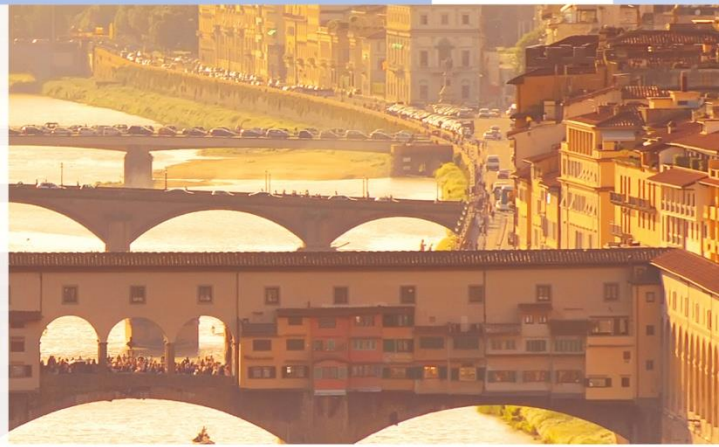
8° WORKSHOP IN EMATOLOGIA TRASLAZIONALE

DELLA SOCIETÀ ITALIANA DI EMATOLOGIA SPERIMENTALE

Firenze - Auditorium CTO - A.O.U. Careggi, 22-23 giugno 2023



Caratterizzazione clinica e molecolare del Linfoma Diffuso a Grandi cellule B: studio del trascrittoma e del microambiente tumorale per l'identificazione di nuovi sottotipi molecolari

Robel Papotti, PhD

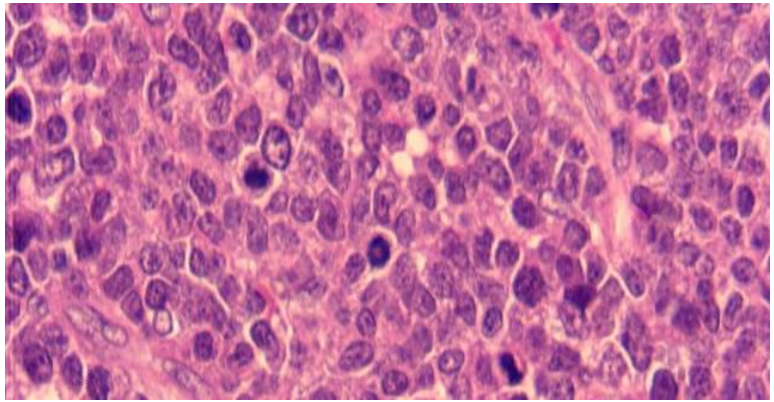
Oncoematologia clinico-sperimentale CRO Aviano

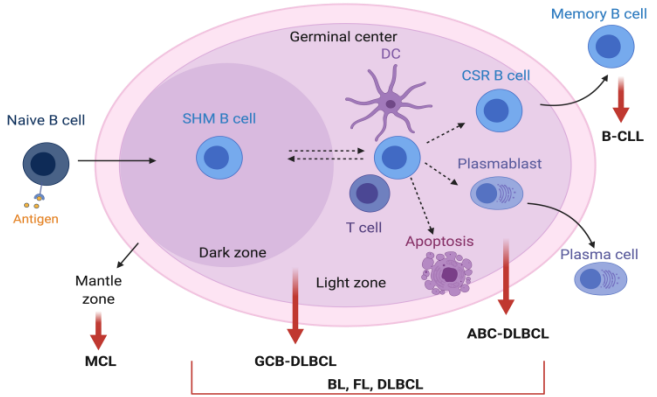
Disclosures of Robel Papotti

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

Diffuse Large B cell Lymphoma (DLBCL)

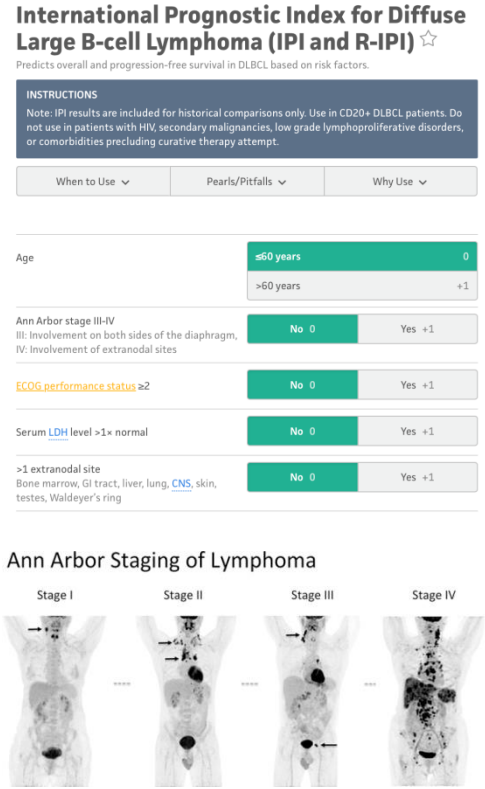
- The most common type of malignant lymphoma
- Classified on the basis of:
  - Morphology
  - Immunophenotype: CD19+, CD5-, CD20+, CD22+, CD79a+, PAX5, rearranged IGH; 40% CD10+, 60% BCL6+, 50% BCL2+
  - Genetic alteration: IGH-BCL2, IGH-BCL6, IGH-MYC, heterogenous genetic landscape
- Treatment: R-CHOP, response rate ~60%





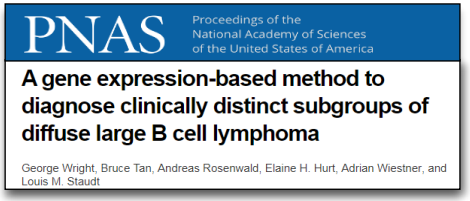
Risk stratification

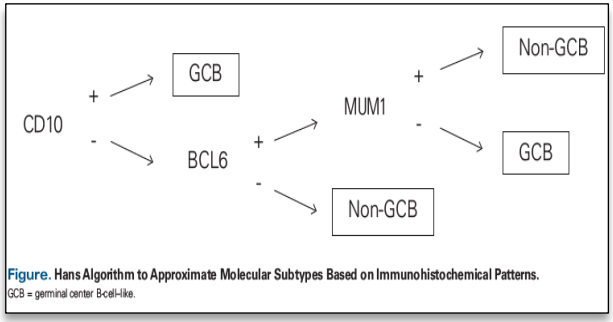
- International Prognostic Index (IPI):
  - age > 60 years
  - stage III/IV disease (Ann Arbor)
  - elevated lactate dehydrogenase [LDH] level
  - ECOG (performance status) ≥ 2
  - extranodal site of disease > 1
- PET-CT scans
- BCL2/MYC over-expression/translocation
- Cell of Origin
  - ABC: gene expression similar to activated B cells
  - GCB: gene expression reminiscent of germinal center B cells
  - Unclassified

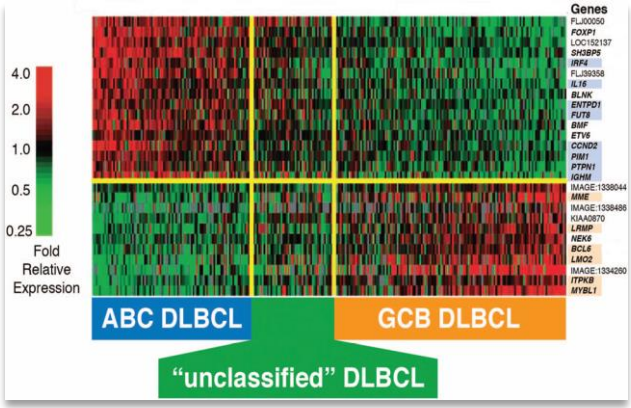


DLBCL molecular subtypes identified with GEP/IHC

- **Microarrays**
  - *Thousands of RNA targets*
  - *RNA from fresh frozen tissues*
- **Immunohistochemistry**
  - *FFPE-Compatible COO classifiers*
  - *Applicable in clinical practice*
- **Lymph2Cx assay**
  - *20-gene signature for COO classification on FFPE (15 core genes, 5 housekeeping genes)*





