Molecular Tools in the Diagnosis of Lymphoma

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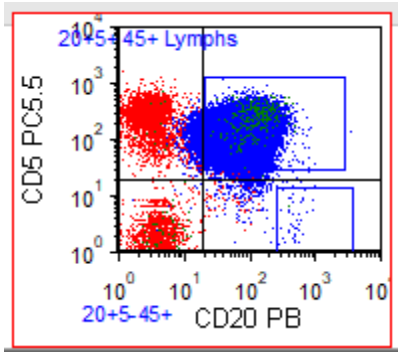
Learning Objectives

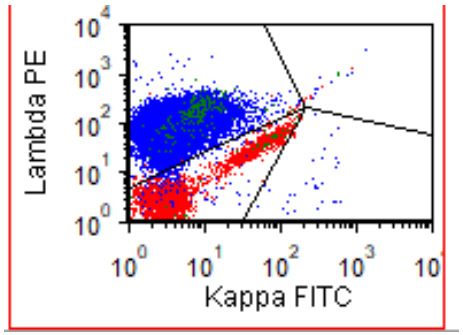
- To be familiar with appropriate work-up for anaplastic large cell lymphoma
- To understand when it is appropriate to use molecular clonality testing in the work up and diagnosis of lymphoma
- To be familiar with the limitations and “pitfalls” of clonality testing
- To know how and when to use the NGS CLL panel in your work-up, diagnosis and prognostication
- To know when to use MYD88 molecular testing in the work-up of suspected lymphoma, and to understand its limitations

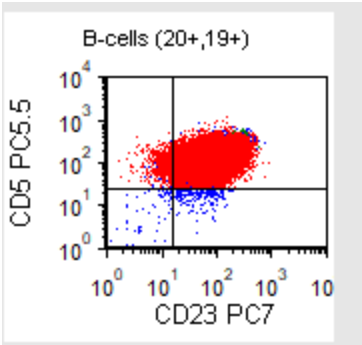
Case-Based Approach

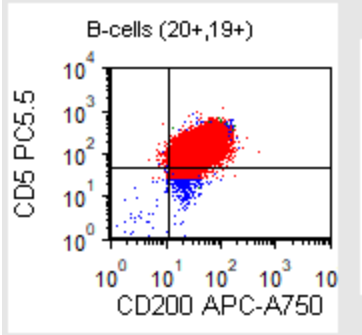
Case #1

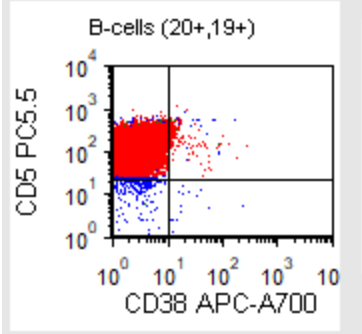
- A 73-year-old male initially presented with mild lymphocytosis and lymphadenopathy in the abdomen and pelvis.
- A left axillary lymph node biopsy was reviewed… (phenotype similar to this peripheral blood flow cytometry:)











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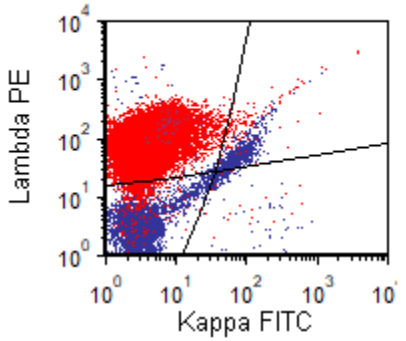
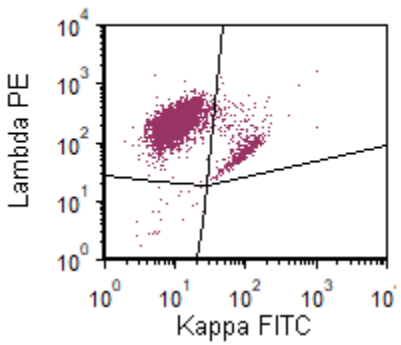


Gate	# of Events	% of all cells	% of gated cells	X Geometric Mean	Y Geometric Mean
UL	185042	63.10	89.33	3.90	79.87
UR	4998	1.70	2.41	82.90	58.25
LL	15702	5.35	7.58	3.39	3.94
LR	1395	0.48	0.67	56.28	16.90

G



Gate	# of Events	% of all cells	% of gated cells	X Geometric Mean	Y Geometric Mean
UL	11867	4.05	84.66	9.97	203.59
UR	2092	0.71	14.92	92.63	88.41
LL	55	0.02	0.39	7.22	5.89
LR	4	0.00	0.03	58.56	11.22

Diagnosis:

- Chronic lymphocytic leukemia/ small lymphocytic lymphoma

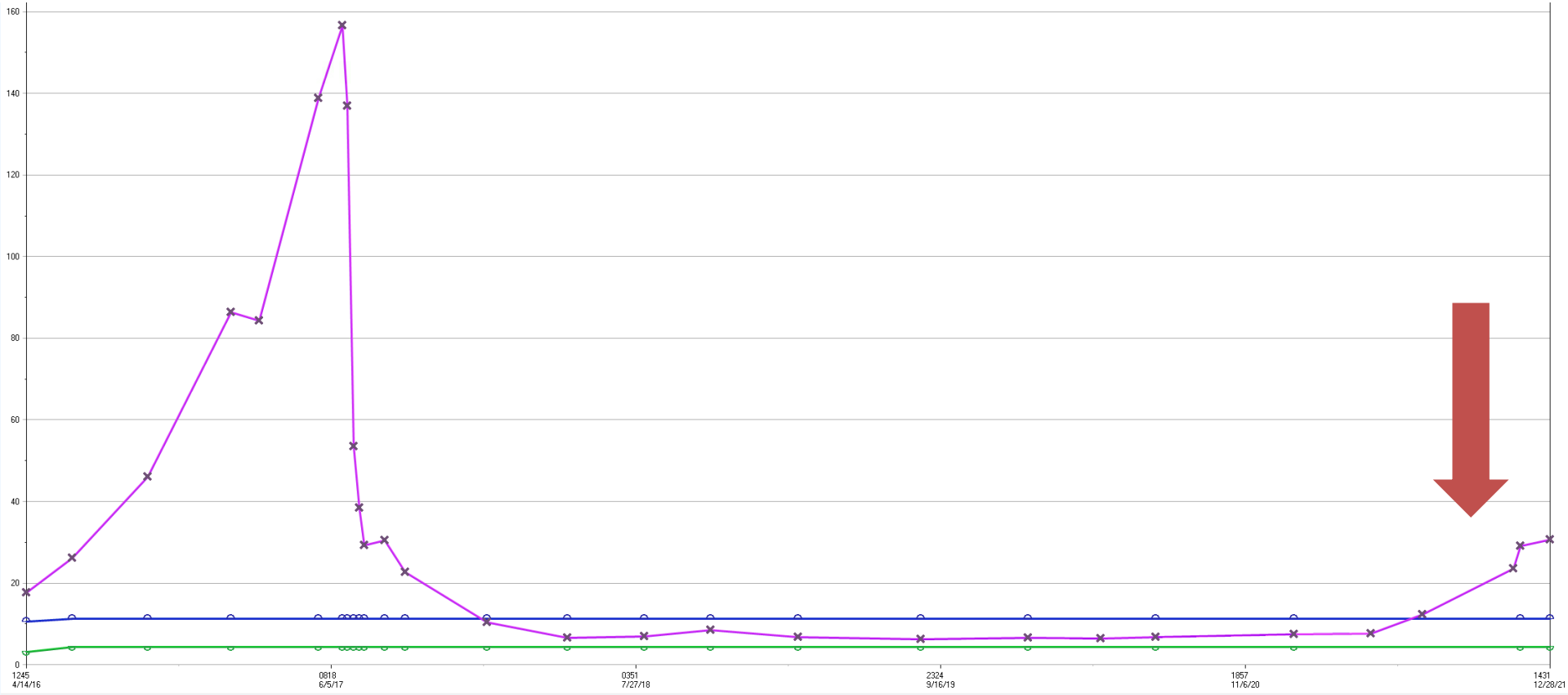
- Ancillary studies:
  - Unmutated IGHV
  - Complex cytogenetics with +12

CLL/SLL Prognostication from NCCN Guidelines

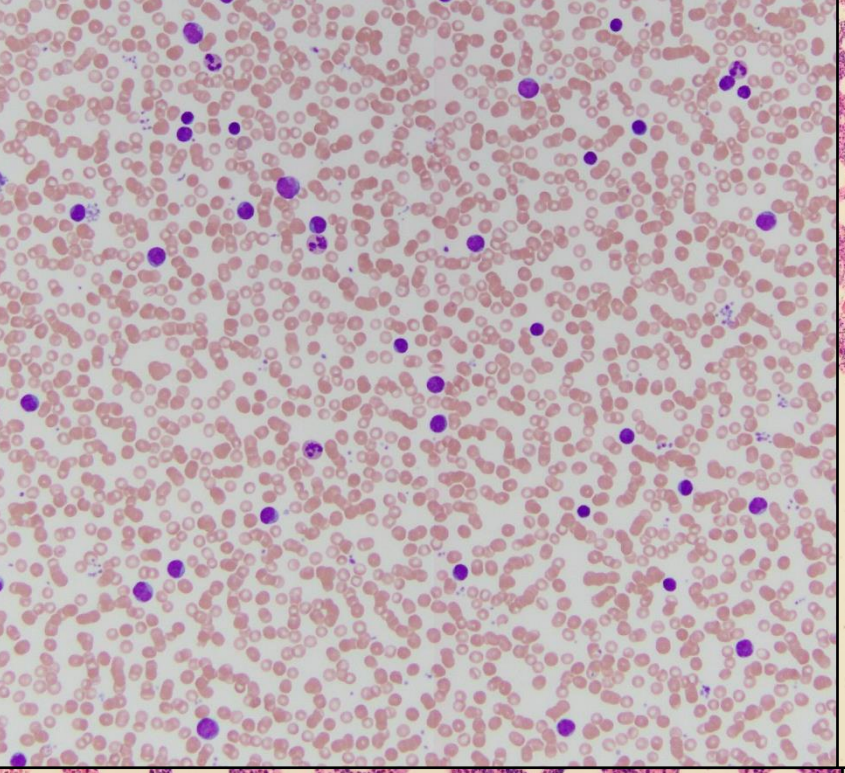
Karyotype/FISH	Del (17p)	Unfavorable
	Del (11q)	Unfavorable
	Complex (>3 abn)	Unfavorable
	Trisomy 12	Intermediate
	Normal	Intermediate
	Del(13q) sole abnl.	Favorable
Molecular	TP53 mutation	Unfavorable
	IGHV unmutated (<2% mutated)	Unfavorable*

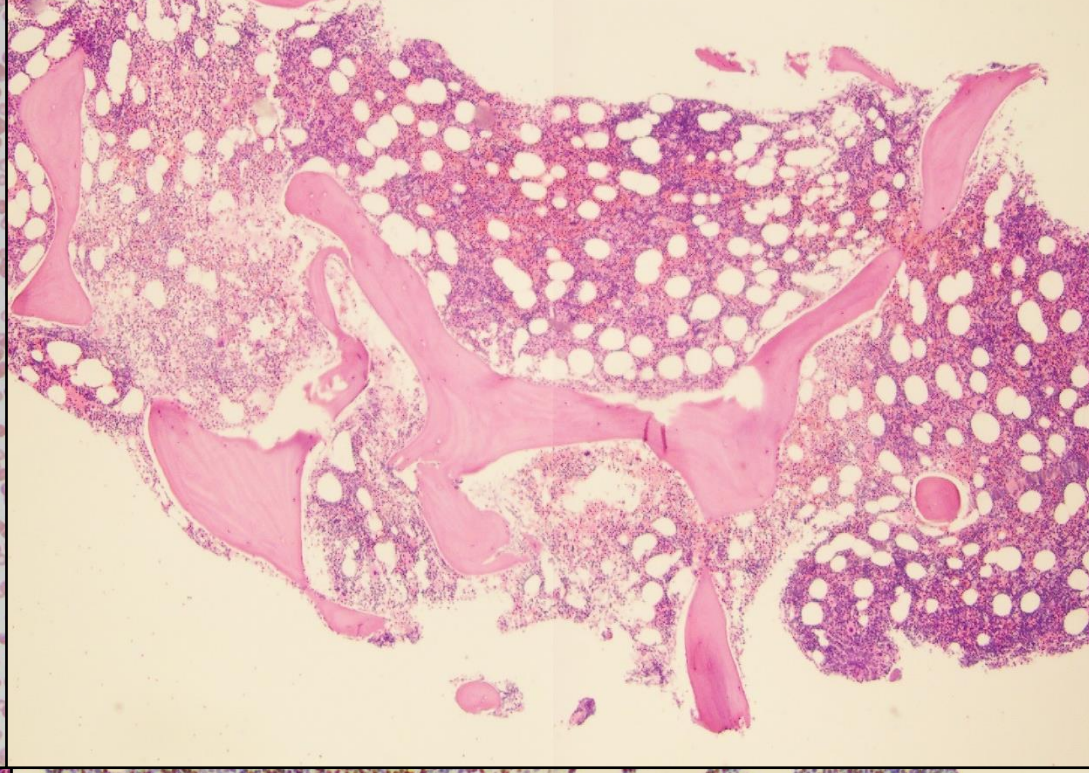
*Rearrangements involving VH3-21 have poor prognosis even if mutated.

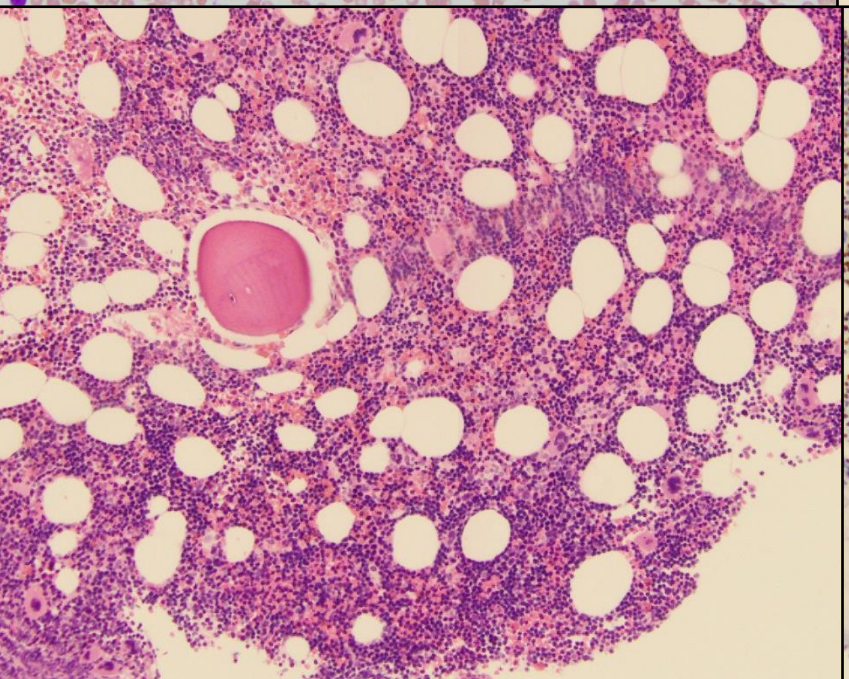
- Patient was started on ibrutinib with excellent response for three years.
- WBC showed the following:

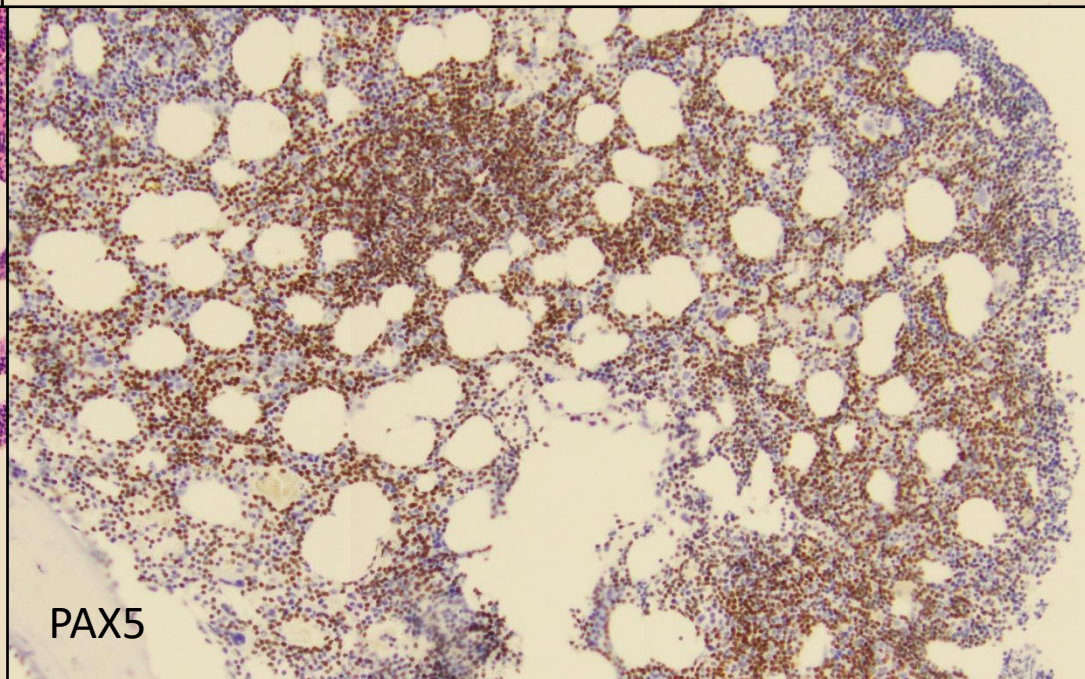


- Findings suggest progression of disease
- Bone marrow biopsy showed the following:








PAX5

NGS testing showed the following…

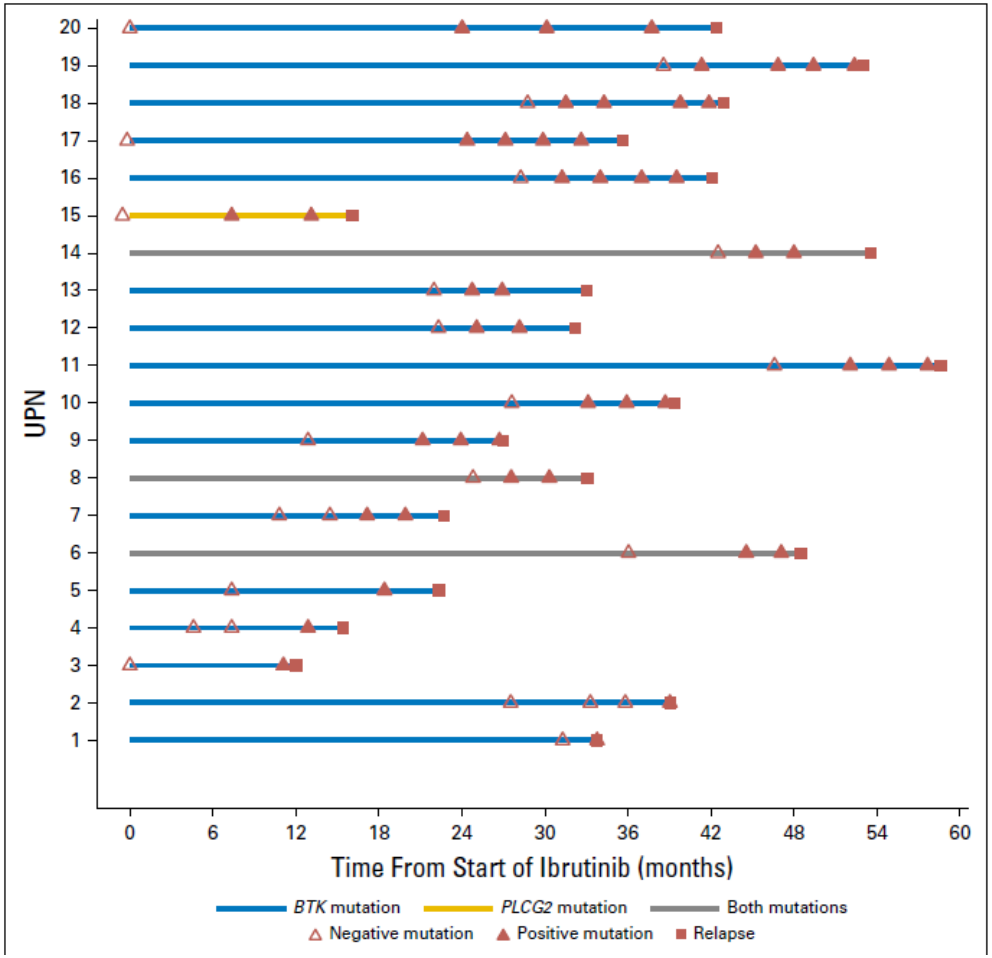
CLL NGS panel results

- Tier 1 variants
  1. BTK c.1441T>A, p.Cys481Ser
     VAF 52.8%
  2. BTK c.1442_1443delinsCT, p.Cys481Ser
     VAF 5.4%
  3. BTK c.1442G>A, p.Cys481Tyr
     VAF 5.2%
  4. BTK c.1442G>C, p.CYs481Ser
     VAF 3.7%
  5. RPS15 c.413C>T, p.Ser138Phe
     VAF 32.4%
  6. MED12 c.130G>A, p.Gly44Ser
     VAF 66.9%


chrX
chrX:100,610,915-100,611,415
Go
p22.33 p22.31 p22.2 p22.13 p22.11 p21.3 p21.2 p21.1 p11.4 p11.3 p11.23 p11.22 p11.21 p11.1 q11.2 q13.1 q13.2 q21.1 q21.2 q21.31 q21.32 q21.33 q22.1 q22.2 q22.3 q23 q24 q25 q26.1 q26.3 q27.1 q27.3
500 bp
100,611,000 bp
100,611,100 bp
100,611,200 bp
100,611,300 bp
NM_000061.2
[0 - 833]
BTK

BTK resistance after ibrutinib

- Acquired resistance usually involves *BTK or PLCG2*
- Mutations can be detected at median of 9 mos up to 15 mos before clinical progression



Woyach et al. 2017. J Clin Oncol 35: 1437-1443.

