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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Abstract

The transcription factor *MYC* is a well-described oncogene with an important role in lymphomagenesis, but its significance for clinical outcome in mantle cell lymphoma (MCL) remains to be determined. We performed an investigation of the expression of MYC protein in a cohort of 251 MCL patients complemented by analyses of structural aberrations and mRNA, in a sub-cohort of patients. Fourteen percent (n=35) of patients showed high MYC protein expression with >20% positive cells (MYC^{high}), among whom only one translocation was identified, and 86% (n=216) of patients showed low MYC protein expression. Low copy number gains of MYC were detected in ten patients, but with no correlation to MYC protein levels. However, *MYC* mRNA levels correlated significantly to MYC protein levels with a R^2 value of 0.76. Patients with a MYC^{high} tumor had both an independent inferior overall survival and an inferior progression-free survival (hazard ratio [HR]=2.03, 95% confidence interval [95% CI]: 1.2-3.4 and HR=2.2, 95% CI: 1.04-4.6, respectively) when adjusted for additional high-risk features. Patients with MYC^{high} tumors also tended to have additional high-risk features and to be older at diagnosis. A subgroup of 13 patients had concomitant MYC^{high} expression and *TP53*/p53 alterations and a substantially increased risk of progression (HR=16.9, 95% CI: 7.4-38.3) and death (HR=7.8, 95% CI: 4.4-14.1) with an average overall survival of only 0.9 years. In summary, we found that at diagnosis a subset of MCL patients (14%) overexpressed MYC protein, and had a poor prognosis but that *MYC* rearrangements were rare. Tumors with concurrent MYC overexpression and *TP53*/p53 alterations pinpointed MCL patients with a dismal prognosis with a median overall survival of less than 3 years. We propose that MYC needs to be assessed beyond the current high-risk factors in MCL in order to identify cases in need of alternative treatment.

Introduction

MYC is a pleiotropic transcription factor that regulates 10-15% of the genome and can simultaneously affect protein-coding genes and non-coding RNA products.[1,2] It is involved in a plethora of essential cellular mechanisms, such as cell growth, metabolism and protein synthesis, cell adhesion, apoptosis, cell cycle, and angiogenesis.[1-3] MYC is an important factor in B-cell proliferation known to be involved in lymphomagenesis,[3] and its deregulation is frequently associated with worse outcome.[2-4] Translocations involving *MYC* and immunoglobulin genes are described in other B-cell lymphomas, particularly Burkitt and double-hit lymphomas, and are associated with aggressive behavior of the malignancy.[2] Overexpression of MYC without genetic rearrangements also has a negative impact on outcome in B-cell lymphomas in general.[5] In mantle cell lymphoma (MCL), mutations of *MYC* have been shown to

occur in around 20% of cases,[6] and *MYC*-regulated pathways are often affected by genetic alterations in subsets of disease.[7] Cytogenetic investigations of *MYC* were recently recommended as part of clinical routine but are not yet clinically implemented in most hospitals.[8] The frequency of translocations involving *MYC* is reportedly low[9-11] and most reports have been case studies describing translocations that juxtapose *MYC* and *CCND1*.[12-15] In general, consensus on the degree of MYC deregulation in MCL is lacking. Here, we aim to describe the frequency of protein expression, mRNA, translocations, and amplifications of *MYC* in primary MCL and relate the findings to clinicopathological parameters and outcome.

MCL is a disease with a heterogeneous clinical behavior characterized by the chromosomal t(11;14)(q13;q32) that juxtaposes *CCND1* to immunoglobulin genes, leading to constitutive cyclin D1 overexpression.[16] An established prognostic tool, the MCL International Prognostic Index (MIPI), integrates information on age, performance status, lactate dehydrogenase levels and white blood cell count and stratifies patients into high, intermediate and low risk.[17] Additional biological risk factors include *TP53* mutations and/or p53 overexpression, high proliferation, and non-classic morphology.[16] Despite recent improvements in treatment,[18] MCL patients often have a poor prognosis and frequently relapse.[19] Thus, identification and improved understanding of alterations in MCL lymphomagenesis, beyond the already established risk factors, are critical in order to be able to individualize therapeutic decision-making.

To date, a limited number of studies have focused on MYC in MCL. In 2017, Hu *et al*. showed that *MYC* rearrangements were present in less than 1.0% of MCL cases at diagnosis, with these cases having a median overall survival (OS) of 31.3 months.[20] When compared with other subtypes of lymphoma, MCL tumors seem to have a lower frequency of structural alterations involving *MYC*.[21] Nonetheless, in an evaluation of 88 patients, Wang *et al*. described 27 with tumors with *MYC* rearrangements and 21 with extra copies of *MYC*. Both subgroups of patients showed a lower OS when compared to cases with no *MYC* aberrations.[22] However, the study by Wang *et al*., and other investigations reporting *MYC* translocations, were selected cohorts enriched for structural alterations involving *MYC*.[9,20,23] An extensive study evaluating 1,214 lymphomas, including 138 cases of MCL, did not find any *MYC* rearrangements in MCL cases and only 2% had MYC protein overexpression >26%.[11]

Overall, *MYC* aberrations in MCL have been associated with a worse prognosis,[10,23,24] non-classic morphology[20,21,23,24] and, reportedly, enrichment for p53 overexpression among MYC-overexpressing tumors,[24-26] but their additive role in MCL prognosis and clinical characteristics has not been determined. A study in diffuse large B-cell lymphoma showed that tumors with dual MYC/*TP53* alterations had distinct clinicopathological characteristics with worse survival compared to wild-type cases.[27]

In the current study, we investigated the frequency of each molecular layer of MYC deregulation in primary diagnostic samples from a cohort of MCL patients and identified its association with both clinical and molecular high-risk factors.

Methods

Patients' material

Two hundred and fifty-two MCL patients were included in this study, and 154 patients were part of a population-based cohort that comprised patients registered in the Swedish Lymphoma Register (SLR) and diagnosed in Uppsala and Southern Sweden between 2000-2017. The additional 98 samples were derived from patients enrolled in the Nordic Lymphoma Group clinical trials MCL2 and MCL3 (N-MCL2/3) (*Online Supplementary Figure S1*). The N-MCL2 trial was registered with ISRCTN.com ID ISRCTN87866680; the N-MCL3 trial was registered with ClinicalTrials.gov ID NCT00514475. Further details are provided in the *Online Supplementary Materials and Methods*.

This study was approved by the Ethical Regional Committee in Lund (Dnr 2011/593) for part of the SLR cohort (BLISS) and by the Ethical Regional Committee in Uppsala for the N-MCL2/3 samples (Dnr 2009/428) and for part of the SLR cohort (U-CAN) (Dnr 2014/233).