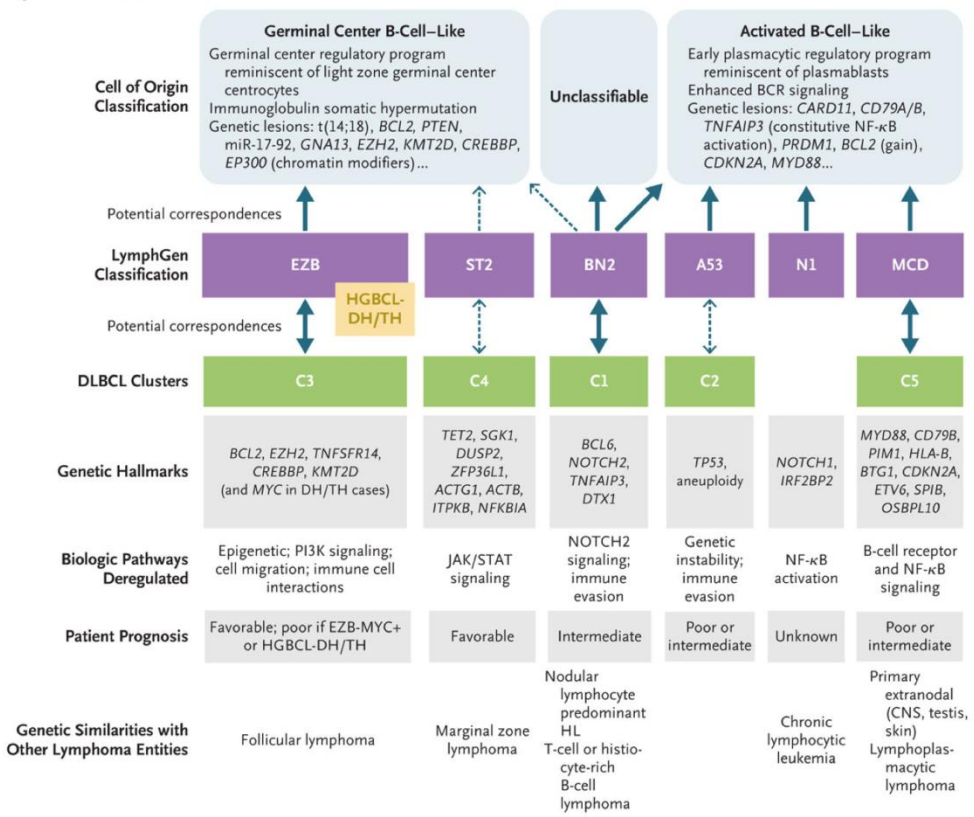


Genomic profiling of DLBCL

Chapuy et al 2018:
C1: BCL6 fusion and NOTCH2 mutations
C2: TP53 mutations and 17p deletion
C3: BCL2 alterations and EZH2 mutations
C4: TET2 and SGK1 mutations
C5: MYD88 (L265P) and CD79B mutations

Schmitz et al 2018:
MCD: MYD88 and CD79B mutations
EZB: EZH2 mutation and BCL2 translocation
BN2: BCL6 fusion and NOTCH2 mutations
N1: NOTCH1 mutations

Wright/Staudt 2020:
EZB – ST2 – BN2 – A53 – N1 – MCD



Aim

The project exploits transcriptomic-based approaches, coupled with existing prognostic tools, to explore:

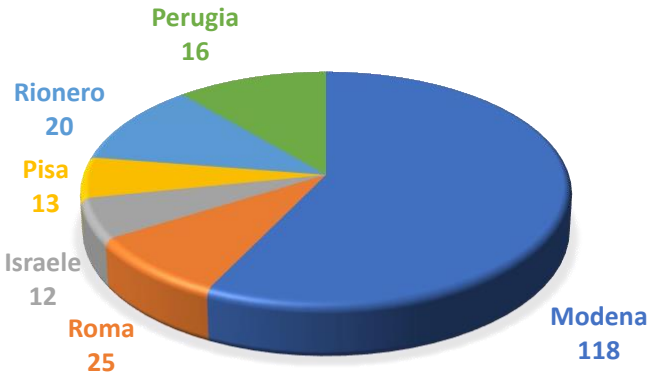
- Gene expression profiling
- Immunoglobulin status
- Mutational landscape
- Microenvironmental interactions of tumor cells (Ecotypes)

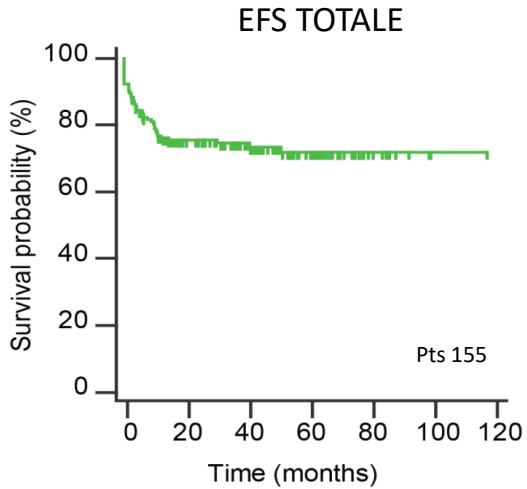
Multicenter cohort characterization

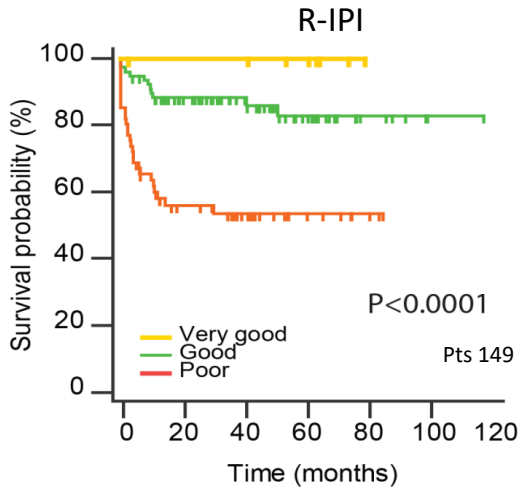
- 204 DLBCL cases with PET-CT-defined stage
- 2 years minimum follow up
- R-CHOP or R-CHOP-like regimens

- Nucleic acids were extracted from Formalin fixed paraffin embedded (FFPE) tissues

- Median follow-up of 32 months
- 3 years EFS of 75% (95CI 68-83%)
- EFS events were in total 40 progression and/or relapses



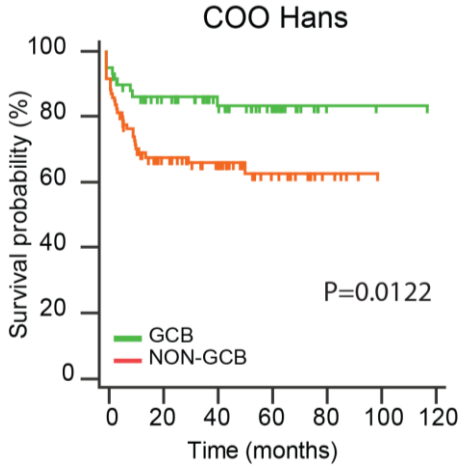


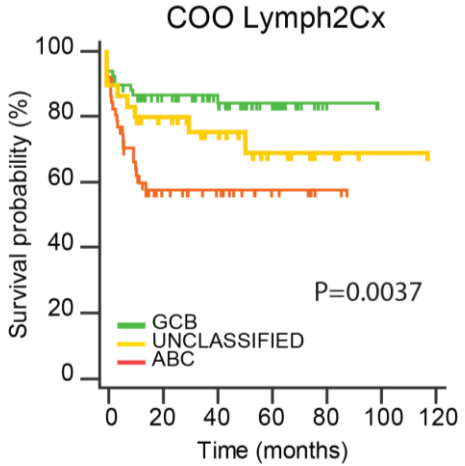


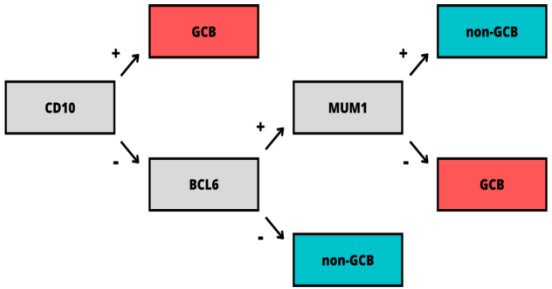
Cell of Origin classification

	Cases	Status	N (%)
COO by Hans' Algorithm	184	GCB	77 (42)
		non-GCB	107 (58)
COO by Lymph2Cx	191	ABC	67 (35)
		GCB	90 (47)
		Unclassified	34 (18)

