MYC protein is a high-risk factor in mantle cell lymphoma and identifies cases beyond morphology, proliferation and *TP53*/p53 – a Nordic Lymphoma Group study

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Supplementary Methods

Patient Material

Patients in the N-MCL2/3 clinical trials were recruited in Denmark, Finland, Norway, and Sweden and were treated with first-line intensive immunochemotherapy and high-dose chemotherapy with autologous stem cell support. Inclusion criteria for MCL2/3 included age at diagnosis 18-65 years, no previous treatment, stage II-IV and cyclin D1 or t(11;14) positive.[1, 2] All cases have been re-evaluated by an haematopathologist. Information regarding Ki-67 and morphology were available for most patients included. *TP53* mutational status and p53 protein expression had been previously published.[3] MIPI was available for all patients included in the N-MCL2/3, and for a subset of patients in the SLR cohort.

Immunohistochemistry (IHC)

A tissue microarray (TMA) from 260 formalin-fixed paraffin-embedded (FFPE) tumor specimens was made as per standard protocols.[4] Representative 1mm tumor areas were transferred into recipient blocks using an automated device (ATA-27, Beecher Instruments, Sun Prairie, WI, USA). Scanning (x20) was performed using a NanoZoomer 60 (Hamamatsu Photonics, Hamamatsu, Shizuoka, Japan). Counts of positive cells for each marker were done by resorting to the Cytonuclear module on the digital pathology tool HALO® (Indica Labs, Albuquerque, NM, USA). The software was trained to identify dark brown nuclei as a positive cell. The mean value of positive cells was used in subsequent analysis when more than one core was available.

mRNA *in situ* hybridization

In brief, tissue sections were deparaffinized and treated with hydrogen peroxide following incubation in citrate buffer. Samples were treated with protease and incubated with an Hs-MYC-C2 probe (1:50). Positive control probes targeting *PPIB* (housekeeping gene) and negative control probes targeting *DapB* (bacterial gene) were used to test mRNA integrity, exclude unspecific staining, and evaluate background auto-fluorescence. All probes were purchased from Advanced Cell Diagnostics (CA, USA). The signals were developed by the addition of horseradish peroxidase (HRP)-C2 and stained with TSA plus Cy3 (PerkinElmer, Massachusetts, USA). 1:10 of Syto13 (Thermo Fisher Scientific, MA, USA) was used for DNA staining. Slides were visualized and scanned with the GeoMx Digital Spatial Profiler (NanoString, Washington, USA).

Analysis of the fluorescent signals was performed using open-source QuPath software.[5] The negative control slide was used to define the background autofluorescence. The scores for each cell were grouped into low (group 1), intermediate (group 2), and high (group 3). Group 1 showed 1 to 3 spots of probe binding, group 2 showed 4 to 9 spots of probe binding and group 3 showed more than 10 spots of probe binding. The final value was calculated based on the sum of each assigned score

multiplied by the percentage of cells per score, resulting in a theoretical range of 0 to 300. The average of duplicate cores per patient was considered for further analysis, and in a single patient with high background, values were corrected using the negative control value.

Multiplexed immunofluorescence (mIF) staining

TMA slides from the SLR cohort (4-5 µm thick) were fixated on glass slides (Superfrost plus, Thermo Fisher Scientific, Massachusetts, USA). Slides were deparaffinized and hydrated before target retrieval and antibody staining.

The multiplex images were uploaded to Aiforia's cloud-based platform as training images. The detection was split into three layers. An independent convolutional neural network (CNN) was created for each layer that was later used to develop a final AI model. The layers aim to detect tissue (segmentation layer), cancer or non-cancer tissue (segmentation layer), and the different immune cells (object detection layer). The layers have a hierarchical order so that a feature can only be seen in its corresponding parent layer. Manual annotations were done throughout selective regions for all the training images for the respective layers. Smaller tissue areas containing only single cells were annotated as background so that they would get ignored in further analysis. Cancer tissue was defined as tissue containing 60-100% B cells when considering a field of view with approximately 100 cells. The algorithm was trained to separately detect immune cells in cancer and non-cancer tissue. The final AI model contained all four nested CNNs. The data used in this project considered all tissue available for analysis, not distinguishing between cancer and non-cancer tissue in the second segmentation layer.

Statistical analysis

For comparison between clinicopathological parameters, χ^2 test was applied when categorical variables were tested. For two-group comparisons, Shapiro Wilk test was used to check for normality and t-test or Wilcoxon signed-rank test was performed in accordance. Yates' continuity correction was used with χ^2 test to prevent overestimation of significance for small values. Pearson correlation coefficient was applied to test the correlation between continuous variables.

Time since diagnosis was the baseline time for the SLR cohort and the underlying time scale for the N-MCL2/3 cohort was the date of inclusion or treatment initiation. For PFS, the endpoint was the date of documented death or progression. As of note, a significant portion of patients included in the SLR cohort did not have information on PFS. Survival was visualized using Kaplan-Meier curves and log-rank test assessed the differences between groups. Univariable and multivariable Cox proportional hazards (PH) models were used to estimate the risk, measured as hazard ratios (HR), with 95% of confidence intervals (CI), for the impact of the covariates. The PH assumption was tested using the Therneau and

Grambsch test of the Schoenfeld residuals. When PH assumption was violated due to few events over a long-follow up time, the time scale was truncated at 3 years follow-up.

Maximally selected rank statistics (Max Rank) in R[6] was used to determine MYC cut-off. Max Rank categorizes a continuous variable into two groups based on the hypothesis that it can be predictive of survival, taking into consideration censored data. Max Rank was applied to overall survival.

Differences were considered statistically significant when the p-value was <0.05. Statistical analysis was performed using the Statistical Package for the Social Sciences (SPSS®) version 25.0 for Windows (IBM Corp., Armonk, NY, USA) and RStudio 2022.12.0+353 "Elsbeth Geranium" Release (R Foundation for Statistical Computing, Vienna, Austria).

References

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Supplementary Tables

Table S1. Clinicopathological characteristics of the patients included in this study, divided by cohort.

		SLR	***N-MCL2/3***
		N (col%)	N (col%)
		155 (100%)	98 (100%)
Sex	Male	118 (776%)	71 (72%)
	Female	37 (24%)	27 (28%)
Age at diagnosis	≤ 65	40 (26%)	98 (100%)
	> 65	115 (74%)	0 (0%)
MIPI	Low risk	20 (20%)	56 (57%)
	Medium risk	36 (37%)	25 (26%)
	High risk	42 (43%)	17 (17%)
	Missing	57	0
Morphology	Classic	130 (90%)	75 (77%)
	Blastoid/ Pleomorphic	15 (10%)	23 (23%)
	Missing	10	0
Ki-67	<30%	111 (74%)	56 (60%)
	≥ 30%	38 (26%)	38 (40%)
	Missing	6	4
MYC	< 20%	125 (81%)	92 (94%)
	≥ 20%	29 (19%)	6 (6%)
	Missing	1	0
TP53	Wild-type	43 (75%)	53 (84%)
	Mutated	14 (25%)	10 (16%)
	Missing	98	35
p53	<30%	128 (88%)	84 (88%)
	≥ 30%	18 (12%)	11 (11%)
	Missing	9	3
TP53/p53	Wild-type/<30% expression	123 (84%)	85 (89%)
	Mutated/≥ 30%	24 (16%)	11 (11%
	Missing*	8	2
Treatment	N-MCL2/3	36 (40%)	98 (100%)
	R-CHOP	3 (3%)	0
	R-Bendamustine	39 (44%)	0
	Chlorambucil	5 (6%)	0
	Others	6 (7%)	0
	Missing	66	0
Median overall survival time (years)		4.5	12.4
Median progression free survival (years)		3.3**	8.7

MIPI: Mantle Cell Lymphoma International Prognostic Index; N: number; N-MCL2/MCL3: Nordic lymphoma group – Mantle Cell Lymphoma 2 and 3 protocol containing dose-intensified cyclophosphamide, vincristine, doxorubicin and prednisone (CHOP) in combination with rituximab (R) alternating with rituximab and high-dose cytarabine; SLR: Swedish Lymphoma Registry cohort;

* Missing information for only one of the variables studied

**PFS information was only available for a portion of patients included in the SLR

Table S2. Clinicopathological characteristics of the patients included in the mRNA analysis through RNAscope®.