- Due to ibrutinib and acalabrutinib resistance, the patient will enroll in a clinical trial
  - Selective PKC-B inhibitor
  - Rituximab/venetoclax is another alternative for those with BTKi resistance mutations

NGS CLL Panel

- When should you use it?
  - May be more useful when relapsing rather than at diagnosis
- What kind of information can it give you?
  - Mutations that indicate drug resistance
  - Some prognostic indicators
  - A few genes are included for other lymphomas

- Genes tested: *ATM, BCL2, BIRC3*, BRAF, BTG1, BTK, CARD11, CD79B, CXCR4, DDX3X, FBXW7, IKZF3, KRAS, MAP2K1, MED12, MGA, MYD88, NOTCH1, NRAS, PLCG2, POT1, RPS15*, SAMHD1, SF3B1, TP53, XPO1, ZMYM3*

CLL Prognostication/ Response to Therapy

Gene	Mutation	Incidence (CLL)	Effect
BTK	C481S	Up to 80% of relapsed; rare in tx-naive	Resistance to BTKi
	Germline		X-linked agammaglobulinemia (XLA)
BIRC3	various	2-8% at dx; 4-25% of relapsed	Higher incidence in relapsed/refractory CLL
NOTCH1	P2514fs	5-22%	Poor prognosis, progression, tx resistance
PLCG2	Various	80-85% of progressive/ relapsed	Often with BTK mutation; Unclear whether independently confers resistance to BTKi
POT1	Germline		Familial CLL
TP53	Missense mutations in DNA binding domain	5-14%	Poor response to tx, progression, shorter OS
BCL2	G101V	rare	Resistance to venetoclax

Genes Useful in Dx other than CLL

- MYD88 and CXCR4
  - MYD88 L265P very common in Lymphoplasmacytic Lymphoma (also some DLBCL – not entirely specific)
    - Helpful when DDx with other small B-cell lymphomas
  - CXCR4 seen in 30-40% of LPL cases
    - Germline -> WHIM syndrome
    - Somatic nonsense/frameshift mutations eliminate Ser339 ->
      - Resistance to BTK inhibitor therapy

- BRAF V600E
  - For heme malignancies, specific for Hairy Cell Leukemia and Langerhans Cell Histiocytosis
  - (Non-V600E mutations seen in CLL)

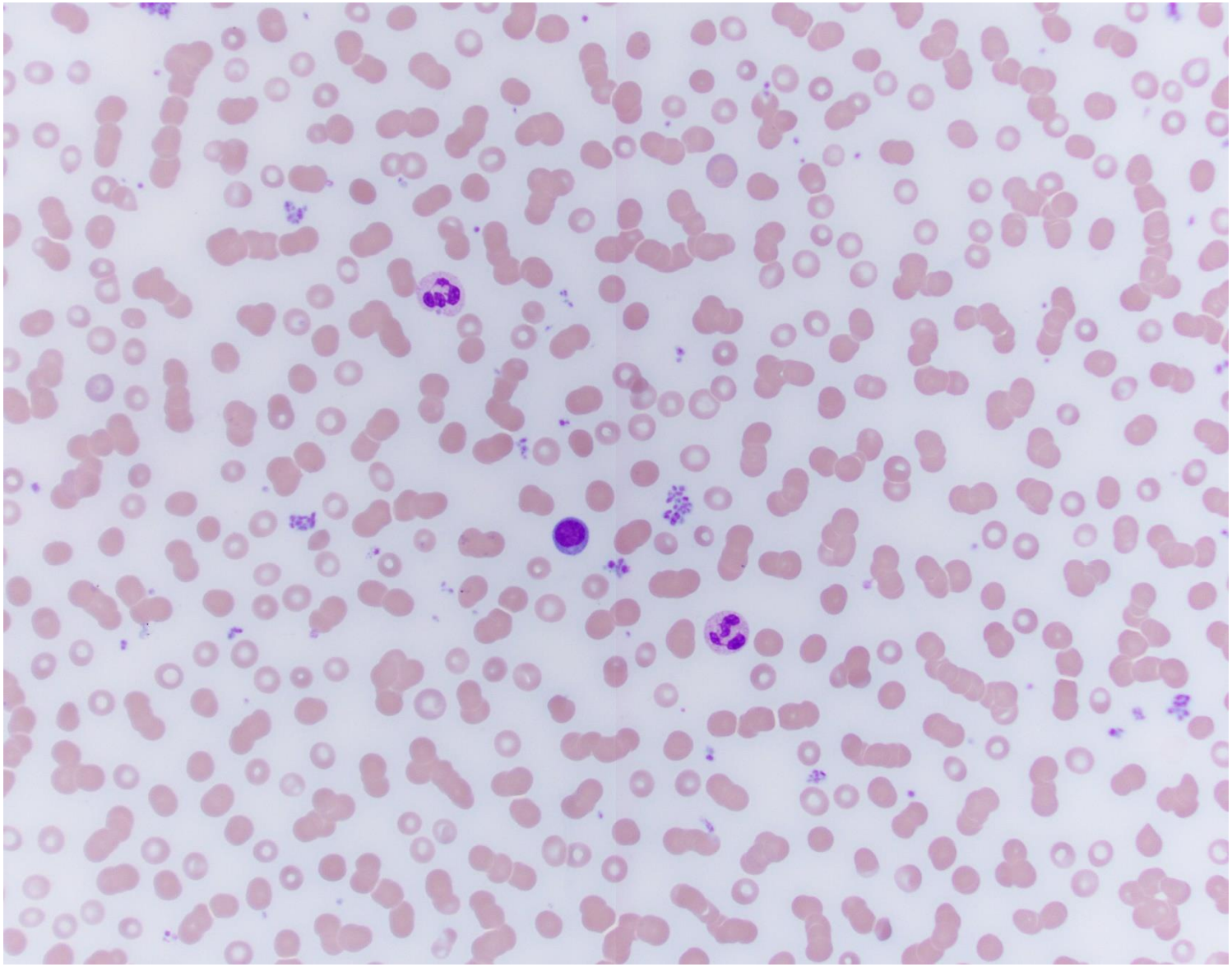
Case #2

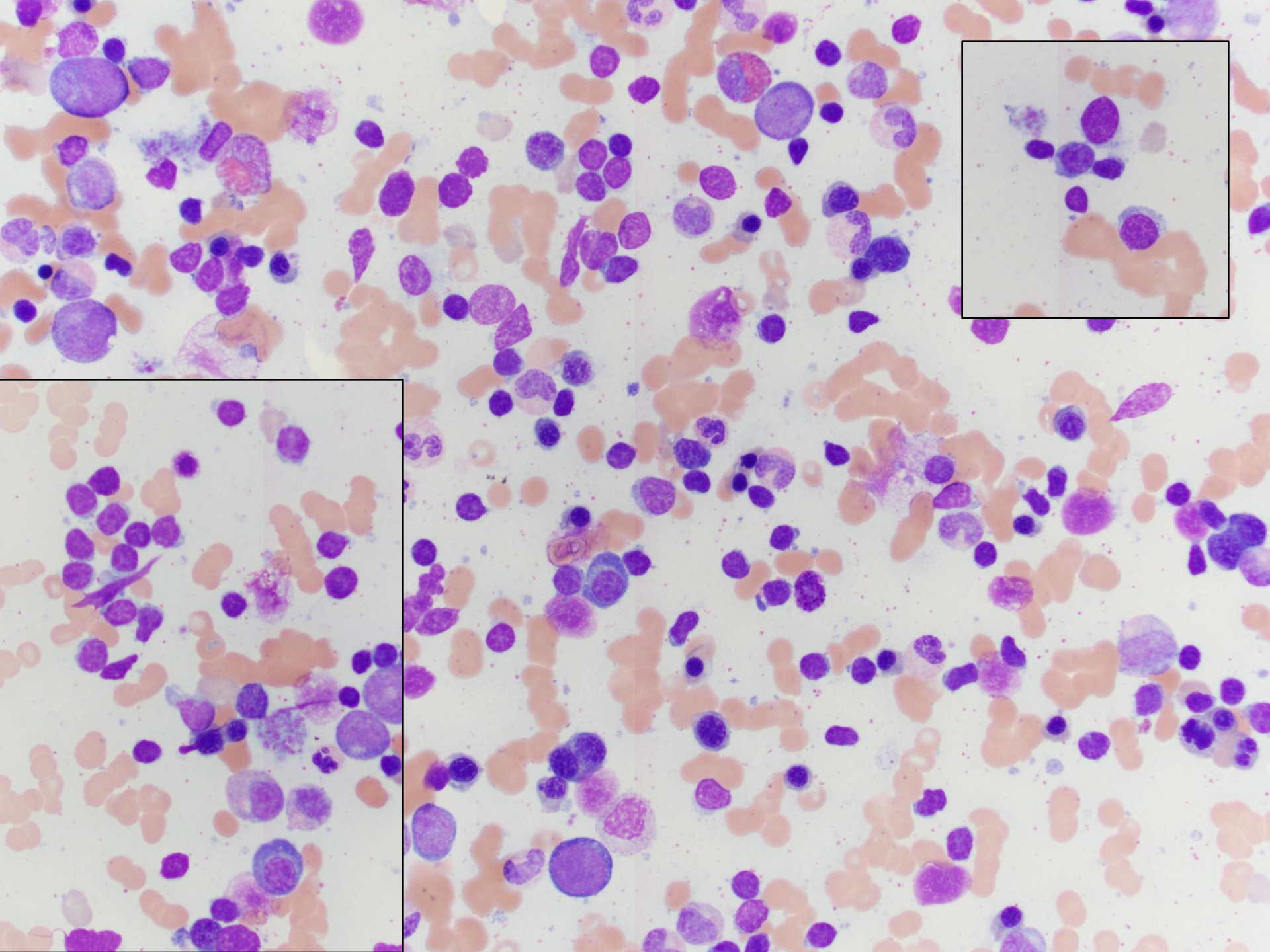
- 65-year-old male presents with abdominal pain that has been worsening over the past six months, and unintentional 15 lb. weight loss
- PET CT:
  - Multiple hypermetabolic lymph nodes throughout the neck, chest, abdomen and pelvis
  - Needle core biopsies are performed: (not shown)
    - Diagnosis: CD5 negative, CD10 negative low grade B-cell lymphoma with plasmacytic differentiation

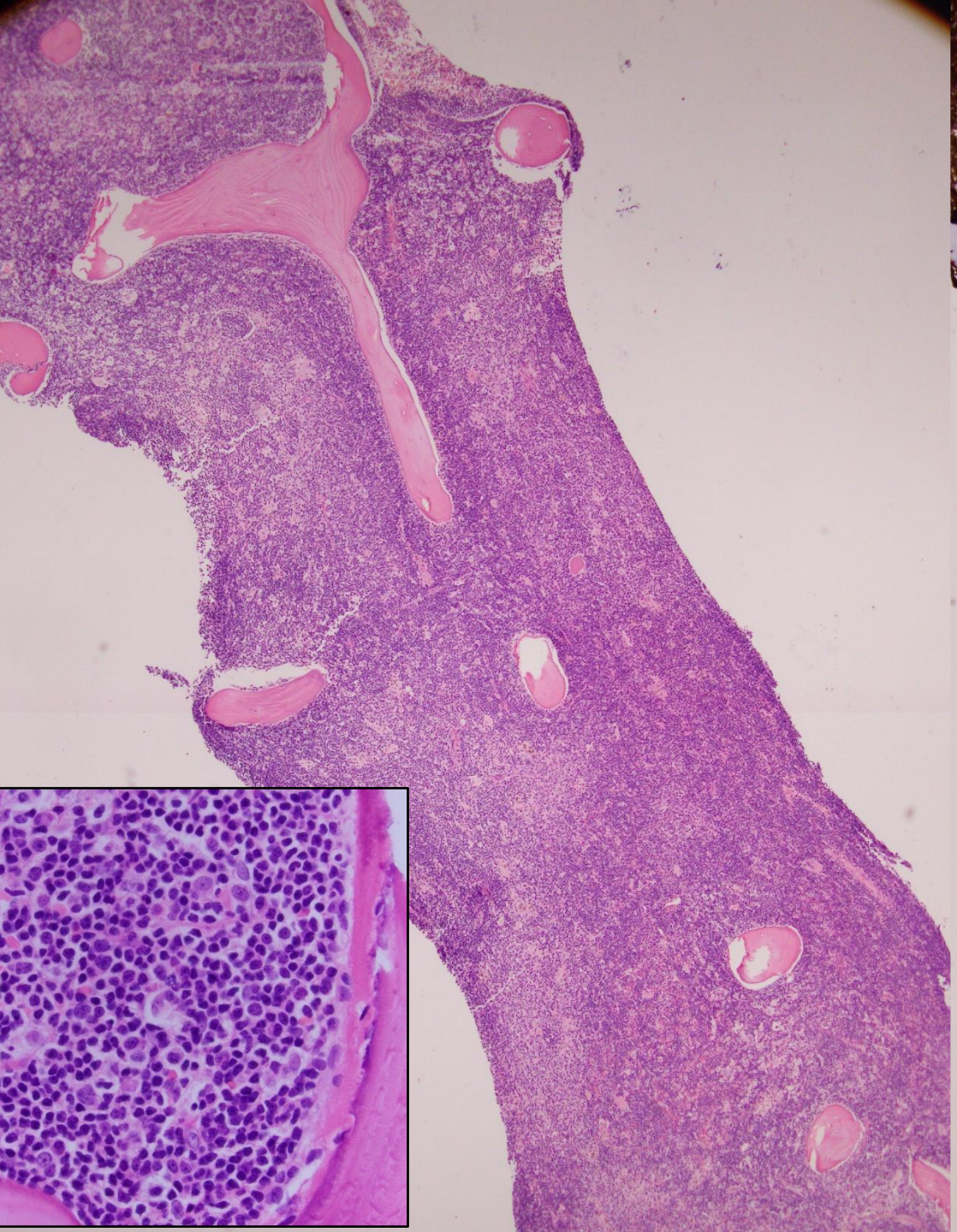
Other studies

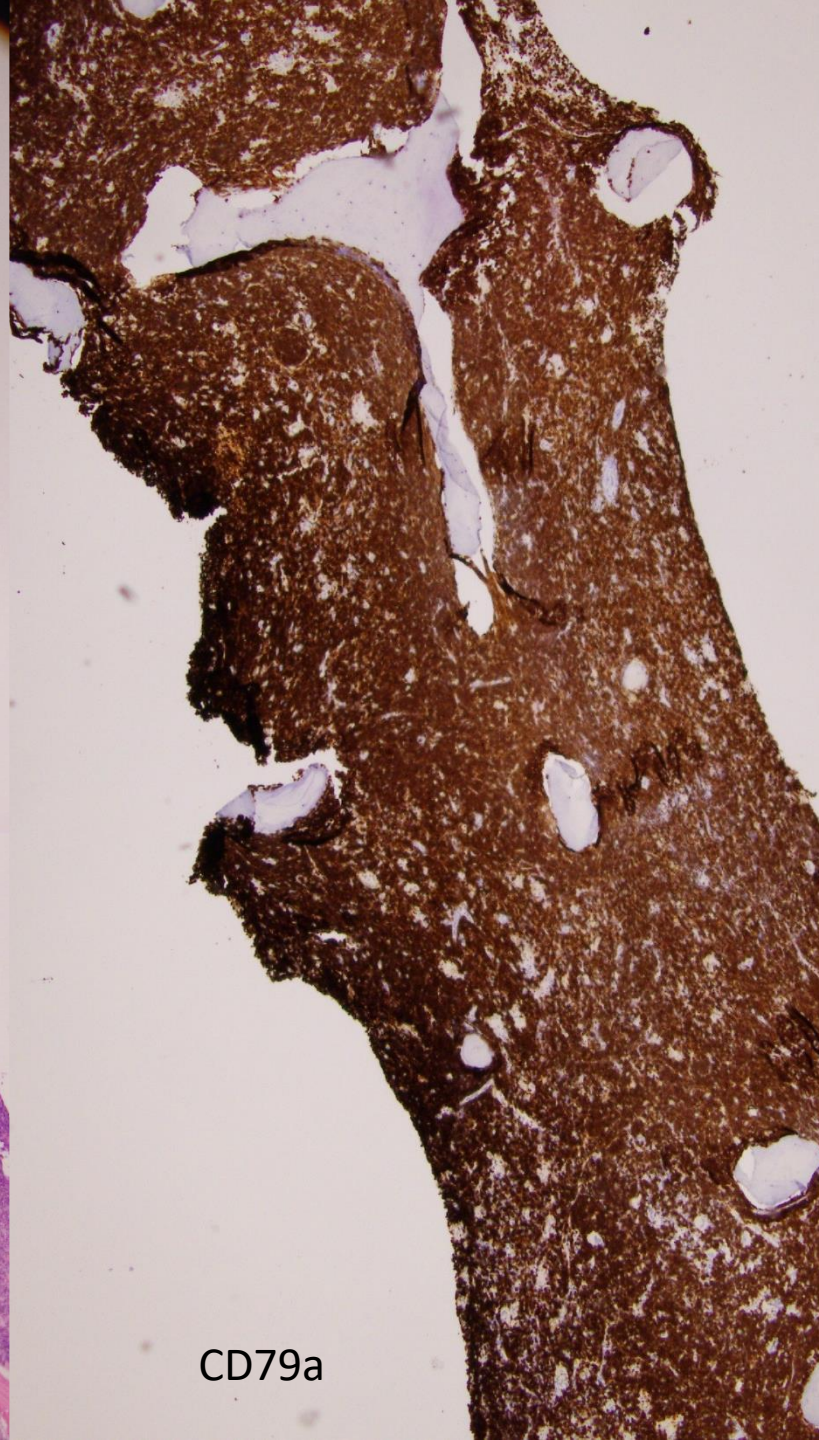
- CBC
  - Mild N/N anemia (Hgb 11.0 g/dl)
  - Normal WBC with normal diff and platelet counts

- SPE/IFE:
  - M-spike in the gamma region. 1.64 g/dl – IgM kappa









CD79a

- MYD88 L265P: Detected

- Diagnosis?