0.719 k-statistic comparison value between the classification based on IHC and Lymph2Cx



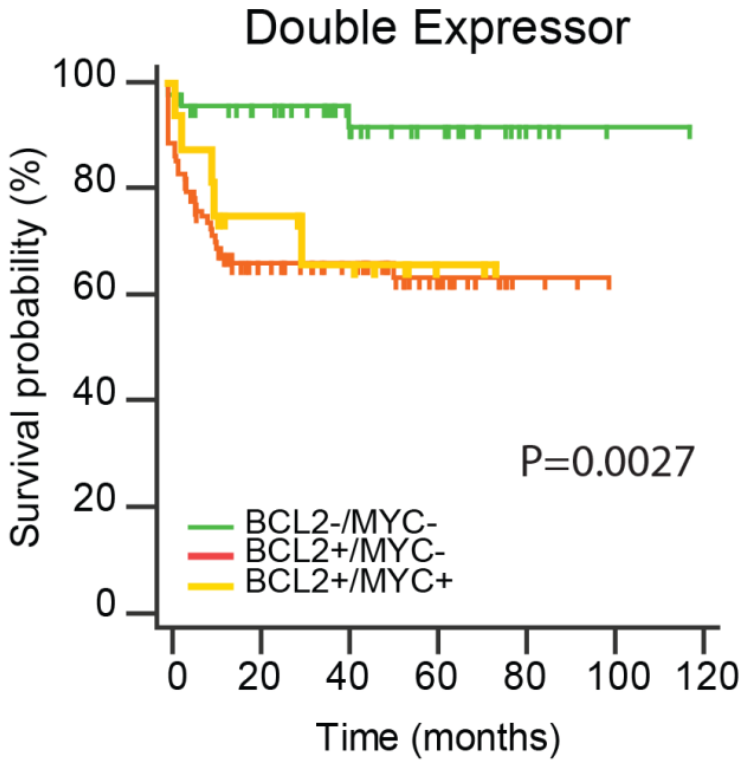




BCL2/MYC expression by IHC

Variable	Status	N	n (%)
BCL2	+	192	129 (67)
MYC	+	190	28 (15)
BCL2/MYC			
	BCL2-/MYC-		57 (30)
	BCL2-/MYC+		5 (3)
	BCL2+/MYC-		104 (55)
	BCL2+/MYC+		23 (12)

Covariate	Status	5-yr EFS (95CI)	HR (95CI)	p-value
BCL2/MYC	BCL2-	91 (71-100)	1.00	
	BCL2+/MYC-	63 (24-68)	5.23 (2.53-10.81)	**=0.0006**
	BCL2+/MYC+	65 (31-88)	4.73 (0.93-20.64)	**=0.0114**



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RNA sequencing

204 total cases

192 with adequate material

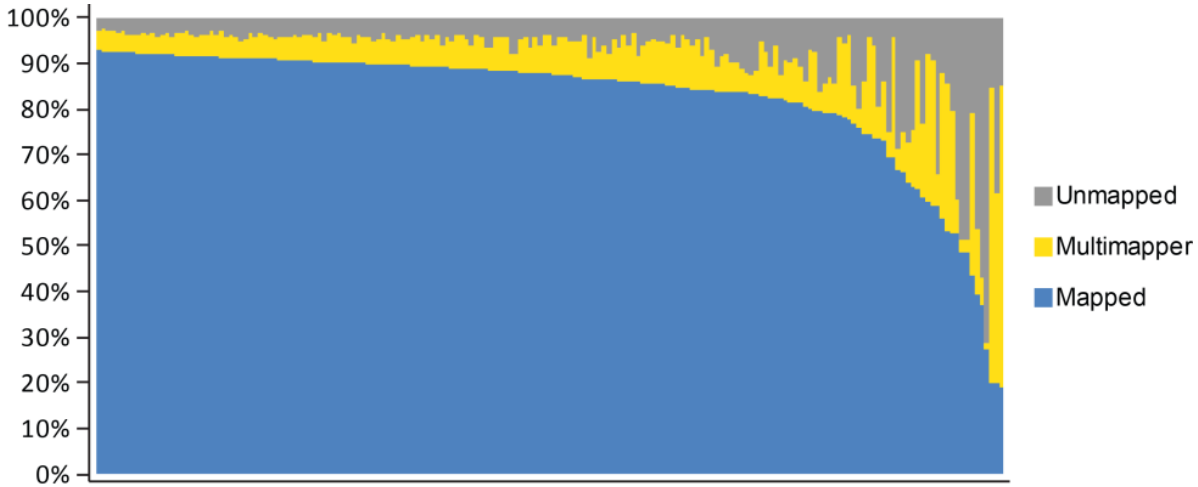
186 with successful library amplification

NovaSeq6000 (ILLUMINA)

S4 ILLUMINA flow-cell
2x100 paired-end reads chemistry
Final output: > 20 billion reads

RNA sequencing statistics

Fastq files (raw reads) ⇨ Alignment on trascriptome STAR 1-pass ⇨ read count with Salmon

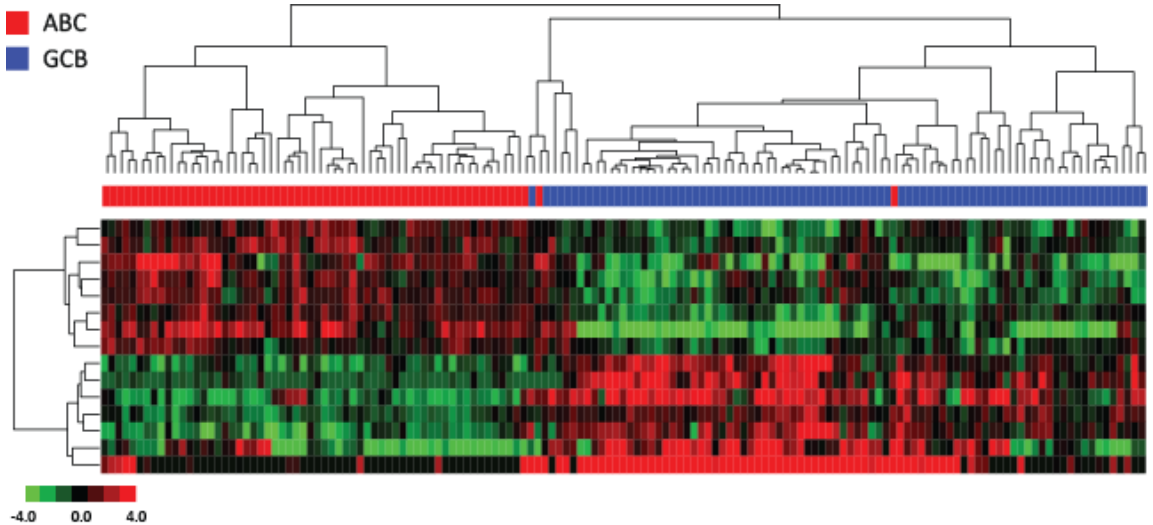


Fraction of reads aligning to **single locus**, **multiple loci** (e.g. rRNA genes) o **no loci**

- RNA HyperPrep with RiboErase (ROCHE), starting from 150 ng of total RNA
- Libraries were globally well balanced
- Average of 2.26×10^8 number of reads
- Median percentage of 82.43% of uniquely mapped reads
- rRNA was efficiently depleted