		N (col%)
		85 (100%)
Sex	Male	68 (80%)
	Female	17 (20%)
Age at diagnosis	≤ 65	22 (26%)
	> 65	63 (74%)
MIPI	Low risk	9 (19%)
	Medium risk	20 (43%)
	High risk	18 (38%)
	Missing	38
Morphology	Classic	75 (90%)
	Blastoid/ Pleomorphic	8 (10%)
	Missing	2
Ki-67	<30%	68 (80%)
	≥ 30%	17 (20%)
MYC	< 20%	72 (85%)
	≥ 20%	13 (15%)
TP53	Wild-type	32 (74%)
	Mutated	11 (26%)
	Missing	42
p53	<30%	70 (85%)
	≥ 30%	12 (15%)
	Missing	3
Treatment	N-MCL2/3	18 (44%)
	R-CHOP	1 (2%)
	R-Bendamustine	2 (49%)
	Chlorambucil	2 (5%)
	NA	44
Median overall survival time (years)		4.5
Median progression free survival (years)		3.8*

MIPI: Mantle Cell Lymphoma International Prognostic Index; N: number; N-MCL2/MCL3: Nordic lymphoma group – Mantle Cell Lymphoma 2 and 3 protocol containing dose-intensified cyclophosphamide, vincristine, doxorubicin and prednisone (CHOP) in combination with rituximab (R) alternating with rituximab and high-dose cytarabine; SLR: Swedish Lymphoma Registry cohort;

*PFS information was only available for a portion of patients included

Table S3. Univariable Cox proportional hazards models for progression free survival truncated at 3 years follow-up time

		Progression Free Survival			
		HR (95% CI)	**n**	**p-value**	**PH**
MYC^{High}	Dichotomized (20% cut-off)	3.16 (1.77 – 5.62)	199	<0.001	>0.05
% MYC positive cells	Continuous	1.04 (1.03 – 1.06)	199	<0.001	>0.05
H-score	Continuous	1.02 (1.01 – 1.02)	51	<0.001	>0.05

CI: confidence interval; HR: Hazard ratio; PH: proportional hazardous; n: number

Table S4. Univariable Cox proportional hazards models for MYC prognostic impact in overall survival and progression free survival divided by cohort

			Overall survival				Progression Free Survival			
			HR (95% CI)	n	p-value	PH	HR (95% CI)	n	p-value	PH
SLR	MYC^{high}	Dichotomized (20% cut-off)	1.91 (1.21-3.03)	154	0.006	<0.05	1.15 (0.63-2.09	101	0.65	<0.05
	% MYC positive cells	Continuous	1.02 (1.01-1.03)	154	<0.001	<0.05	1.03 (1.01-1.04)	101	0.003	<0.05
N-MCL2/3	MYC^{high}	Dichotomized (20% cut-off)	2.37 (0.83-6.72)	98	0.11	>0.05	2.58 (1.01-6.56)	98	0.05	>0.05
	% MYC positive cells	Continuous	1.03 (1.00-1.06)	98	0.02	>0.05	1.04 (1.02-1.06)	98	0.001	>0.05

Table S5. Difference in clinicopathological characteristics with focus on established high-risk factors comparing MYC^{high} tumors with overall survival (OS) longer or shorter than 3 years.

		OS> 3 years	***OS< 3 years***
		15 (43%)	20 (57%)
Gender	Female	1 (7%)	8 (40%)
	Male	14 (93%)	12 (60%)
Age	<65	8 (53%)	4 (20%)
	>65	7 (47%)	16 (80%)
Morphology	Classic	9 (75%)	11 (58%)
	Non-classic	3 (25%)	8 (42%)
	Missing	3	1
Ki-67	High	7 (58%)	14 (74%)
	Low	5 (42%)	5 (26%)
	Missing	3	1
MIPI	Low	1 (8%)	0 (0%)
	Intermediate	9 (75%)	0 (0%)
	High	2 (17%)	11 (100%)
	Missing	3	9
***TP53*/p53**	WT	11 (92%)	8 (40%)
	Mut/high	1 (8%)	12 (60%)

MIPI: Mantle Cell Lymphoma International Prognostic Index

Table S6. Univariable Cox proportional hazards models results including only patients with MIPI information

		Overall survival				Progression Free Survival			
		HR (95% CI)	n	p-value	PH	HR (95% CI)	n	p-value	PH
MYC^{high}	Dichotomized (20% cut-off)	2.04 (1.20-3.47)	195	0.009	<0.05	1.84 (1.10-3.08)	192	0.02	<0.05
% MYC positive cells	Continuous	1.03 (1.01-1.4)	195	<0.001	<0.05	1.04 (1.02-1.05)	192	<0.001	<0.05

CI: confidence interval; HR: Hazard ratio; MIPI: MCL prognostic index; PH: proportional hazardous; n: number

Table S7. Overview of clinicopathological characteristics of patients with tumors that showed structural aberrations for *MYC*.

Patient ID	Cohort	% of MYC positive cells	MYC status	H-score	TMA evaluation	% of cells with alteration (TMA)	Whole-slide evaluation	% of cells with alteration (Whole-slide)	Overall survival (OS)*	OS (years)	Progression Free Survival (PFS)**	PFS (years)
MCL2126	N-MCL2/3	3.39	MYC^{low}	NA	Copy Gain > 20%	44	Copy Gain > 20%	52	0	13.3	0	13.43
MCL3015	N-MCL2/3	5.25	MYC^{low}	NA	Copy Gain > 20%	25	Copy Gain > 20%	50	1	5.01	1	3.186
MCL3033	N-MCL2/3	29.71	**MYC^{high}**	NA	Copy Gain > 20%	38	Copy Gain > 20%	48	1	2.4	1	1.075
MCLB031	SLR	21.39	**MYC^{high}**	81.71	Translocation	55	Translocation	45	1	6.49	1	5.002
MCLB048	SLR	7.16	MYC^{low}	30.06	Copy Gain > 20%	49	Copy Gain > 20%	63	1	5.74		
MCLB059	SLR	57.89	**MYC^{high}**	196.49	Copy Gain > 20%	66	Copy Gain > 20%	79	1	0.92		
MCLB116	SLR	8.72	MYC^{low}	NA	Copy Gain > 20%	21	Copy Gain > 20%	57	0	8.94	0	8.945
MCLB139	SLR	5.57	MYC^{low}	79.12	Copy Gain > 20%	35	NA	59	0	13.6		
MCLB172	SLR	8.54	MYC^{low}	68.81	Copy Gain > 20%	59	Copy Gain > 20%	34	1	1.22		
MCLB185	SLR	8.67	MYC^{low}	69.195	Copy Gain < 20%	4	No amplification	0	1	4.97	1	4.507
MCLB186	SLR	9.90	MYC^{low}	64.035	Copy Gain < 20%	10	Copy Gain < 20%	14	1	0.82	1	0.816
MCLB191	SLR	11.94	MYC^{low}	86.78	Copy Gain < 20%	15	Copy Gain > 20%	24	1	1.56	1	0.257
U081	SLR	5.44	MYC^{low}	NA	Copy Gain > 20%	71.5	Copy Gain > 20%	57	1	1.03	1	0.392
U102	SLR	NA	NA	NA	Copy Gain > 20%	46.5	Copy Gain > 20%	44	1	2.01	1	1.808

* 1 = death, 0 = alive
** 1 = progression, 0 = no progression
NA: Not available; TMA: tissue microarray

