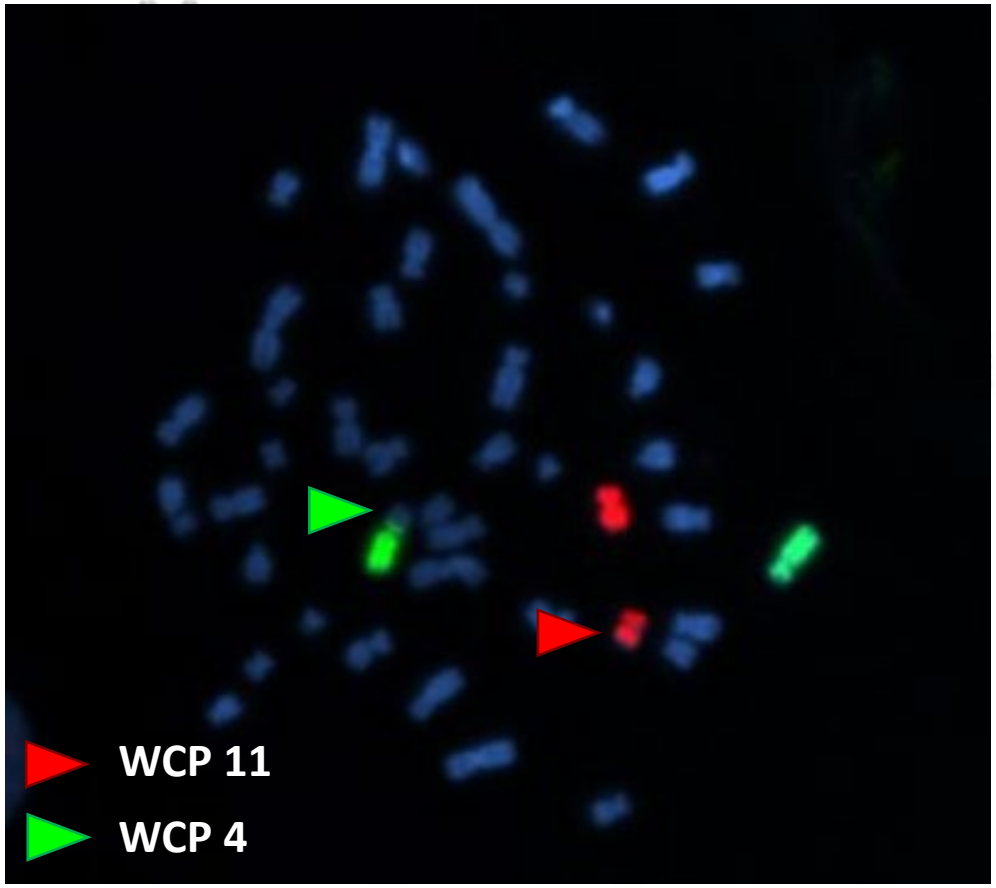
72h + mitogeni

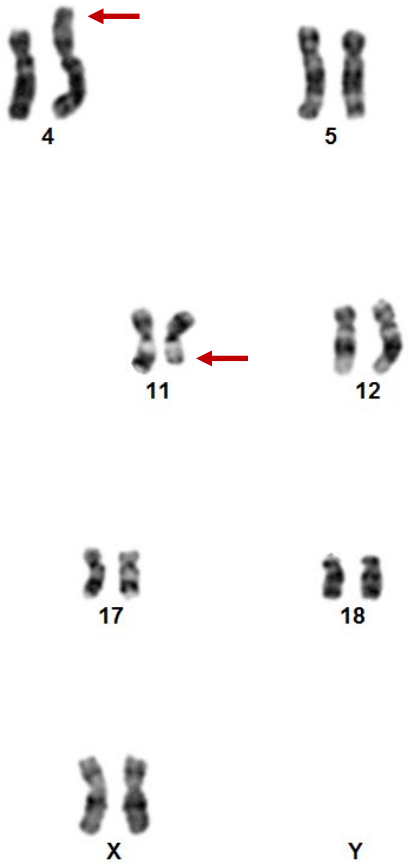
WORKFLOW CBA/FISH

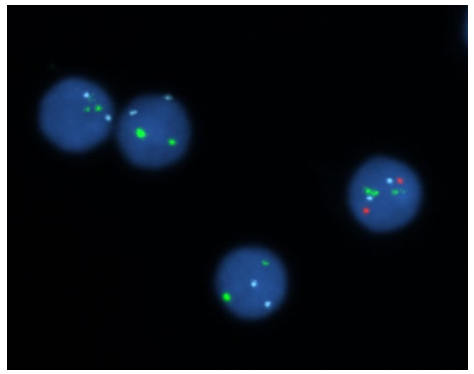


20 metafasi
~ 300 nuclei in interfase

CASE REPORT



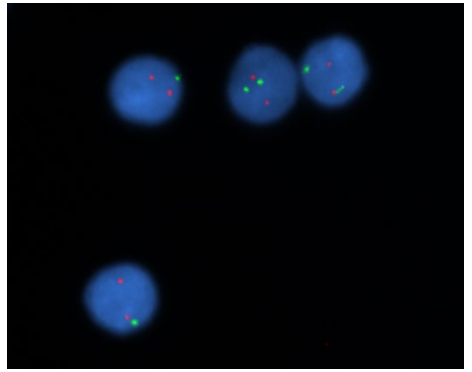




CEN12 x2

DLEU1 (13q14) x0

LAMP (13q34) x2



TP53 (17p13) x2

ATM (11q22.3) x1

46,XX,add(4)(p16),del(11)(q21q24)[4]/46,XX[16].nuc ish(ATMx1,TP53x2)[26/389],(D12Z3x2,DLEUx0,LAMP1x2)[144/368]

CARIOTIPO (CBA)

VANTAGGI

Analisi genomica completa
Analisi su singola cellula
Identificazione di anomalie numeriche e strutturali
Sensibilità del 10-15%
Identificazione del cariotipo complesso
Identificazione di cloni multipli/evoluzione clonale

LIMITI

Lenta e laboriosa
Morfologia dei cromosomi inadeguata
Risoluzione 10-20 Mb
Necessità di ottenere metafasi

FLUORESCENT IN SITU HYBRIDIZATION (FISH)

VANTAGGI

Rapida
Risoluzione 150-900kb
Sensibilità del 3-5%
Applicabile in interfase

LIMITI

Analisi mirata
Necessità di usare pannelli per l'identificazione di più anomalie
Complementare al CBA