Immunohistochemistry

The patients' tissue microarrays were stained with anti-MYC antibody (clone Y69 1:50, Abcam; Cambridge, UK), as used in the clinical setting and in previous publications.[10,24] Tissue samples were considered to overexpress MYC when the percentage of cells with a dark brown nucleus was ≥20%, in agreement with published data.[24] Details about the immunohistochemistry investigaions are provided in the *Online Supplementary Materials and Methods*.

Fluorescent *in-situ* hybridization

Fluorescent *in-situ* hybridization (FISH) was performed on 4 μm tissue sections using split-signal DNA probes for *MYC* with Vysis MYC Break Apart FISH Probe (Abbott Laboratories; Green Oaks, IL, USA) according to instructions from the manufacturer. An Olympus BX-51 microscope (Prior Lumen200 light source) and GenASIs Capture and Analysis Platform software (Applied Spectral Imaging; Carlsbad, CA, USA) were used to capture digital images of tumor areas. Cases without representative tumor material or with no representative signals were excluded. Positive FISH results on tissue microarrays were validated by evaluating whole-tissue sections.

mRNA *in situ* hybridization

MYC mRNA was evaluated in 85 fresh-frozen, paraffin-embedded samples from the SLR cohort with the RNAscope® assay (Advanced Cell Diagnostics; Newark, CA, USA) following the manufacturer's instructions. The H-score was calculated

for each sample, and was defined as the dynamic range of *MYC* expression based on the quantification of the probe signal on a cell-by-cell level. The workflow is described in detail in the *Online Supplementary Materials and Methods*.

Multiplexed immunofluorescence staining

Tissue microarray slides were stained with anti-CD20 (11.9 μg/mL, clone IGEL/773, Novus Biologicals, Littleton, CO, USA), anti-CD3 (2 μg/mL, clone UM500048CF, OriGene; Rockville, MD, USA) conjugated with AlexaFluor 532 antibody labeling kit (Thermo Fisher Scientific, Waltham, MA, USA), anti-CD163 (1.25 μg/mL, clone EPR14643-36, Abcam; Cambridge, UK) conjugated with AlexaFluor 647 antibody labeling kit (Thermo Fisher Scientific), and Syto13 (500 nM, Nanostring, Seattle, WA, USA).

A software for deep learning artificial intelligence, Aiforia Create Version 5.3 (Aiforia Technologies Plc, Helsinki, Finland), was used for image analyses as further described in the *Online Supplementary Materials and Methods*.

Statistical analysis

A χ^2 test, *t* test or Wilcoxon signed-rank test was used to evaluate differences between groups. The Pearson correlation coefficient was applied to test correlations between continuous variables. The outcome variables considered in the study were OS and PFS. Maximally selected rank statistics (Max Rank) in R[28] was used to determine a cutoff for MYC. Differences were considered statistically significant when the *P* value was <0.05. A detailed description of the statistical analysis is provided in the *Online Supplementary Materials and Methods*.

Results

Patients' clinicopathological characteristics

A total of 252 patients were included in this study (Table 1), with 98 patients belonging to the N-MCL2/3 clinical trials and 154 patients being part of the SLR cohort. Male patients were predominant (75%) and the median age at diagnosis was 63 years. Patients were evenly distributed among the different MIPI risk groups, but this information was only available for 196 of the 252 patients. Thirty-one percent (76/242) of the patients had highly proliferative tumors

Table 1. Clinicopathological characteristics of the patients included in this study.

Characteristic	Full set
Patients, N (%)	252 (100)
Sex, N (%) Male Female	 188 (75) 64 (25)
Age at diagnosis, N (%) ≤65 years >65 years	 137 (55) 115 (45)
MIPI risk group, N (%) Low risk Medium risk High risk Missing	 76 (39) 62 (31) 59 (30) 56
WBC x10^9/L, median (range)	7.8 (2.6-520)
LDH >ULN, median (range)	2.2 (0.5-13)
ECOG performance status, N (%) 0 1 2-4 Missing	 125 (63) 62 (31) 13 (7) 52
Morphology, N (%) Classic Blastoid/pleomorphic Missing	 204 (84) 38 (16) 10
Ki67 index, N (%) <30 ≥30 Missing	 166 (69) 76 (31) 10
MYC expression, N (%) <20% ≥20% Missing	 216 (86) 35 (14) 1
TP53, N (%) Wildtype Mutated Missing	 95 (80) 24 (20) 133
p53 expression, N (%) <30% ≥30% Missing	 211 (88) 29 (12) 12
TP53/p53, N (%) Wildtype/<30% expression Mutated/≥30% expression Missing*	 207 (86) 35 (14) 10
Treatment, N (%) N-MCL2/3 protocols R-CHOP Rituximab-bendamustine Chlorambucil Others Missing	 134 (72) 3 (2) 39 (21) 5 (3) 6 (3) 65
OS in years, median	6.2
PFS in years, median,	4.8**

Percentages might not add up to 100 due to rounding. *Missing information for only one of the variables studied. **Progression-free survival information was only available for 198 patients. N: number; MIPI: Mantle Cell Lymphoma Prognostic Index; WBC: white blood cell; LDH: lactate dehydrogenase; ULN: upper limit of normal; ECOG: Eastern Cooperative Oncology Group; N-MCL2/3: Nordic-Mantle Cell Lymphoma 2/3 clinical trials; R-CHOP: rituximab, cyclophosphamide, doxorubicin, vincristine, prednisone; OS: overall survival; PFS: progression-free survival.

(Ki67 >30%) and 16% (38/242) had non-classic morphology. A subgroup of patients had tumors with high expression of p53 (12%, 29/240) and/or *TP53* mutations (20%, 24/119). Thus, 35/242 cases had either high p53 protein expression or a *TP53* mutation. The discrepancy in frequency between the p53 and *TP53* evaluation is mostly due to the lower number of samples sequenced, restricted by the availability of high-quality material. Overall, patients had a median OS of 6.2 years and a median PFS of 4.8 years. Of note, OS information was available for all patients included, whereas PFS was calculated based on 200 patients. The patients' clinicopathological characteristics, divided by cohort, can be found in *Online Supplementary Table S1*.

MYC overexpression

Based on previous studies in which MYC expression was assessed in a cohort of 65 cases of MCL,[24] a cutoff of 20% was used to define patients with MYC protein overexpression (MYC^{high}). Immunohistochemistry was used to determine MYC protein expression. The mean expression was 13.1% (range, 0.14-82.9%) and the median expression was 8.7%. Max Rank statistics showed that OS outcome differences were maximized when groups were dichotomized with a cutoff at 21.4%, supporting the applicability of the 20% cutoff used to define MYC overexpression.[24]

Using the 20% cutoff, MYC was overexpressed in 14% of all tumor samples studied (35/252) (Table 1). *Online Supplementary Figure S2* shows representative immunohistochemistry staining for MYC.

