



MYD88 mutations identify a molecular subgroup of diffuse large B-cell lymphoma with an unfavorable prognosis

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Haematologica 2020
Volume 105(2):424-434

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Received: December 24, 2018.

Accepted: May 22, 2019.

Pre-published: May 23, 2019.

doi:10.3324/haematol.2018.214122

Check the online version for the most updated information on this article, online supplements, and information on authorship & disclosures: www.haematologica.org/content/105/2/424

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ABSTRACT

The 2016 World Health Organization classification defines diffuse large B-cell lymphoma (DLBCL) subtypes based on Epstein-Barr virus (EBV) infection and oncogenic rearrangements of *MYC/BCL2/BCL6* as drivers of lymphomagenesis. A subset of DLBCL, however, is characterized by activating mutations in *MYD88/CD79B*. We investigated whether *MYD88/CD79B* mutations could improve the classification and prognostication of DLBCL. In 250 primary DLBCL, *MYD88/CD79B* mutations were identified by allele-specific polymerase chain reaction or next-generation-sequencing, *MYC/BCL2/BCL6* rearrangements were analyzed by fluorescence *in situ* hybridization, and EBV was studied by EBV-encoded RNA *in situ* hybridization. Associations of molecular features with clinicopathologic characteristics, outcome, and prognosis according to the International Prognostic Index (IPI) were investigated. *MYD88* and *CD79B* mutations were identified in 29.6% and 12.3%, *MYC*, *BCL2*, and *BCL6* rearrangements in 10.6%, 13.6%, and 20.3%, and EBV in 11.7% of DLBCL, respectively. Prominent mutual exclusivity between EBV positivity, rearrangements, and *MYD88/CD79B* mutations established the value of molecular markers for the recognition of biologically distinct DLBCL subtypes. *MYD88*-mutated DLBCL had a significantly inferior 5-year overall survival than wild-type *MYD88* DLBCL (log-rank; $P=0.019$). DLBCL without any of the studied aberrations had superior overall survival compared to cases carrying ≥ 1 aberrancy (log-rank; $P=0.010$). *MYD88* mutations retained their adverse prognostic impact upon adjustment for other genetic and clinical variables by multivariable analysis and improved the prognostic performance of the IPI. This study demonstrates the clinical utility of defining *MYD88*-mutated DLBCL as a distinct molecular subtype with adverse prognosis. Our data call for sequence analysis of *MYD88* in routine diagnostics of DLBCL to optimize classification and prognostication, and to guide the development of improved treatment strategies.

Introduction

Diffuse large B-cell lymphoma (DLBCL) is characterized by substantial heterogeneity in tumor biology and clinical behavior.^{1,2} Currently, rituximab, cyclophosphamide, doxorubicin, vincristine, and prednisone (R-CHOP) is used as a 'one-size-fits-all' treatment. Unfortunately, a considerable percentage of patients will experience chemorefractory disease or relapse, resulting in a 5-year overall survival (OS) of approximately 60%.³ Particularly, patients with chemorefractory disease or an early relapse have a poor prognosis. For optimal counseling, DLBCL patients are categorized in risk groups according to the IPI.⁴ The IPI consists of clinical and biochemical parameters, but does not include tumor biological characteristics or provide any indication for precision medicine.⁵

The recently updated WHO classification of lymphoid neoplasms (2016) recognizes this heterogeneity by including selected drivers of lymphomagenesis for subclassification of DLBCL, *i.e.* the delineation of high-grade B-cell lymphomas (HGBL) with *MYC* and *BCL2* and/or *BCL6* rearrangements, and of Epstein-Barr virus-positive (EBV⁺) DLBCL.⁶ *MYC*, *BCL2*, and *BCL6* rearrangements are found in respectively 4-14%, 20-30%, and ~20% of DLBCL.⁷⁻⁹ HGBL comprise approximately 5-10% of all DLBCL.^{9,11} It is thought that the combination of *MYC*-stimulated cell proliferation and anti-apoptotic effects of *BCL2* in HGBL cause aggressive growth, relative resistance to therapy, and inferior OS.¹² In addition, Asian studies showed a frequency of 1-14% EBV positivity in DLBCL and an association with inferior survival.^{13,14} EBV-associated viral proteins, such as latent membrane proteins (LMP)-1/2 and nuclear antigens, stimulate proliferation of B-cells via activation of nuclear factor-kappa-B (NFκB), regulate immune evasion, and inhibit apoptosis.¹³