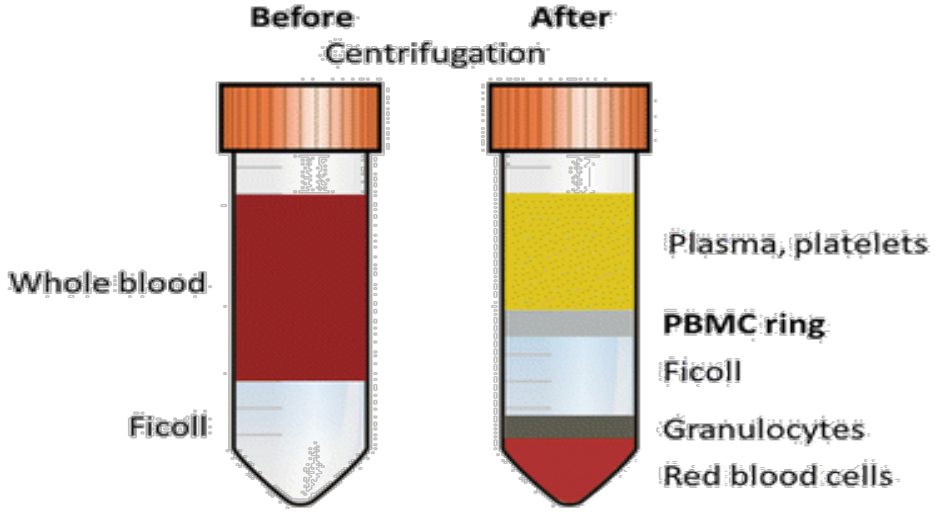
CARATTERISTICHE DEL CAMPIONE

TIPOLOGIA

Sangue periferico

ANTICOAGULANTE

EDTA



TEMPO

Entro 24h

VOLUME

0,5-5ml

1 - Technical considerations for the determination of IGHV somatic hypermutation status in clonotypic IGHV-IGHD-IGHJ gene rearrangements in CLL.
Abbreviations: EDTA: ethylenediaminetetraacetic acid; CPT: citrate/pyridoxal 5'-phosphate/Tris; gDNA: genomic DNA; cDNA: complementary DNA; PAGE: polyacrylamide gel electrophoresis.
**Agathangelidis et al. Blood 2012 (ref. 7)*

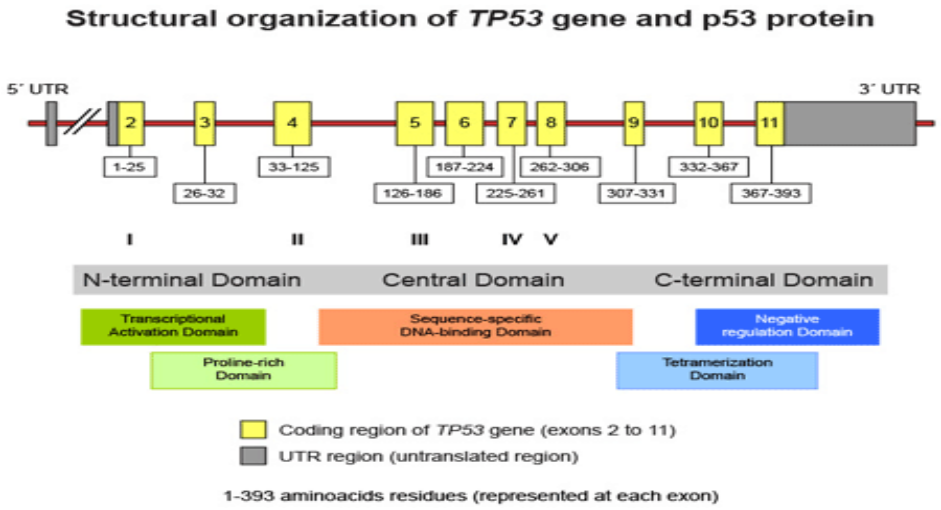
Item	Recommendations	Remarks
Material		
Anticoagulants	EDTA (or CPT)	
Cells / Tissue	Blood, bone marrow, tissue biopsy	purification of B cells usually not necessary unless low fraction of leukemic cells
Nucleic acid	gDNA or cDNA	cDNA useful when mutations on the IGHJ gene impair amplification

Estrazione DNA

Langerak AW et al , Leukemia 2011

Diagnosi di Leucemia Linfatica Cronica: Il laboratorio di Biologia Molecolare

Valutazione stato mutazionale TP53



Analisi Ipermutazione Somatica:IGHV

Gene IGH, cromosoma 14

Non si fa diagnosi, ma solo PROGNOSI con questi marcatori. Quando viene richiesta l'Ipermutazione somatica, siamo in presenza di una patologia già diagnosticata.

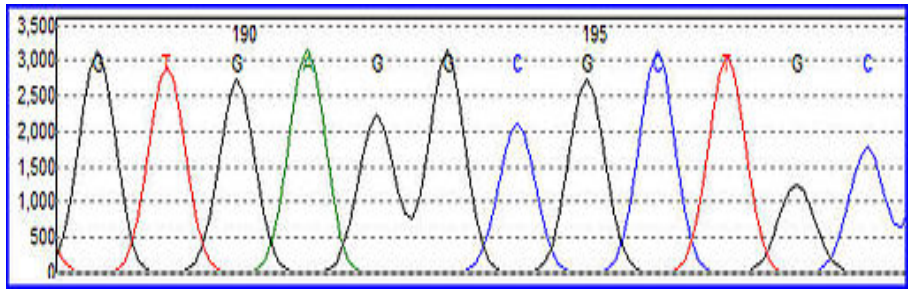
Metodiche utilizzate

Metodica Classica:

PCR e sequenziamento Sanger

Relativamente poco costosa
Flusso non semplice
Dati spesso soggettivi o difficili da interpretare.

Reazioni multiple simultanee per singolo gene.
Uso per analisi di regioni geniche con specifici primer.



Metodica nuova Generazione

Next Generation sequencing

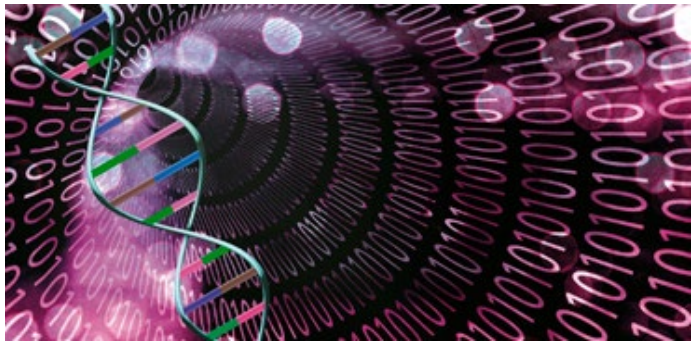
Può essere più costosa

Meno standardizzata (se fatta con metodiche home made)

Restituisce dati oggettivi e ben interpretabili.