MYD88 L265P specificity

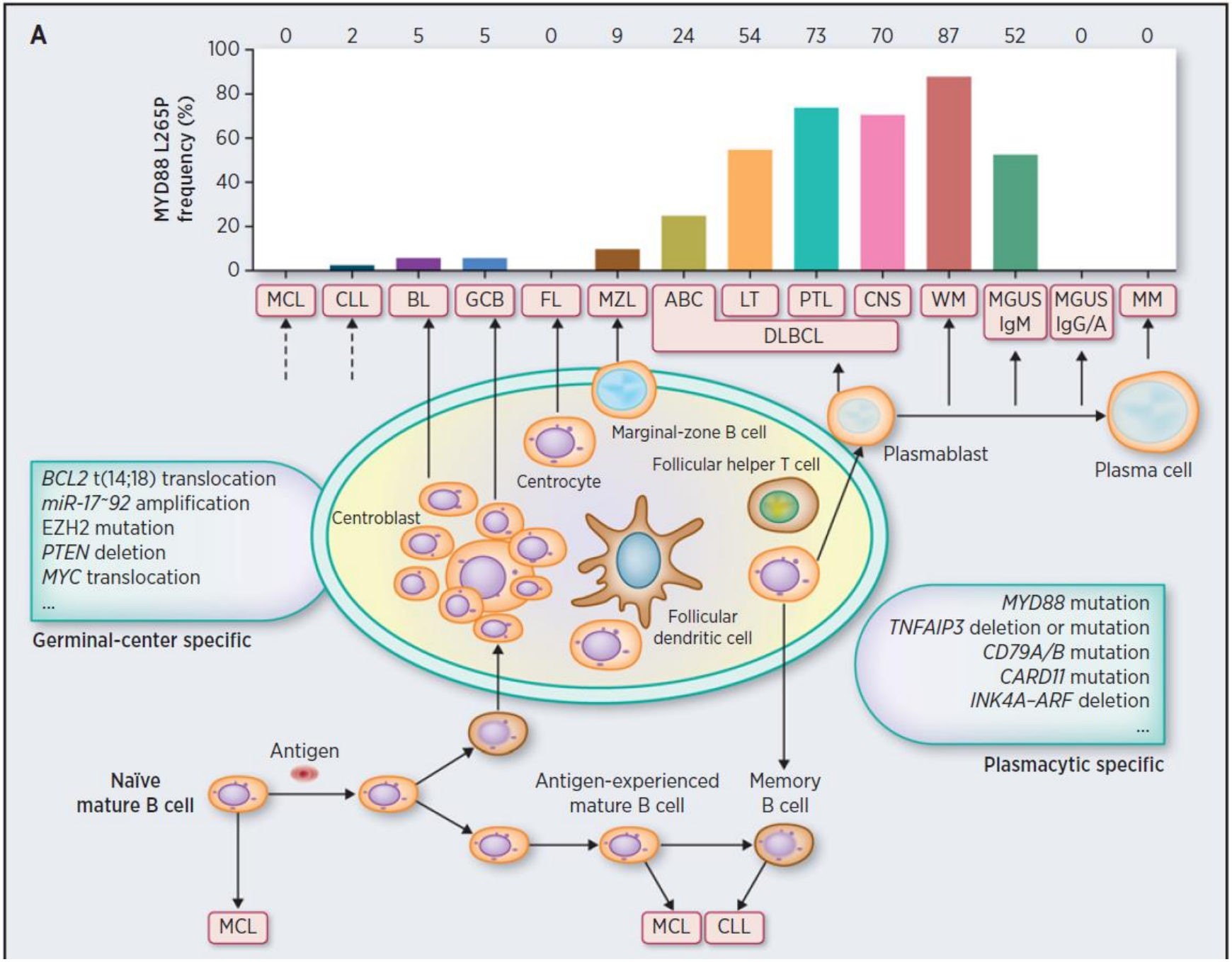
Table 1. Selected Prior Studies of *MYD88* Mutation Prevalence in Key Subtypes of Low-Grade B-Cell Lymphoma

Source, y	Assay Methodology	LPL, WM, or Both, %	MZL,[a] %	CLL/SLL, %
Puente et al,[13] 2011	NGS			3
Wang et al,[14] 2011	NGS			10
Li et al,[15] 2012	Sanger		6	
Treon et al,[6] 2012	Sanger	91	7	
Gachard et al,[16] 2013	RE digestion	67	4	
Xu et al,[28] 2013	AS-PCR	93	10	4
Varettoni et al,[29] 2013	AS-PCR	100	6	
Ondrejka et al,[23] 2013	AS-PCR	100	8	0
Mori et al,[7] 2013	Sanger	30		
	RE digestion	74		
	AS-PCR	70		
Jiménez et al,[8] 2013	AS-PCR	80	21	0
Traverse-Glehen et al,[18] 2013	Sanger		5	
Tren et al,[17] 2013	SNaPshot/Sanger		4	
Poulain et al,[9] 2013	Sanger[b]	79	6	0
Ogura et al,[19] 2013	Sanger	78	0	0
Current study, 2015	Pyrosequencing	94	4	3

Abbreviations: AS-PCR, allele-specific polymerase chain reaction; CLL/SLL, chronic lymphocytic leukemia/small lymphocytic lymphoma; LPL, lymphoplasmacytic lymphoma; MZL, marginal zone lymphoma; NGS, next-generation sequencing; RE, restriction enzyme; WM, Waldenström macroglobulinemia.

[a] Various combinations of MZL subtypes were assessed in the different studies.
[b] Performed on purified B-cell population after immunomagnetic sorting.

Beltran et al 2015



Yu et al. Cancer Res 2018

Diagnosis

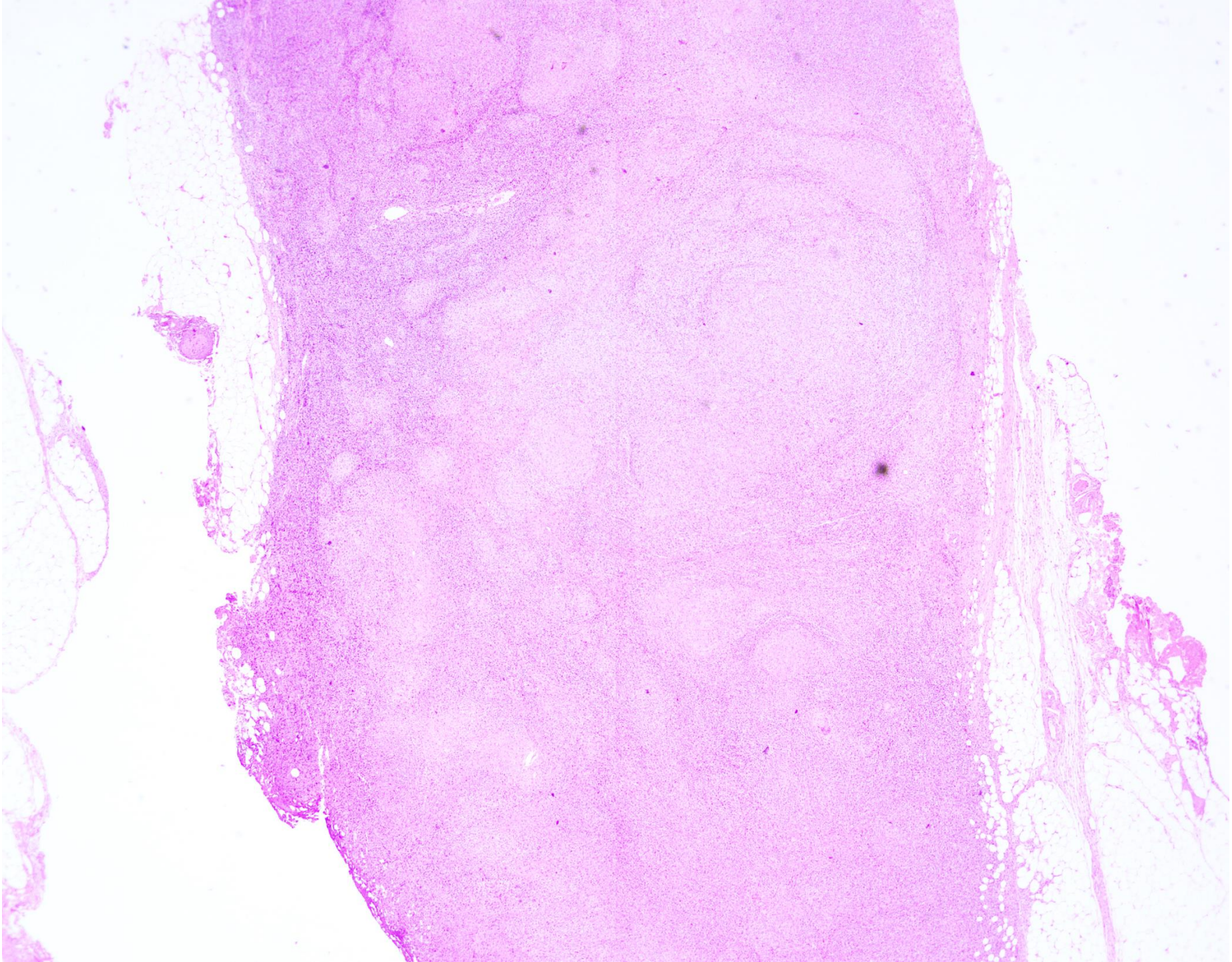
- Lymphoplasmacytic lymphoma
- (Waldenstrom macroglobulinemia)

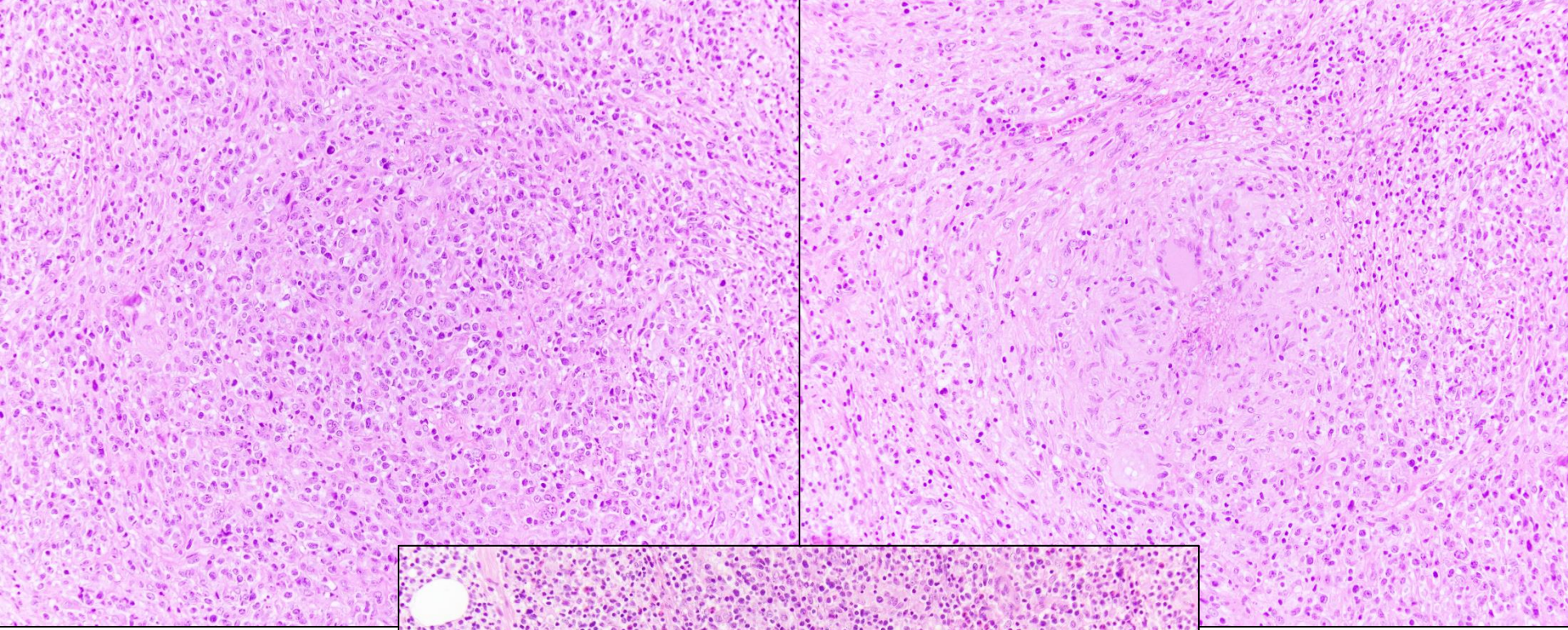
- Patient is currently being followed with observation
- Indications for treatment:
  - B symptoms
  - Threatened end organ function
  - Progressive bulky disease
  - Progressive anemia (Hgb <10 g/dl)
  - Progressive thrombocytopenia (plt <100K/ul)
  - Hyperviscosity
  - Peripheral neuropathy
  - Symptomatic cryoglobulinemia
  - Symptomatic cold agglutinin anemia
  - Autoimmune hemolytic anemia
  - Nephropathy or amyloidosis related to WM

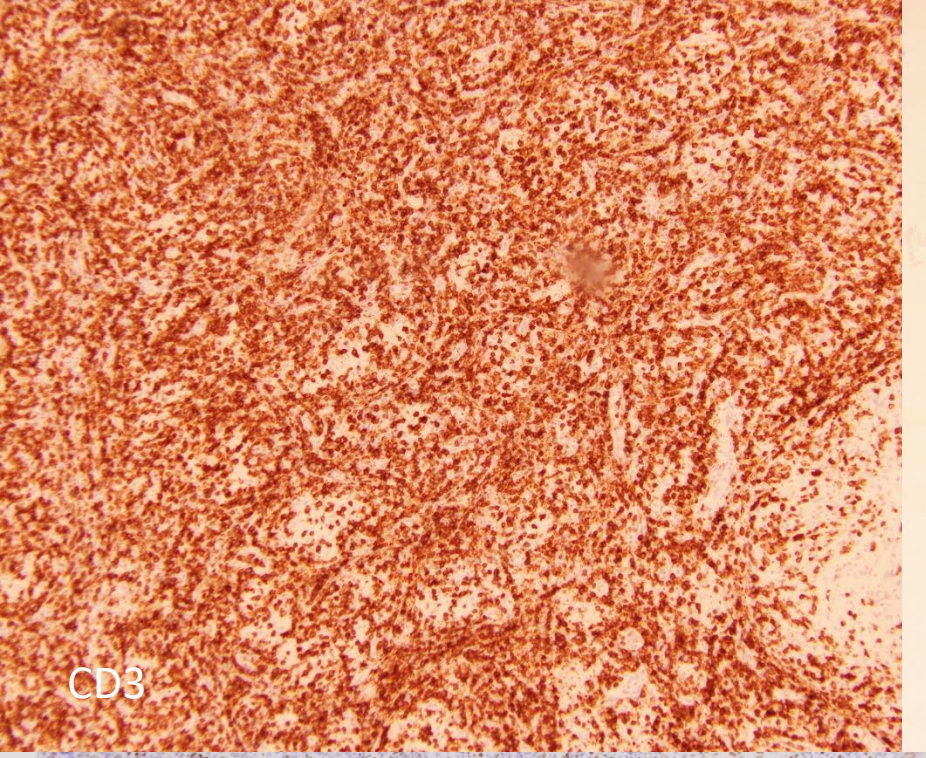
Case #3

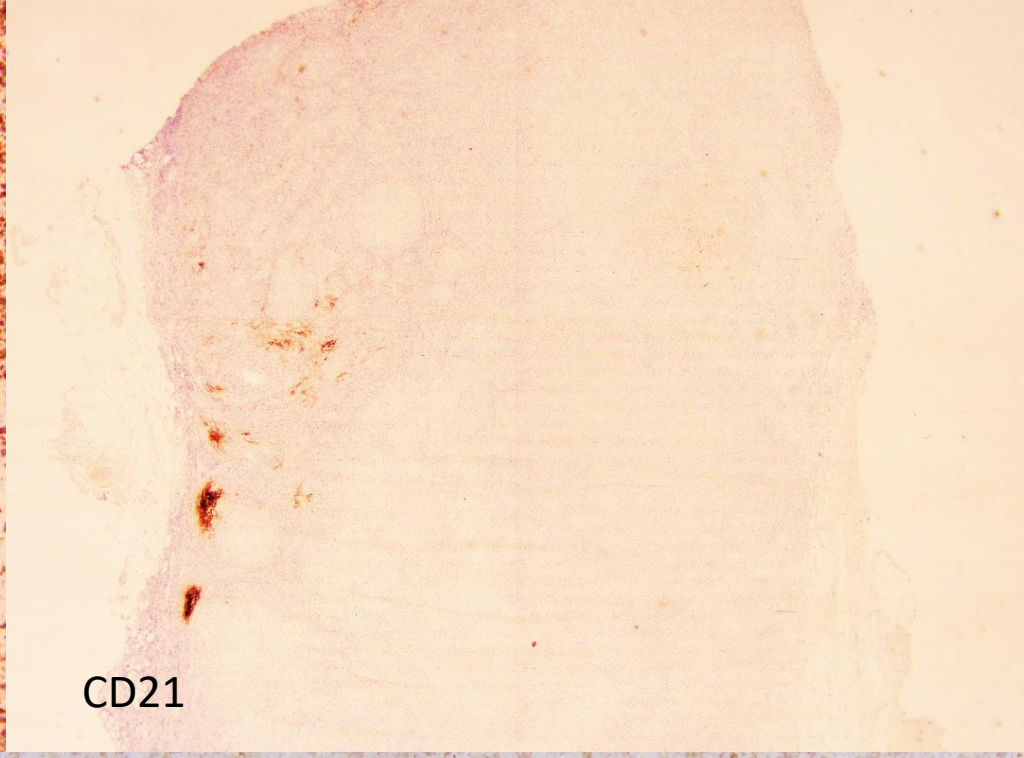
- 59-year-old female presented with bilateral neck and axillary lymphadenopathy, fever, night sweats and weight loss

- A mesenteric lymph node biopsy is performed:






CD3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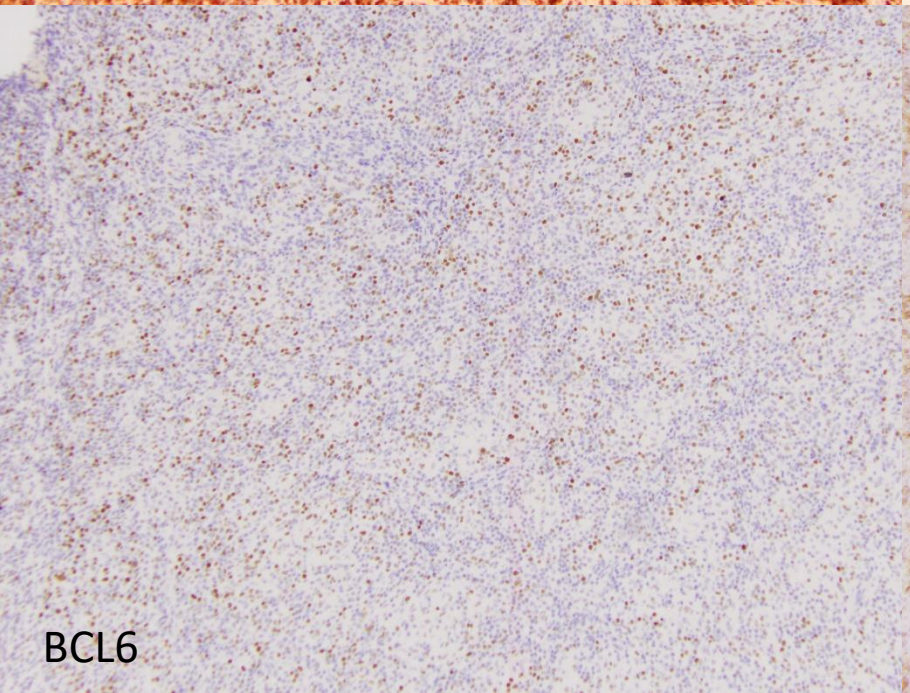
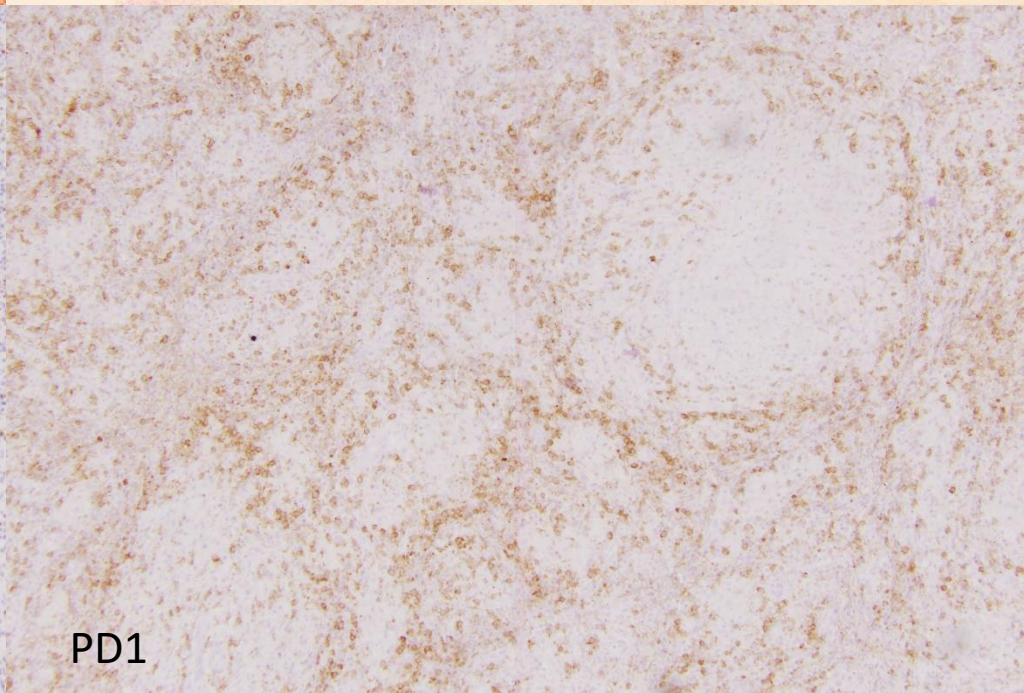

BCL6

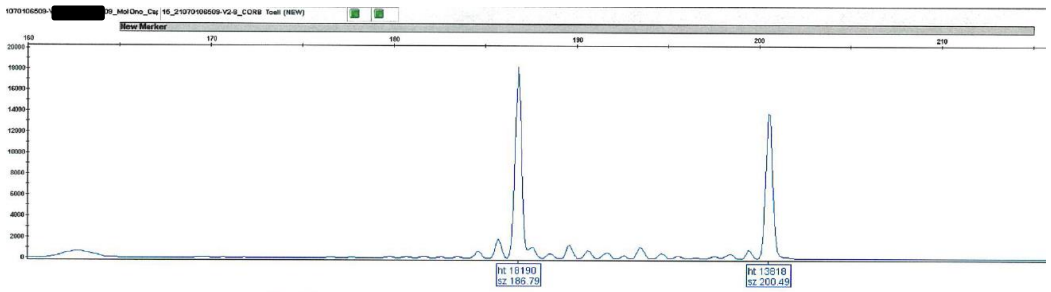

PD1

IHC Markers of TFH phenotype

- CD10
- BCL6
- PD1
- CXCL13
- ICOS

T-cell receptor gene rearrangement

V2-8



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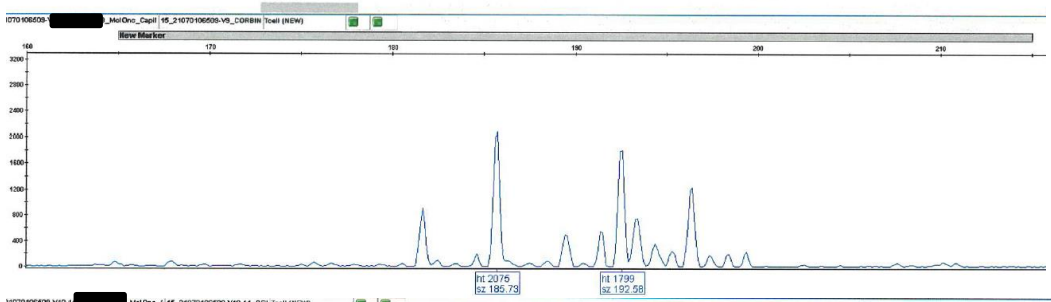
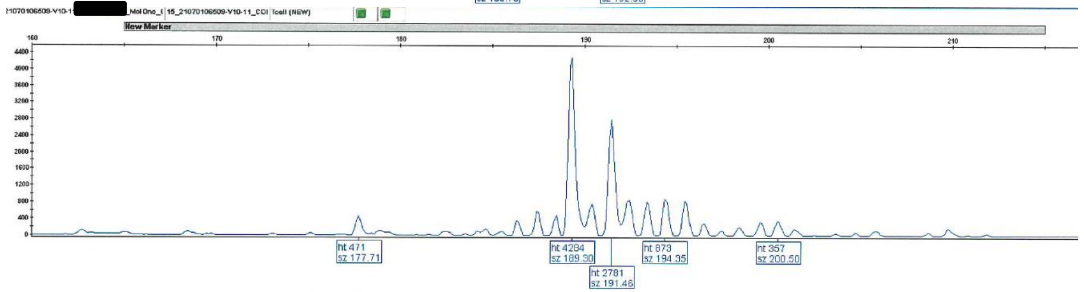
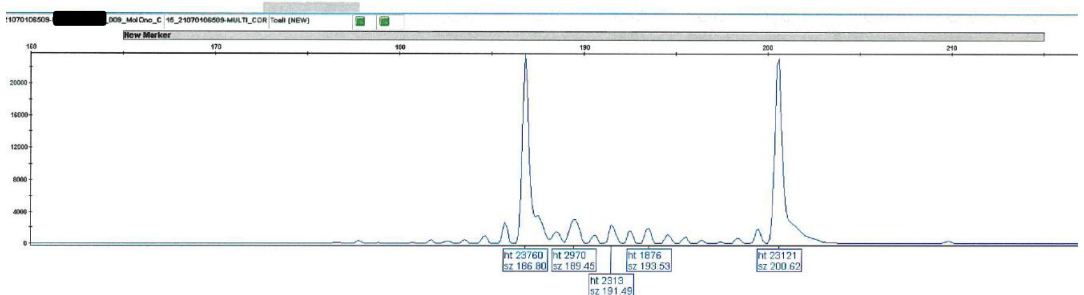
V9



V10-11



Multi



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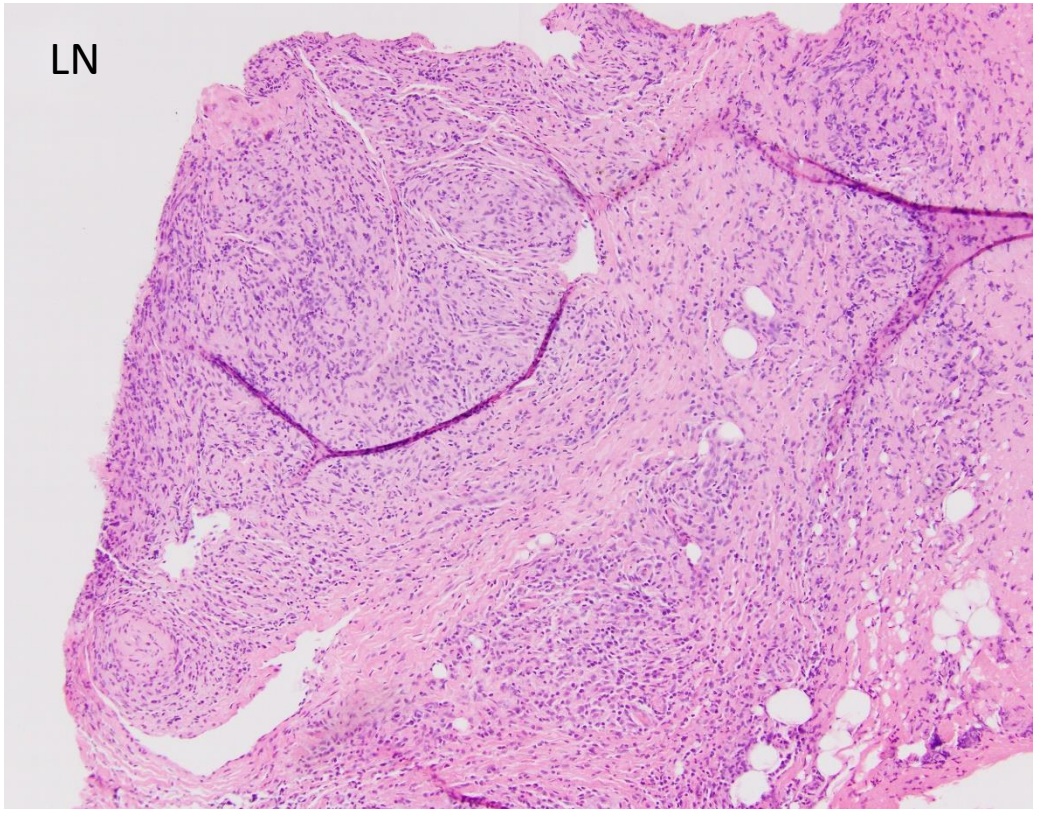
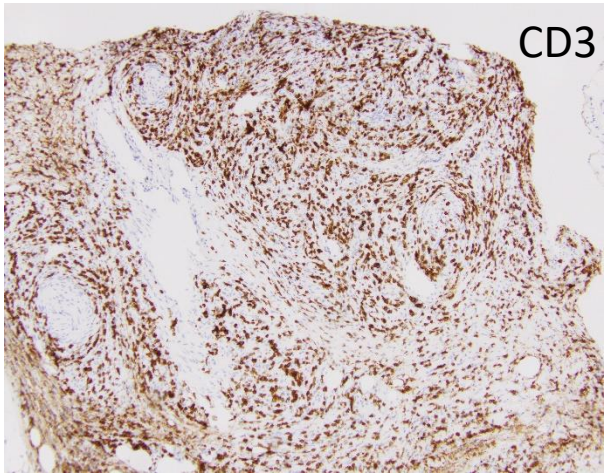
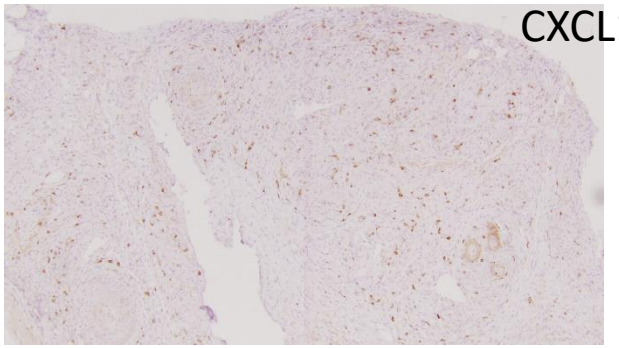
Diagnosis

- Lymph node, mesenteric:
  - Angioimmunoblastic T-cell lymphoma

2 months later PET CT showed progression with suspicious muscle findings

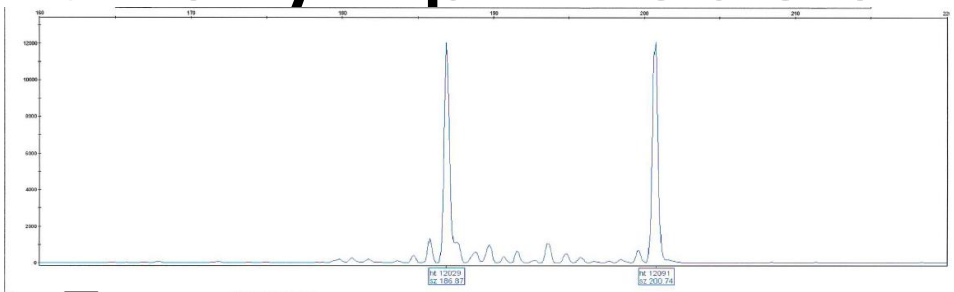






TCR on this lymph node showed:

V2-8



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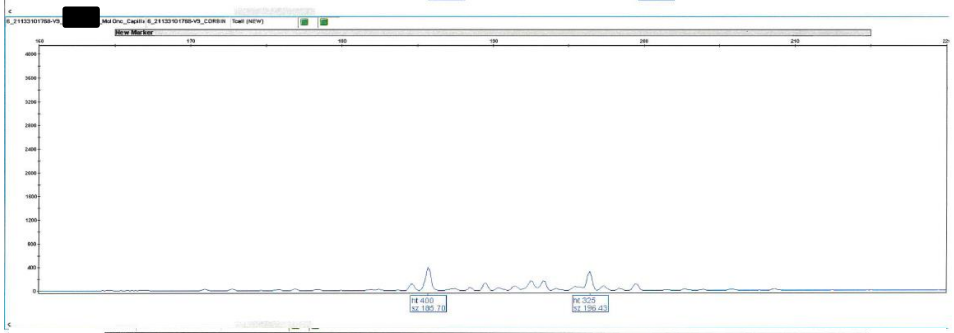
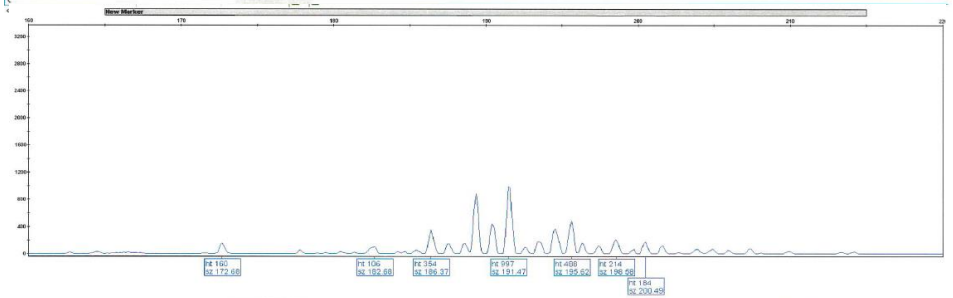
V9



V10-11



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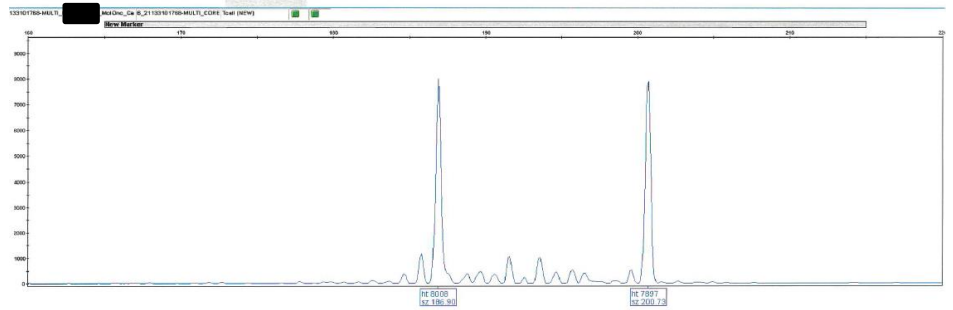

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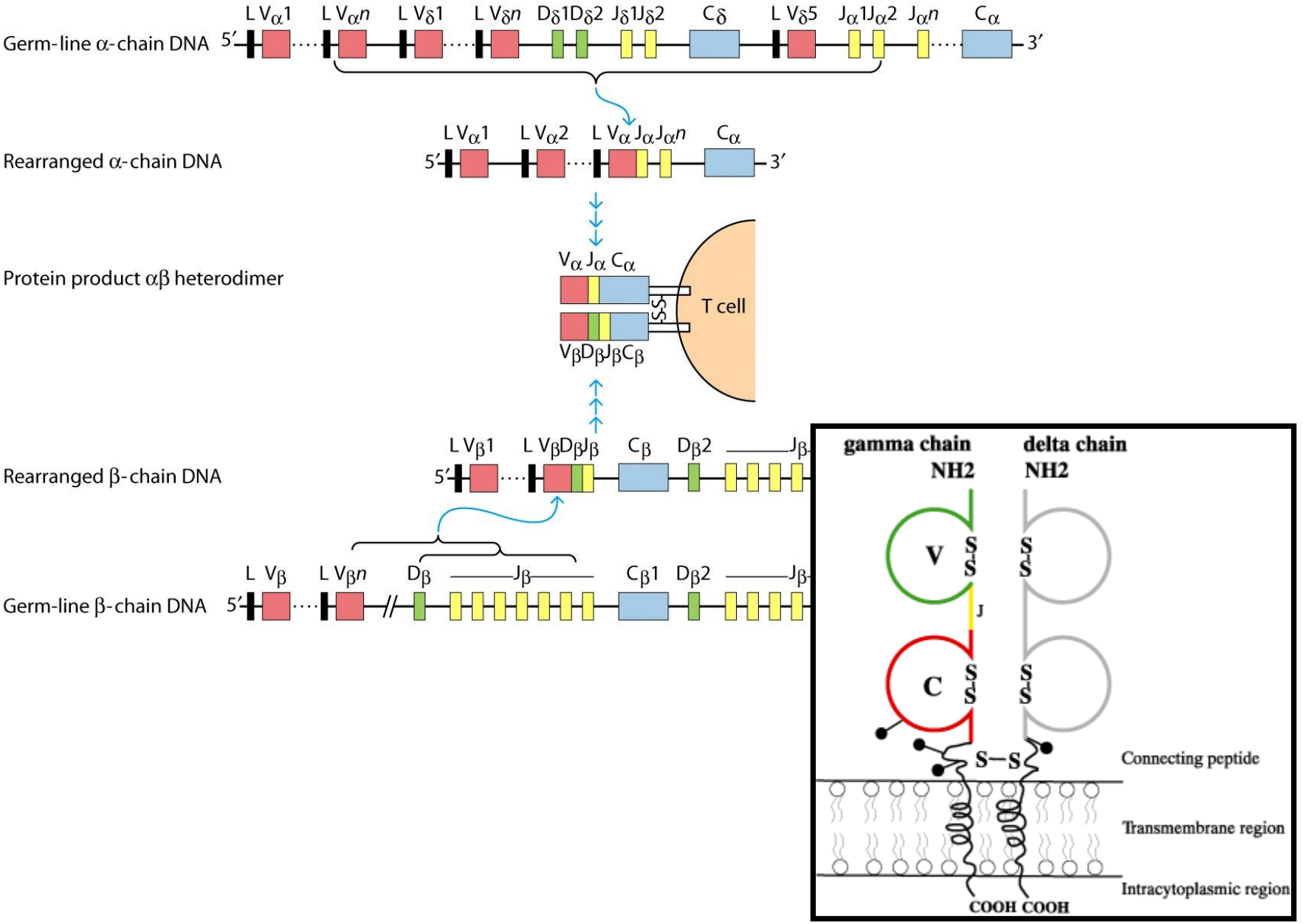
Clonality Testing

When to use T-cell clonality testing?

- There are MANY examples of clonal T-cell proliferations that are NOT neoplastic
- Still can be very helpful in tissues (lymph node, etc.) that look like a T-cell lymphoma, but more evidence/support is needed.