In the search for additional oncogenic drivers and to discriminate different molecular DLBCL subtypes, large next-generation-sequencing (NGS) studies have revealed specific mutational profiles that reflect the dysregulation of distinct intracellular pathways, including epigenetic regulation and NF-κB, Toll-like receptor (TLR), and B-cell receptor (BCR) signalling.^{1,2,15,16} Recurrent 'hotspot' mutations in *MYD88* (L265P) and *CD79B* (Y196) belong to the most prevalent sequence alterations in DLBCL. By altering the toll/interleukin-1 receptor domain of MYD88, the L265P increases interaction and consecutive phosphorylation of downstream targets, potentially without external stimuli from the TLR.¹⁷ The connection of MYD88 with BCR signalling within the so-called 'My-T-BCR' supercomplex facilitates activation of the NF-κB pathway via TLR9.² Hotspot mutations, such as Y196, in the CD79B subunit of the BCR lead to increased BCR expression and inhibition of feedback in the BCR signalling pathway by attenuating downstream Lyn kinase. Therefore, *CD79B* mutations are thought to contribute to lymphomagenesis by enhancing chronic active BCR signalling.¹⁸

Both *MYD88* and *CD79B* mutations are more prevalent in the so-called non-germinal center B-cell (GCB)-type DLBCL according to the cell-of-origin (COO) concept, originally developed on the basis of gene expression profiling.^{1,2,19} In addition, the prevalence of these mutations varies greatly among DLBCL originating at different anatomical sites. We recently described a high percentage of *MYD88* L265P and *CD79B* Y196 mutations in intravascular large B-cell lymphomas (44% *MYD88* and 26%

CD79B).²⁰ A high frequency of these mutations has also been found in other extranodal DLBCL, such as primary cutaneous DLBCL, leg type,²¹ orbita/vitreoretinal DLBCL,²²⁻²⁴ primary breast DLBCL,²⁵ and DLBCL presenting at immune-privileged (IP) sites, *i.e.* primary testicular DLBCL (PTL)²⁶ and primary central nervous system B-cell lymphoma (PCNSL).²⁷⁻²⁹ Several studies have shown that *MYD88* mutations are associated with inferior OS in DLBCLs compared to wild-type *MYD88*.^{30,31}

Despite the increasing knowledge of the landscape of genetic drivers in DLBCL, the clinical implications of different oncogenic driver mutations remain unclear,³² and the R-CHOP regimen is used as a uniform treatment. Since patients with chemorefractory disease or relapses after R-CHOP have a poor outcome, the global 5-year OS in DLBCL is approximately 60%.³ While HGBL patients have been recognized as a particularly unfavorable subgroup, prognostication for the remaining DLBCL is based on clinical and biochemical parameters that define the IPI as well as primary extranodal manifestations.^{4,5} In contrast, the prognostic significance and interaction of mutations in *MYD88* and *CD79B* with standard molecular aberrations (as designated by the WHO 2016) have not yet been conclusively elucidated. Therefore, the present study investigated whether the assessment of the mutational status of *MYD88* and *CD79B* would improve the classification and prognostication of DLBCL.

Methods

Patient cohort

This retrospective study investigated a cohort of 250 primary DLBCL. DLBCL patients were diagnosed between 2000-2016 at the Amsterdam University Medical Center (AUMC), the Leiden University Medical Center (LUMC), and their affiliated hospitals. In all cases, diagnosis was centrally revised following the WHO classification 2008. A subset of this cohort was previously published without survival analysis.^{28,29} As our academic hospitals are tertiary referral centers, this cohort is enriched for IP locations. Formalin-fixed and paraffin-embedded (FFPE) tissue samples were obtained during standard diagnostic procedures. The study was performed in accordance with the Dutch Code for Proper Secondary Use of Human Tissue in accordance with the local institutional board requirements and the revised Declaration of Helsinki 2008 and was approved by the medical ethics committees of both the AUMC (W15_213#15.0253) and the LUMC (B16.048). Patients were eligible in case tissue was available and *MYD88* mutational analysis was successful.