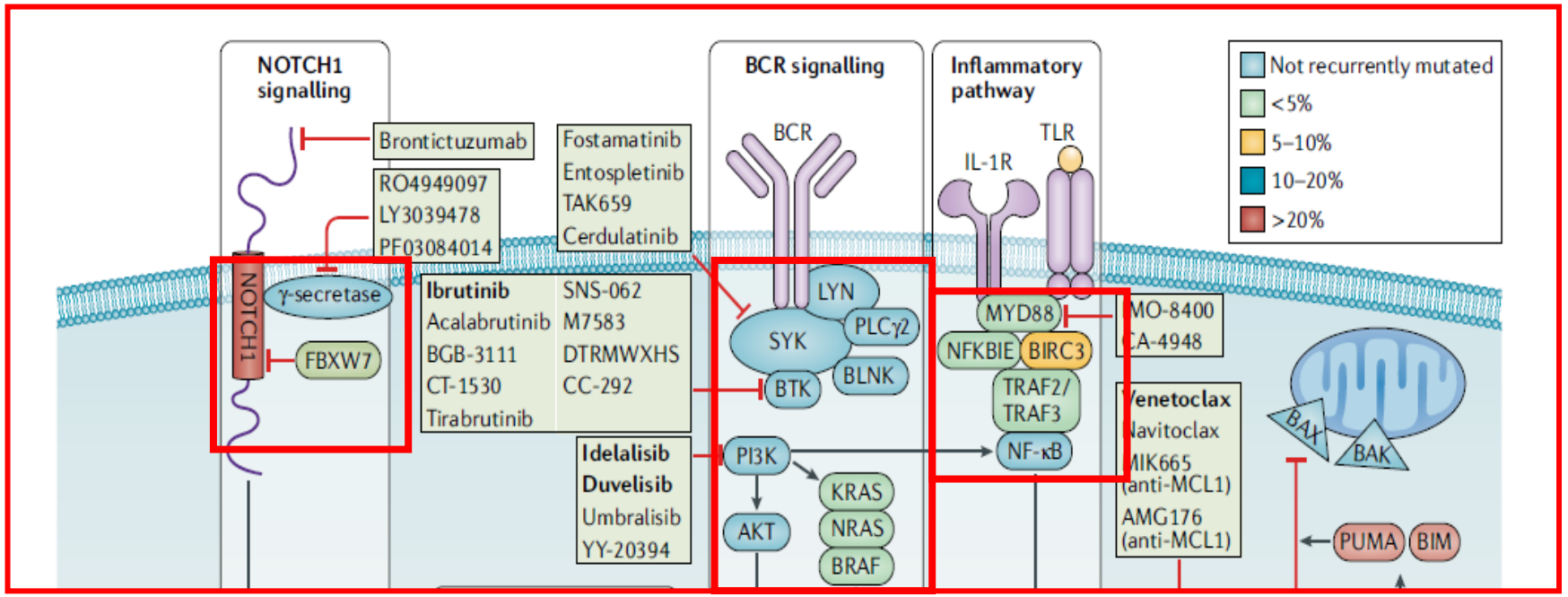
Sequenziamento massivo in parallelo di frammenti di larghe regioni genomiche

Necessita di analisi Bioinformatiche.



Chronic Lymphocytic Leukemia – Recurrent genetic lesions

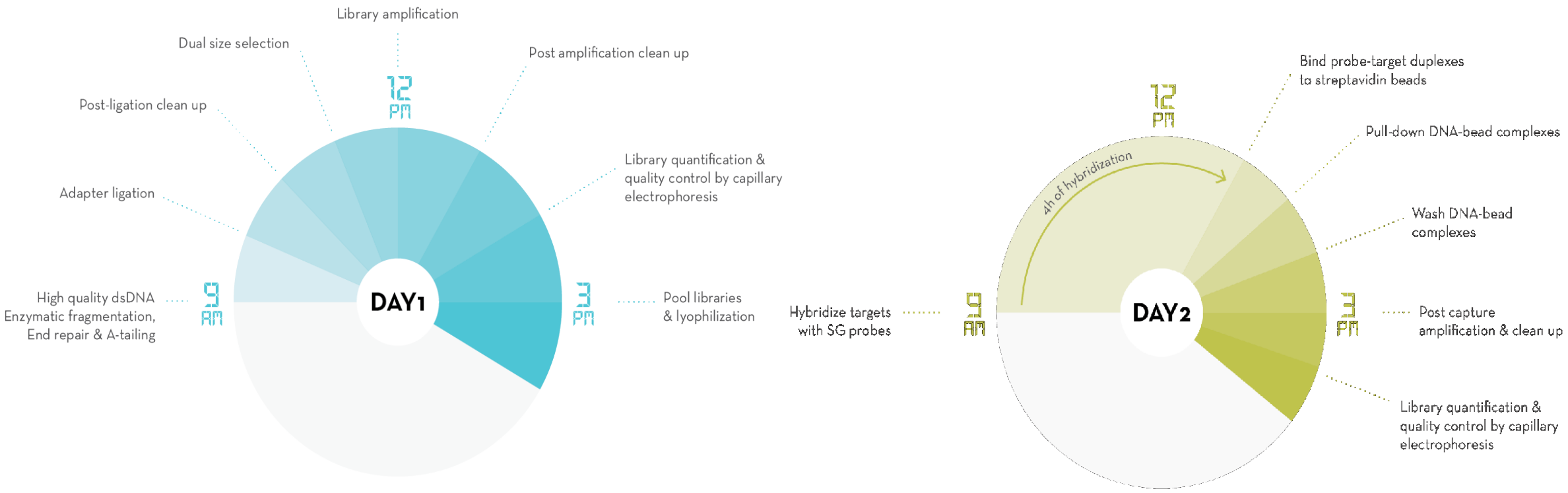
- Geni coinvolti nella regolazione del Ciclo Cellulare e Apoptosi (DLEU1, BCL2)
- Geni coinvolti nel signalling di NOTCH1(NOTCH1, ICN1, FBXW7)
- Geni coinvolti nella risposta al danno su DNA (TP53, ATM, POT1)
- Geni coinvolti nel RNA Processing (SF3B1, XPO1)
- Geni coinvolti nel Pathway Infiammatorio(MYD88, NFKBIE, BIRC3)
- Geni coinvolti nel Signalling di BCR(KRAS, BRAF, AKT, BTK, PLCγ2)



Bosh F. et al Nature Nov. 2019

Custom Chronic Lymphocytic Leukemia - NGS Workflow

Hybrid-Capture Library Preparation. . . Manual Workflow



21 GENES: AFT1, ATM, BIRC3, BTK, CDK4, CXCR4*, CUL4A*, DLEU1, EGR2, FBXW7, KLF5, KRAS, MYD88, NFKBIE, NOTCH1, PLCG2, POT1, PROZ, SF3B1, TP53, XPO1

Custom Chronic Lymphocytic Leukemia - NGS Workflow

Hybrid-Capture Library Preparation . . .Automated Workflow

Custom Chronic Lymphocytic Leukemia - NGS Workflow

Sample Mutiplexing

	Flow Cell/Sequencing Kit	Recommended samples per run (for 1000x coverage depth)
 MiSeq®*	v3 (2x300bp)	24
	v2 (2x250bp)	16

Analisi Stato Mutazionale

Analisi Copy Number Variation (CNV)

Custom Chronic Lymphocytic Leukemia - Performance

Overall Performance after Internal Validation (32 samples)

Institute	CEINGE_Lab Izzo
Test	CCLL_A_v1
Platform	Illumina MiSeq
Sensitivity	100% [88.88%]
Specificity	99.99% [99.97%]
Accuracy	99.99% [99.97%]
Precision	93.54% [73.47%]

Institute	CEINGE_Lab Izzo
Test	CCLL_A_v1
Platform	Illumina MiSeq
Observed Sensitivity	100% [90.9%]

Institute	CEINGE_Lab Izzo
Test	CCLL_A_v1
Platform	Illumina MiSeq
Repeatability	99.98% [99.97%]
Reproducibility	99.97% [99.94%]

CASE REPORT

L'analisi delle sequenze degli esoni 2-11 del gene TP53 ha evidenziato la presenza di una mutazione missenso in esone 7
p.Tyr234Cys (c.707A>G) 5%

L'analisi delle sequenze del gene SF3B1 ha evidenziato la presenza di una mutazione missenso p.Lys700Glu al 44,5%.