COO classification through RNASeq data

Clustering on 15 genes corresponding to Nanostring COO signature



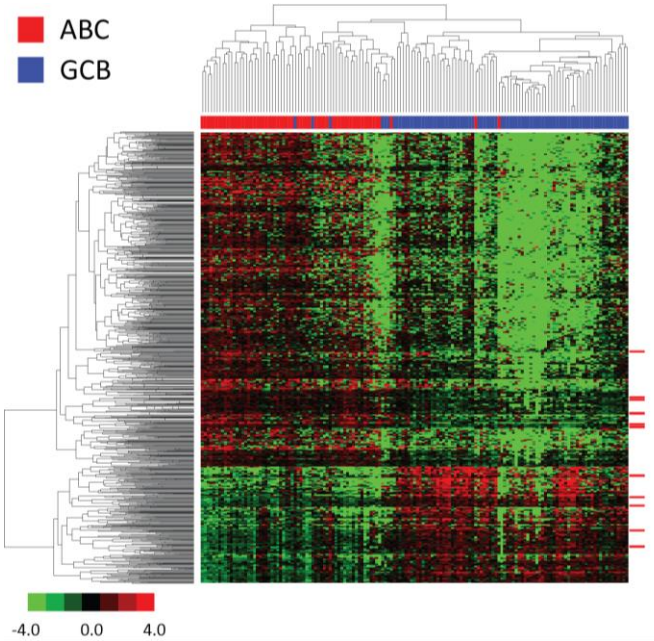
Clustering analyses on the 147 cases correctly split the ABC subtypes from GCB, showing a good concordance with Lymph2Cx (0.97 Kappa Cohen)

Good performance of FFPE RNASeq for gene expression

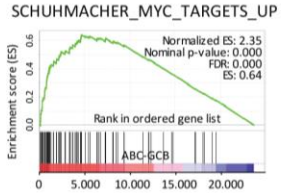
Gene expression profile analysis

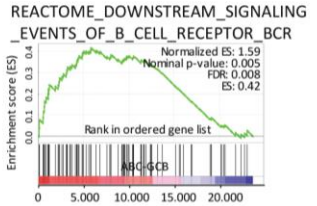
62 ABC vs 85 GCB
cutoff value a p<0.05 and a fold change (FC)>2

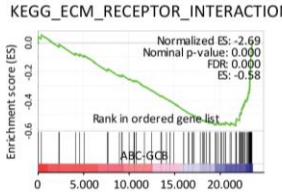
1.328 differentially expressed genes
985 over-expressed in ABC
343 over-expressed in GCB

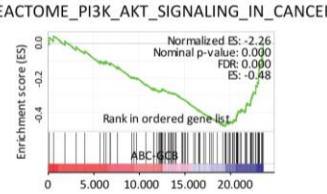


GSEA
Gene Set Enrichment Analysis









ABC: MYC upregulation, and B cell receptor (BCR) signaling activation
GCB: extracellular matrix regulation, and PI3K/AKT pathway

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Immunoglobulin reconstruction

STAR Alignment

GGACGTACGTACGTAGTGCTAGTGGT
ACGTACGTACGTAGTGCTA
GTACGTACGTAGTGCTAG
CGTACGTAGTGCTAGTG
TACGTAGTGCTAGTGGT

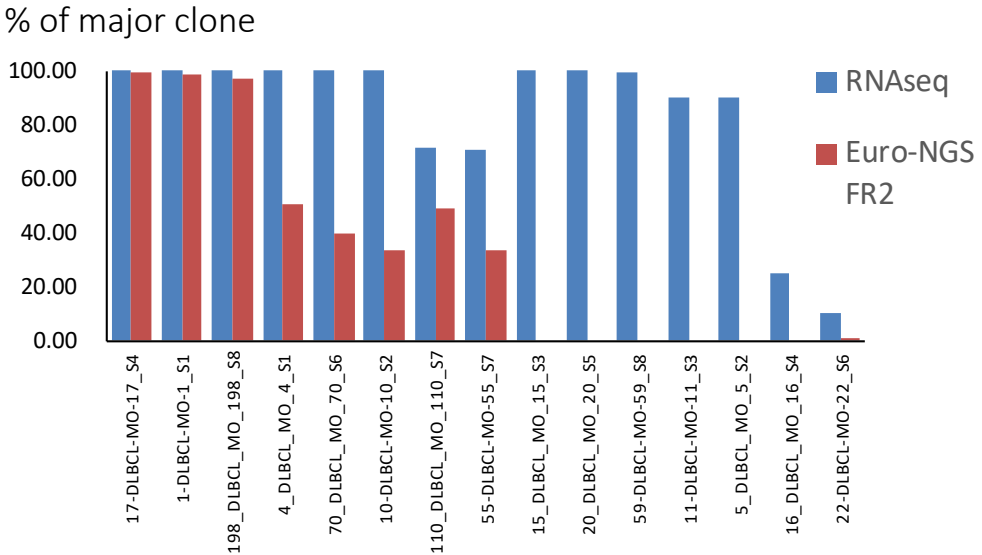
De novo IGH Assembly

Refinement

Targeted realignment

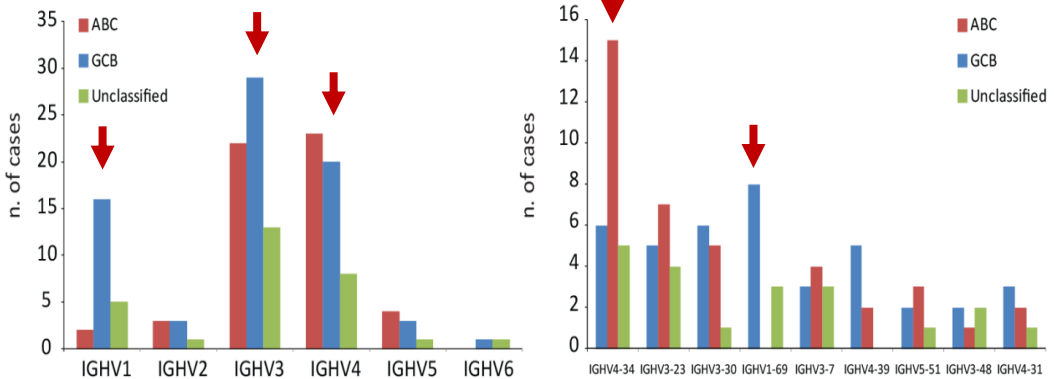
Pilot experiments based on 15 DLBCL samples sequenced both for RNASeq, and for the immunoglobulin according to standard sequencing methods

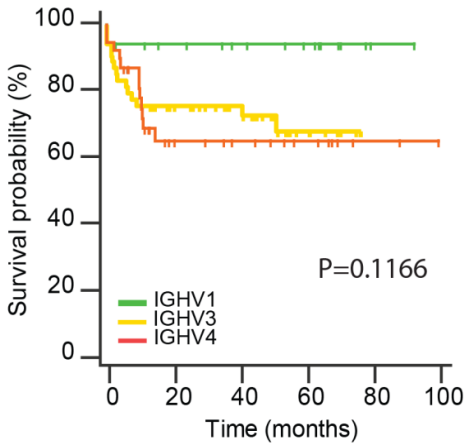
RNAseq identifies IGH clones from degraded material even better than targeted amplicon method



Courtesy of Filippo Vit

IGHV families usage





- IGHV reconstruction on 173 out of 186 cases (93%)
- 159 cases showed a single major clone
- IGHV3 (41%), IGHV4 (33%), and IGHV1 (15%) were the most used IGHV families
- IGHV4-34 was significantly over-represented in ABC (p=0.0039)
- IGHV1-69 was represented only in the GCB (p=0.0117)
- IGHV1 and IGHV4 presented the best and worst outcome, respectively (IGHV1 vs IGHV4 p=0.032)

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- **Mutational landscape**
- Microenvironmental interactions of tumor cells (Ecotypes)