Histopathologic and molecular characterization

In the majority, immunohistochemistry was performed for CD20, CD10, BCL6, MUM1, and BCL2. The Hans' algorithm was used for the COO classification.³³ The EBV status was assessed by EBER-ISH. *MYC*, *BCL2*, and *BCL6* rearrangements were analyzed by FISH using break-apart probes. Antibodies and probes are depicted in the *Online Supplementary Table S1*.^{20,29} In the AUMC, DNA was isolated using the QIAamp DNA Micro kit (Qiagen) and mutational status of *MYD88* and *CD79B* was established by allele-specific PCR, followed by mutation-specific primers and confirmed by Sanger sequencing, as described before.^{28,29} In the LUMC, DNA isolation was automatically performed with the TPS robot (Siemens Healthcare Diagnostics), as presented previously.³⁴ The Ampliseq Cancer Hotspot Panel V.2-V.4 (Thermo Fisher Scientific) was used for the detection of variants in *MYD88* (exons

3&5) and CD79B (exons 5&6). The minimum coverage threshold was 100 on-target reads with a minimum variant allele frequency of $\geq 10\%$ of the reads. Variants were analyzed using Geneticist Assistant NGS Interpretative Workbench (v.1.4.15, SoftGenetics, State College). As described, identified variants were classified into five classes based on potential pathogenicity and only class 4 (possibly pathogenic) and class 5 variants (pathogenic) were reported.³⁵

Statistical analysis

The correlation between the clinicopathologic parameters and biological aberrations was examined with the Chi-square test or ANOVA. The Kaplan-Meier method was applied to estimate 5-year OS and progression free survival (PFS). The starting point for time-to-event analysis was the date of the histological diagnosis. An event for OS was defined as death by any cause. An event for PFS was determined as relapse, disease progression, or death by any cause (whatever came first). If patients received palliative treatment and no remission evaluation was performed during the follow-up, an event for progression was defined at three weeks before patients succumbed to their disease. Observational intervals of patients without any event at the time of the last follow up or at 5 years after diagnosis were censored. The median follow up time for the whole cohort was determined by the use of reverse Kaplan-Meier.³⁶ The log-rank test was performed to compare risk groups. The Cox proportional-hazards model was used to estimate hazard ratios (HR) including 95% confidence intervals (95%-CI). Adjusted HR were obtained in a multivariable Cox model. Competing risks analysis was used to estimate the cumulative incidences of relapse/progression, with non-relapse mortality considered as competing risk. Gray's test was performed to compare cumulative incidences, whereas a cause-specific Cox proportional-hazards model was used to estimate the impact of risk factors on them.³⁷ The incremental prognostic value of *MYD88* and/or *CD79B* was assessed by comparing Harrell's cross-validated C statistic for Cox models with and without *MYD88* and/or *CD79B*.³⁸ All statistical analyses were performed using SPSS software (version 23, IBM SPSS statistics) and RStudio (version 1.1442, RStudio, Inc. packages survival, prodlm, dynpred and cmprsk). P-values were two-sided and $P < 0.05$ was considered statistically significant.

Results

Patient characteristics

Table 1 depicts the baseline characteristics of the 250 DLBCL patients (AUMC N=224 patients and LUMC N=26 cases). The median age at diagnosis was 61.4 years (range 18.6-89.6). A total of 38 DLBCL patients were immune-compromised, due to inherited conditions (severe combined immunodeficiency disorder, common variable immunodeficiency disorder), human immunodeficiency virus (HIV) infection, or extended use of therapeutic immunosuppression necessitated by organ transplantation or auto-immune disorders. Based on anatomical locations, 75 patients (30.0%) had strictly nodal DLBCL and in 67 patients (26.8%) the lymphoma presented in IP sites: 33 patients with PTL and 35 patients with PCNSL of whom one patient had testicular and CNS locations synchronously. The remaining 108 patients (43.2%) had extranodal disease in non-IP sites (with or without nodal involvement). With respect to staging, PCNSL was considered as advanced disease equivalent to Ann Arbor Stage IV for assignment of the IPI and subsequent statisti-

Table 1. Patient characteristics at time of diagnosis.