Inoltre, sono presenti CNV di delezione sul cromosoma 13 a livello del gene DLEU1

Bosh F. et al Nature Nov. 2019

Table 1 | **Prognostic influence of CLL genetic lesions at diagnosis**

Genetic lesion	Study	Number of patients	Frequency (%)	TTFT (months)	5-Year OS (%)	Additional features
Isolated del13q14	Döhner et al.[50]	325; 52% with early stage CLL	55[a]	~90[b]	>90[b]	• Enriched in early stages • Enriched in *IGHV*-M
NOTCH1 mutation	Rossi et al.[63]	637; 75% with early stage CLL	11	NA	50[b]	• Enriched in advanced stages • Associated with trisomy 12 • Enriched in *IGHV*-UM
	Weissmann et al.[116]	643; 0% with early stage CLL	12	42[b]	55[b]	
	Baliakas et al.[64]	2697; 78% with early stage CLL	7	40[b]	NA	
	Nadeu et al.[82]	396; 84% with early stage CLL	22	<12[b]	~70[b,c]	
	Jeromin et al.[125]	908; 0% with early stage CLL	12.3	40[b]	80[b]	
	Mansouri et al.[185]	359; 77% with early stage CLL	5	5[b]	~50[b]	
FBXW7 mutation	Jeromin et al.[125]	908; 0% with early stage CLL	2.5	NA	NA	NA
del17p or *TP53* disruption	Döhner et al.[50]	325; 52% with early stage CLL	7[a]	<12[b]	40[b]	• Enriched in advanced stages • Enriched in *IGHV*-UM
	Zenz et al.[71]	125; 65% with early stage CLL	10[d]	NA	40	
	Puente et al.[40]	452; 68% with early stage CLL	5	<12[b]	NA	
	Rossi et al.[15]	637; 75% with early stage CLL	8.5	27[b]	50[b]	
	Baliakas et al.[64]	1691; 78% with early stage CLL	9	36	35[b]	
del11q or *ATM* disruption	Döhner et al.[50]	325; 52% with early stage CLL	18[a]	13[b]	68[b]	• Enriched in advanced stages • Associated with bulky disease • Associated with young age • Enriched in *IGHV*-UM
	Nadeu et al.[82]	398; 84% with early stage CLL	11	<12[b]	NS	
	Wierda et al.[186]	930; 87% with early stage CLL	9[a]	<24[b]	NA	
POT1 mutation	Ramsay et al.[83]	127; 82% with early stage CLL	3	NA	NA	Enriched in *IGHV*-UM
SF3B1 disruption	Wang et al.[37]	91[e]; 79% with early stage CLL	15	<12[b]	NA	• High male:female ratio • Associated with high white blood cell count • Enriched in advanced stages • Associated with del11q • Enriched in *IGHV*-UM
	Rossi et al.[63]	637; 75% with early stage CLL	6.8	<12[b]	2.5 years[b,f]	
	Baliakas et al.[64]	1715; 78% with early stage CLL	8	36[b]	NA	
	Jeromin et al.[125]	1160; 0% with early stage CLL	9	40[b]	64[b]	
	Mansouri et al.[185]	360; 77% with early stage CLL	3.6	3[b]	~55[b]	
BIRC3 disruption	Rossi et al.[187]	637; 75% with early stage CLL	6.2	NA	3.1 years[b,f]	• Enriched in advanced stages • Associated with del11q • Enriched in *IGHV*-UM
	Baliakas et al.[64]	919; 78% with early stage CLL	2.5	32[b]	NA	
	Nadeu et al.[82]	399; 84% with early stage CLL	4	NS	NA	
MYD88 mutation	Martínez-Trillos et al.[87,88]	587; 81% with early stage CLL	4	NS	100[b]	• Increased prevalence in males • Associated with young age[g] • Enriched in advanced stages • Enriched in *IGHV*-M
	Baliakas et al.[188]	1080; 78% with early stage CLL	2.2	~79[b]	NA	
	Rossi et al.[63]	637; 75% with early stage CLL	4.1	NA	96[b]	

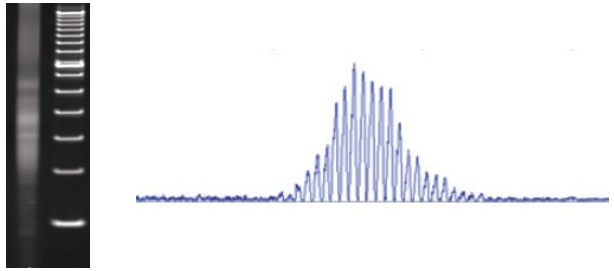
CLL, chronic lymphocytic leukaemia; NA, not available; NS, not significant; OS, overall survival; TTFT, time to first treatment. [a]Fluorescence in situ hybridization; [b]Statistical prognostic significance of the lesion; [c]Not statistically significant at the subclonal level; [d]Sanger sequencing; [e]Two-thirds untreated; [f]Median survival; [g]Controversial data.

IGHV: Ipermutazione Somatica

L'analisi dell'Ipermutazione Somatica consiste nell'andare ad individuare la presenza di cloni a livello dei riarrangiamenti delle immunoglobuline per poi valutarne lo stato mutazionale.