Supplementary Figures

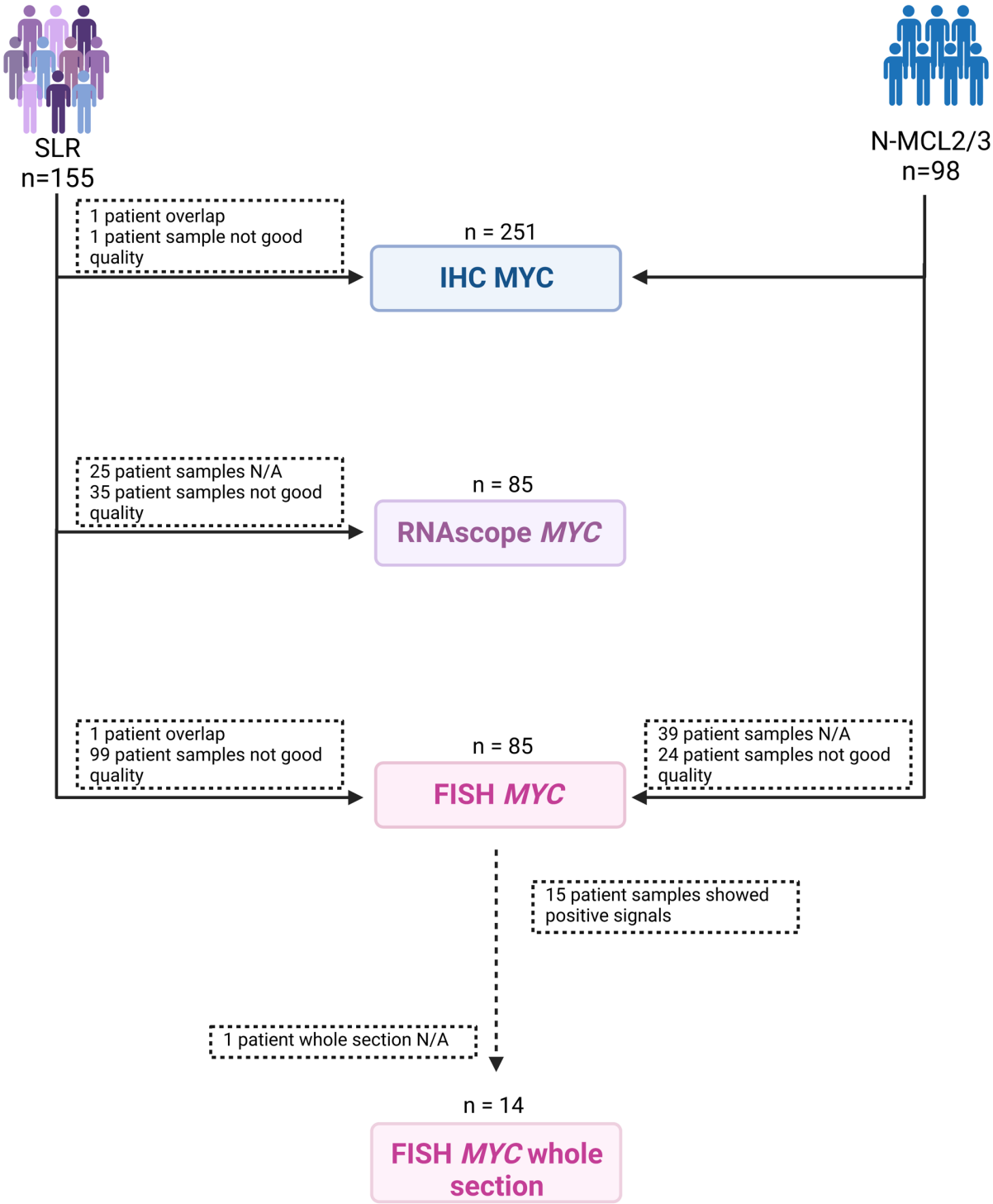
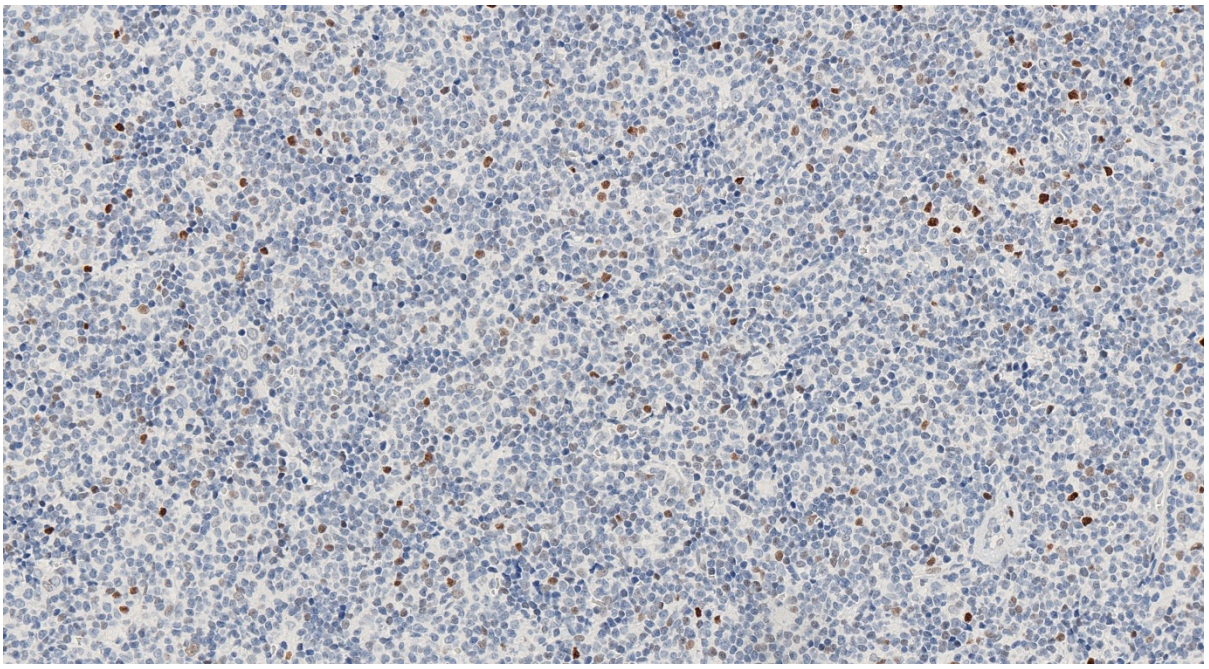


Figure S1. Flowchart of the main analysis performed in this study. For FISH, all cases with high MYC protein expression and/or identified amplifications/translocations identified on TMA analyses were validated on whole tissue sections. FISH: Fluorescent *in situ* hybridization; IHC: immunohistochemistry; N/A: Not available; N-MCL2/3: Nordic lymphoma group – Mantle Cell Lymphoma 2 and 3 protocol; SLR: Swedish Lymphoma Registry cohort;

a)