Imgt.org

www.sbs.utexas.edu

T-cell receptor rearrangement

- TRD -> TRG -> TRB -> TRA
- This happens in all T-cells, regardless of αβ or γδ expression
- Thus, all αβ T-cells (the most common subset) will have identifiable (but not expressed) TRG rearrangements

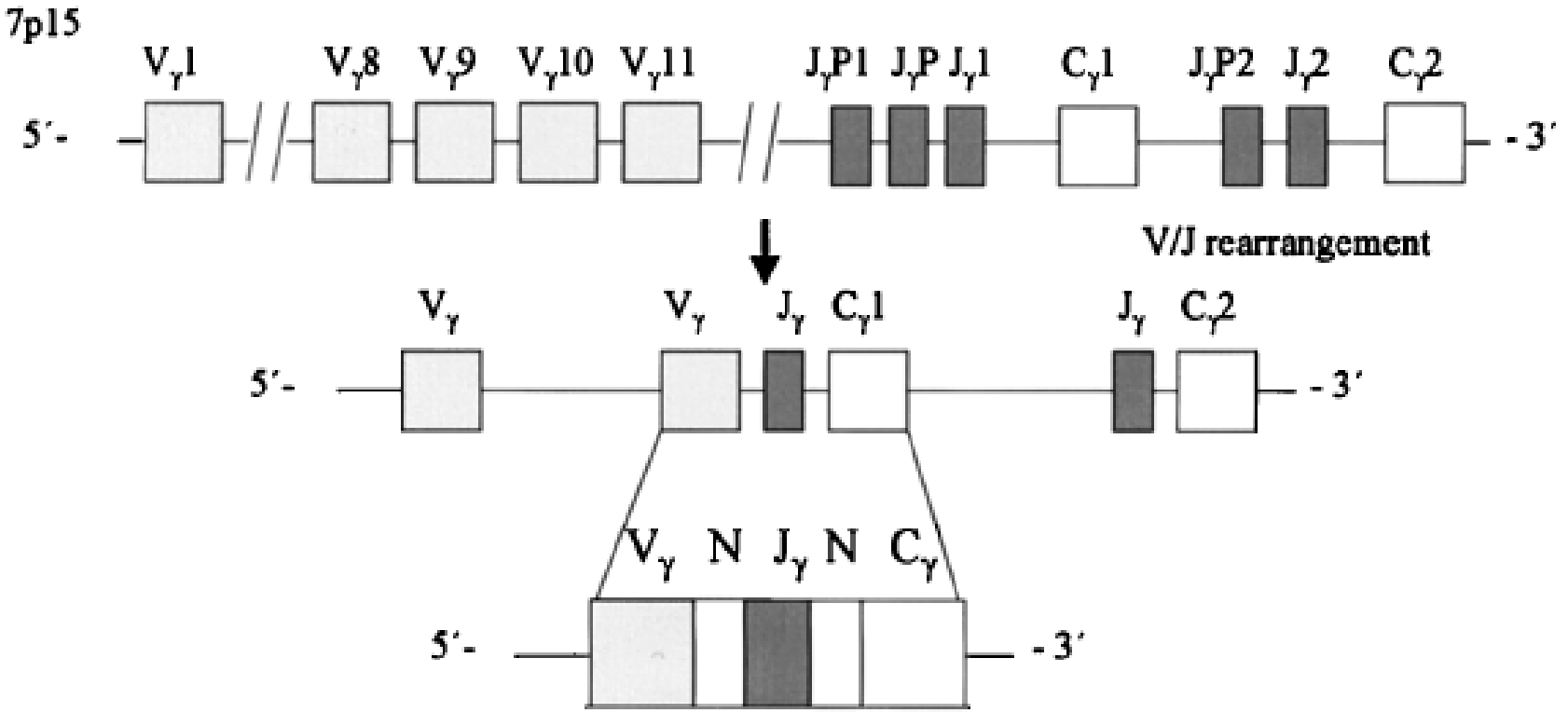
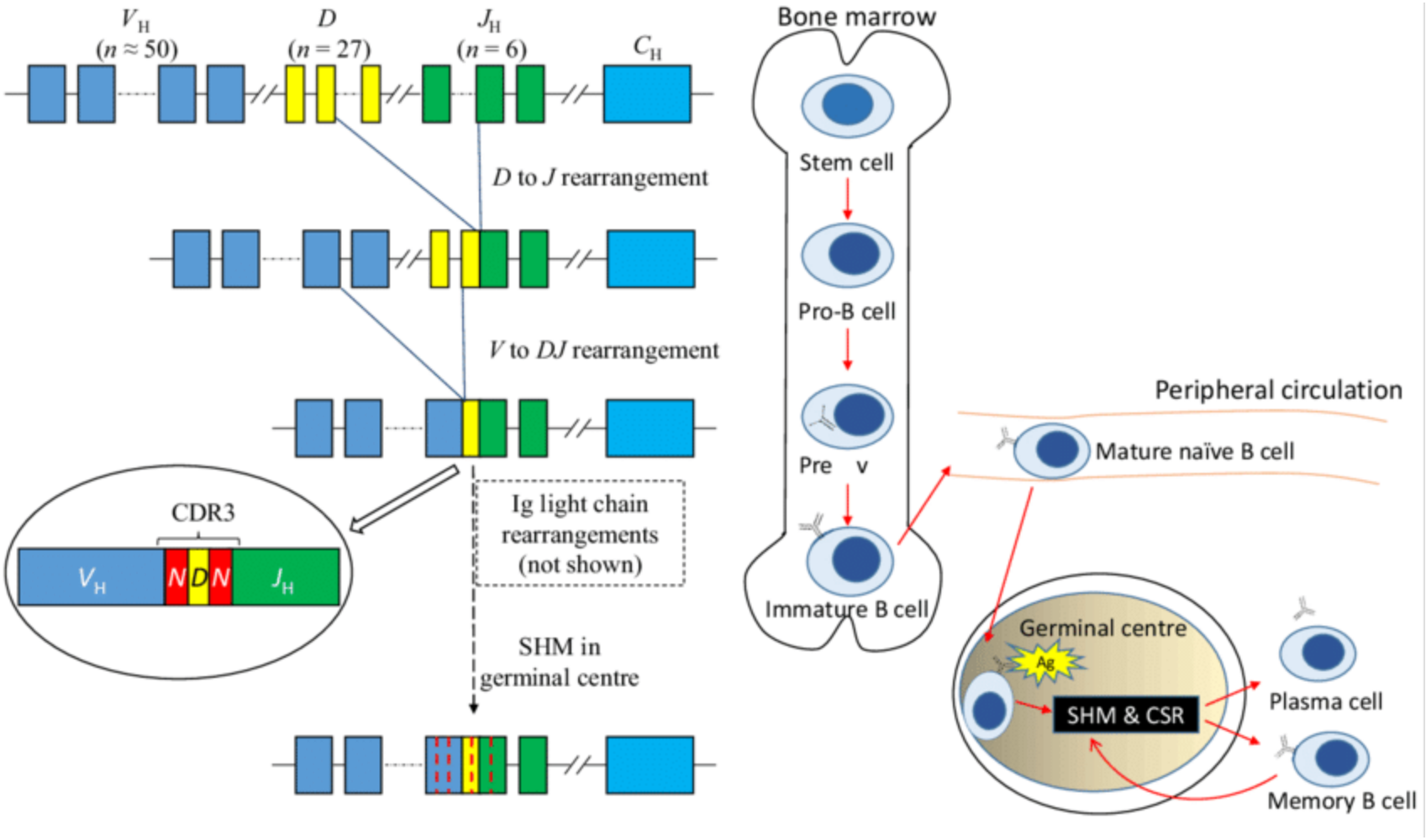


Figure 6. The T cell receptor γ chain locus on chromosome region 7p15 contains a limited number of variable and joining region genes that make it ideal for PCR amplification of the rearrangements.

Arber, JMD 2000

B-cell clonality testing operates under the same principles



BJH 2018; 181: 11-26.

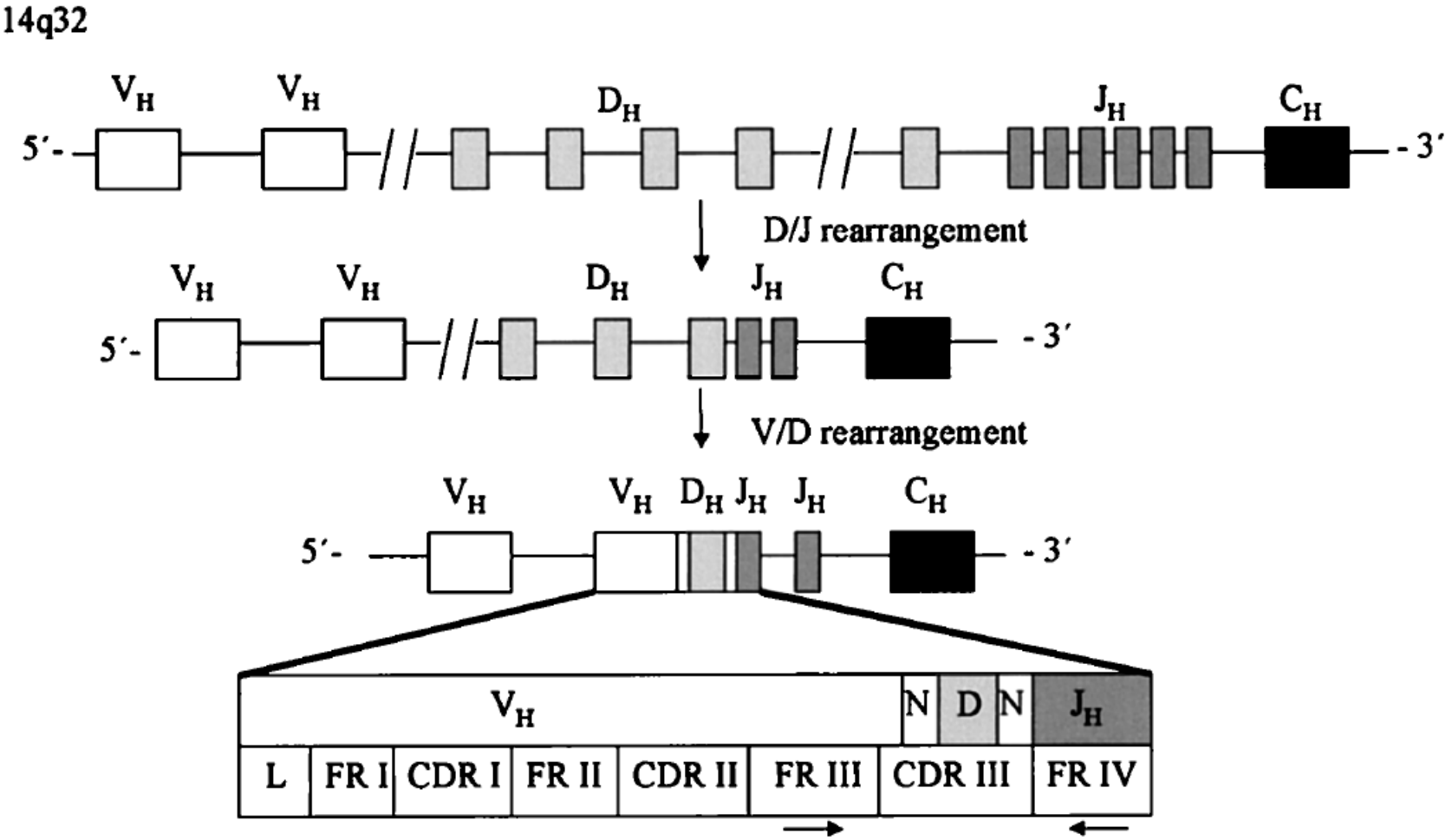
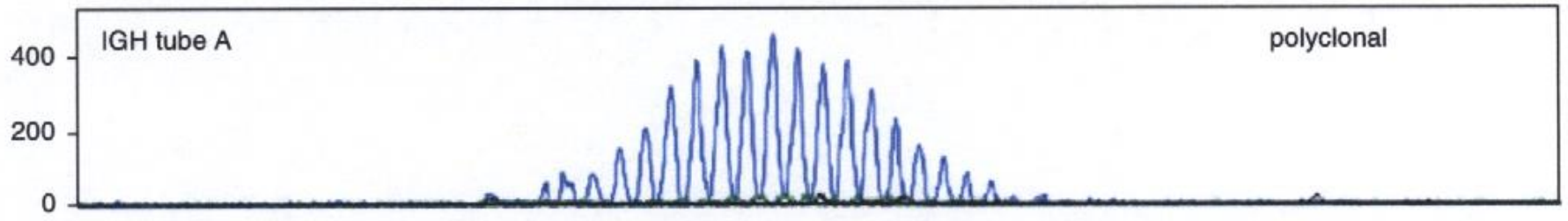
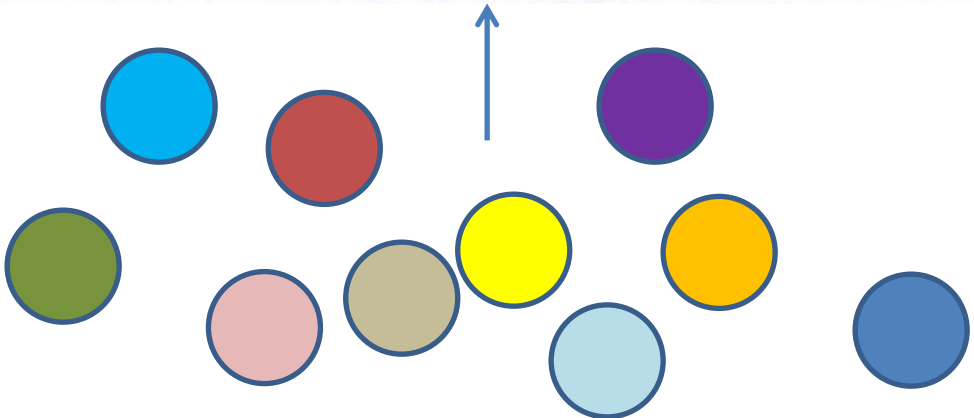


Figure 1. Immunoglobulin heavy chain gene rearrangement. Most PCR tests for this rearrangement use consensus primers directed against the framework three (FRIII) region and the heavy chain joining (J_H or FRIV) region of the rearranged product.

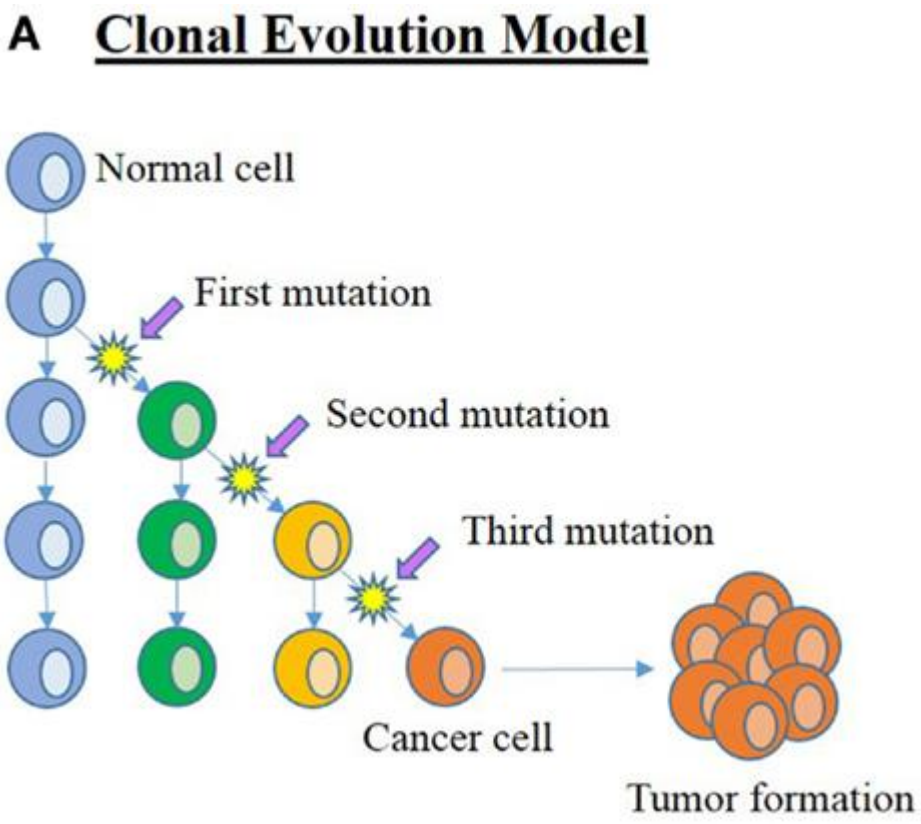
Arber, JMD 2000

Physiologic (“normal”) B- or T-cell populations

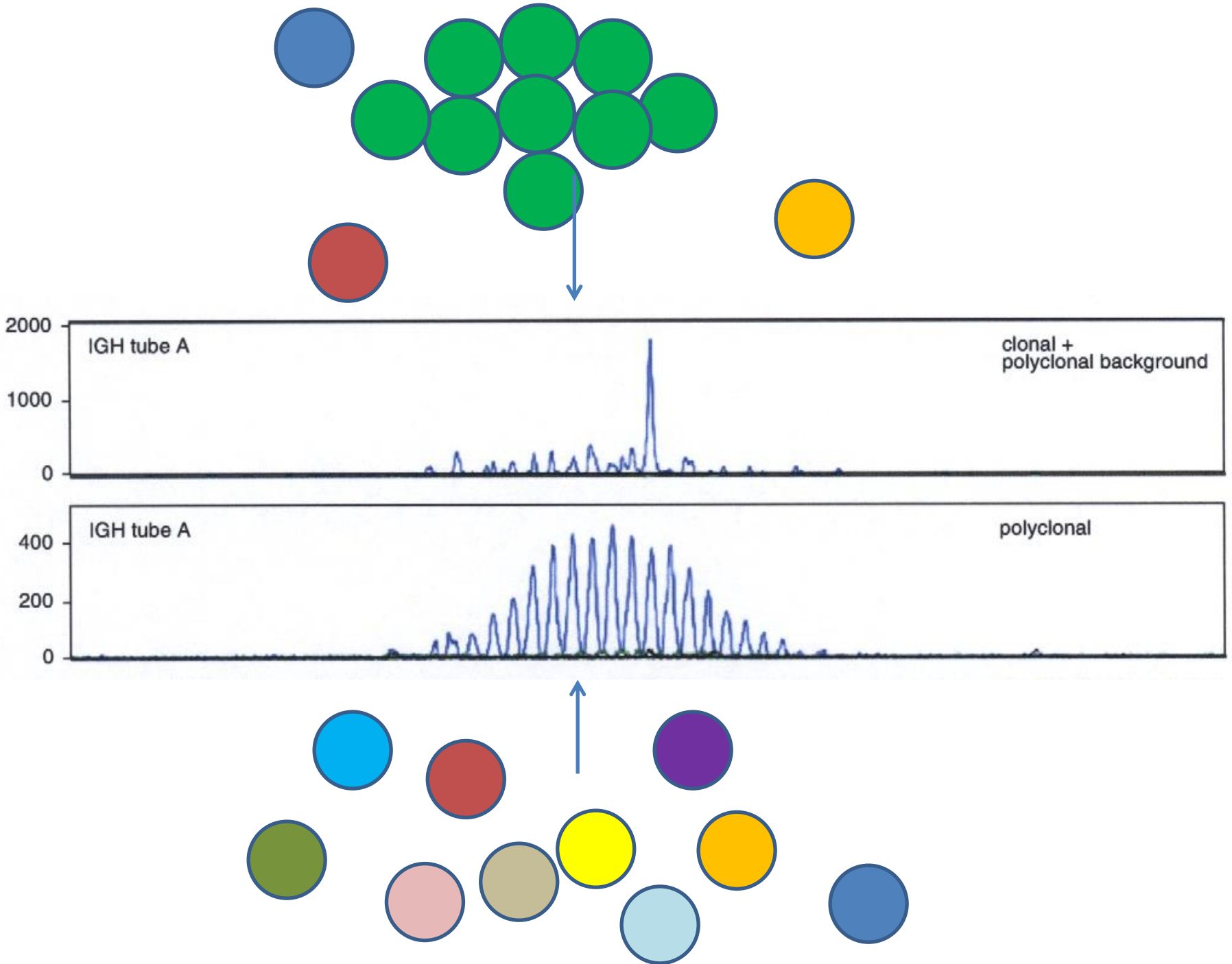




Assumption of Clonality in Cancer is Critical to Diagnostic Tools (Flow, Molecular)