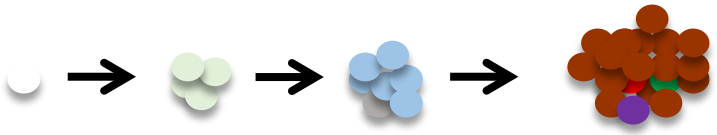
«Le regioni variabili, costituite da un dominio Ig per entrambe le catene contengono le cosiddette regioni ipervariabili tratte della catena polipeptidica dove si riscontrano le maggiori variabilità amminoacidiche che donano a ciascun anticorpo la specificità unica verso un antigene»

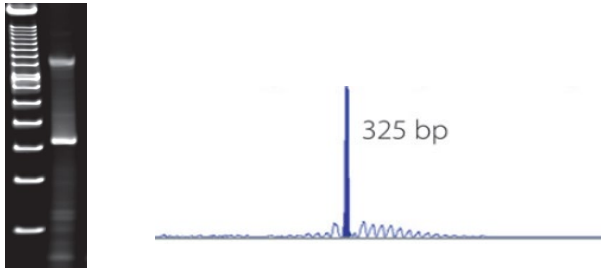
Polyclonal Progression



Polyclonal population – each V-D-J molecule differs in length and sequence

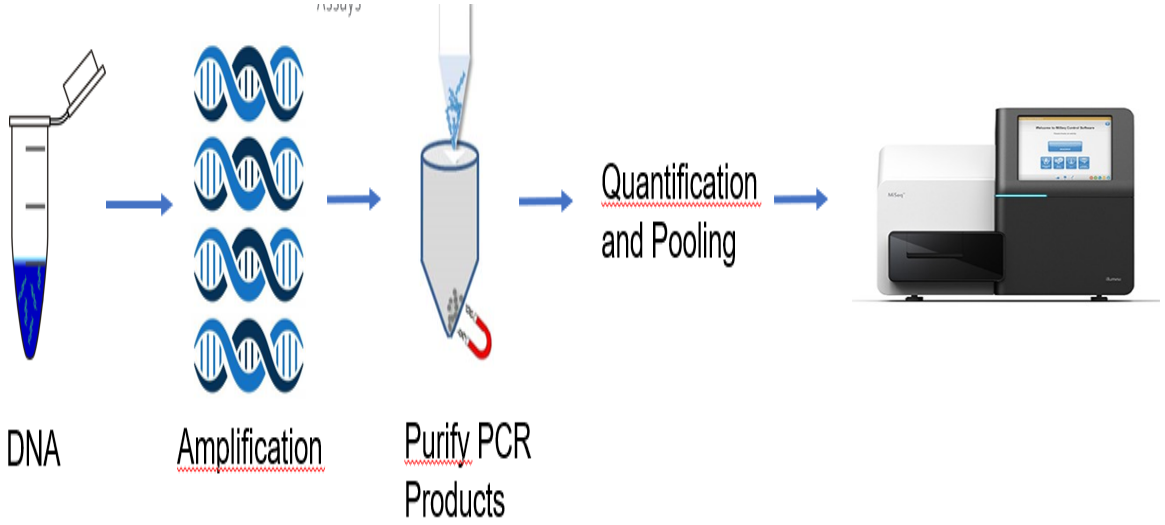
Clonal Progression



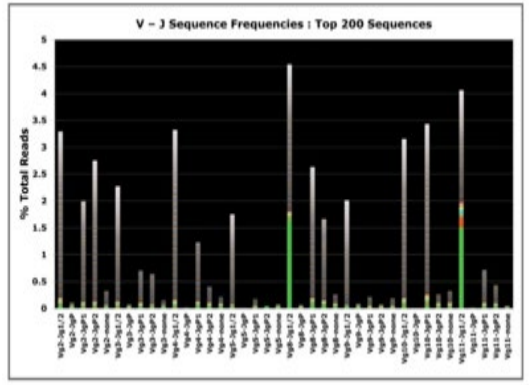


Individuato il clone, si procede con il sequenziamento: la sequenza ottenuta viene caricata su un database e paragonata alla sequenza normale.

IGHV: Ipermutazione Somatica…Workflow in NGS



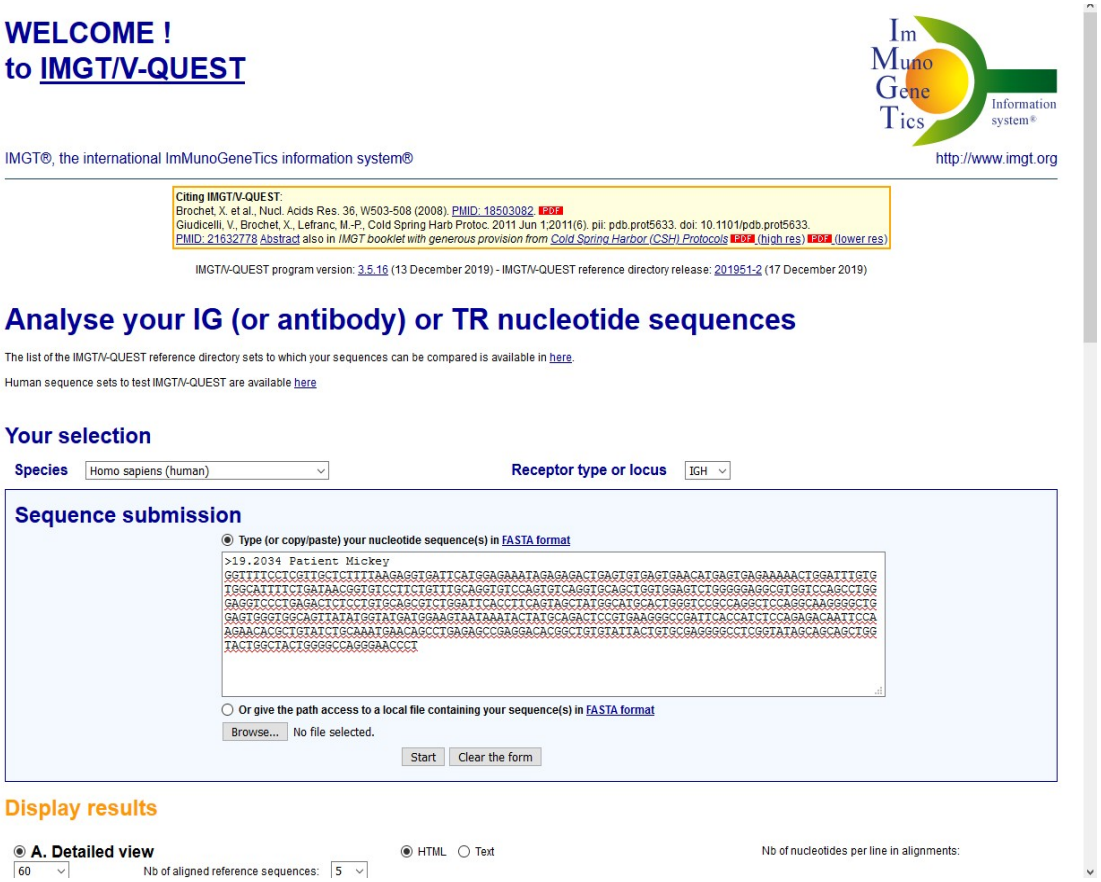
FASTQ File → **ANALYSIS** →



Simple workflow with one-step PCR for library prep
Controls are included with the kits
Following sequencing the FASTQ file can be easily analyzed using the included software solution
Fast analysis does not require bioinformatics knowledge
Software can run on most standard computers

E quindi l'IGHV SHM cos'è? E a cosa serve?

IGHV SHM: fattore prognostico di malattia. Si basa sulla presenza di mutazioni a livello della regione variabile delle Immunoglobuline sul clone individuato rispetto alla sequenza germinale.