MYC mRNA and immunohistochemistry results are concordant

To evaluate *MYC* mRNA expression levels, we performed RNAscope®; representative images of the staining are shown in *Online Supplementary Figure S3*. An H-score for *MYC* mRNA expression was calculated, which showed a strong correlation (Pearson) to the frequency of positive cells detected by immunohistochemistry (*Online Supplementary Figures S4* and *S5*) with a R^2 value of 0.76 (P<0.001), in the same range as previously reported for MCL.[10] Information about the cohort used for RNAscope® can be found in *Online Supplementary Table S2*.

MYC^{high} is associated with poor outcome

Outcome in patients with MYC^{high} tumors was inferior compared to that of patients with MYC^{low} tumors (Figure 1). Patients with MYC^{high} MCL had a median OS of 2.2 years and PFS of 1.8 years, whereas patients with MYC^{low} tumors had a median OS of 7.3 years and PFS of 5.2 years. In concordance, patients with MYC^{high} tumors also had a significantly higher risk of death (hazard ratio [HR]=2.34, 95% confidence interval [955 CI]: 1.55-3.57) and disease progression (HR=1.73, 95% CI: 1.05-2.86) compared to patients with MYC^{low} tumors. The frequency of MYC-positive cells was also significantly associated with poor outcome as a continuous variable, for both OS and PFS. However, per increased percentage of MYC-positive cells, the risk of death or progression only increased by 3% and 4%, respectively (Table 2). Likewise, for *MYC* mRNA (as a continuous value), a higher H-score was associated with inferior survival and shorter progression-free survival, albeit with a low hazard ratio per H-score unit increase (Table 2). MYC protein measurements violated the proportional hazards assumption, showing a greater impact on short-term survival (*Online Supplementary Figure S6*). Of the cases classified as MYC^{high}, 57% died and 62% progressed within the first 3 years (Figure 1). In a follow-up of 3 years, the proportional hazards assumption for PFS was not violated and a negative impact on survival could be observed (*Online Supplementary Table S3*). Similar trends were seen when evaluating the effect of MYC^{high} and MYC frequency

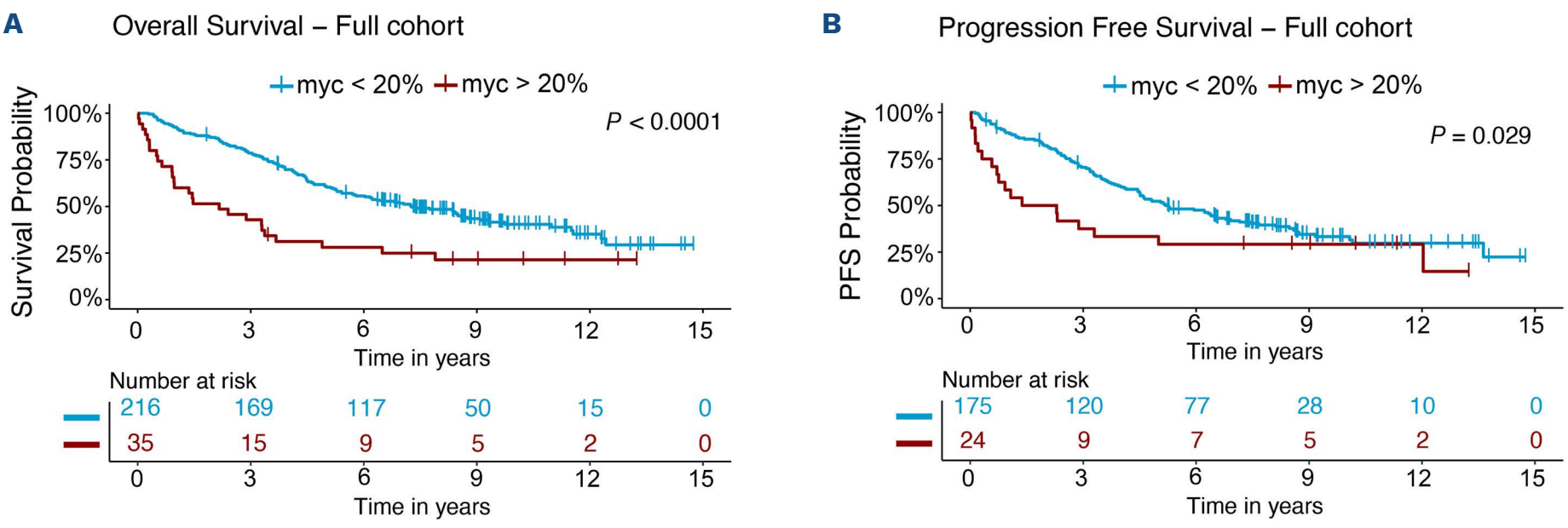

Figure 1. Patients with tumors with MYC^{high} protein overexpression have a worse outcome than patients with MYC^{low} tumors. (A, B) Prognostic impact of MYC protein expression on overall survival (A) and progression-free survival (B). Samples (N=252) were categorized based on total percentage of MYC-expressing cells, with a cutoff of 20%. Kaplan-Meier estimates were calculated and are shown. Log-rank statistics were used to evaluate the statistical significance. PFS: progression-free survival.

as a continuous value on outcome for the different cohorts separately (*Online Supplementary Figure S7, Online Supplementary Table S4*). However, in the SLR cohort, MYC^{high} was not statistically prognostic for PFS, probably because of the heterogenous treatment protocols used in this population-based cohort. In the N-MCL2/3 cohort, only six patients were classified as MYC^{high} and the group reached statistical significance as predictor for PFS, but not OS (*Online Supplementary Table S4*).

To understand if there were consistent differences among MYC^{high} cases depending on whether they had an OS shorter or longer than 3 years, we compared the main clinicopathological parameters of these two groups of patients (*Online Supplementary Table S5*). The patients with an OS less than 3 years had additional high-risk factors, being in a high-risk MIPI group and the majority also with high proliferation and/or *TP53*/p53 aberrations. Surprisingly, the male/female ratio showed a major difference, with only one out of nine female MYC^{high} patients surviving for more than 3 years.

MYC^{high} tumors remained associated with OS (HR=2.03, 95% CI: 1.22-3.40) and PFS (HR=2.20, 95% CI: 1.04-4.64) when adjusting for gender, age and established high-risk factors (Table 3). MYC^{high} was not significant when adjusting for MIPI group (Table 3). Of note, evaluation of MYC protein expression on prognosis in MCL remained significant only when considering patients with MIPI information (*Online Supplementary Table S6*). MYC as a continuous variable was not independent of high-risk factors (*data not shown*).