Genetic mutations

Deep evaluation of somatic mutations in the context of our real-world cohort

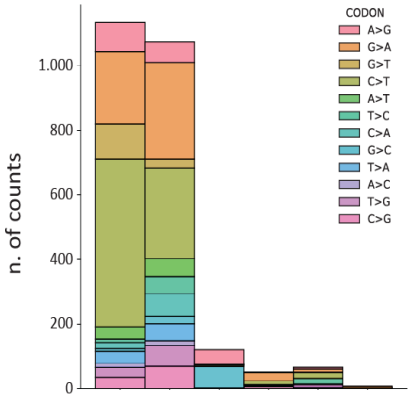
Variant calling: LoFreq

↓

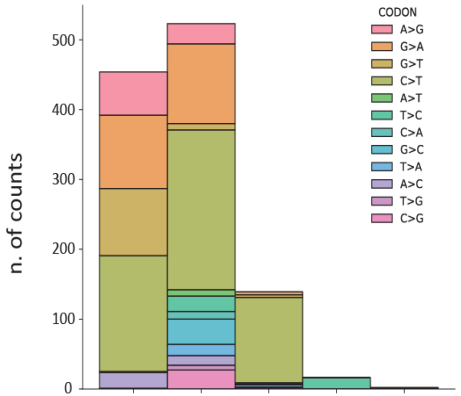
Filtering on dbSNP138 and COSMIC

↓

VAF cut-off ≥ 20%



Transcriptome effect

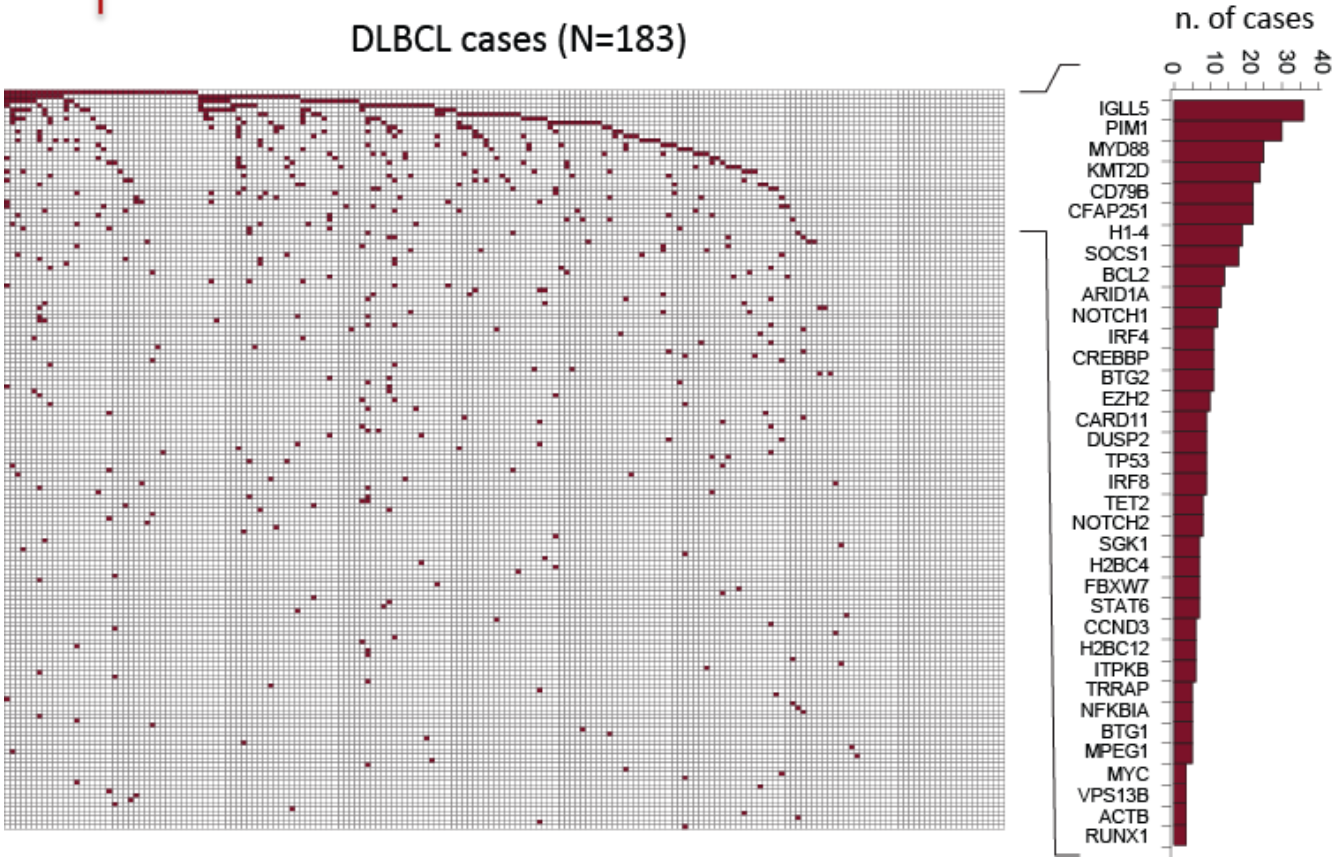


Exonic effect

Effect of variants identified in exonic positions

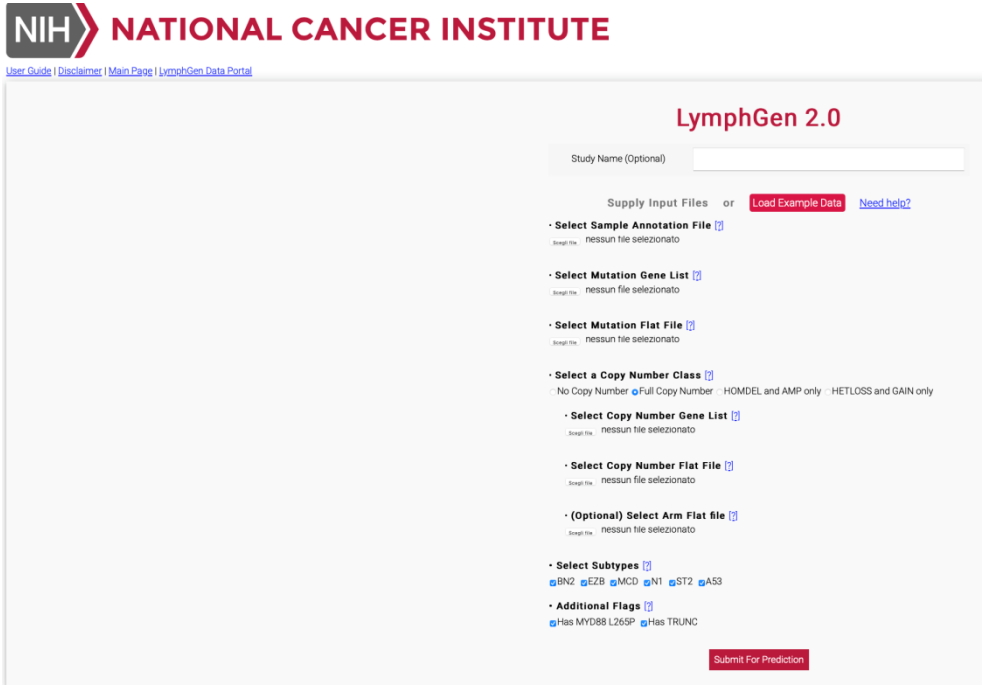
Courtesy of Filippo Vit

Frequently mutated genes



- 1.135 exonic mutations:
  - 455 synonymous
  - 523 missense
  - 139 stop/gain
  - 16 stop/loss
  - 2 start/loss
- Mean mutation rate was 4 (range 0-21)
- Among the most frequently mutated genes: *IGLL5, PIM1, MYD88, KMT2D, CD79B, SOCS1, BCL2, ARID1A, NOTCH1*

LymphGen classifier



Wright et al., 2020

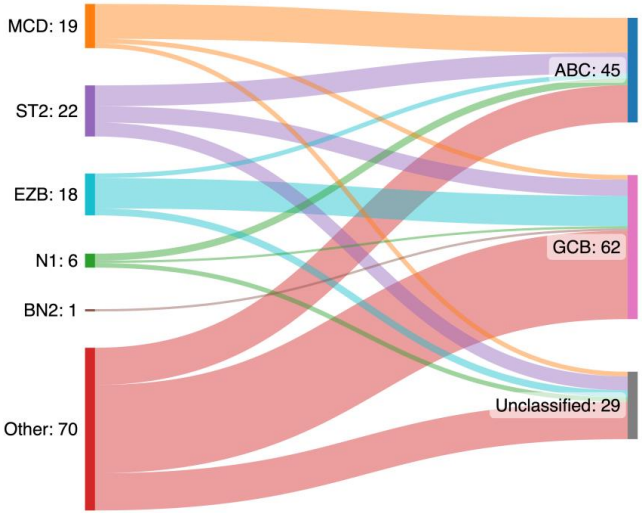
Algorithms able to categorize DLBCLs based on specific key genetic alterations in the MCD, BN2, ST2, EZB and N1 subtypes