Si effettua SEMPRE e con la ricerca di Ins/del.

IMGT/V-QUEST
http://www.imgt.org/IMGT_vquest/analysis;jsessionid=F298C4B3E3DC042BF283FB526A287425

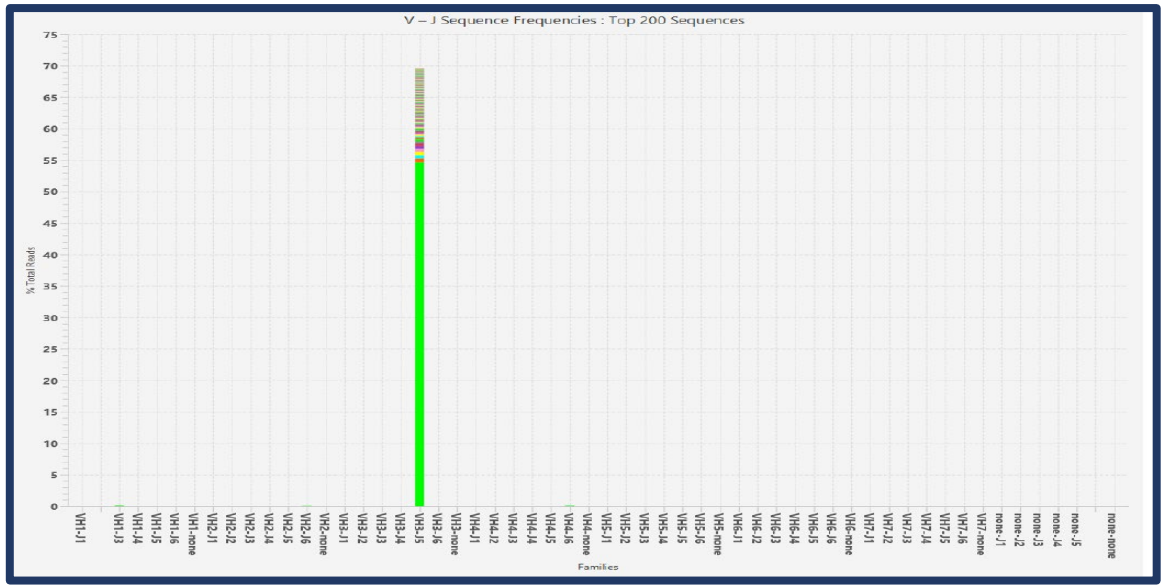
Agathangelidis A. et al Nature May 2022

CASE REPORT

≥ 98% di identità rispetto alla sequenza IgHV germ-line :
Non Mutato (UM)

< 98% di identità rispetto alla sequenza IgHV germ-line :
Mutato (M)

97-97.99 % di identità rispetto alla sequenza IgHV germ-line :
Borderline



Sequence: 1 igh

Analysed sequence length: 499.
Sequence analysis category: 2 (indel search & correction).
Sequence compared with the *Homo sapiens* (human) IG set from the IMGT reference directory (set: F+ORF+ in-frame P)

>igh
ggttttccttgttgctattttaaaaggtaattcatggtgtactagagatactgagtgtga
ggggacatgagtggtagaaacagtggatatgtgtggcagtttctgaccttggtgtttctg
tgtttgcaggtgtccaatgtgaggtgcagctggtggagtctgggggaggcttggtacagc
cagggcggtccctgagactctcctgtacagcttctggattcacctttggtgattatgcta
tgagctgggtccgccaggctccagggaaggggctggagtgggtaggtttcattagaagca
aagcttatggtgggacaacagaatacgccgcgtctgtgaaaggcagattcaccatctcaa
gagatgattccaaaagcatcgcctatctgcaaatgaacagcctgaaaaccgaggacacag
ccgtgtattactgtactagaggatttttggagtggttattcggggataactggttcgacc
cctggggccagggaaccct

In questo caso la % di identità è del 100% ed essendo la % di mutazione pari a 0% il campione non è ipermutato

Result summary: igh	Productive IGH rearranged sequence (no stop codon and in-frame junction)		
V-GENE and allele	Homsap IGHV3-49*04 F	score = 1470	identity = **100.00%** (294/294 nt)
J-GENE and allele	Homsap IGHJ5*02 F	score = 161	identity = 97.06% (33/34 nt)
D-GENE and allele by IMGT/JunctionAnalysis	Homsap IGHD3-3*01 F	D-REGION is in reading frame 1	
FR-IMGT lengths, CDR-IMGT lengths and AA JUNCTION	[25.17.38.5]	[8.10.16]	CTRGFLEWLFGDNWFDPW
JUNCTION length (in nt) and decryption	54 nt = (10)-1{0}-7(19)-5{7}-2(18)	(3'V)3'{N1}5'(D)3'{N2}5'(5'J)	

J-REGION partial 3' missing nt nb: **16**

Prognosi sfavorevole

I Subset

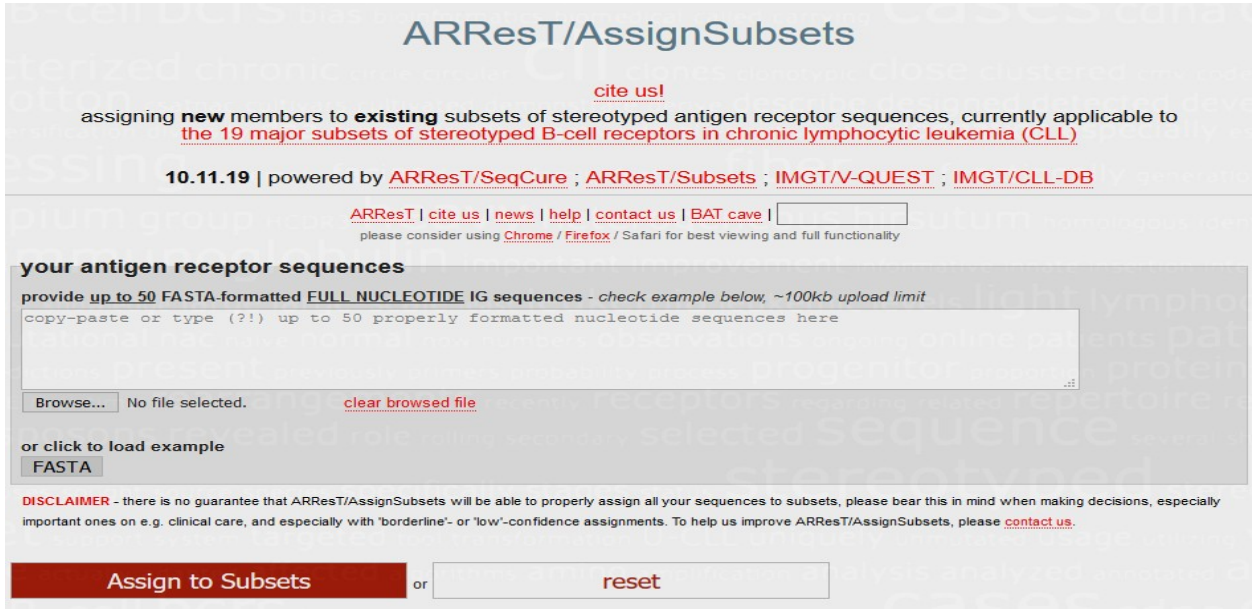
Grazie al sequenziamento dei geni Ig riarrangiati in tanti casi di LLC, si è raggiunta la costruzione di particolari subset di riarrangiamento VDJ chiamati STEREOTIPI, associabili a differenze di patogenesi e decorso di malattia. La profondità degli studi molecolari condotti fino ad oggi nella LLC, permette di stabilire un diverso comportamento clinico in categorie di pazienti MUTATED e UNMUTATED con particolari stereotipi già stratificati per alterazioni citogenetiche quali del17p e/o 11q.