MYC^{high} is associated with high-risk factors

Patients with MYC^{high} tumors were enriched in other high-risk factors such as age, Ki-67 proliferation index, non-classic morphology, MIPI group and *TP53*/p53^{+} aberrations (Figure 2A, Table 4). Patients with MYC^{high} tumors were on average older at diagnosis (Figure 2B), with a median age at diagnosis of 70.7 years *versus* 63.6 years for patients with MYC^{low} tumors. MYC protein overexpression (MYC^{high}) was found to be associated with the presence of *TP53* mutations and/or p53 overexpression (hereon referred to as *TP53*/p53^{+} tumors, n=35). Dual alterations of MYC and *TP53*/p53^{+} (hereon referred to as MYC^{high} *TP53*/p53^{+}) were detected in 13 out of 250 patients (Table 4). Tumors with alterations in *TP53* had a higher median expression of MYC-positive cells compared to wild-type tumors (*P*<0.001) (Figure 2C) and MYC^{high} *TP53*/p53^{+} cases were more likely to have high proliferation, non-classic morphology and be in a high-risk MIPI group, similar to the MYC^{high} group (Table 4).

MYC^{high} *TP53*/p53^{+} tumors have a dismal prognosis

A total of 13 patients had tumors classified as MYC^{high} *TP53*/p53^{+}. These patients had a very short median survival (0.9 years) and a shorter median PFS (0.5 years) compared to patients who did not show concomitant alterations in these molecules. The majority died within 3 years of diagnosis (Figure 3A). This was significantly lower compared to either one of the individual high-risk groups (Figure 3B). All patients with PFS information available with MYC^{high} *TP53*/p53^{+} tumors progressed within 2 years (Figure 3C) and had a shorter time to progression than either of the patient groups presenting with only one of the individual risk factors (Figure 3D). *Online Supplementary Figure S8* depicts the process for classification of tumors based on MYC and *TP53*/p53 status. Double aberrations conferred an increased risk of death (HR=7.83, 95% CI: 4.35-14.09) and disease progression (HR=16.87, 95% CI: 7.43-38.31) (Table 2). MYC^{high} *TP53*/p53^{+} aberrations remained prognostic, for both OS and PFS, when adjusting for additional high-risk factors (Table 5). Of note, prognostic analysis considering only *TP53* mutation and MYC^{high} tumors remained significant for both OS and PFS (*Online Supplementary Figure S9*).

MYC protein expression is not correlated with genomic aberrations

To explore the hypothesis that MYC protein overexpression can be associated with genomic rearrangements of *MYC*, a total of 85 cases mounted in tissue microarrays were evaluated by FISH with a *MYC* break-a-part probe. Of those 85 patients, 70 (82%) showed no signs of genomic alterations of *MYC*. Among the remaining 15 cases, three showed copy gains in less than 20% of the evaluated cells, 11 showed copy gains in more than 20% of the evaluated cells and one case showed the presence of a translocation (*Online Supplementary Figure S10*).

To validate the FISH findings, whole tissue sections were used. Diagnostic tissue blocks from 14 out of the 15 patients were available for further evaluation (*Online Supplementary*

Table 2. Univariable Cox proportional hazards models results.

		Overall survival				Progression-free survival			
		HR (95% CI)	N	*P*	PH	HR (95% CI)	N	*P*	PH
MYC^{high}	Dichotomized (20% cutoff)	2.34 (1.55-3.57)	251	<0.001	<0.05	1.73 (1.05-2.86)	199	<0.001	<0.05
% MYC positive cells	Continuous	1.03 (1.02-1.04)	251	<0.001	<0.05	1.04 (1.00-1.05)	199	<0.001	<0.05
MYC H-score	Continuous	1.01 (1.01-1.02)	85	<0.001	<0.05	1.01 (1.004-1.02)	51	<0.001	<0.05
MYC^{high} *TP53*/p53^{+}	Categorical	7.83 (4.35-14.09)	250	<0.001	>0.05	16.9 (7.43-38.31)	198	<0.001	>0.05

HR: hazard ratio; 95% CI: 95% confidence interval; N: number; *P*: probability value; PH: proportional hazard.

Table 3. MYC protein multivariable Cox proportional hazards models.

Variable	Overall survival			Progression-free survival*		
	HR (95% CI)	*P*	N	HR (95% CI)	*P*	N
MYC^{high} *vs.* MYC^{low}	2.03 (1.22-3.40)	0.007	229	2.20 (1.04-4.64)	0.04	182
Age	1.08 (1.06-1.10)	<0.001	229	1.08 (1.05-1.12)	<0.001	182
Non-classic *vs.* classic morphology	1.14 (0.65-2.00)	0.65	229	1.20 (0.59-2.40)	0.62	182
High Ki-67 (>30%) *vs.* low (<30%)	1.52 (0.97-2.37)	0.07	229	2.79 (1.48-5.26)	0.002	182
Female *vs.* male	1.08 (0.72-1.61)	0.72	229	1.12 (0.61-2.05)	0.71	182
p53 overexpression *vs.* wt p53 expression	2.52 (1.52-4.19)	<0.001	229	4.97 (2.57-9.63)	<0.001	182
MYC^{high} *vs.* MYC^{low}	1.34 (0.69-2.61)	0.38	179	1.64 (0.78-3.47)	0.20	176
MIPI risk group Intermediate High	 2.17 (1.21-3.88) 8.57 (4.87-15.1)	 0.009 <0.001	179	 2.16 (0.95-4.90) 6.90 (3.25-14.64)	 0.07 <0.001	176
Non-classic *vs.* classic morphology	1.26 (0.70-2.28)	0.44	179	0.99 (0.50-1.95)	0.98	176
High Ki-67 (>30%) *vs.* low (<30%)	1.16 (0.70-1.93)	0.57	179	2.56 (1.37-4.79)	0.003	176
Female *vs.* male	1.28 (0.80-2.04)	0.30	179	1.14 (0.69-2.10)	0.67	176
p53 overexpression *vs.* wt p53 expression	1.85 (1.05-3.26)	0.03	179	4.40 (2.32-8.35)	<0.001	176

*Progression-free survival analysis truncated at 3 years. HR: hazard ratio; 95% CI: 95% confidence interval; *P*: probability value; N: number; wt: wild-type; MIPI: Mantle Cell Lymphoma International Prognostic Index.

Table 4. Differences in clinicopathological characteristics with a focus on established high-risk factors comparing MYC^{high} tumors with MYC^{low} tumors and comparing MYC^{high}*TP53*/$p53^{+}$ tumors to all others.