	All patients (N=250)
Gender	
Male	168 (67.2 %)
Female	82 (32.8 %)
Median age in years (range)	61.4 (18.6-89.6)
History of immune deficiency	38 (15.2 %)
HIV	16 (6.4 %)
Organ transplantation with prolonged use of immune suppressive drugs	7 (2.8 %)
SCID/CVID	3 (1.2 %)
Other ^a	13 (5.2 %)
Anatomical lymphoma location	
Nodal	75 (30.0 %)
Extranodal ^b (with or without nodal location)	108 (43.2 %)
Immune-privileged	67 (26.8 %)
CNS location ^c	35 (14.0 %)
Testis location	32 (13.2 %)
Ann Arbor ^d	(N = 248)
I	51 (20.6 %)
II	32 (12.9 %)
III	26 (10.5 %)
IV	139 (56.0 %)
IPI ^e	(N = 241)
0	20 (8.3 %)
1	41 (17.0 %)
2	90 (37.3 %)
3	58 (24.1 %)
4	24 (10.0 %)
5	8 (3.3 %)
First line treatment	
R-CHOP	160 (64.0 %)
CHOP	25 (10.0 %)
Other chemotherapy ^f	5 (2.0 %)
Radiotherapy only	1 (0.4 %)
Surgery only	2 (0.8 %)
None /Palliative	34 (13.6 %)
High-dose methotrexate regimens (HD-MTX) ^g	23 (9.2 %)
Radiotherapy	
With curative intent	
Palliative care only	77 (30.8 %)
	60 (24.0 %)
	17 (6.8 %)
Response to first line treatment	
Complete response	166 (66.4 %)
Partial response	14 (5.6 %)
Stable disease	2 (0.8 %)
Progressive disease	67 (26.8 %)
Too early to call	1 (0.4 %)

HIV: human immunodeficiency virus; SCID: severe combined immunodeficiency disorder; CVID: common variable immunodeficiency disorder; CNS: central nervous system; IPI: international prognostic index; (R-)CHOP: (rituximab), cyclophosphamide, doxorubicin, vincristine, prednisone. ^aOthers include inflammatory bowel disease, Sjögren, sarcoidosis, atopic dermatitis, and/or auto-immune haemolytic anaemia. ^bExtranodal comprised lung, liver, spleen, bone marrow, breast, soft tissue, thyroid, bone, (ad)renal, orbital, stomach, skin, pancreas, bowel, bladder, ovary, and naso-/oropharynx locations. ^cOne patient experienced both CNS and testicular locations. ^dPCNSL were classified as advanced stage (Ann-Arbor stage IV) and subsequently received one risk point for IPI. ^e(R-)C(E)OP: (rituximab), cyclophosphamide, (etoposide), vincristin, prednisone. ^fSpecific regimens include HD-MTX + cytarabine + carmustine, HD-MTX + cytarabine, rituximab + HD-MTX + prednisone (RMP), cyclophosphamide + doxorubicin + teniposide + prednisone + vincristine + bleomycin (CHVMP/BV), HD-MTX + procarbazine + lomustine, HD-MTX + cytarabine + thiopeta + rituximab (MATRiX), HD-MTX + teniposide + carmustin + prednisone (MBVP) (+ rituximab).

cal analyses. With this definition, 83 patients (33.5%) were categorized as having regional disease (Ann Arbor stage I-II) and 165 patients (66.5%) had advanced disease (stage III-IV). Sixty-one patients (25.3%) had an IPI risk score of 0/1, 148 patients (61.4%) an IPI of 2-3, and 32 patients (13.3%) an IPI of 4-5. The IPI of nine patients was

unknown. The majority of (extra)nodal and testicular DLBCL patients were treated with R-CHOP (N=160), CHOP (N=25), or (R)CHOP-like treatments (N=5) with curative intent. Curative treatment regimens incorporating high-dose methotrexate were initiated for 23 patients with PCNSL. Because of older age, poor clinical Eastern