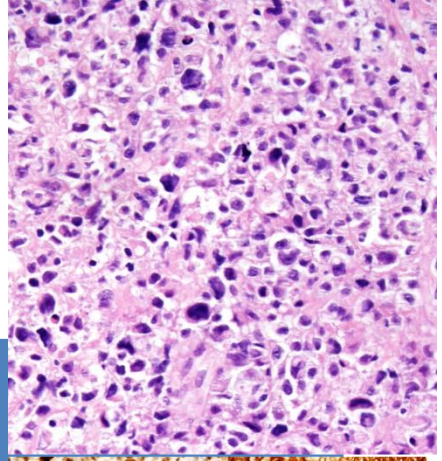


https://doi.org/10.3389/fsurg.2016.00021

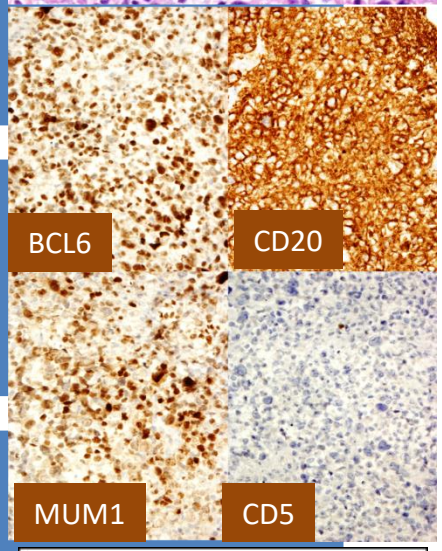

2000
1000
0
IGH tube A
clonal +
polyclonal background
400
200
0
IGH tube A
polyclonal

Lymphoma Diagnosis

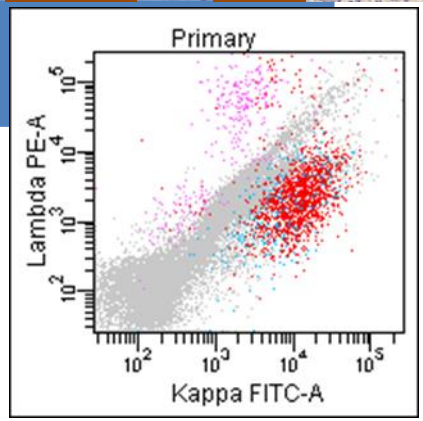
Morphology



Immunohistochemistry



Flow cytometry

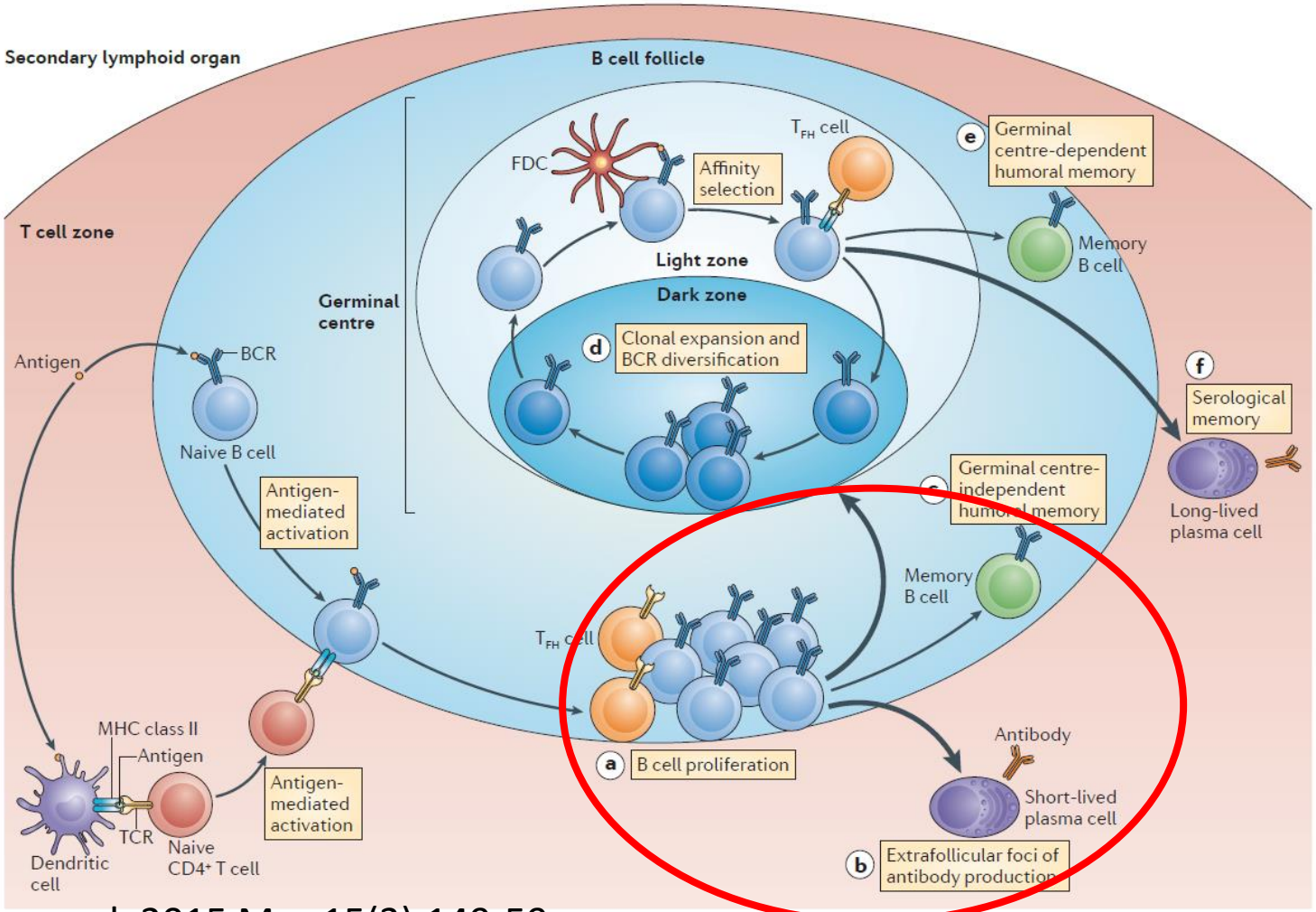


- This is enough! (Most of the time…)

Pitfalls of Clonality Testing

- Failed amplification
  - Low quantity
  - Poor quality (FFPE)
- Sampling
  - Pseudoclones
  - Wrong area
- False negatives
  - Somatic hypermutation (Follicular lymphoma)
  - Sampling wrong area
  - Clone too small; high reactive background
- "False positives"
  - Clonal proliferation in non-neoplastic processes

Clonal expansion as part of normal immune response

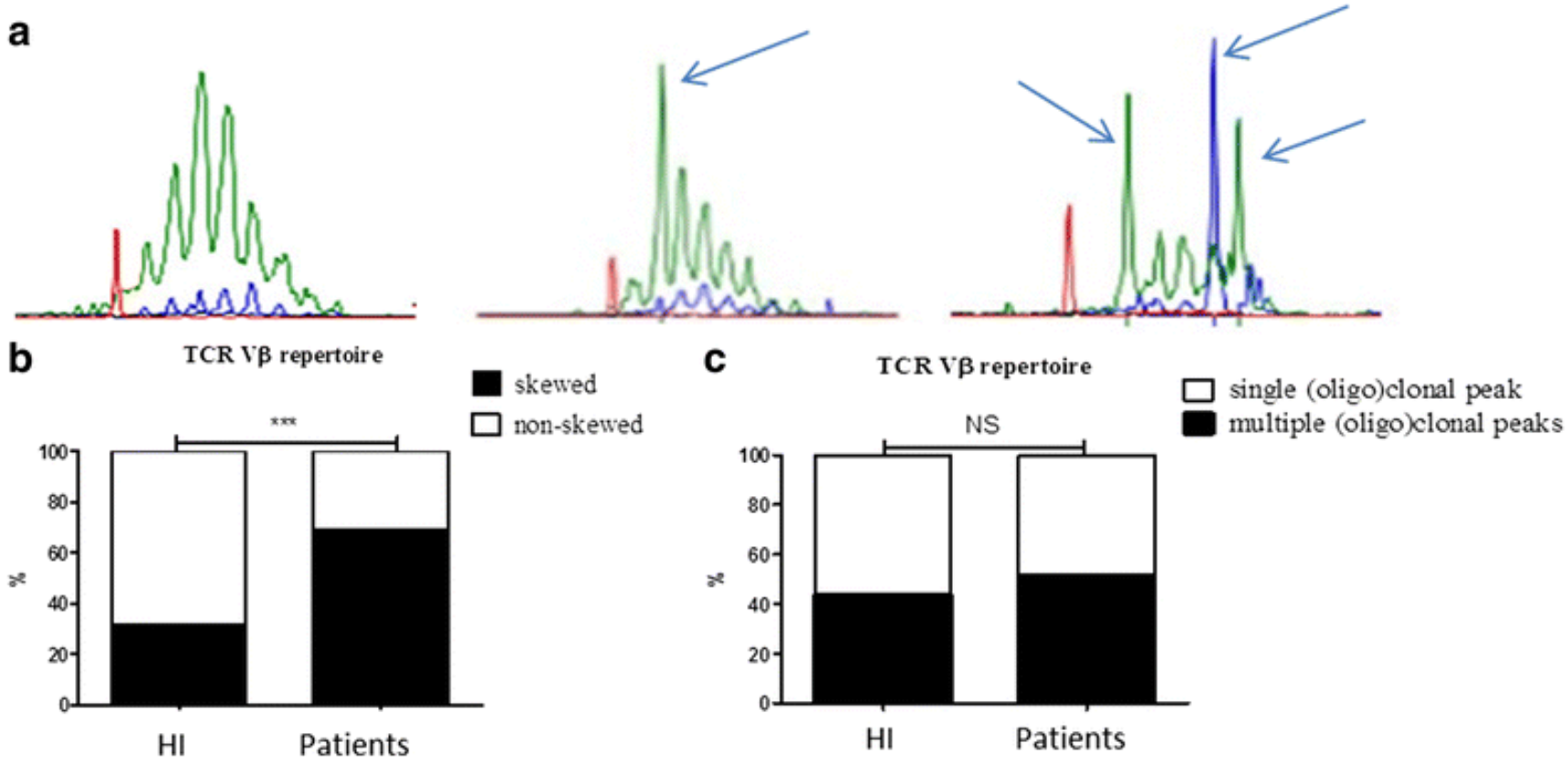


Nat Rev Immunol. 2015 Mar;15(3):149-59.

Non-Neoplastic Clonal T-cells

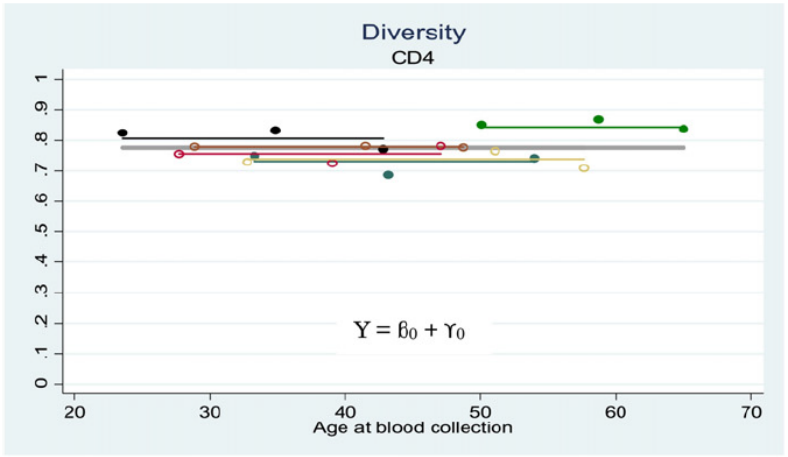
- There are MANY examples of clonal T-cell proliferations that are NOT neoplastic
  - Commonly skin, peripheral blood
  - Post transplant
  - Various immune responses
    - Inflammatory (Crohn's etc.)
    - Malignancy (CLL/SLL, etc.)

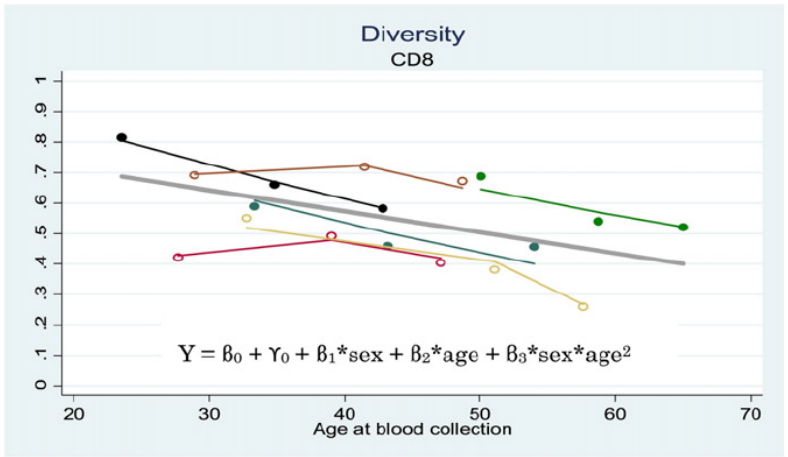
Example from ESRD patients – Peripheral blood T-cells

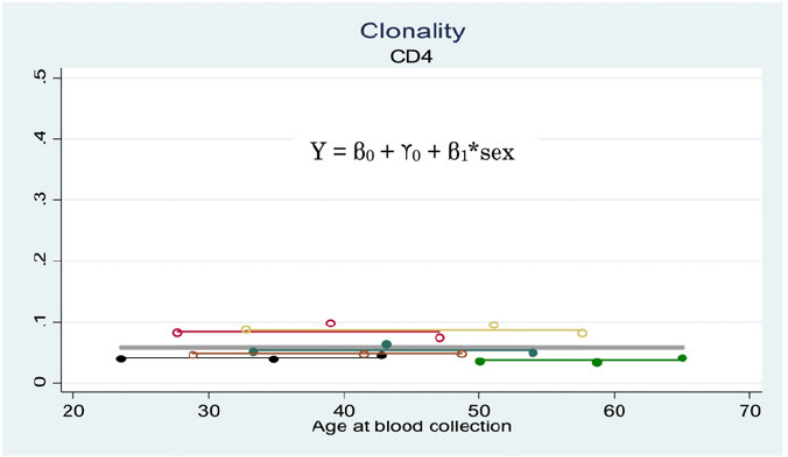


Huang et al. Immunity & Ageing 2015;12:28.

T-cell repertoire decreases with age







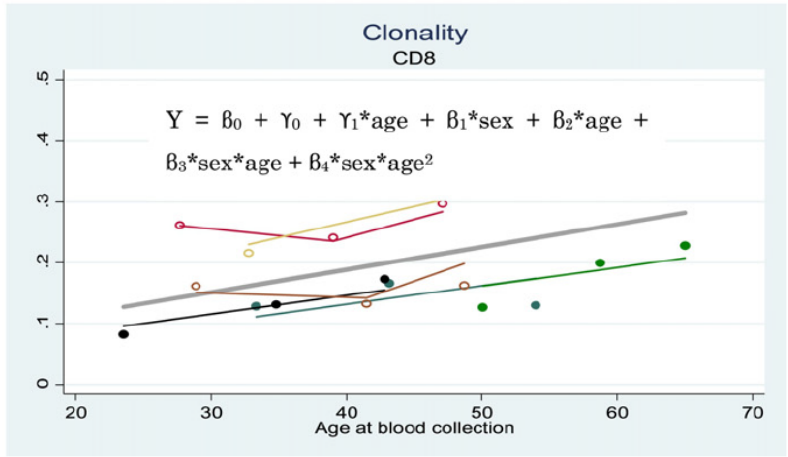
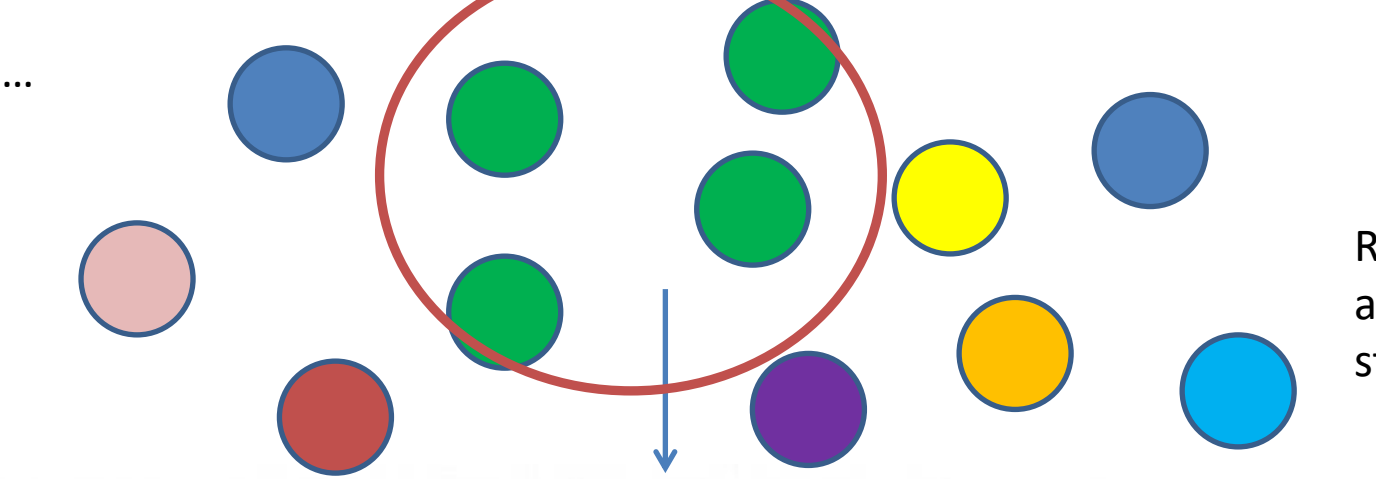


Fig. 1. CD4 and CD8 TCR diversity and clonality. Points are observed, repeated values: solid circles represent males (color coded in blue, green, and black); open circles represent females (color coded in brown, red, and tan). Solid lines connect the fitted values of the best-fitting models at the observed age points. The single gray line is the population-average trajectory, deviations from which reflect differences in overall level, slope, or both.