Characteristic		MYC^{high} N (row %)	MYC^{low} N (row %)	*P**	MYC^{high} *TP53*/$p53^{+}$ N (row %)	All others N (row %)	*P**
	Total N (%)	35 (14)	216 (86)		13 (5)	237 (95)	
Age ≤65 years >66 years	 137 (54) 115 (46)	 12 (34) 23 (66)	 124 (57) 92 (43)	0.01	 4 (31) 9 (69)	 132 (56) 105 (44)	0.08
Sex Male Female	 188 (75) 64 (25)	 26 (74) 9 (26)	 162 (75) 54 (25)	0.93	 7 (54) 6 (46)	 179 (76) 58 (24)	0.08
Ki-67 <30% ≥30% Missing	 166 (69) 76 (31) 10	 10 (32) 21 (68) 4	 156 (74) 54 (26) 6	<0.001	 3 (23) 10 (77) 0	 163 (71) 66 (29) 8	<0.001**
Morphology Classic Non-classic Missing	 204 (84) 38 (16) 10	 20 (65) 11 (35) 4	 183 (87) 27 (23) 6	0.001	 7 (54) 6 (46) 0	 197 (86) 32 (14) 8	0.007**
MIPI risk group Low Intermediate High Missing	 76 (39) 61 (31) 59 (30) 56	 1 (4) 9 (39) 13 (57) 12	 75 (44) 52 (30) 45 (26) 44	<0.001	 0 (0) 1 (12) 7 (88) 5	 76 (41) 59 (32) 52 (28) 50	0.001**
TP53/p53 Wild-type Mutated/overexpressed Missing	 194 (85) 35 (15) 23	 19 (59) 13 (41) 3	 174 (89) 22 (11) 20	<0.001	-	-	-

Percentages for some variables do not add up to 100% due to rounding. *A χ^2 statistical test was performed to evaluate significant differences between patients with different levels of MYC expression or those double-positive for MYC and *TP53*/p53 and the clinicopathological characteristics. **A χ^2 statistical test was performed with Yates continuity correction to adjust for small values. N: number; *P*: probability value; MIPI: Mantle Cell Lymphoma International Prognostic Index.

Table S7). Results were consistent between tissue microarrays and full tissue sections. No correlation between copy number gains of *MYC* and protein overexpression was observed, as most of tumors with *MYC* copy number gains had a low frequency of cells expressing MYC protein (*Online Supplementary Table S7*). The presence of copy number gains was not associated with outcome (*data not shown*). The single *MYC*-translocated MCL case had an OS of over 6 years, no other high-risk factors but a classic morphology, low Ki-67 expression, and was *TP53*/p53 wild-type.

In line with the lack of genomic alterations, *MYC* showed genetic mutations in only one patient, determined by targeted sequencing of part of the SLR cohort as previously published.[29] This *MYC* mutated case only had 4% of cells expressing MYC protein.

MYC^{high} tumors showed an increase in M2-like macrophage infiltration

In a recent study, we showed that the frequencies of T cells and M2 macrophages are prognostic in MCL[30] and hypothesized that the composition of the microenvironment would be different in MYC^{high} and MYC^{low} cases. T-cell and M2 macrophage frequencies were determined based on multiplexed immunofluorescence staining using machine learning (Aiforia software). The multiplexed immunofluorescence staining included stains for CD3, CD163 and CD20. For a total of 117 patients, information was available for both multiplexed immunofluorescence and MYC status. T-cell infiltration, marked as CD3 positivity, ranged from 1.48% to 52.58% of total cells. The presence of CD163^{+} cells, as a surrogate marker for M2 macrophages, varied between 0.02% and 23.09% of total cells. The abundance of T cells and M2-like macrophages and the density of tumor cells in the tumor regions in MYC^{high} and MYC^{low} cases were compared to assess the association with MYC status. MYC^{high} cases had a higher infiltration of CD163^{+} macrophages, whereas no difference was seen for T-cell infiltration or density of tumor cells (*Online Supplementary Figure S11*).

Discussion

The impact of *MYC* deregulation on outcome in MCL is consistently reported in the literature,[10,22,23] but most studies include limited or selected cohorts. The present study of 252 MCL patients is, to the best of our knowledge, the largest study so far to evaluate clinical impact by exploring MYC protein expression and its association with clinicopathological features including established risk factors. The study is focused on the impact of MYC protein, and the