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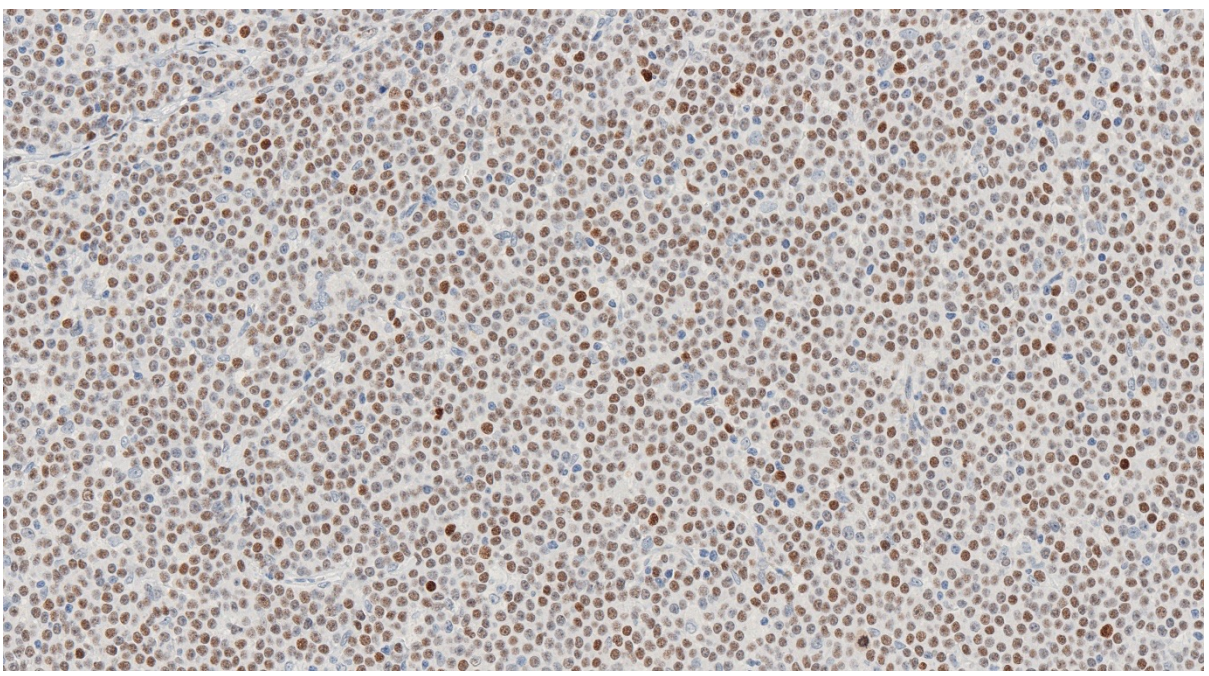
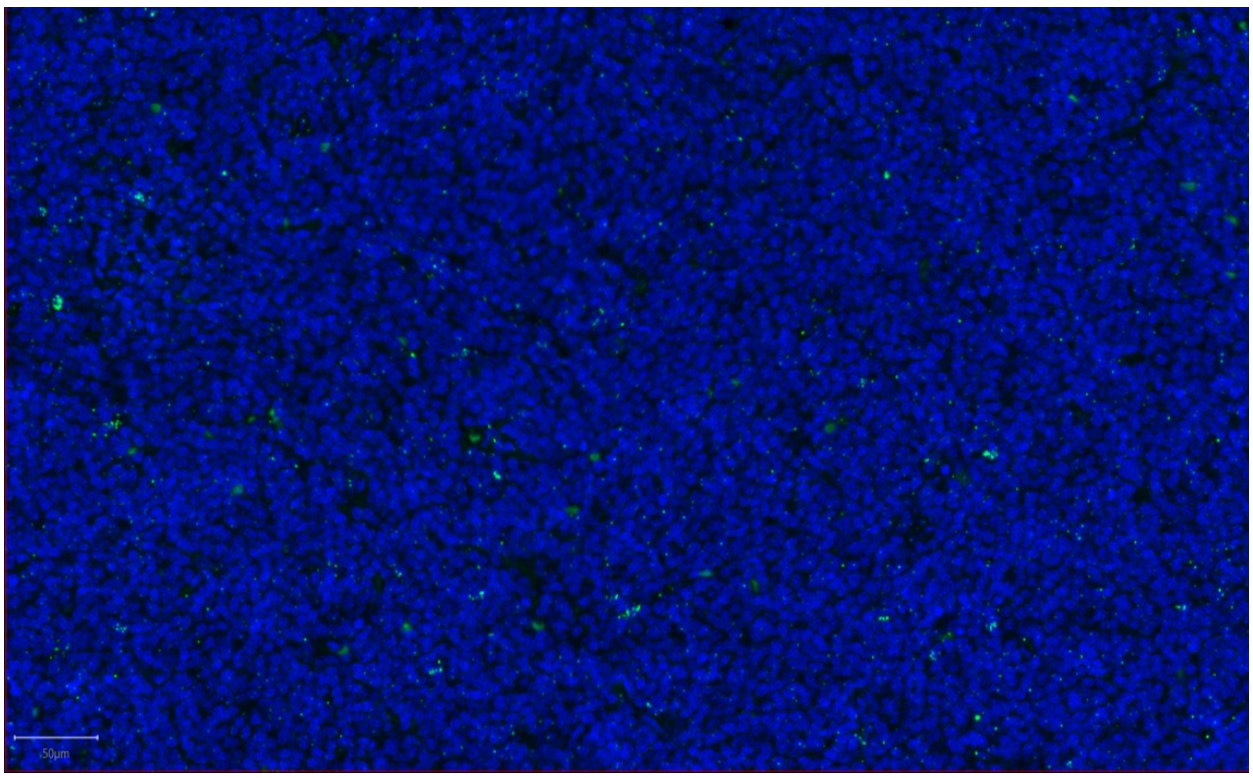


Figure S2. Representative immunohistochemistry stainings of MYC^{high} and MYC^{low} MCL cases. a) Tissue biopsy showing MYC^{low} tumor. b) Tissue biopsy showing MYC^{high} tumour. Slides were scanned at 20X.

a)



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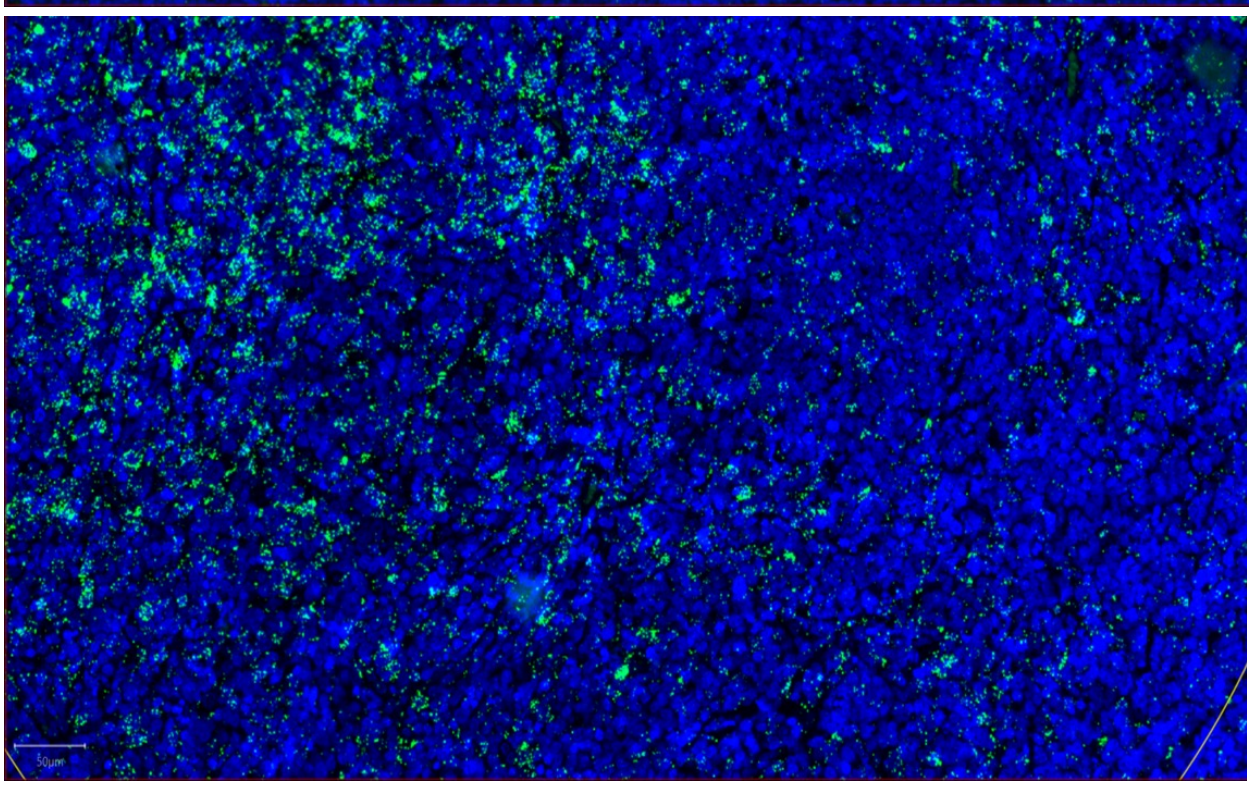


Figure S3. Representative mRNA quantification using RNAscope® of MYC^{high} and MYC^{low} MCL cases. a) Low expression of *MYC* mRNA, H-score < 88; b) High expression of *MYC* mRNA, H-score > 88. Slides were scanned at 20X.