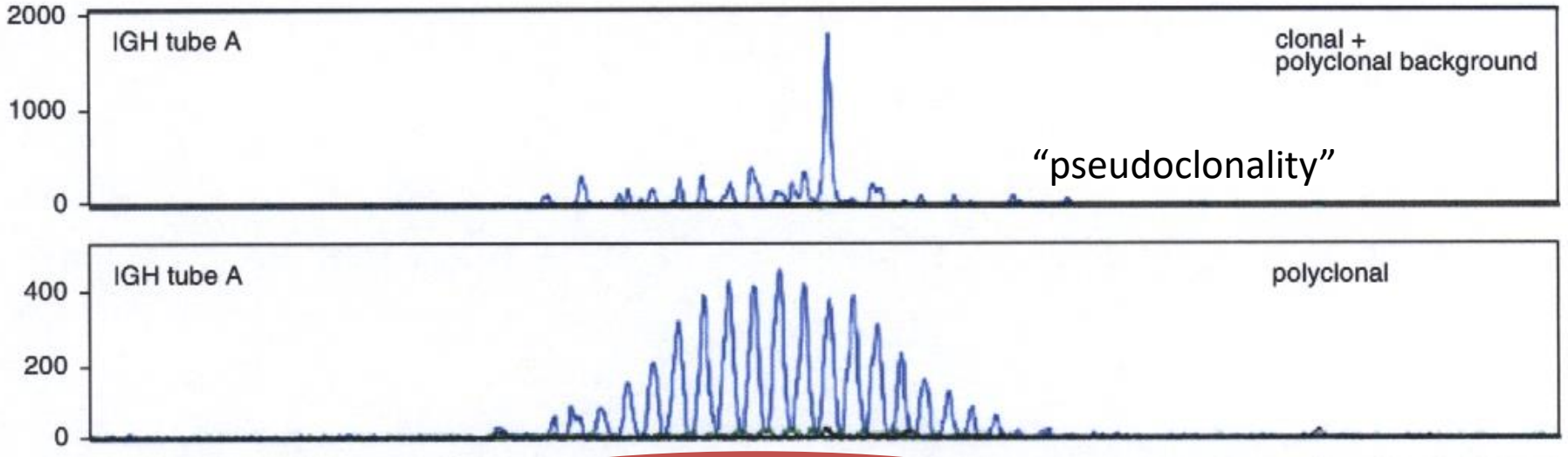
Pitfalls of Clonality Testing

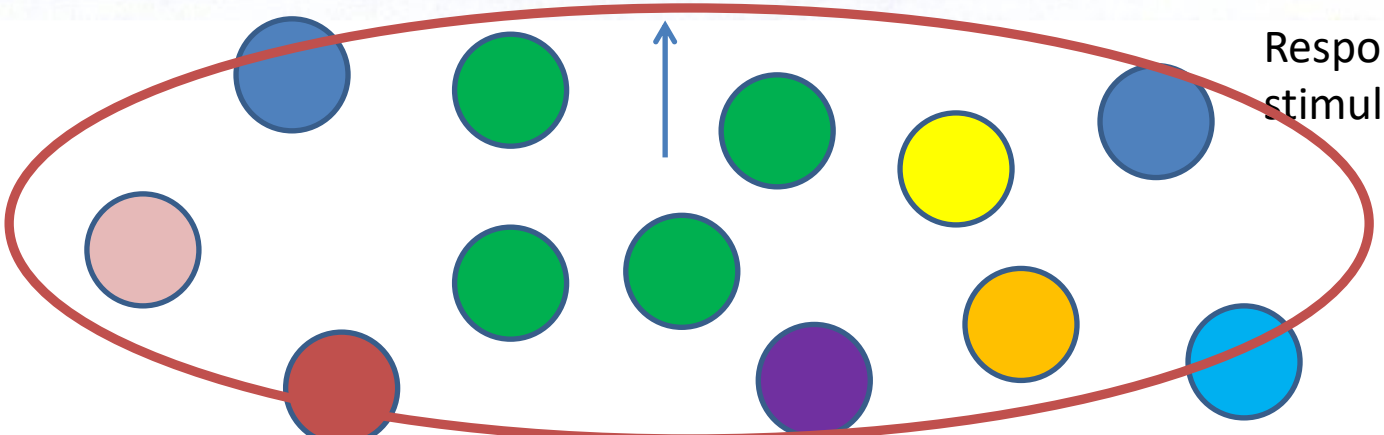
- Failed amplification
  - Low quantity
  - Poor quality (FFPE)
- Sampling
  - Pseudoclones
  - Wrong area
- False negatives
  - Somatic hypermutation (Follicular lymphoma)
  - Sampling wrong area
  - Clone too small; high reactive background
- “False positives”
  - Clonal selection in non-neoplastic processes

Sampling…



Response to antigenic stimulation



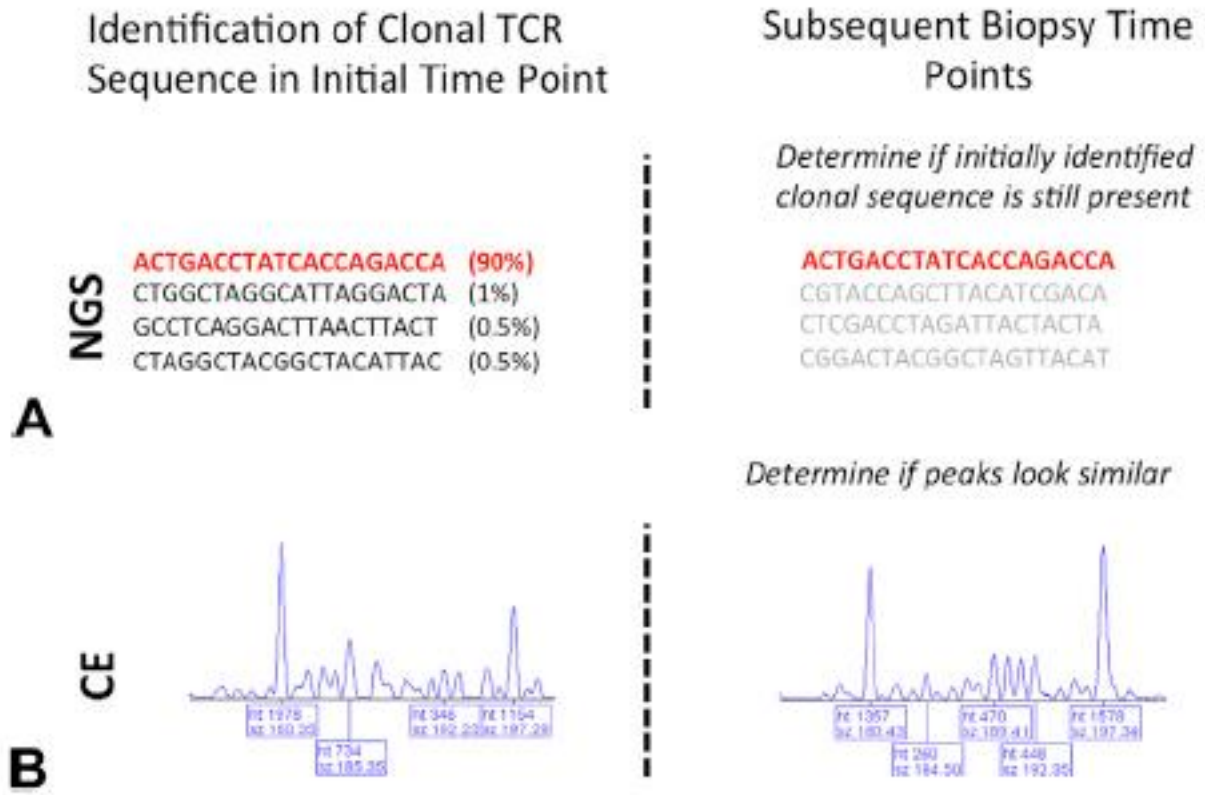


Response to antigenic stimulation

Clonality testing: The future…

- Using NGS data for T-cell clonality
  - More powerful
  - Not just used for clonality, but can examine different types of T-cell immune responses in other non-hematologic malignancies
    - May alter therapy choices; immune checkpoint inhibitors
- The downside
  - Longer TAT
  - Higher cost
  - Clones may be readily identified and still does not solve the problem that clonality ≠ lymphoma!

NGS in recurrence



J Am Acad Dermatol 2015;73:228-36.

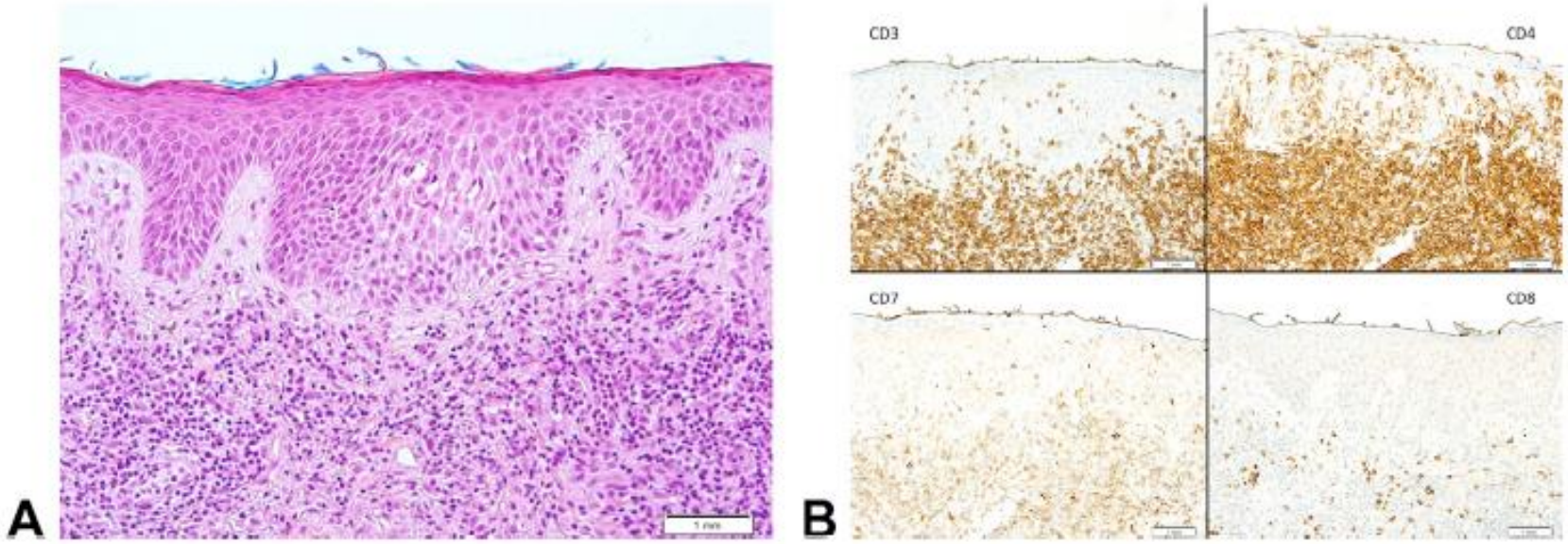
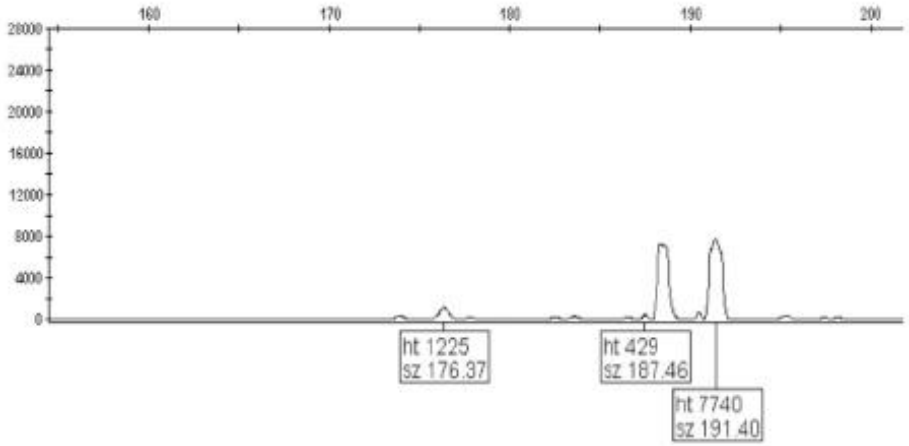


Fig 4. Mycosis fungoides. **A**, Representative skin biopsy specimen characterized by a lymphocytic infiltrate composed of small to medium atypical cerebriform cells demonstrating epidermotropism. Haloed cells are notable within the epidermis. (Hematoxylin-eosin stain; original magnification: ×20.) **B**, Lymphocytes were immunoreactive for CD2, CD3, and CD5, with reduced CD7 positivity. As CD4 also stains Langerhans cells in the epidermis, there are more $CD4^+$ cells than $CD3^+$ cells in the epidermis. Because of this, it is important to compare CD3 and CD8 when examining the epidermal compartment. The $CD3^+CD8^-$ cells likely correspond to $CD4^+$ T cells. CD4 expression was greater than that of CD8. (Immunohistochemistry, original magnification: ×20.)

J Am Acad Dermatol 2015;73:228-36.