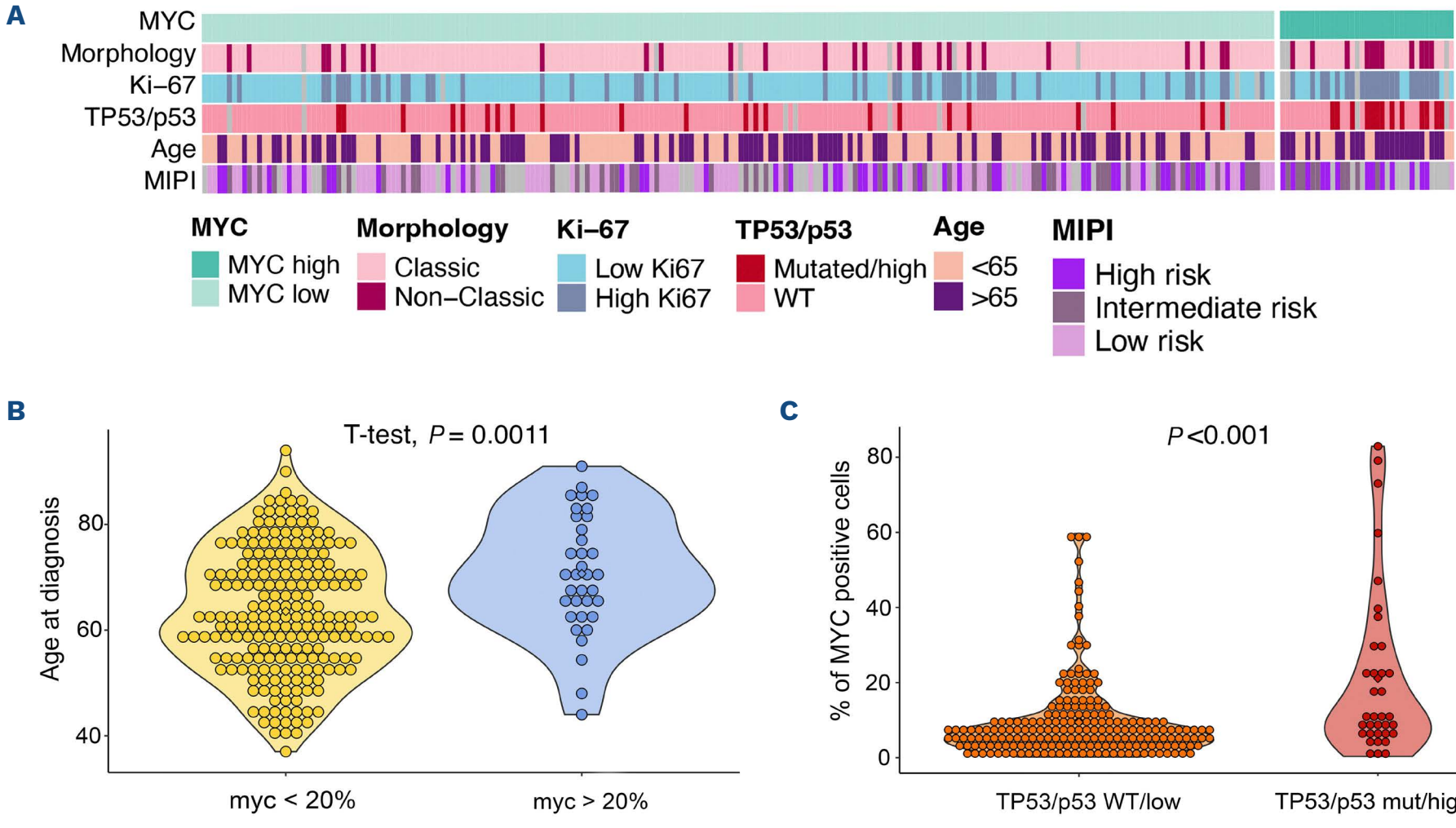

Figure 2. High-risk mantle cell lymphoma is enriched for MYC^{high} tumors. (A) Heatmap representing the distribution of patients with high and low MYC protein expression, *TP53* mutation/p53 expression, morphology variant, proliferation (Ki-67) and age at diagnosis. (B) Association between age at diagnosis and MYC^{high} tumors. (C) Association between total percentage of MYC-positive cells and *TP53*/p53 status. Wilcoxon and *t*-test *P* values were used to evaluate statistical significance, and *P* values are shown. WT: wild-type; MIPI: Mantle cell lymphoma International Prognostic Index.

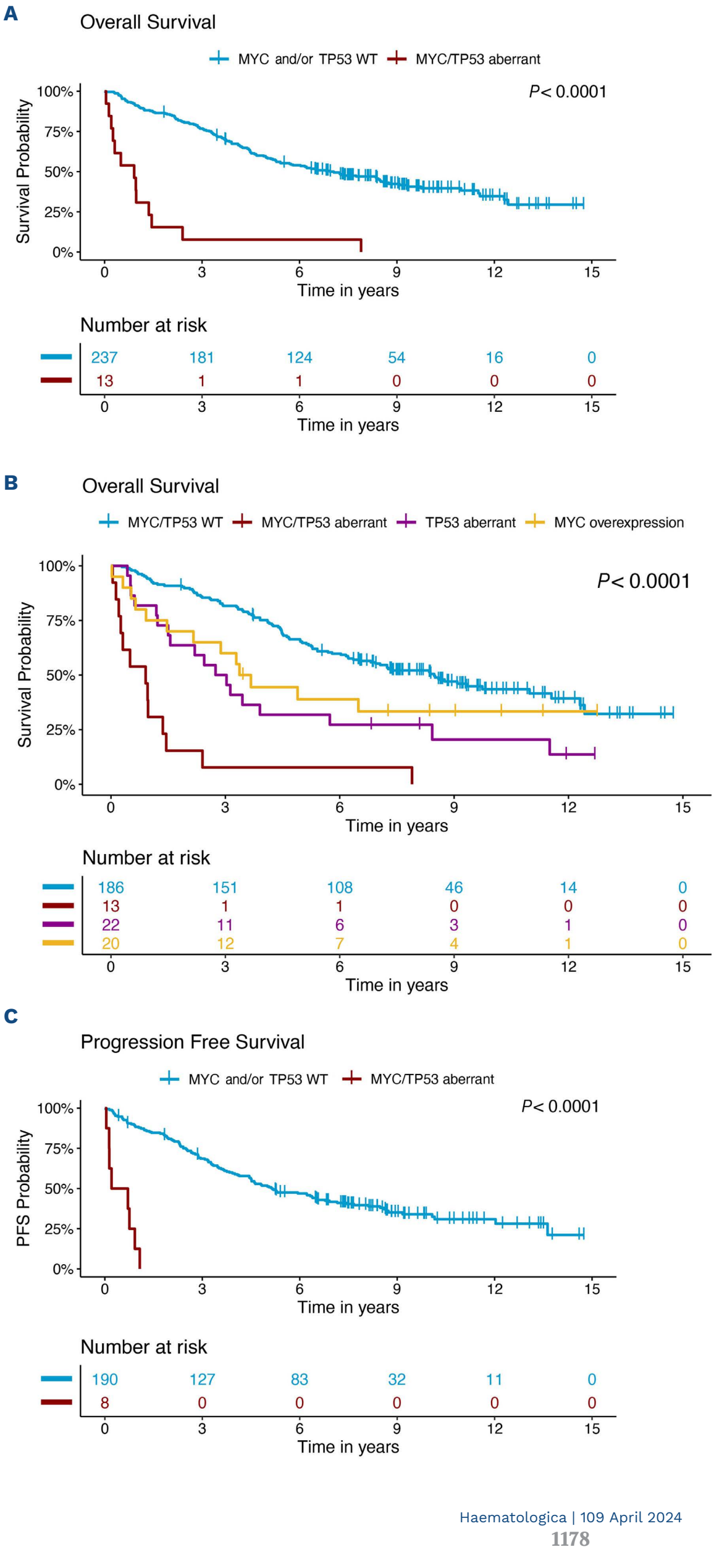

Figure 3. Patients with MYC^{high}*TP53*/p53^{+} form a subgroup with adverse prognosis. (A) Prognostic impact of MYC^{high}*TP53*/p53^{+} *versus* all other states on overall survival. (B) Prognostic impact on overall survival for patients with tumors that were classified only as MYC^{high}, only *TP53*/p53^{+}, both MYC^{high}*TP53*/p53^{+} or wild-type for both markers. (C) Prognostic impact of MYC^{high}*TP53*/p53^{+} *versus* all other patients on progression-free survival. (D) Prognostic impact on progression-free survival for patients with tumors that were classified only as MYC^{high}, only *TP53*/p53^{+}, both MYC^{high}*TP53*/p53^{+} or wild-type for both markers. Kaplan-Meier estimates were calculated and are shown. Log-rank statistics were used to evaluate the statistical significance of differences. WT: wild-type; PFS: progression-free survival.