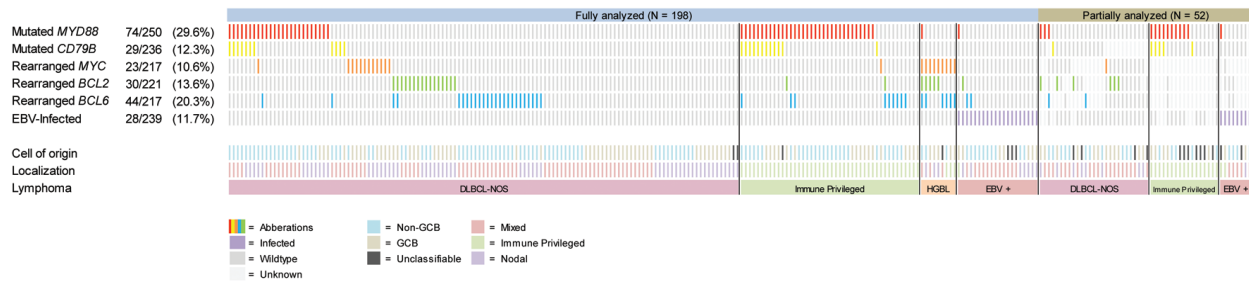


Figure 1. OncoPrint plot of the molecular analysis of 250 cases with diffuse large B-cell lymphoma (DLBCL). EBV: Epstein-Barr virus; GCB: germinal center B-cell; IP: immune-privileged. Of 52 cases, molecular analysis was not complete due to results that were ambiguous to interpret or no FFPE material was left for subsequent analysis.

Table 2. Hans' algorithm and molecular analysis at time of diagnosis.

	All patients (N=250)	Nodal (N=75)	Extranodal with/without nodal (N=108)	Immune-privileged (N=67)	P*
Cell-of-origin, according to Hans' algorithm (N=250)					
GCB	100 (40.0 %)	36 (48.0 %)	38 (58.3 %)	26 (38.8%)	0.228
Non-GCB	130 (52.0 %)	35 (46.7 %)	63 (35.2 %)	32 (47.8 %)	
Unclassifiable	20 (8.0 %)	4 (5.3 %)	7 (6.5 %)	9 (13.4 %)	
MYD88 (N=250)					
Wildtype	176 (70.4 %)	62 (82.7 %)	92 (85.2 %)	22 (32.8 %)	<0.001
Mutated	74 (29.6 %)	13 (17.3 %)	16 (14.8 %)	45 (67.2 %)	
CD79B (N=236)					
Wildtype	207 (87.7 %)	70 (95.9 %)	88 (90.7 %)	49 (74.2 %)	<0.001
Mutated	29 (12.3 %)	3 (4.1 %)	9 (9.3 %)	17 (25.8 %)	
MYC (N=217)					
Wildtype	194 (89.4 %)	59 (85.5 %)	89 (90.8 %)	46 (92.0 %)	0.434
Rearranged	23 (10.6 %)	10 (14.5 %)	9 (9.2 %)	4 (8.0 %)	
BCL2 (N=221)					
Wildtype	191 (86.4 %)	53 (74.6 %)	89 (89.9 %)	49 (96.1 %)	0.001
Rearranged	30 (13.6 %)	18 (25.4 %)	10 (10.1 %)	2 (3.9 %)	
BCL6 (N=217)					
Wildtype	173 (79.7 %)	57 (82.6 %)	78 (79.6 %)	38 (76.0 %)	0.675
Rearranged	44 (20.3 %)	12 (17.4 %)	20 (20.4 %)	12 (24.0 %)	
High grade B-cell lymphoma (N=221)					
Negative	212 (95.9 %)	66 (95.7 %)	98 (97.0 %)	48 (94.1 %)	0.686
Positive	9 (4.1 %)	3 (4.3 %)	3 (3.0 %)	3 (5.9 %)	
EBV status (N=239)					
Negative	211 (88.3 %)	65 (89.0 %)	88 (83.8 %)	58 (95.1 %)	0.091
Positive	28 (11.7 %)	8 (11.0 %)	17 (16.2 %)	3 (4.9 %)	
Genetic aberrations (N=198)					
None	51 (25.8 %)	21 (31.8 %)	27 (32.1 %)	3 (6.3 %)	0.002
One or more	147 (74.2 %)	45 (68.2 %)	57 (67.9 %)	45 (93.8 %)	

EBV: Epstein-Barr virus. *P-value indicating a difference in distribution between the three subgroups as calculated by Pearson's χ^2 test. The number between brackets in the left hand column represents the number of patients from whom this information was available.