Stereotyped subsets: beyond mutated / unmutated status

Andreas Agathangelidis *et* al, Blood (2012) *119* (*19*): 4467–4475.

http://tools.bat.infspire.org/arrest/assignsubsets/

Agathangelidis A. et al Nature May 2022



Five major IGHV stereotyped subsets:

- Subset #1: poor prognosis, aggressive clinical course

Riarrangement IGHV3-21

- Subset #2: poor prognosis
- Subset #4: indolent course
- Subset #5: indolent course
- Subset #8: higher risk of Richter's transformation

Riarrangement IGHV4-39

Agathangelidis A. et al Nature May 2022

CASE REPORT ARResT/AssignSubsets

assigning new members to existing subsets of stereotyped antigen receptor sequences

we're running ARResT/AssignSubsets - please follow our progress below...

```
(?) monitoring the resources used (your quota: 300 sec and 1000 megabytes RAM)
(?) checking IMGT accessibility
(?) running ARResT/SeqCure with your sequences...
(=) ARResT/SeqCure report
(?) model is running...

(=) 0 / 1 / 1 were assigned / 'healthy' / submitted
```

DISCLAIMER - there is no guarantee that ARResT/AssignSubsets will be able to properly assign all your sequences to subsets, please bear this in mind when making decisions, especially important ones on e.g. clinical care, and especially with 'borderline'- or 'low'-confidence assignments. To help us improve ARResT/AssignSubsets, please contact us.

plain-text-formatted results table (best viewable in a spreadsheet), or see below

click to open/close quick help »

assignment frequencies table

CLL#2	CLL#1	CLL#4	CLL#6	CLL#5	CLL#3	CLL#8	CLL#31	CLL#16	CLL#77
2.8%	2.4%	1.0%	0.9%	0.7%	0.6%	0.5%	0.4%	0.3%	0.3%

CLL#7H	CLL#28A	CLL#201	CLL#12	CLL#59	CLL#14	CLL#64B	CLL#99	CLL#202
0.3%	0.3%	0.3%	0.3%	0.3%	0.3%	0.3%	0.3%	0.3%

assignment report table

label [+ heat map, if appl.]	SeqCure	subset	confidence	score
89503	OK	unassigned	extreme	-Inf

hosted at the Bioinformatics Analysis Team / BAT

Interpetazione dati: Linee guida

Guidelines from European group

ERIC = European Research Initiative on CLL

Leukemia (2011) 25, 979–984

www.nature.com/leu

ORIGINAL ARTICLE

Immunoglobulin sequence analysis and prognostication in CLL: guidelines from the ERIC review board for reliable interpretation of problematic cases

AW Langerak[1], F Davi[2], P Ghia[3], A Hadzidimitriou[4], F Murray[5], KN Potter[6], R Rosenquist[5], K Stamatopoulos[4,7] and C Belessi[8] on behalf of the European Research Initiative on CLL (ERIC)

[1]Department of Immunology, Erasmus MC, University Medical Center Rotterdam, Rotterdam, The Netherlands; [2]Laboratory of Hematology and Université Pierre et Marie Curie, Hôpital Pitié-Salpètrière, Paris, France; [3]Laboratory of B Cell Neoplasia and Unit of Lymphoid Malignancies, Division of Molecular Oncology and Department of Oncology, Università Vita-Salute San Raffaele and Istituto Scientifico San Raffaele, Milan, Italy; [4]Institute of Agrobiotechnology, Center for Research and Technology Hellas, Thessaloniki, Greece; [5]Department of Immunology, Genetics and Pathology, Rudbeck Laboratory, Uppsala University, Uppsala, Sweden; [6]Cancer Sciences Division, Somers Cancer Research Building, University of Southampton, Southampton, UK; [7]Hematology Department and HCT Unit, G. Papanicolaou Hospital, Thessaloniki, Greece and [8]Hematology Department, Nikea General Hospital, Pireaus, Greece

OPEN

Leukemia (2017) **31**, 1477–1481

www.nature.com/leu

EDITORIAL

Immunoglobulin gene sequence analysis in chronic lymphocytic leukemia: updated ERIC recommendations

OPEN

Citation: Blood Cancer Journal (2016) **6**, e440; doi:10.1038/bcj.2016.49

www.nature.com/bcj

LETTER TO THE EDITOR

Double IGHV DNA gene rearrangements in CLL: influence of mixed-mutated and -unmutated rearrangements on outcomes in CLL

Blood Cancer Journal (2016) **6,** e440; doi:10.1038/bcj.2016.49; published online 1 July 2016

treatment or death. Primary end points for the study were time to treatment (TTT) and overall survival (OS). OS was from the time of

Immunoglobulin gene sequence analysis in chronic lymphocytic leukemia: the 2022 update of the recommendations by ERIC, the European Research Initiative on CLL

Andreas Agathangelidis[1,2], Anastasia Chatzidimitriou[1,3], Thomas Chatzikonstantinou[1,4], Cristina Tresoldi[5], Zadie Davis[6], Véronique Giudicelli[7], Sofia Kossida[7], Chrysoula Belessi[8], Richard Rosenquist[3,9], Paolo Ghia[10✉], Anton W. Langerak[11], Frédéric Davi[12], Kostas Stamatopoulos[1,3] and on behalf of ERIC, the European Research Initiative on CLL