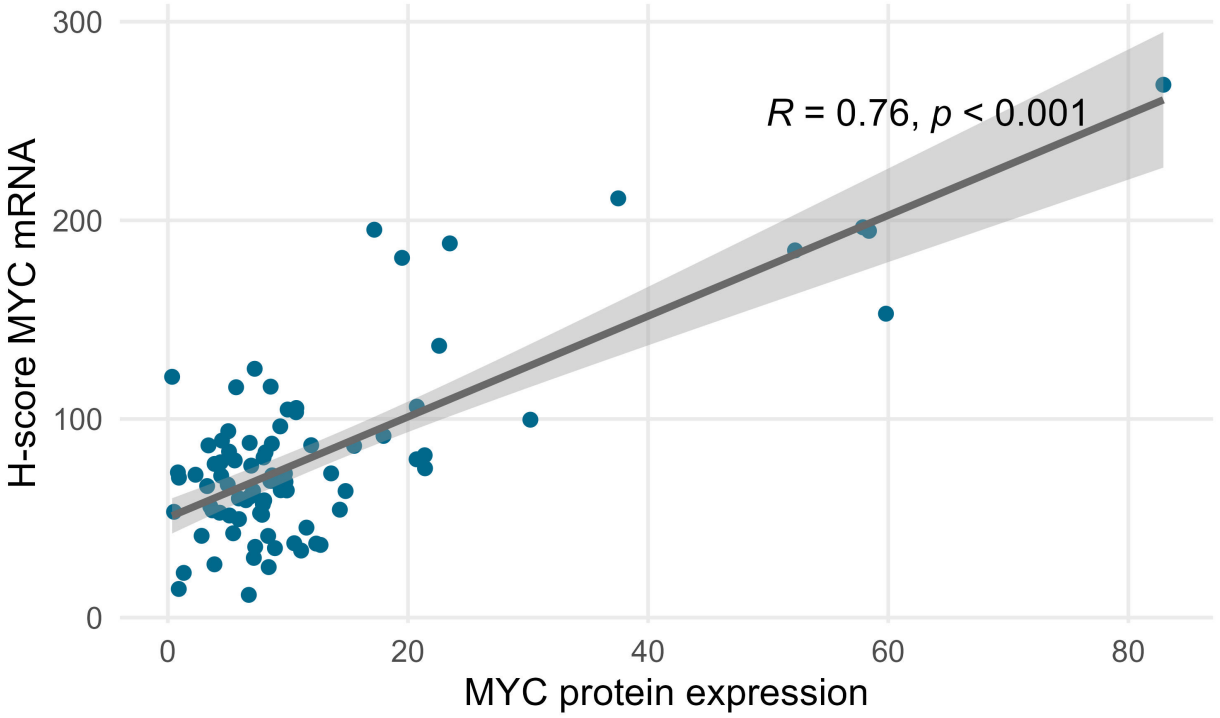


Figure S4. Correlation between H-score (RNAscope®) and percentage of MYC-positive cells (immunohistochemistry, IHC). Spearman correlation coefficient and p-value are shown.

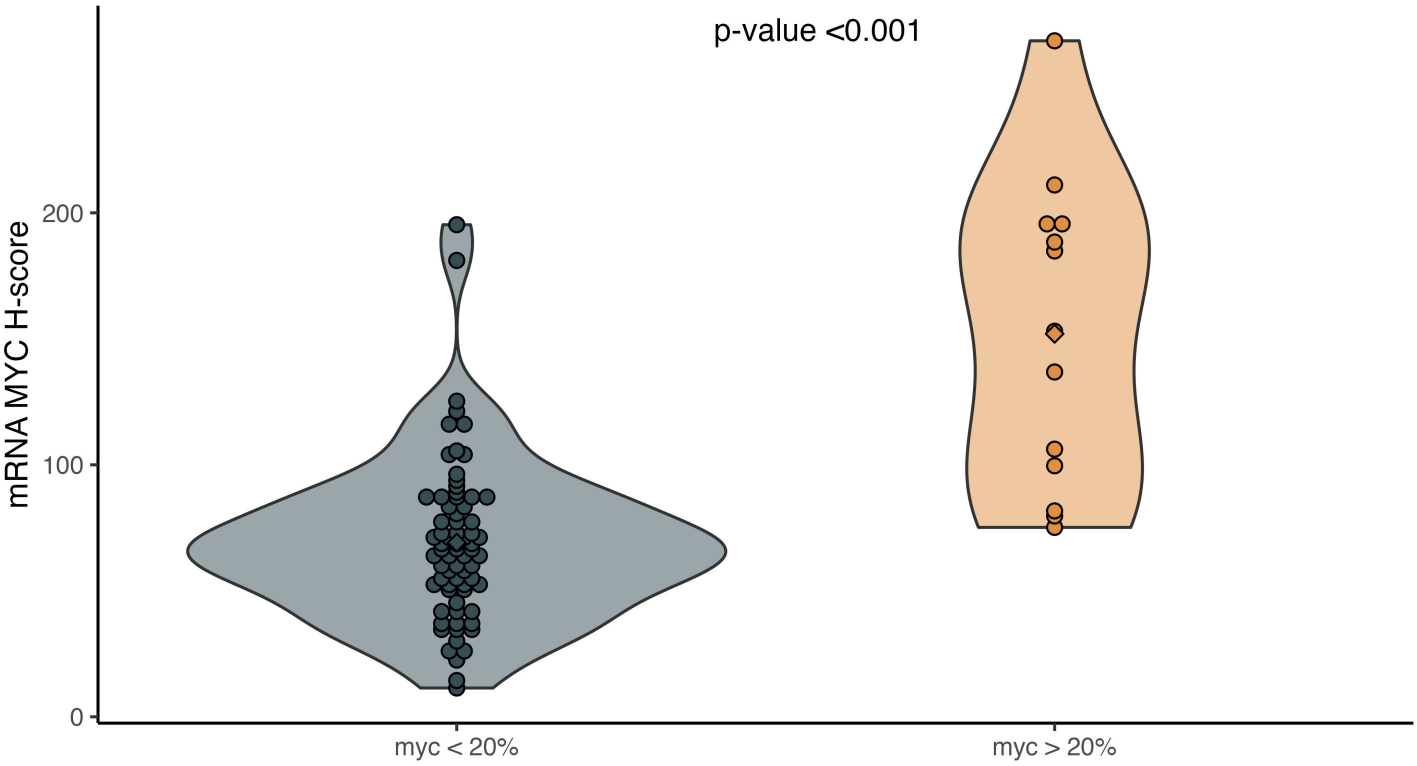


Figure S5. Boxplots showing distribution of MYC mRNA H-score (RNAscope®) and MYC protein expression categories. Wilcoxon test was used to evaluate significance, and p-values are shown.