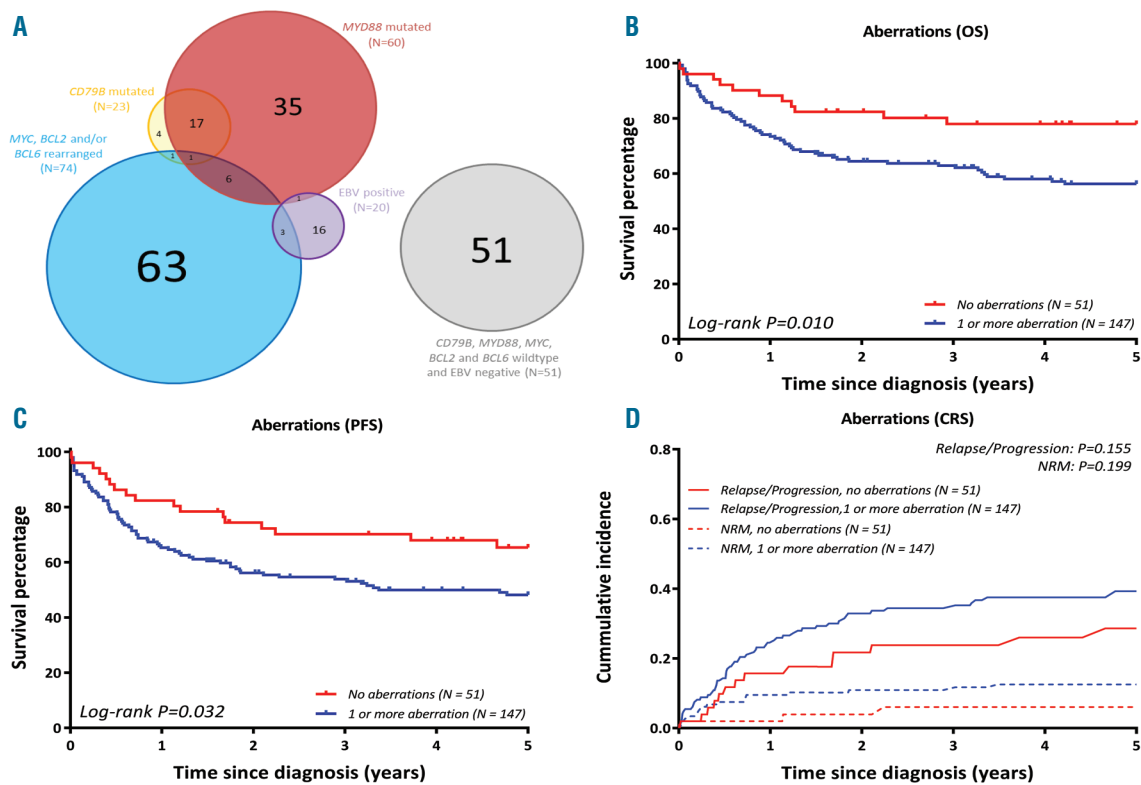


Figure 2. Molecular characterization discriminates distinct DLBCL subgroups with prognostic impact. (A) Venn diagram demonstrating the overlap of aberrations for 198 fully analysed DLBCL. (B) DLBCL without detected aberrations showed a superior overall survival compared to DLBCL with ≥ 1 affected aberrations (for cases with complete aberration analysis), identifying a novel good-risk group. (C) PFS of the novel identified risk group (for cases with complete driver analysis). (D) Cumulative incidences of novel identified risk group (for cases with complete driver analysis). CRS: competing risk; PFS: progression free survival.

Cooperative Oncology Group Performance Status (ECOG-PS), or patients' refusal of treatment, 34 patients received palliative care only, mainly with steroids or (local) radiotherapy. The median follow up time was 6.6 years (range 0.1-15.7).³⁶

Molecular characterization: mutated MYD88 discriminates a distinct DLBCL subgroup

According to the Hans' algorithm, DLBCL were classified as GCB (N=100, 40.0%), non-GCB (N=130, 52.0%), or unclassifiable (N=20, 8.0%), with no statistical difference between nodal, extranodal, and IP locations ($P=0.228$)(Table 2).³³

In 198 patients (79.2%), molecular analysis for MYD88 and CD79B mutations, MYC, BCL2, and BCL6 rearrangements, and EBV infection was complete, whereas in 52 patients, partial data sets were available (Figure 1; Table 2). MYD88 mutations were identified in 74 cases (29.6%), of whom 67 harbored the hotspot L265P mutation. The other MYD88 variants were S219C (N=5) and S243N (N=2). In line with a published meta-analysis,³⁰ mutated MYD88 was significantly correlated with older age (≥ 65 years), anatomical lymphoma location, and non-GCB subtype ($P=0.006$; $P<0.001$; $P=0.042$, respectively). CD79B mutations were detected in 29 patients (12.3%), including the hotspot Y196 mutation (N=28) and the L188 mutation (N=2, one patient had both mutations). MYC, BCL2, and BCL6 were rearranged in 23 (10.6%), 30 (13.6%), and 44 (20.3%) DLBCL, respectively, with a total of nine HGBL patients (4.1%).