It relies on data from exome or targeted sequencing, either with or without copy number variant (CNV) data

81 cases (47%) were successfully categorized:

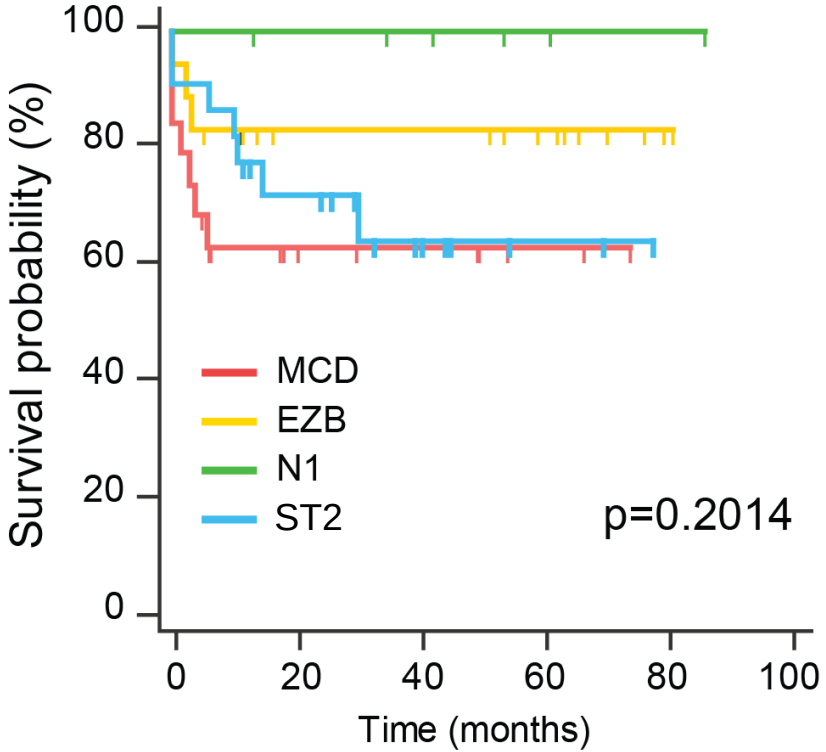
- **25 (14%) cases were MCD**
- **25 (14%) cases were ST2**
- **23 (13%) cases were EZB**
- **7 (4%) cases were N1**
- **1 (0.6%) case was BN2**

Clinical significance of LymphGen classification



MCD subtype shows the worst outcome

MCD subtype is enriched in ABC cases and presents frequent alterations of MYD88 and CD79B



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- **Microenvironmental interactions of tumor cells (Ecotypes)**

EcoTyper algorithm

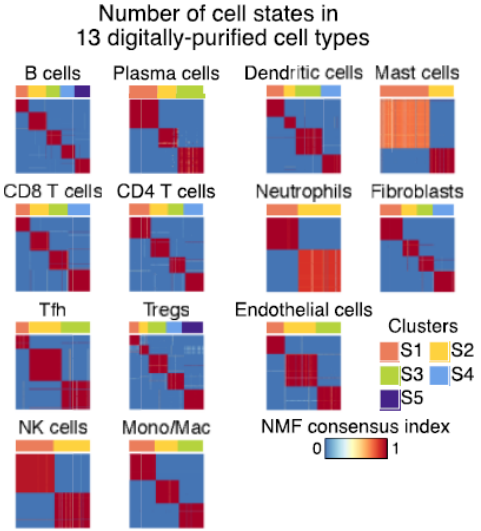
> Cancer Cell. 2021 Oct 11;39(10):1422-1437.e10. doi: 10.1016/j.ccell.2021.08.011.
Epub 2021 Sep 30.

The landscape of tumor cell states and ecosystems in diffuse large B cell lymphoma

Chloé B Steen [1], Bogdan A Luca [2], Mohammad S Esfahani [3], Armon Azizi [4], Brian J Sworder [3], Barzin Y Nabet [5], David M Kurtz [3], Chih Long Liu [3], Farnaz Khameneh [4], Ranjana H Advani [3], Yasodha Natkunam [6], June H Myklebust [7], Maximilian Diehn [5], Andrew J Gentles [8], Aaron M Newman [9], Ash A Alizadeh [10]

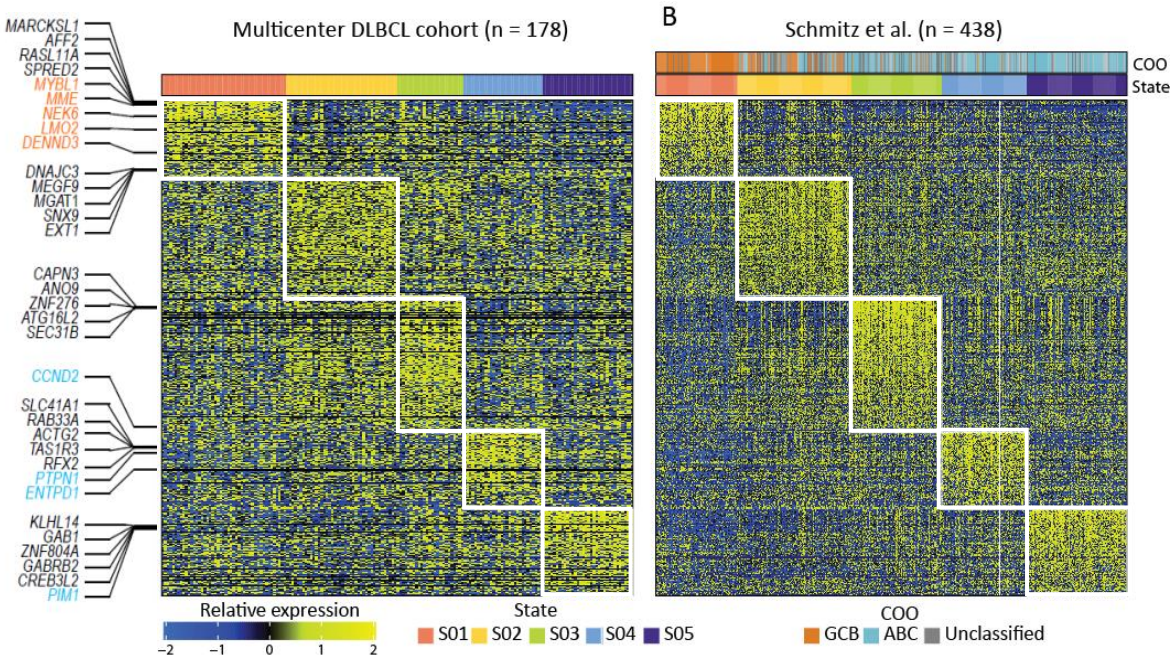
Machine-learning approach for dissecting cellular heterogeneity in DLBCL from RNAseq data

For 13 different cell types (B cells + TME cells), identifies specific and discrete transcriptional programs known as **cell states**