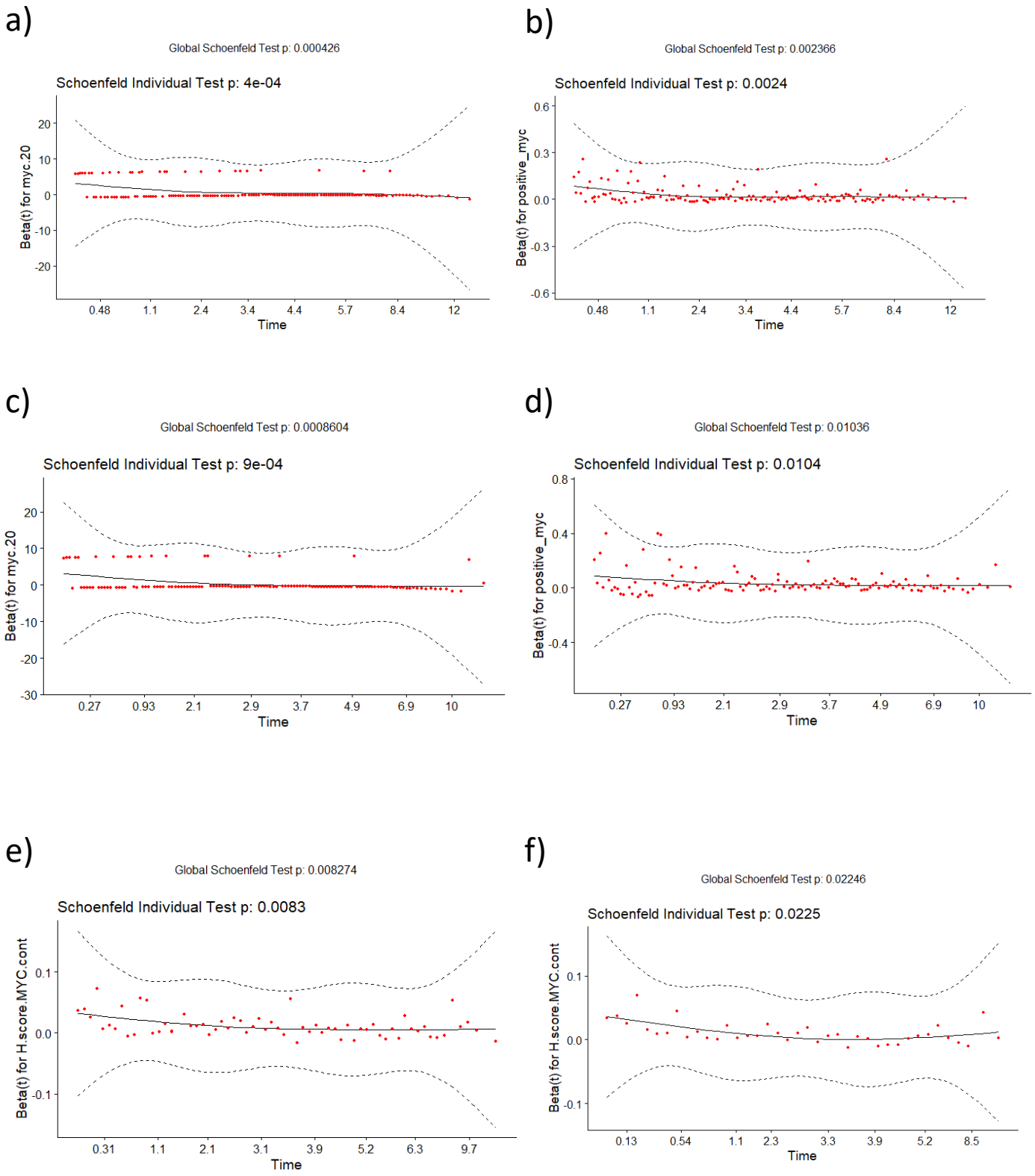


Figure S6. Residuals plots with Schoenfeld test p-value for variables that violated proportional hazards assumption. a) MYC^{high} for variable outcome overall survival (OS). b) MYC^{high} for variable outcome progression free survival (PFS). c) % of MYC-positive cells for variable outcome OS. d) % of MYC-positive cells for variable outcome PFS. e) H-score for *MYC* mRNA for variable outcome OS. f) H-score for *MYC* mRNA for variable outcome PFS.

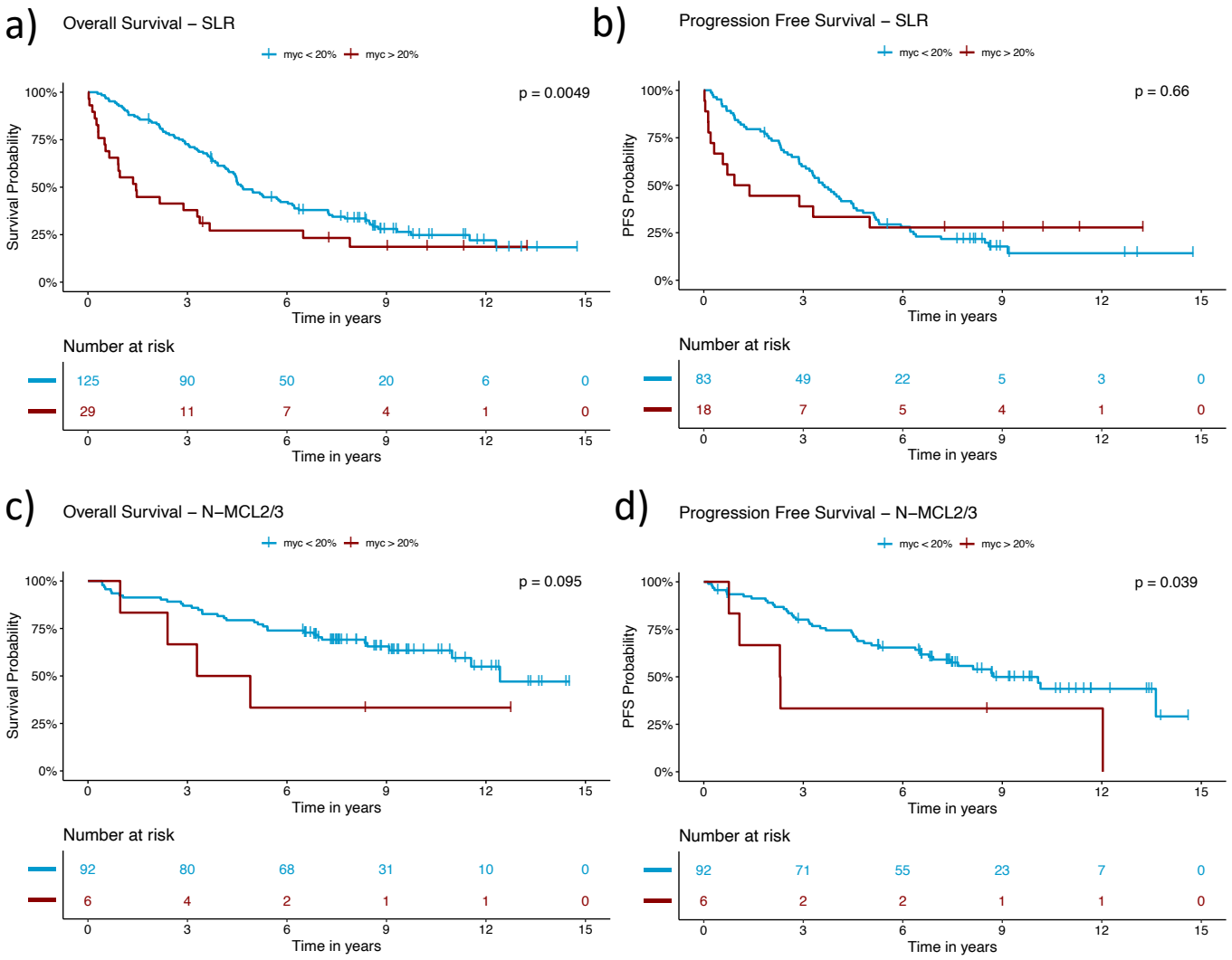


Figure S7. Prognostic impact of MYC overexpression (>20%) per cohort. Impact on a) overall survival (OS) and b) progression free survival (PFS) in the SLR cohort. Impact on c) OS and d) PFS in the N-MCL2/3 cohort. Kaplan-Meier estimates were calculated and are shown. Log-rank statistics were used to evaluate the significance.