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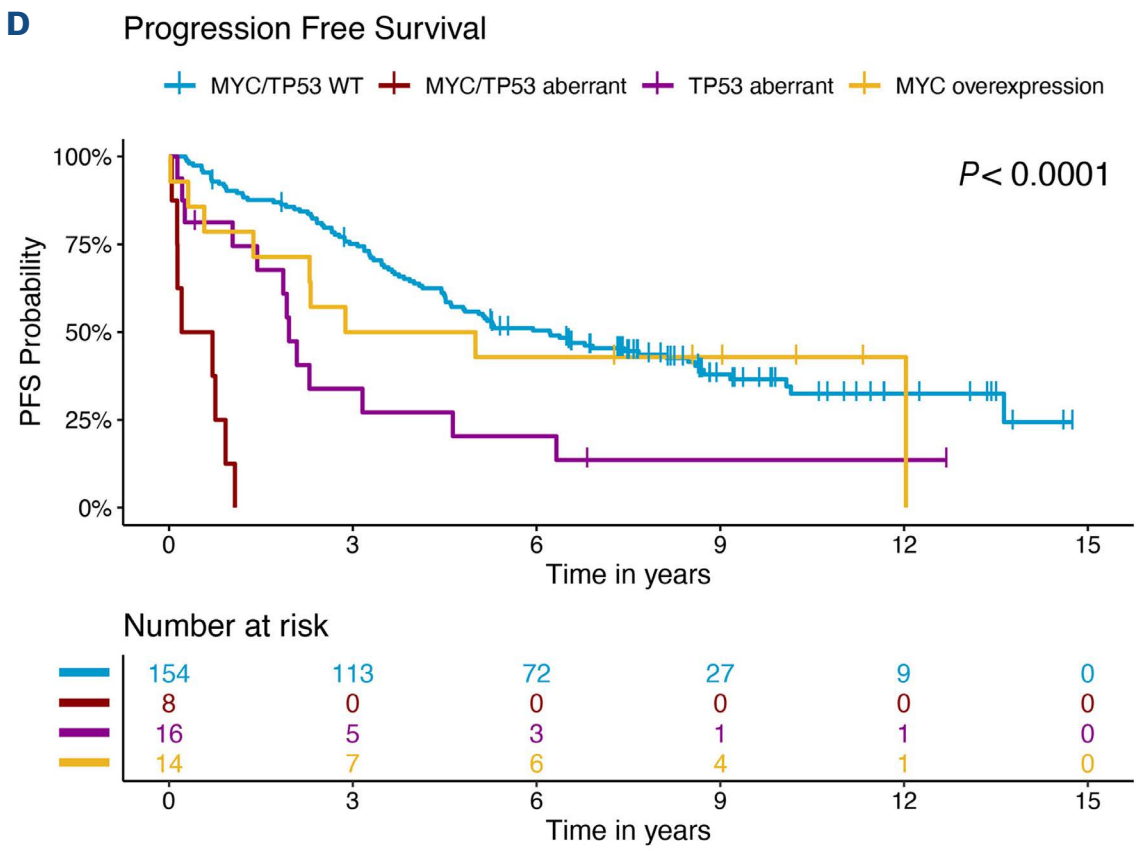

Table 5. MYC^{high} *TP53*/p53^{+} multivariable Cox proportional hazards models.

Variable	Overall survival			Progression-free survival		
	HR (95% CI)	*P*	N	HR (95% CI)	*P*	N
MYC^{high} *TP53*/p53^{+}	4.49 (2.32-8.70)	<0.001	237	9.78 (3.93-24.32)	<0.001	188
Age	1.071 (1.05-1.09)	<0.001	237	1.06 (1.04-1.08)	<0.001	188
Non-classic *vs.* classic morphology	1.23 (0.72-2.09)	0.45	237	1.35 (0.79-2.32)	0.27	188
High Ki-67 (>30%) *vs.* low (<30%)	1.70 (1.11-2.60)	0.02	237	1.34 (0.84-2.14)	0.22	188
Male vs. female	1.12 (0.77-1.64)	0.56	237	0.83 (0.53-1.30)	0.41	188
MYC^{high} *TP53*/p53^{+}	3.995 (1.71-9.31)	0.001	185	9.56 (3.75-24.34)	<0.001	182
MIPI risk group Intermediate High	 2.16 (1.22-3.84) 8.64 (4.92-15.19)	 0.008 <0.001	185	 1.87 (1.15-3.03) 5.54 (3.40-8.99)	 0.01 <0.001	182
Non-classic *vs.* classic morphology	1.22 (0.67-2.03)	0.52	185	1.13 (0.65-1.95)	0.66	182
High Ki-67 (>30%) *vs.* low (<30%)	1.16 (0.71-1.91)	0.71	185	1.09 (0.69-1.72)	0.69	182
Male *vs.* female	1.22 (0.77-1.92)	0.78	185	0.74 (0.46-1.18)	0.46	182

HR: hazard ratio; 95% CI: 95% confidence interval; *P*: probability value; N: number.

association with gene amplifications, and rearrangements, and transcriptional activity.

Current literature on MYC in MCL has not reached a consensus on a cutoff for the definition of MYC^{high}.[10,25,31,32] Previous studies used 10%, 20% or 26%,[10,11,24] which hampers direct comparison of the impact of dichotomized groups. We applied a 20% cutoff based on the findings of a previous study on 65 MCL patients by Choe *et al.*[24] To validate the applicability of the applied cutoff in our cohort, we further used Max Rank statistics that identified 21% as the cutoff maximizing the difference in OS in the present cohort.

Most MCL patients had tumors with a low frequency of MYC-positive cells, but 14% (n=35) of the tumors had >20% positive cells and were defined as MYC^{high}. The patients with MYC^{high} tumors had a median OS of 2.2 years and PFS of 1.8 years. These were significantly shorter than the OS of 7.3 years and the PFS of 5.2 years for patients with MYC^{low} tumors. The impact on outcome is similar to that previously reported by Oberley *et al.*[10] Both elevated mRNA and protein MYC levels were associated with poor prognosis. Of interest, MYC levels violated the proportional hazards assumption, indicating that the prognostic effect of MYC protein is time-dependent, with a mainly negative impact in the first 3 years after diagnosis. To understand whether there were other high-risk features that separated MYC^{high} patients with OS shorter or longer than 3 years, the clinicopathological characteristics were compared and confirmed that additional high-risk factors such as being in a

high-risk MIPI group, having *TP53*/p53 aberrations and high proliferation were more common in MYC^{high} patients with an OS shorter than 3 years. Of note, women with MYC^{high} tumors seemed to do even more poorly than men, with only one out of nine women having an OS longer than 3 years. Although numbers are small, this indicates that there might be a sex-related difference in the adverse effects mediated by MYC.

Comparison of MYC^{high} and MYC^{low} tumors with clinicopathological parameters showed that there is a positive correlation between high age and MYC overexpression in MCL. This has not been reported before. In the study by Aukema *et al*.[26] most patients with high MYC expression were older than 65 years, although this was not specifically mentioned. High expression of MYC is also associated with older age in patients with anaplastic lymphoma kinase-positive large cell lymphomas[33] and diffuse large B-cell lymphoma.[34]

Besides age, patients with MYC^{high} tumors were also enriched for other high-risk features such as high-risk MIPI group, non-classic morphology, and high proliferation. However, the negative impact of MYC on outcome was independent of these high-risk factors, with the exception of MIPI group, emphasizing the additive effect between molecular factors, which was explored further. The impact of *TP53* mutations and/or p53 overexpression in MCL has been widely documented by us and others[35,36] and assessment of *TP53* status should be performed in routine clinical diagnostics.[16] Wild-type p53-mediated apoptosis can be induced by MYC,[37] and as MYC overexpression in cancer is believed to lead to deregulation of its physiological targets,[38] altered *TP53*/p53 could synergize with MYC and lead to a more aggressive variant of the disease. Here we show that simultaneous alterations in MYC and *TP53*/p53 (MYC^{high}*TP53*/p53^{+}) did indeed have an additive negative prognostic effect compared to either of the aberrations alone, being associated with a median OS and PFS of only 0.9 and 0.5 years, respectively. The MYC^{high}*TP53*/p53^{+} subgroup of patients had unfavorable clinical characteristics, with most having highly proliferative tumors, with non-classic morphology and being in a high-risk MIPI group. The presence of tumors with both aberrations had been noted in other studies,[23,26,39,40] but this is the first time that an association with prognostic and clinicopathological parameters in MCL is reported. The negative effect of MYC overexpression combined with *TP53* mutations, excluding patients with wild-type *TP53* but high levels of p53 protein, was also confirmed. The mechanisms behind *MYC* and *TP53*/p53 crosstalk remain to be explored, but these aberrations are known to influence each other by involvement of proteins, such as BMI-1, ARF, and microRNA, including microRNA34a.[41] Den *et al*. studied the synergetic effect of MYC and *TP53*/p53 abnormalities on outcome in patients with diffuse large B-cell lymphoma, similarly to this study on MCL.