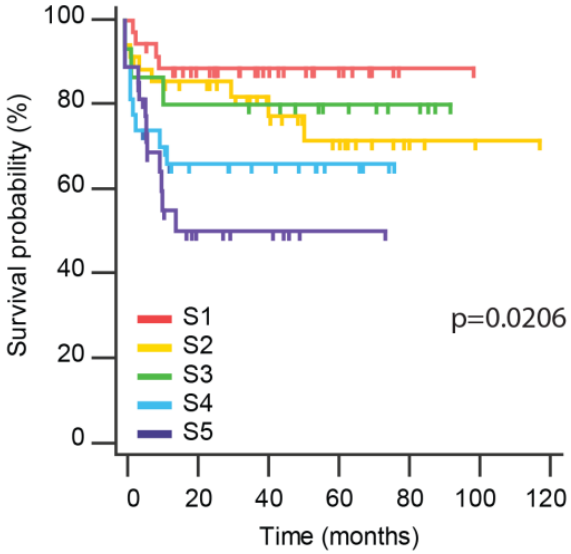
Steen et al., 2021

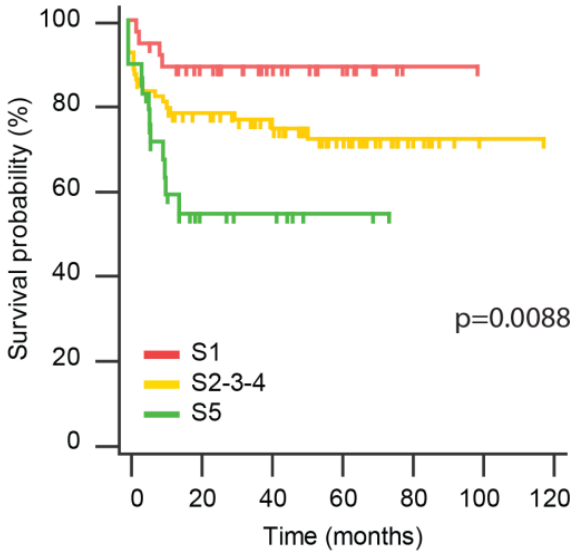
EcoTyper: B cell states



- EcoTyper algorithm on 178/186 (96%) samples
- B cells: 5 different cell states named S1-S5
- S1: transcriptional signature attributable to germinal center cells
- S5: associated with a plasma cell signature
- S2-S3-S4: gene programs resembling the maturation processes of B cells

B cell states survival curves

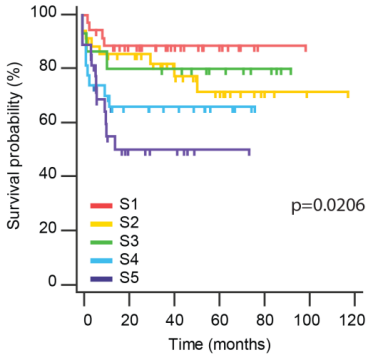




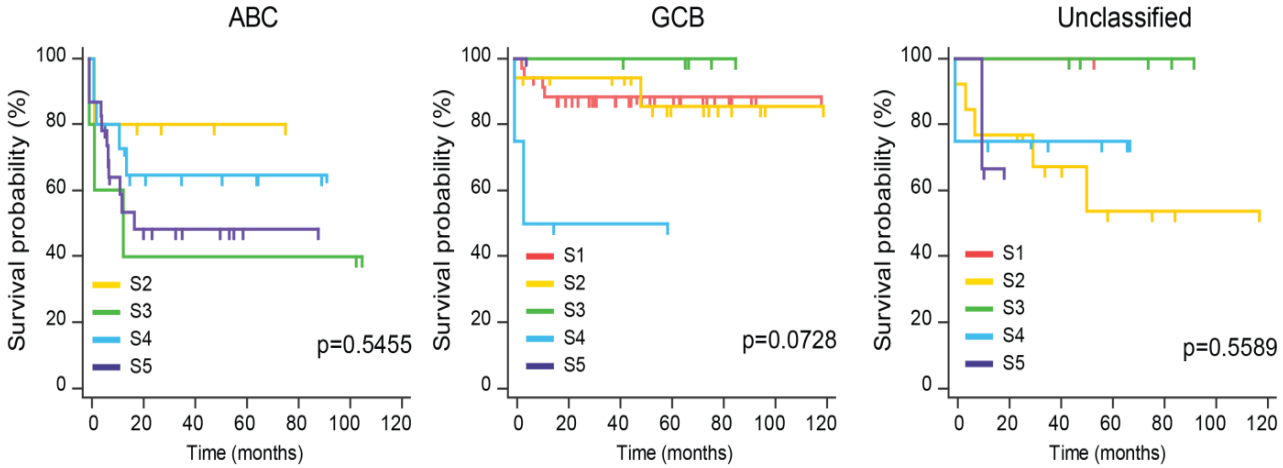
- B cell states associated survival curves had a specific distinct outcome
- S1: best prognosis; S5: worst prognosis (S1 vs S5 p=0.0003)
- Through recursive partitioning: 3 different subsets

B cell states and Nanostring

B cell states



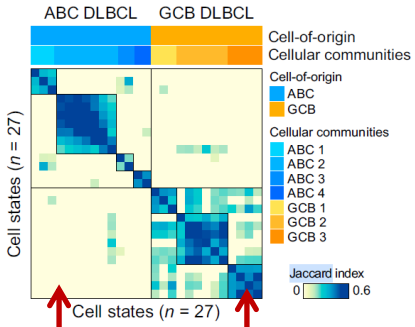
B cell states + COO Nanostring



To note that these cell states further stratify within COO
e.g. S2 in ABC or S3 in Unclassified