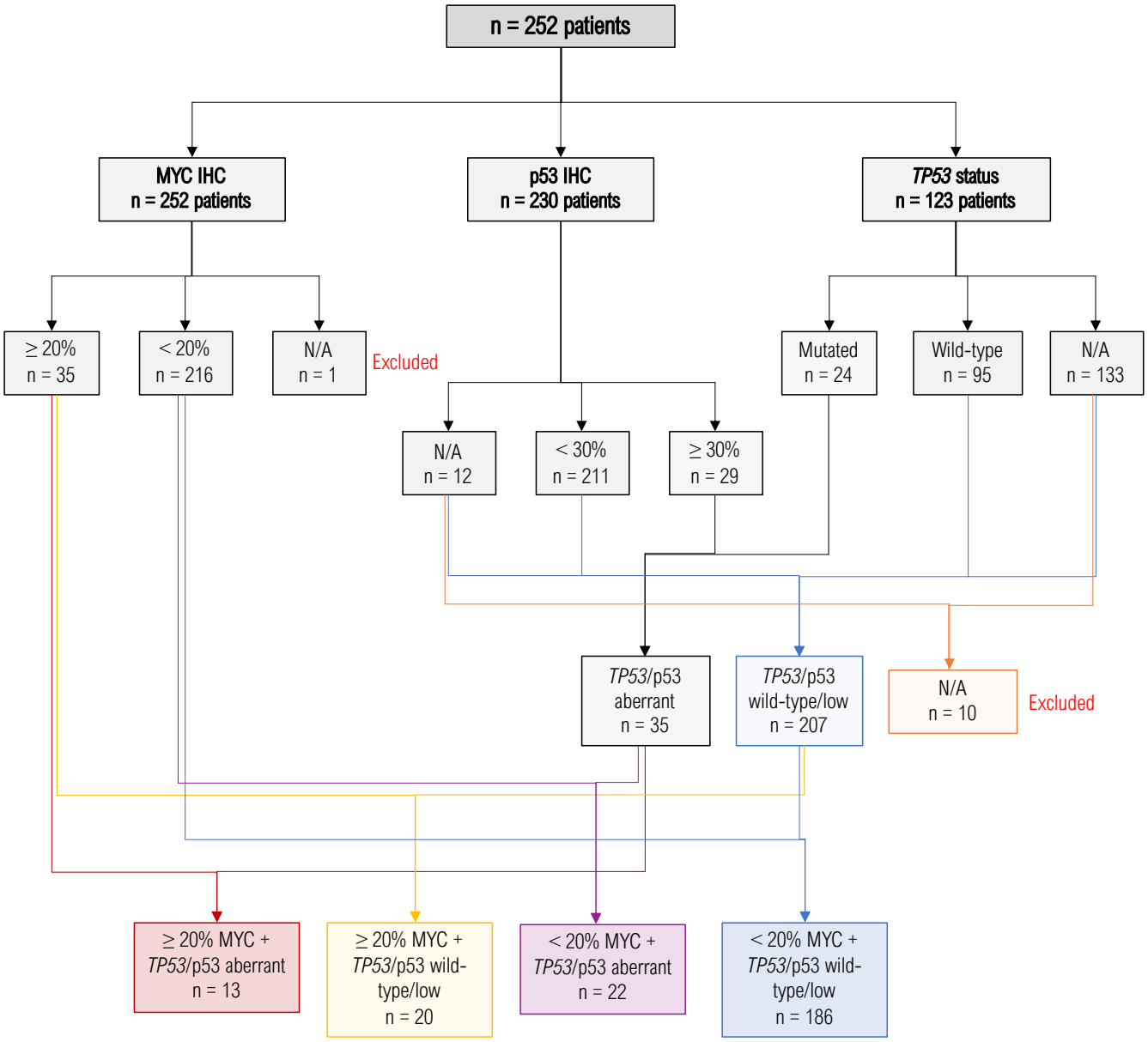


Figure S8. Flowchart showing process for classification of tumors based on their MYC protein expression and *TP53* mutational status/p53 overexpression.

IHC: immunohistochemistry; N/A: not available

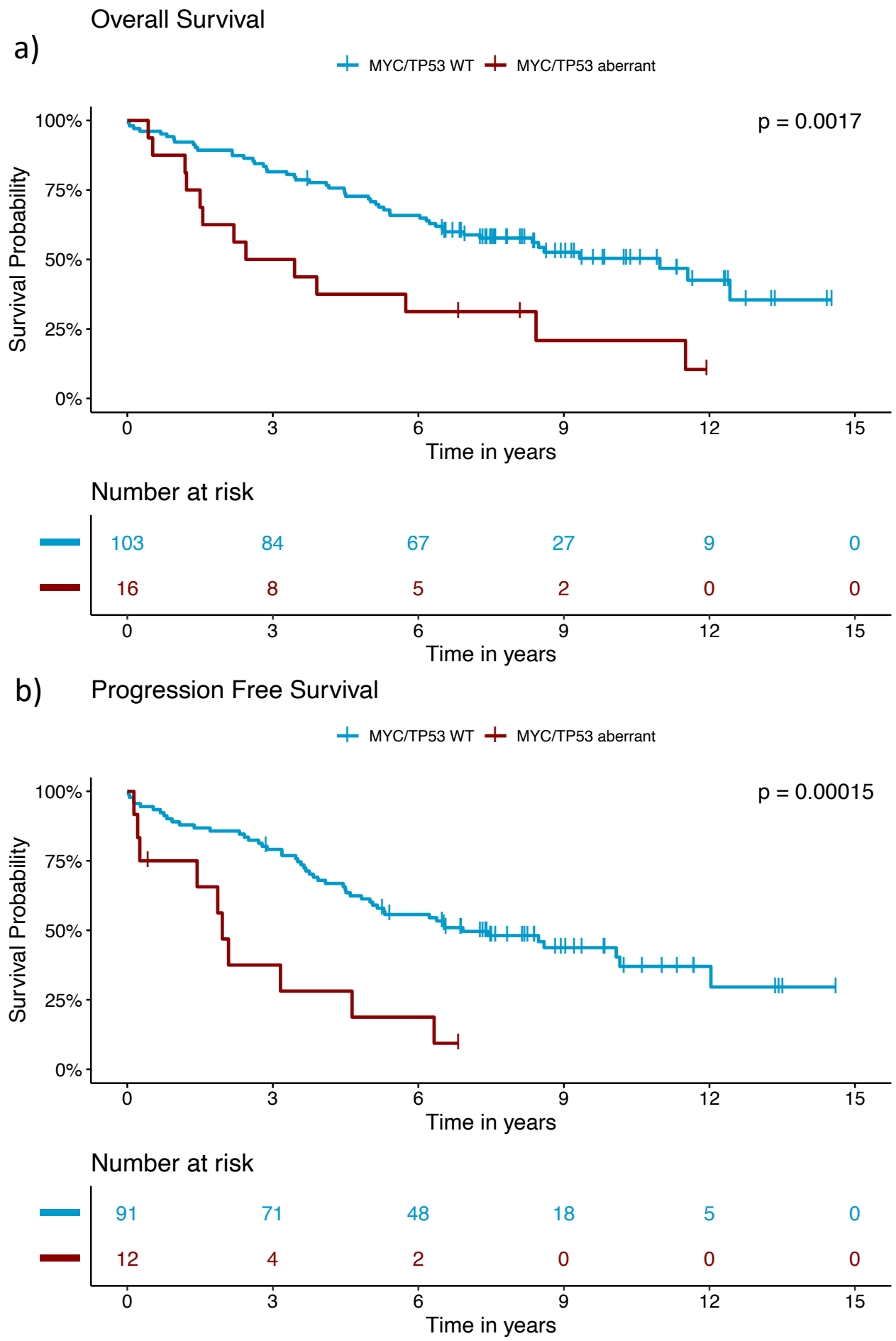
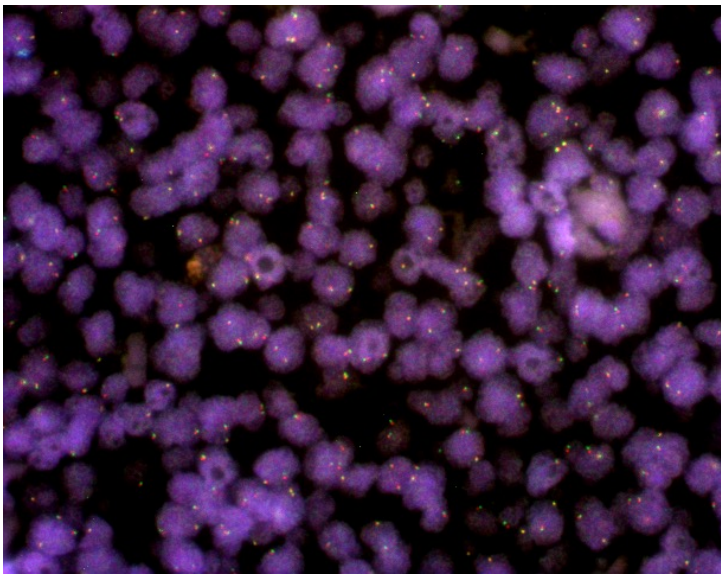
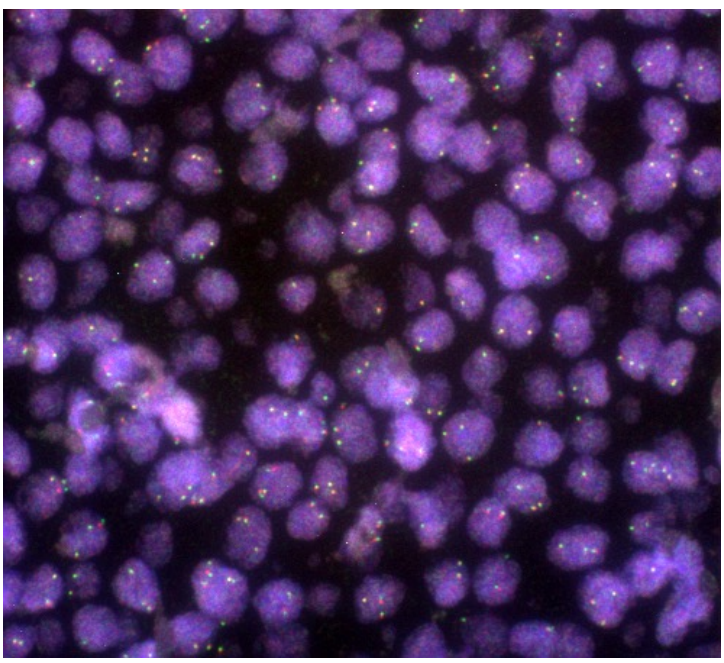