In our cohort we identified only one case with *MYC* rearrangements, but a few cases (1.3% of the 85 evaluated cases) showed copy gains of *MYC*. These results are in line with prior studies in which *MYC* rearrangements were rare.[10,20,42] When genetic alterations have been observed, they have mainly been amplifications/copy gains rather than chromosomal translocations of *MYC*.[31] A high frequency of *MYC* abnormalities has been found in only one selected cohort.[23] Similarly, concurrent translocations of *MYC* and *CCND1* have been reported only as single cases, except in a selected cohort with overrepresentation of leukemic MCL in which 5% of MCL tumors had these dual aberrations.[43] Thus, we can conclude that translocations or other rearrangements involving *MYC* are rare in MCL at diagnosis, corresponding to less than 2% of all cases in most studies. However, in the present study MYC protein overexpression identified 14% of MCL patients with a poor prognosis and added information on high risk beyond *TP53*/p53, morphology and proliferation.

In other B-cell lymphomas, such as Burkitt lymphoma and diffuse large B-cell lymphoma, structural alterations involving *MYC* are a predominant mechanism leading to *MYC* overexpression.[25] In MCL, we found that *MYC* copy number gains were not correlated with outcome or *MYC* mRNA or protein overexpression. The expression seems to be driven by transcriptional dysregulation and mRNA and protein expression were highly correlated with similar effects on outcome. Investigations of MYC-driven lymphomagenesis in MCL support this notion, by showing that miRNA, such as miR33b, miR96, and miR503, are pivotal in the regulation of MYC[44] and that histone deacetylation is involved in the repression of transcription mediated by *MYC*.[45] In addition, MALT1 has also been proposed as an alternative mechanism for MYC protein stabilization in MCL.[46] Studies in other lymphomas show that hotspot mutations in regions that can affect protein stability are selected during lymphomagenesis and are associated with a negative impact on outcome.[47] It has also been shown by Nadeau *et al*. that *MYC* and *TP53* are the only genes whose alterations have an impact on outcome beyond that of the total genomic complexity.[48] However, mutations of *MYC* do not seem to drive relapse in MCL as MYC has been shown to be less mutated in relapsed cases compared to cases at diagnosis.[6]

In other cancers, *MYC* has a role in shaping the tumor immune microenvironment, through several mechanisms. Indeed, MYC is capable of immune checkpoint regulation, like PD-L1 and MHC class I and II molecules, and promotes cytokine secretion, leading, as an example, to re-programing M1- to M2-like macrophages.[49,50] We hypothesized that MYC^{high} tumors would have an altered immune microenvironment. Image analyses showed no differences in T-cell infiltration but MYC^{high} tumors were associated with increased infiltration of CD163^{+} cells. This suggests that M2 macrophages may contribute to an adverse outcome in such tumors and that MYC is associated with both intrinsic and extrinsic high-risk features.

Previous studies in cancer have shown the potential of MYC

inhibition to promote antitumor effects. Nonetheless, due to intrinsically disordered domains and lack of enzymatic sites, MYC has been considered undruggable.[51] Several approaches have been proposed to inhibit MYC both directly and indirectly at all its levels of regulation. Recently, the first direct MYC inhibitor, the blocking peptide OMO-103 (Peptomyc), reached clinical phase studies. OMO-103 has been shown to alter the tumor microenvironment, potentiating an antitumor immune response,[52] providing hope for future successful clinical use of this agent. MYC has been shown to have a role in ibrutinib resistance.[53,54] Thus, also in the ibrutinib era in MCL, we expect that MYC will remain a high-risk marker and that MYC-targeting therapies may play an important role in both the diagnostic and relapsed setting. The current study included 252 MCL patients evaluated at diagnosis; their treatment during the follow-up was not homogenous. As both MYC and p53 aberrations affect a limited group of patients, results need to be validated in independent cohorts of patients, ideally under the same treatment protocol. The analyses are further limited by the fact that no mutational analyses were performed, so no correlation between different mutational sites that may affect protein stability and protein expression could be identified.

In summary, MYC protein is a high-risk marker and, in this study, was overexpressed in a significant subgroup of cases of MCL (14%). Overexpression of MYC (>20% expression) adds prognostic information beyond that of established molecular risk factors, such as *TP53*/p53, morphology and proliferation, for risk stratification of MCL patients. MCL patients carrying tumors with both MYC^{high} and *TP53*/p53 aberrations constitute a subgroup with a dismal prognosis, indicating additive negative effects. Previous efforts at risk stratification have included the presence of upregulation of *MYC* at the bulk mRNA level together with other markers in a five-gene signature that predicts survival in MCL.[55] However, we propose that MYC may be assessed through routine immunohistochemistry, using a cutoff at 20% to separate high from low expression, together with routine assessment of *TP53*/p53 status, proliferation and MIPI group.

Disclosures

MJ has received research support from Abbvie, AstraZeneca, Janssen, Gilead, BMS and Roche, and honoraria from Abbvie, AstraZeneca, BMS, Genmab, Janssen, Novartis, Incyte, EUSApharma, Gilead, and Roche. IG has participated in educational sessions for Jansen Cilag, and has received research support from Takeda and Lokon Pharma. KG has received research support from Janssen, and has participated in advisory boards for GSK and Nanexa. DK is employed by Aiforia Technologies Plc. The remaining authors do not have any conflicts of interest to disclose.

Contributions

JMR was involved in the conception and design of the study, performed data and statistical analyses, and wrote the manuscript. PH and MR were involved in the pathology review and FISH evaluation. LS and EG took part in the experimental and data analyses. DK was involved in data generation and technical support. CG, RR, AK, IG, and MJ were involved in the collection of clinical information and responsible for the cohorts used in this study. CWE and KG performed sequencing analysis of the N-MCL2/3 cohort. CS and AP were involved in the pathology review. SE designed the study and wrote the manuscript. All authors approved the final version of the manuscript.

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Data-sharing statement

Original data and protocols are available upon request.

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