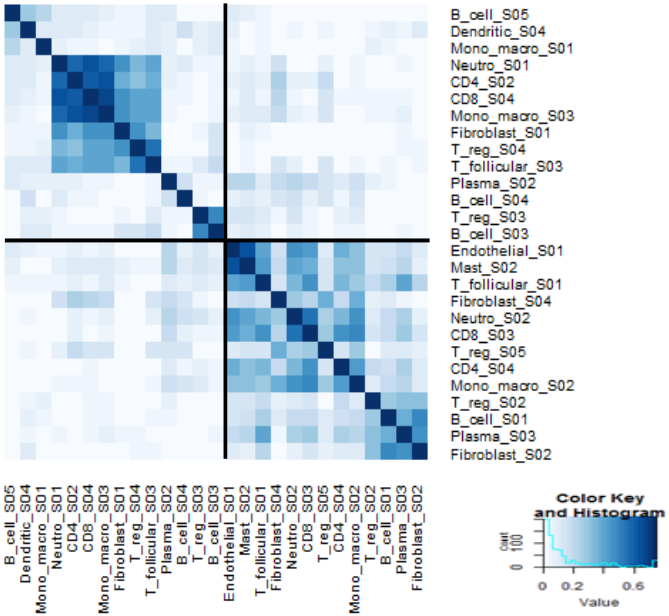
Co-occurrence between cell states

Steen et al., 2021

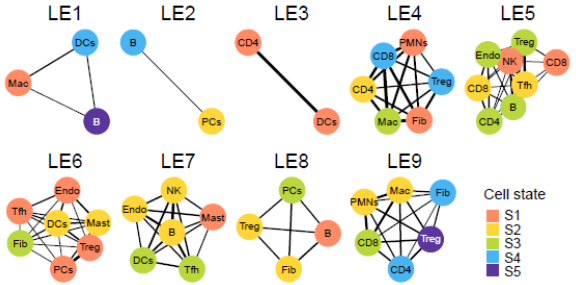


White: states are mutually exclusive

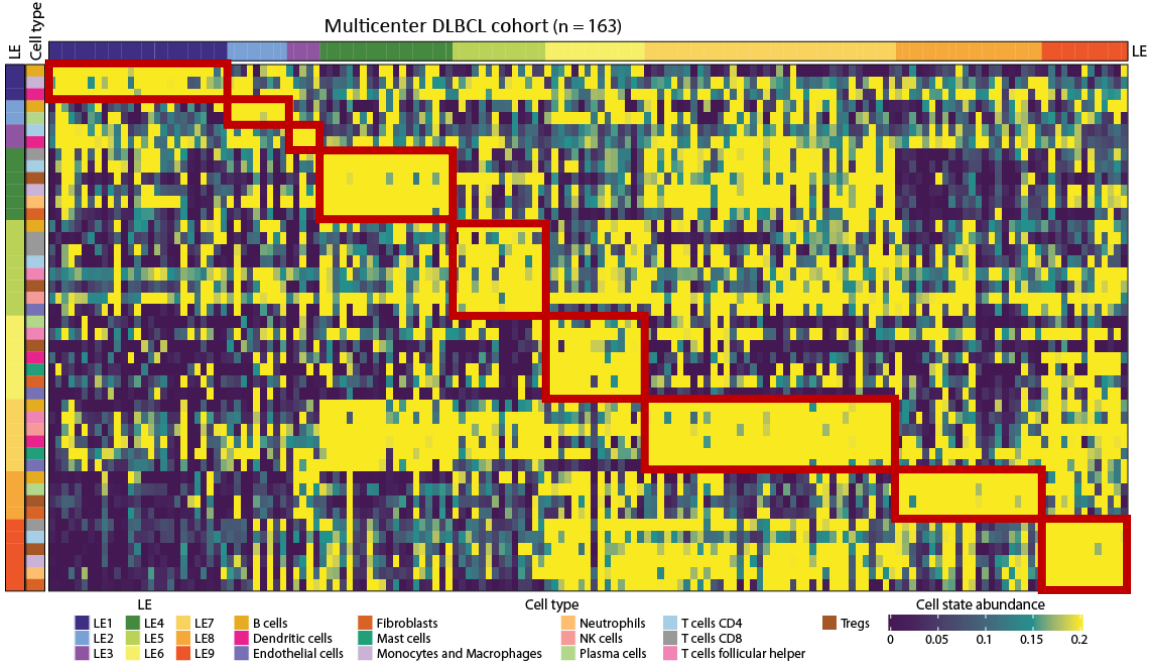
Blue: states tend to co-occur



- Co-occurrence estimation between different cell states through the Jaccard index
- Some of them were likely to be found co-occurring, others were mutually exclusive
- B cell states: S1 displayed the highest interaction with TME cells

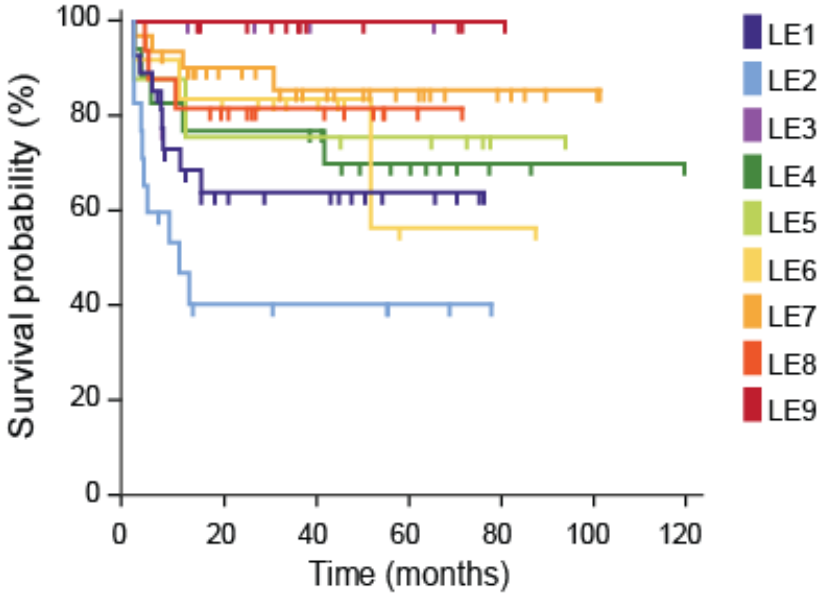


Lymphoma Ecotypes (LEs)



- EcoTyper assembles multiple cell states into 9 Lymphoma Ecotypes (LEs)
- LE1-LE2: prevalent infiltration of B cells; mainly ABC cases
- LE4: immunoreactive T cell states enrichment
- LE8: enriched in GCB
- LE6-LE7-LE9: significant stromal content
- LE9: abundant in fibroblasts and strongly associated with other TME elements

Lymphoma Ecotypes survival curves



- LEs classification had clinical significance
- LEs can be interpreted as TME-informed subgroups
- Gradient of risk from LE1-LE2 to LE9

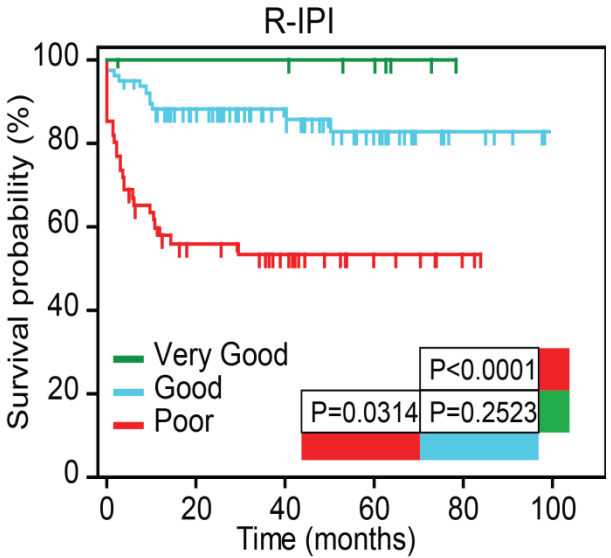
Univariate and multivariate analysis

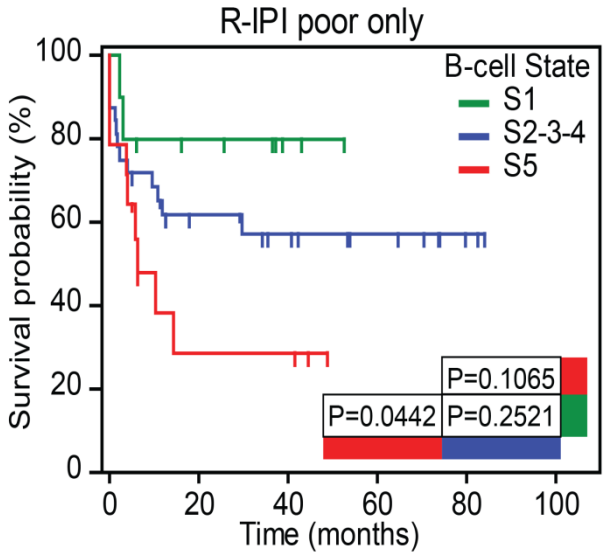
EFS (n=138)

	UVA				MVA with B cell state				MVA with Lymphoma Ecotypes			
	HR	LCI	UCI	P	HR	LCI	UCI	P	HR	LCI	UCI	P
R-IPI (poor)	3,69	1,90	7,18	**0,0001**	4,07	1,99	8,31	**0,0001**	3,53	1,69	7,36	**0,0008**
R-IPI (very good)	0,48	0,26	1,82	0,9542	-				-			
COO Nanostring (ABC)	3,19	1,54	6,60	**0,0017**	ni				ni			
COO Nanostring (Unclassified)	1,68	0,67	4,18	0,2623	-				-			
B cell state (S2-3-4)	2,57	0,88	7,54	0,0839	-				not used			
B cell state (S5)	5,01	1,61	15,60	**0,0054**	2,3	1,14	4,65	**0,0203**	not used			
Lymphoma Ecotype (LE1-2-3)	2,15	1,52	8,90	**0,0103**	not used				2,13	1,08	4,18	**0,0246**
Lymphoma Ecotype (LE4-5-6)	1,76	0,75	6,52	0,1503	not used				-			
Lymphoma Ecotype (LE7-8)	1,52	0,62	4,92	0,3452	not used				-			

Notes and abbreviations: R-IPI, Revised international prognostic index; COO, cell of origin according to Lymph2Cx; B cell state as identified by EcoTyper. EFS, event free survival from diagnosis; HR, Hazard Ratio; CI, confidence interval; LCI, 95% lower CI; UCI, 95% Upper CI; -: not used in the final model; n.i.: not included in the final model.

B cell states refine R-IPI risk stratification





- B cell states further stratifies R-IPI poor patients
- **S1 state identifies a subgroup with an outcome comparable to R-IPI very good/good cases**
- **S5 state identifies the cases with the worst outcome**

Conclusions

- COO classification (Lymph2Cx/Hans' algorithm/RNAseq) and BCL2 over-expression have prognostic significance
- IGHV can be reconstructed from RNASeq overcoming standard primer-based Ig sequencing approaches
- IGHV4-34 overexpressed in ABC supports the idea that ABC might rely on antigen-dependent BCR signalling driven by self-antigens
- Mutational analysis can be obtained from RNASeq on FFPE samples and indicates different survival outcomes
- Different states (B cells and TME-cells) and their co-association refine DLBCL risk stratification

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