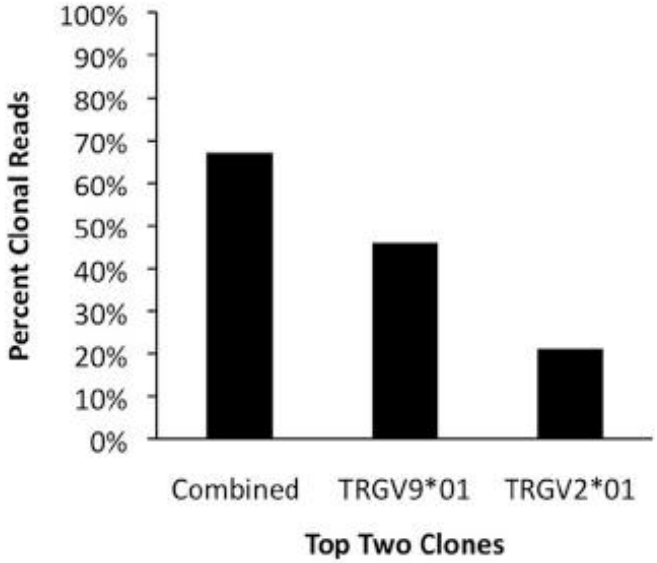
Case 1

CE



Clonal

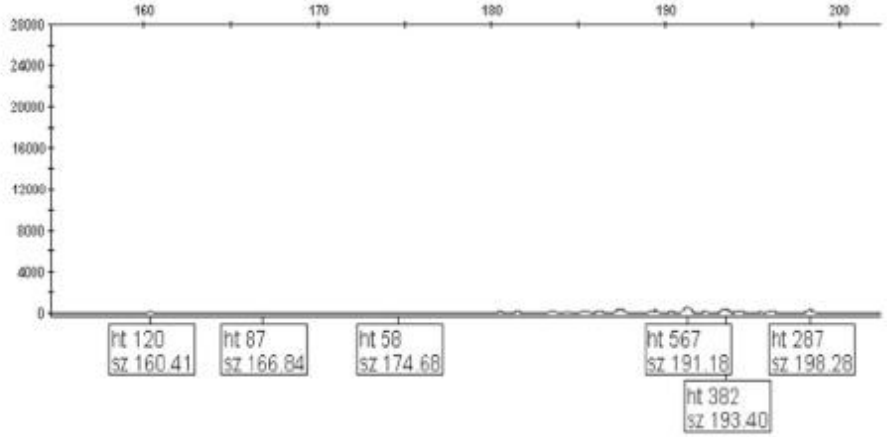
NGS



Clonal

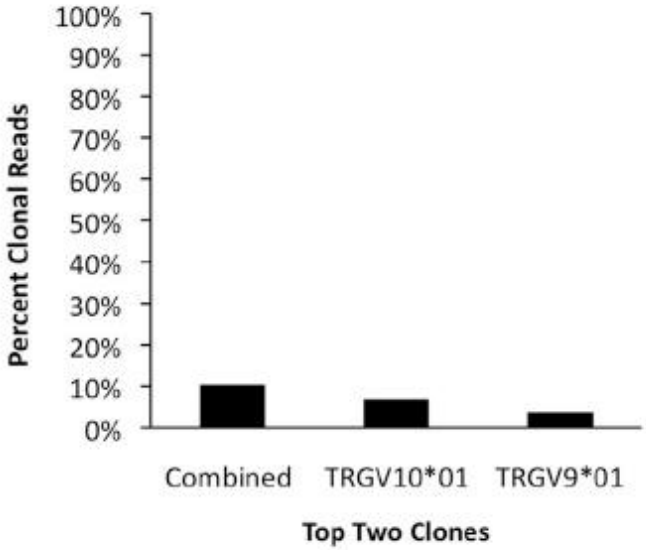
Case 2

CE



Polyclonal

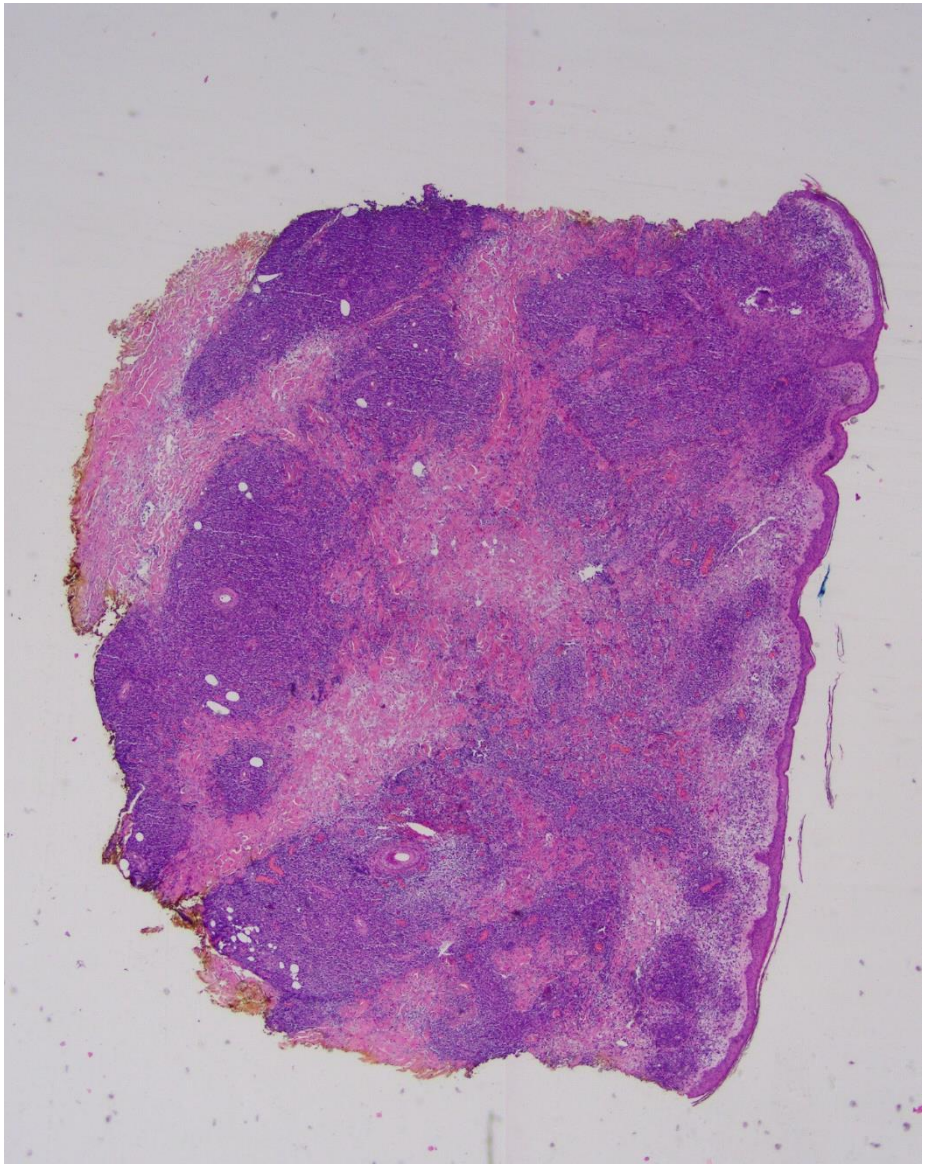
NGS

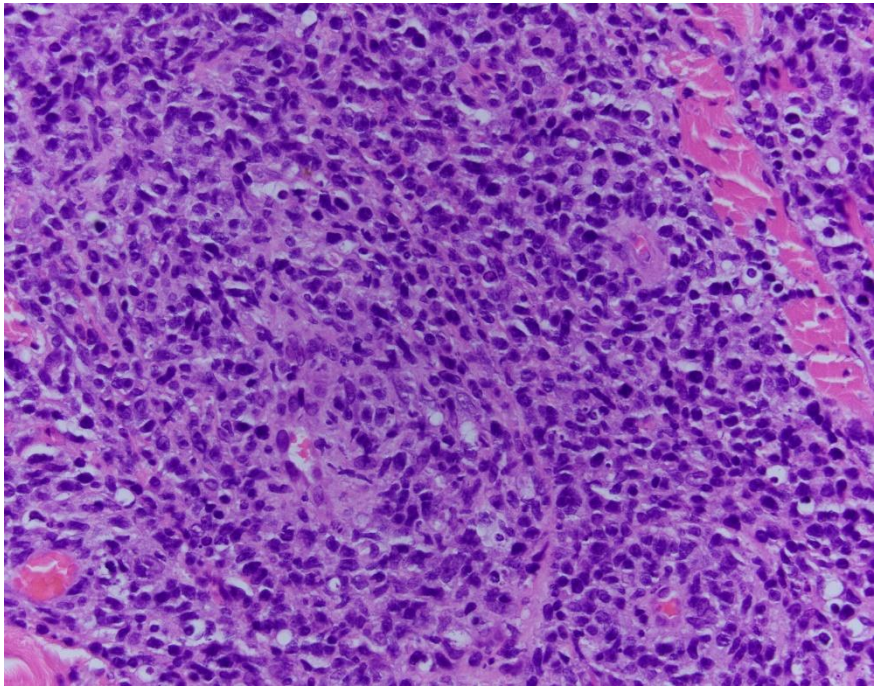


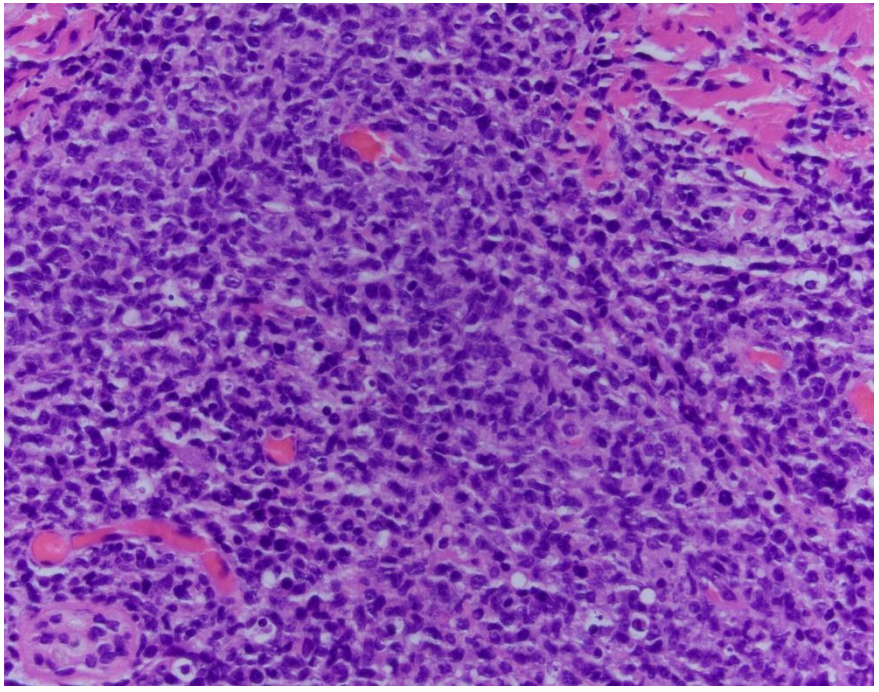
Clonal

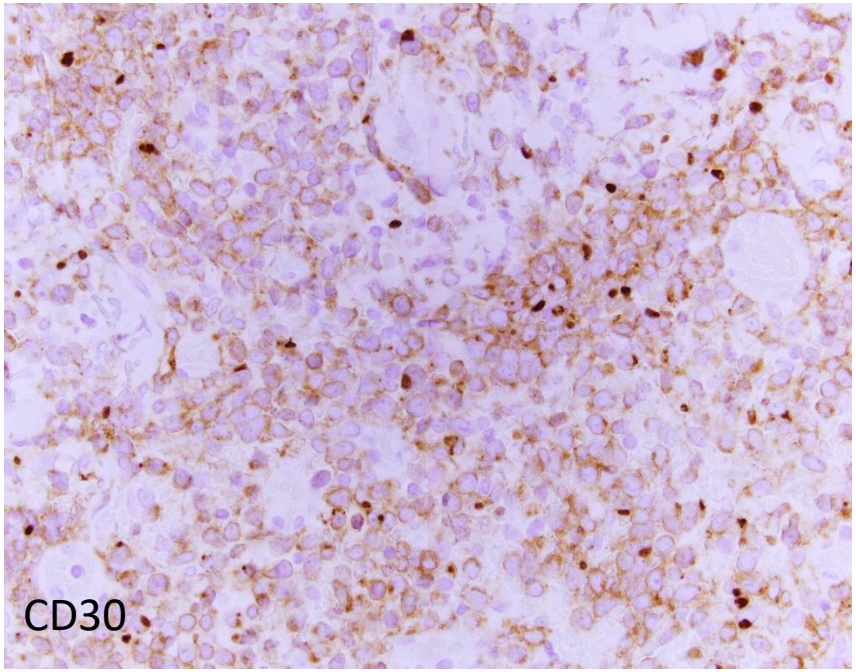
Case #4

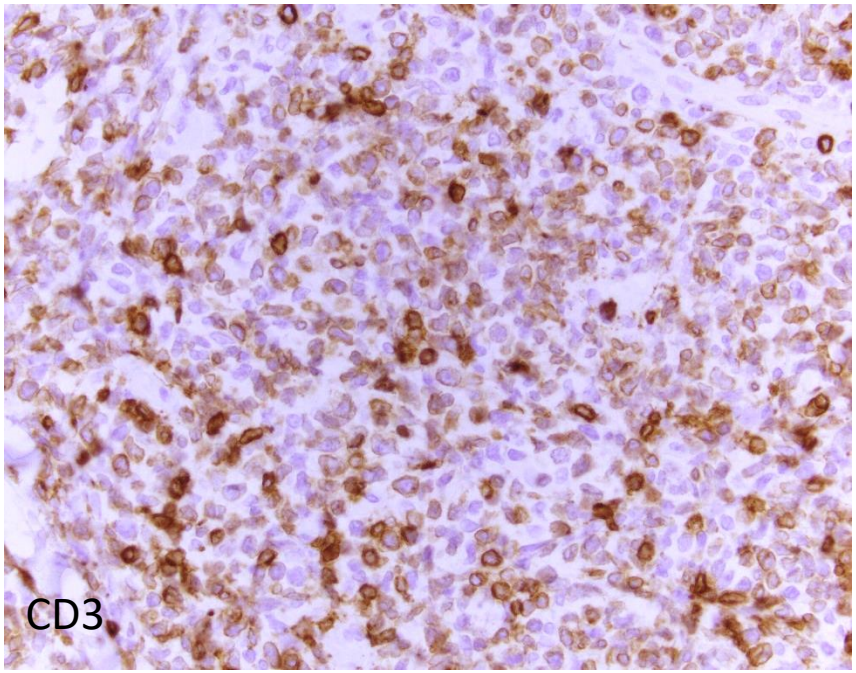
A 73-year-old male presents with multiple skin lesions.









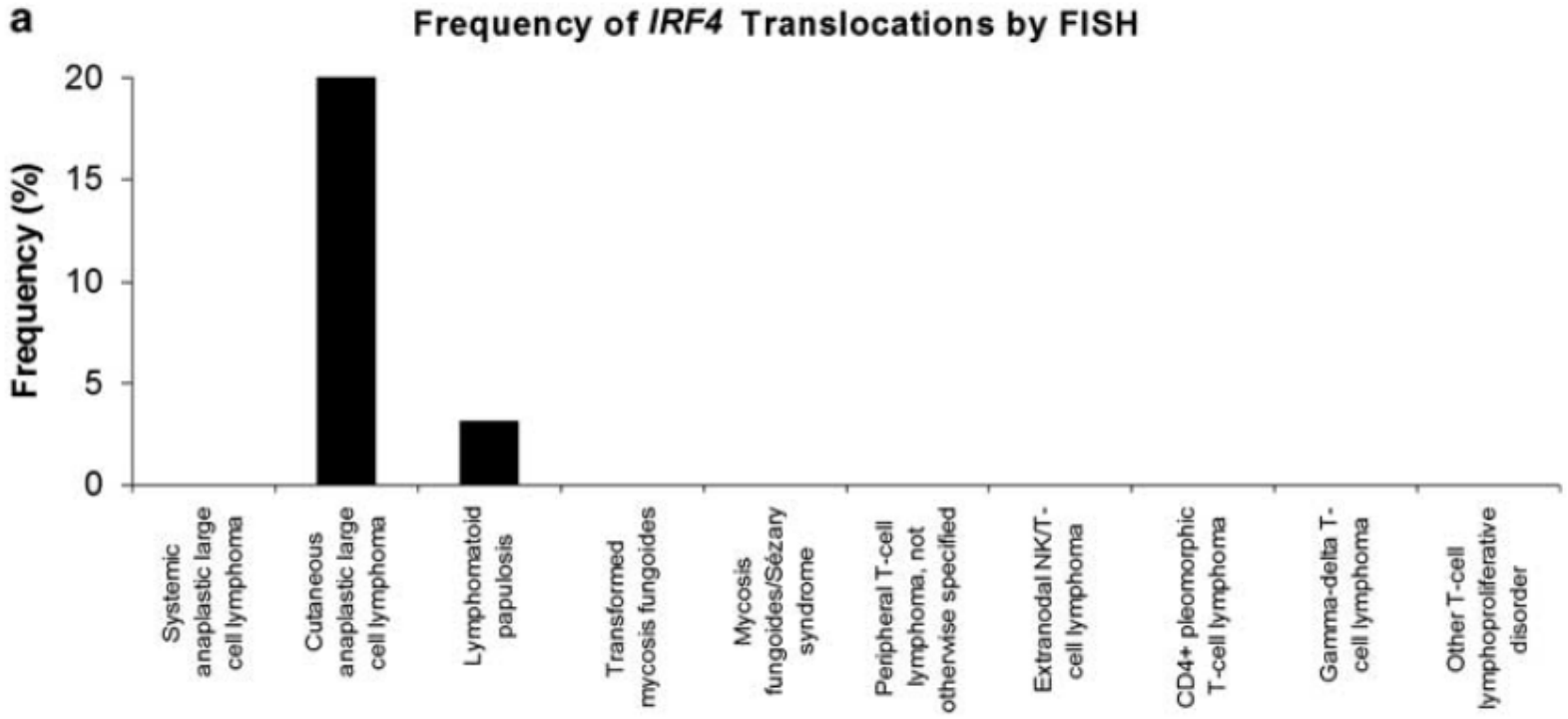


- Positive:
- CD30, CD3, CD4
- Negative:
- ALK, P63, CD8

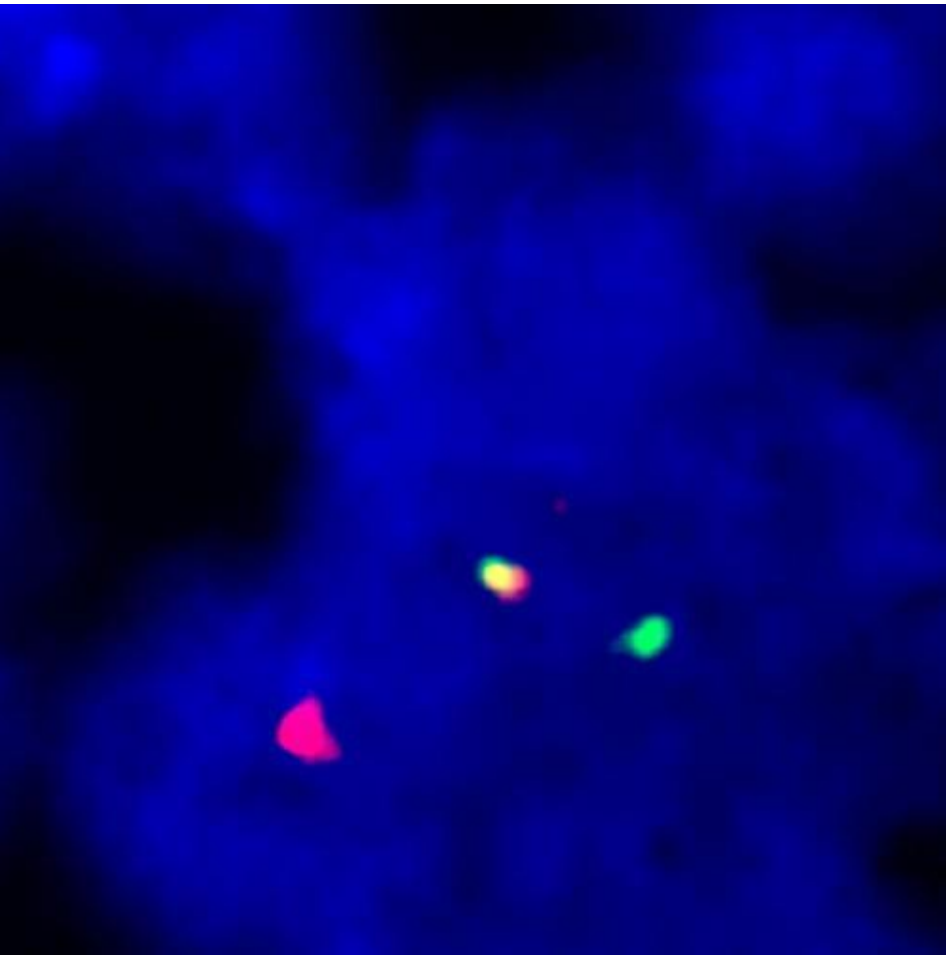
Diagnosis

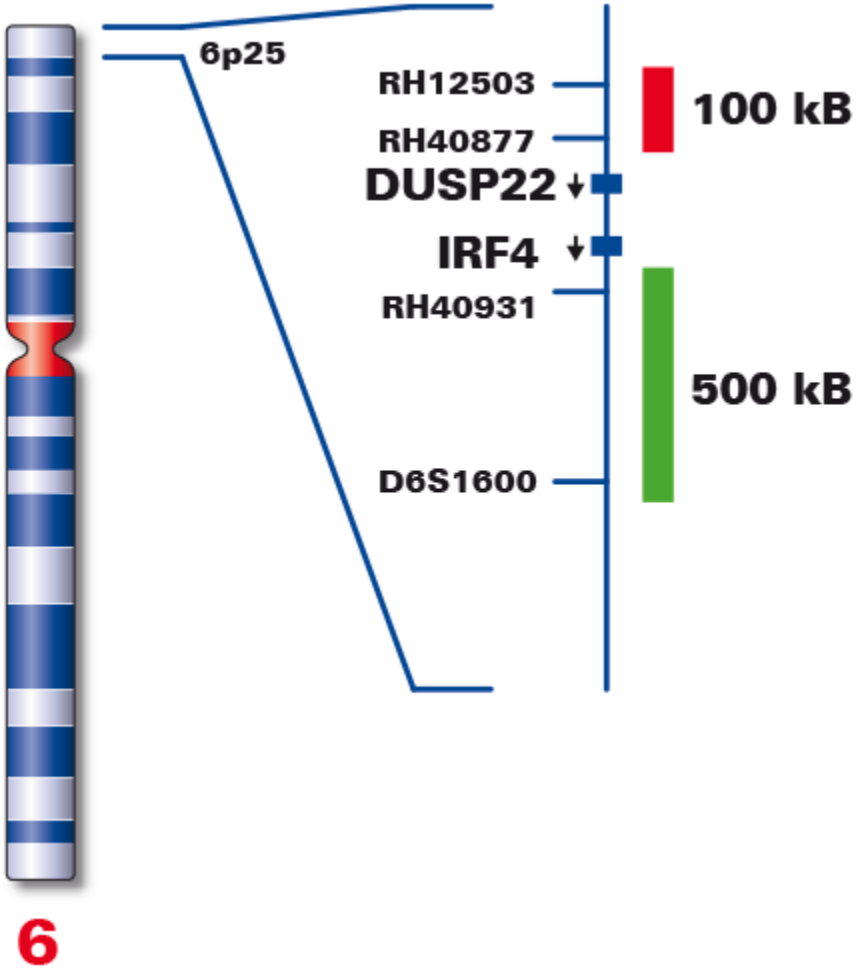
- Skin, biopsy:
  - Anaplastic large cell lymphoma, ALK-negative
  - ?Systemic or primary cutaneous?
  - Prognosis?

Specificity of IRF4/DUSP22 rearrangement



Wada et al Mod Path 2011

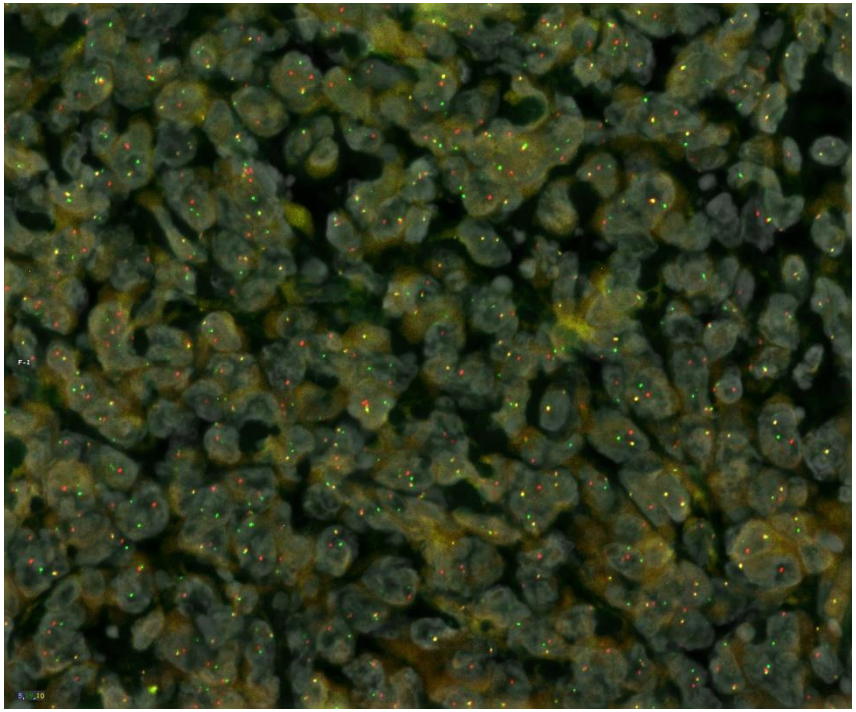




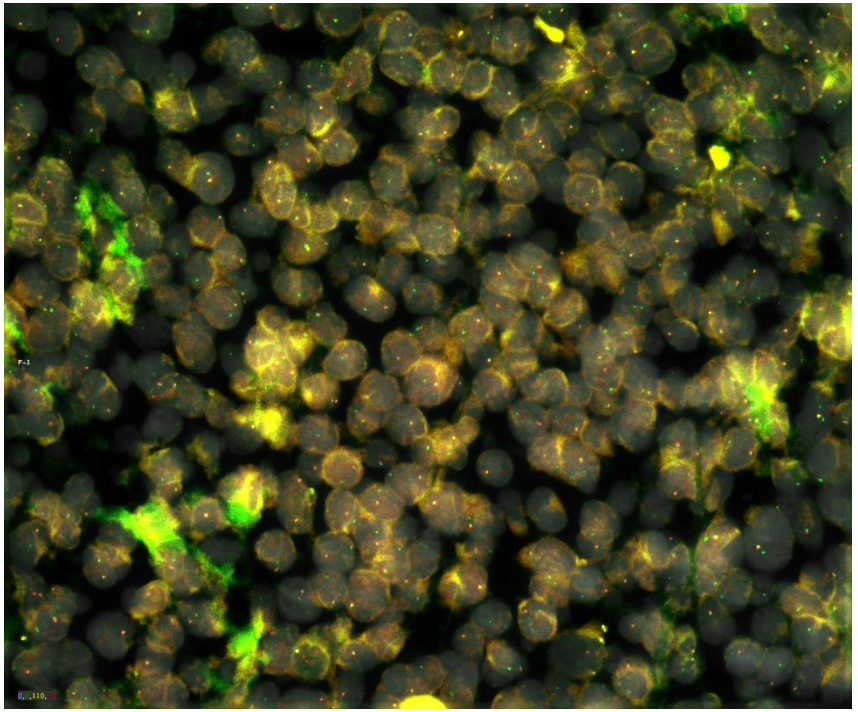
From Leica Biosystems

FISH for IRF4/DUSP22

Positive (1F, 1G, 1O)



Our Patient (Atypical: 1F, 1G)



Algorithm for ALCL work-up and Prognosis

Marker	Frequency	Prognosis (5yr OS)
ALK1 (IHC)	~50%*	85%
DUSP22 (FISH)	30% of ALK neg	90%
TP63 (p63 IHC to screen, then FISH)	8% of ALK neg	17%
None of these	~30%*	42%

Castellar et al. Blood. 2014 Aug 28; 124(9): 1473–1480.

Conclusions

- CLL NGS panel can be very useful in CLL patients with relapsed or refractory disease
  - Treatment management
- MYD88 mutation testing can be helpful in confirming the diagnosis of lymphoplasmacytic lymphoma in the appropriate clinical and histomorphologic context.
- Molecular clonality assays can be very helpful in lymphoma diagnosis, if used in the right context, with an awareness of possible “pitfalls”.
  - Most importantly they should be combined with impression from all other studies and history
  - Can investigate “relatedness” of tumors
- DUSP22 FISH should be used routinely in the work-up of anaplastic large cell lymphoma, especially if there is skin involvement

Thank You!