Figure S9. Prognostic impact of MYC overexpression (>20%) and *TP53* mutation double aberrations (without including p53 overexpression) compared to MYC low and *TP53* wt on a) overall survival (OS) and b) progression free survival (PFS). Kaplan-Meier estimates were calculated and are shown. Log-rank statistics were used to evaluate the significance.

a)



b)



c)

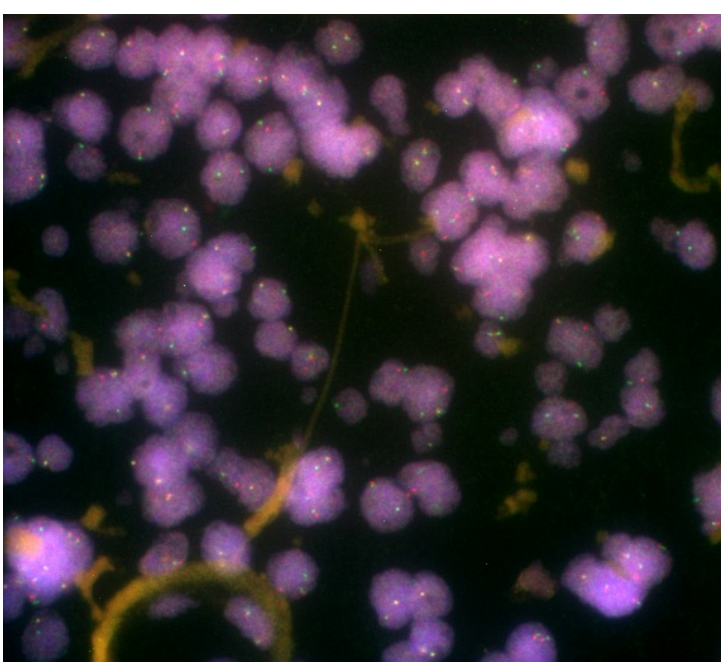


Figure S10. Representative FISH staining using the LSI MYC Break Apart Probe. a) Tumor with no signs of structural alterations. b) Tumor with copy gains in more than 20% of the analyzed cells. c) Tumor with split signals.

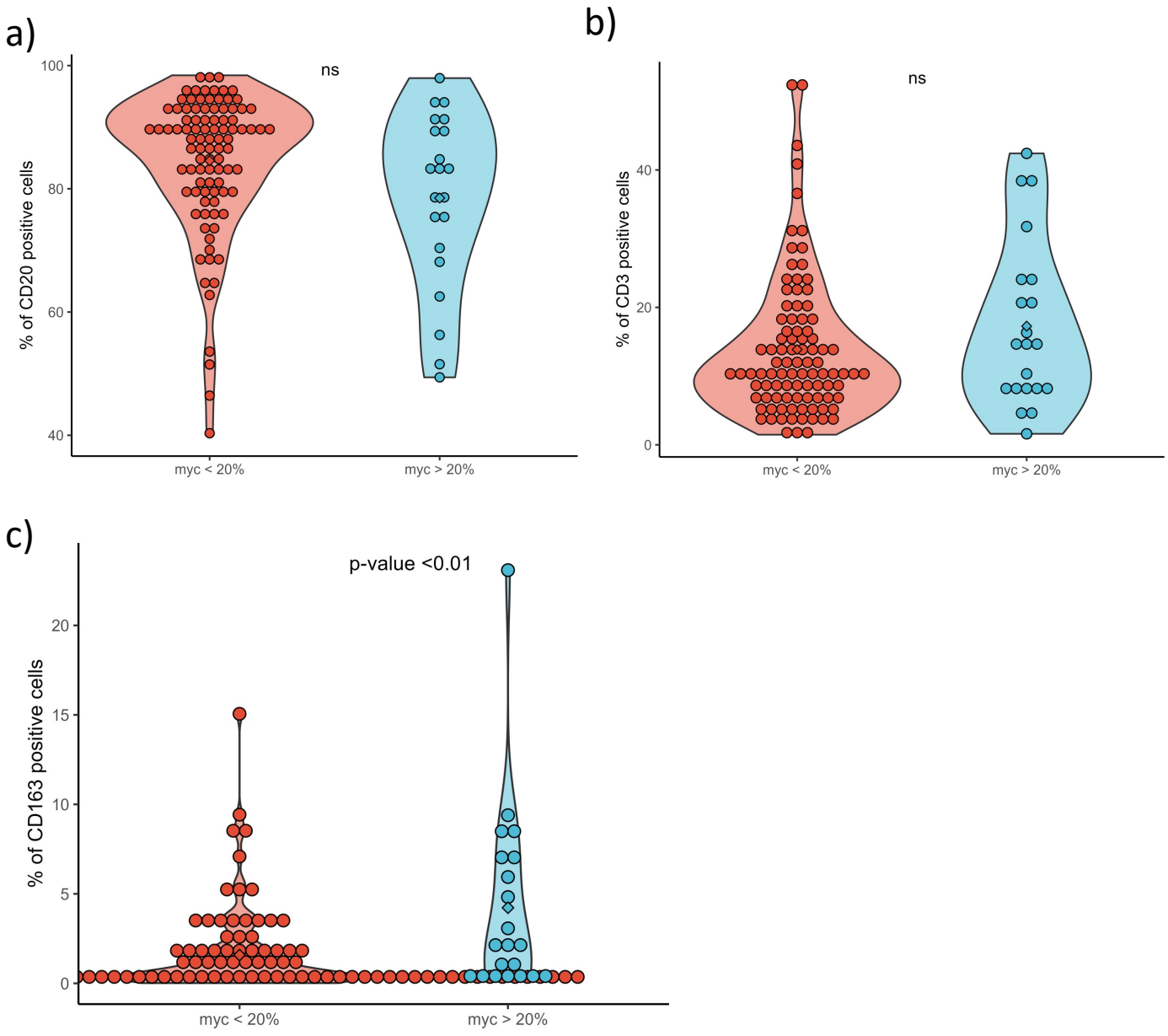


Figure S11. Association between immune microenvironment markers and MYC^{high} tumors. a) Frequency (%) of CD20 positive cells in MYC^{low} and MYC^{high} tumors. b) Frequency (%) of CD3 positive cells in MYC^{low} and MYC^{high} tumors. c) Frequency (%) of CD163 positive cells in MYC^{low} and MYC^{high} tumors. Wilcoxon test was used to evaluate significance, and p-values are shown.