Risultati analisi NGS

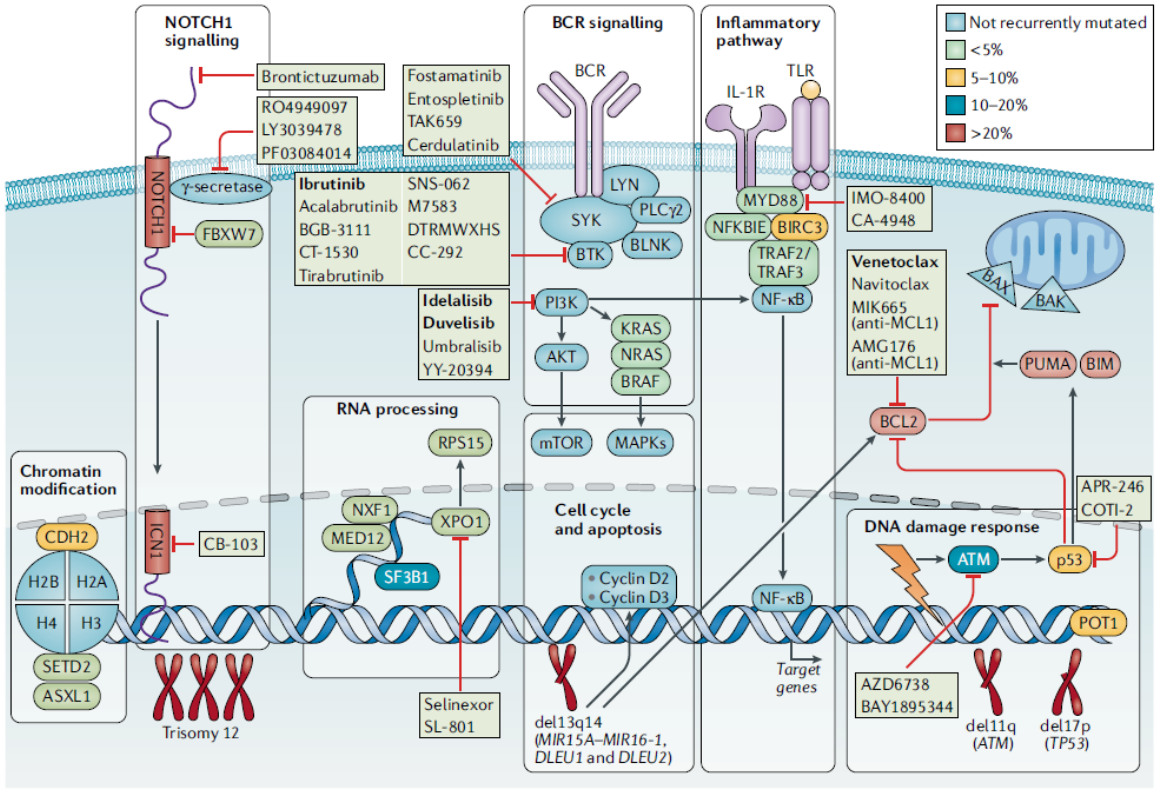
Questo caso appartiene alla categoria IG-UNMUTATED, che è generalmente associata a prognosi sfavorevole.

Lo stato mutazionale del gene TP53 risulta mutato.

L'analisi delle sequenze del gene SF3B1 ha evidenziato la presenza di una mutazione missenso p.Lys700Glu al 44,5%.

Inoltre, sono presenti CNV di delezione sul cromosoma 13 a livello del gene DLEU1

CONCLUSIONI



Bosch, F., Dalla-Favera, R.
Chronic lymphocytic leukaemia: from genetics to treatment.
Nat Rev Clin Oncol 16, 684–701 (2019). https://doi.org/10.1038/s41571-019-0239-8

Il rapido progresso nelle tecniche di genetica e biologia molecolare ha significativamente contribuito alla comprensione della patogenesi della CLL e potrebbe portare ad una tailored therapy sulla base delle caratteristiche di ciascun paziente

La profonda caratterizzazione biologica dei pazienti offre nuove prospettive per un corretto management della CLL.

I pannelli genici NGS rappresentano oggi una delle metodiche di maggior valore nella diagnostica molecolare della CLL.

PROSPETTIVE FUTURE….

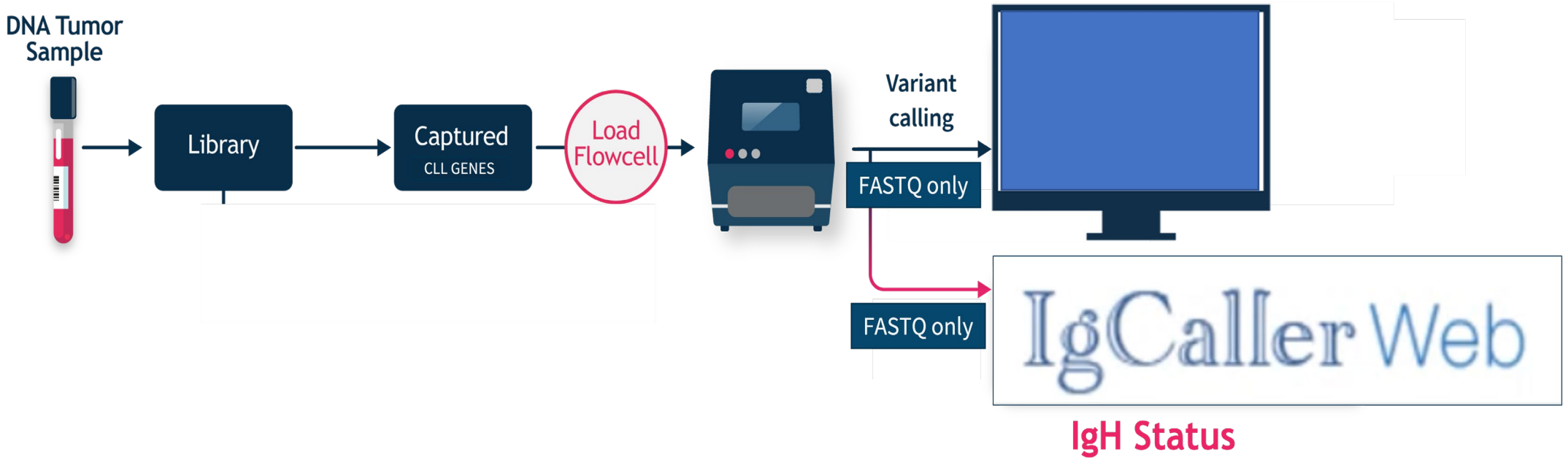
Custom Chronic Lymphocytic Leukemia – V2
Main updates

- **Gene Panel**
  - 2 Extra genes added for all protein-coding exons for : **RB1** (Improve Del13q14 detection), **BCL2** (Drug: Venetoclax)
  - **Extra probes for Ig rearrangements** in chr14 and chr22 - hg38 format
  - Multiplexing
    - Adjusted to new size of the panel (73Kb to 112 Kb)
    - Pure Runs:
      - 12 samples per run on a MiSeq (v2, 2x250pb)
      - 16* samples per run on a MiSeq (v3, 2x300pb)

    *Recommended→ IgCaller works better on long reads with 97% Sensitivity and 100% Specificity, based on validation program)

- **IgCaller Web** (analysis outside DDM)
  - IgCaller Web has been design to perform automatically all the bioinformatic steps required to extract from raw data (FASTQ files) the DNA sequence of :
    1. the heavy chain gene rearrangement
    2. light chain gene rearrangements involving the IGLV3-21 gene

IgH Hypermutation status - Workflow



ALL-IN-ONE workflow

Variant calls + CNVs + IgH Hypermutation Status

GRAZIE!