As suggested by previous reports and other studies, MYD88 and CD79B mutations were significantly more common in IP-DLBCL (67.2% resp. 25.8%) compared to nodal (17.3% resp. 4.1%) and other extranodal sites (14.8% resp. 9.3%)($P<0.001$ and $P<0.001$).^{26,29,39} In contrast, BCL2 rearrangements were more prevalent in nodal and extranodal DLBCL ($P=0.001$), whereas MYC and BCL6 rearrangements were evenly distributed across the anatomical sites. EBV was positive in 28 patients (11.7%) and was not associated with an anatomical location ($P=0.091$).

In the 198 cases with complete molecular analysis, hardly any overlap between the presence of oncogenic rearrangements, EBV positivity, or MYD88 and/or CD79B mutations was observed (Figure 2A), suggesting that they represent distinct DLBCL subgroups with different drivers of lymphomagenesis. CD79B mutations co-occurred with MYD88 mutations in 18 of 23 cases (78.2%). In contrast, MYD88 mutations co-occurred with any rearrangement in only 7 of 60 patients (11.7%) and with EBV positivity in only one case (1.7%). EBV infection was detected in only 3 of 71 cases (4.2%) with a rearrangement. In 51 patients (25.8%) with full molecular characterization, no aberrancy was detected.

Mutated MYD88 predicts inferior survival

All outcomes are reported at the 5-year survival. For the entire cohort, the OS was 61.0% (95%-CI 55.1-67.5) and the PFS was 52.6% (95%-CI 46.6-59.3). Cumulative incidences of relapse/progression and non-relapse mortality

Table 3A. Prognostic impact of molecular aberrations and IPI risk factors on overall survival: univariable and multivariable analysis.

	Overall survival							
	Univariable		Multivariable Model 1 (IPI)		Multivariable Model 2 (IPI + molecular aberrations WHO 2016)		Multivariable Model 3 (IPI + molecular aberrations WHO 2016 + MYD88 + CD79B)	
	HR	95%-CI	HR	95%-CI	HR	95%-CI	HR	95%-CI
IPI: >2 Extranodal Yes (<i>vs.</i> No)	1.37	0.91-2.07	1.41	0.90-2.22	1.49	0.94-2.37	1.71	1.07-2.74
IPI: Stage III/IV (<i>vs.</i> I/II)	2.33	1.41-3.85	1.67	0.98-2.84	1.71	0.97-3.00	1.84	1.04-3.25
IPI: ECOG Performance Score >2 (<i>vs.</i> <1)	8.15	5.23-12.7	7.53	4.67-12.15	8.69	5.23-14.45	8.16	4.90-13.59
IPI: Age >60 (<i>vs.</i> <60)	1.54	1.00-2.37	1.35	0.85-2.13	1.38	0.87-2.19	1.33	0.83-2.12
IPI: LDH >Upper limit (<i>vs.</i> Normal)	1.53	1.01-2.31	1.14	0.74-1.77	1.15	0.73-1.81	1.29	0.82-2.05
<i>MYC</i>								
Rearranged (<i>vs.</i> Wildtype)	1.62	0.88-3.00			1.71	0.89-3.27	1.86	0.93-3.69
<i>BCL2</i>								
Rearranged (<i>vs.</i> Wildtype)	0.74	0.37-1.47			0.51	0.24-1.08	0.57	0.26-1.24
<i>BCL6</i>								
Rearranged (<i>vs.</i> Wildtype)	1.21	0.71-2.04			0.94	0.53-1.65	1.00	0.55-1.83
EBV Status								
Positive (<i>vs.</i> Negative)	1.54	0.86-2.78			1.29	0.67-2.47	1.65	0.82-3.30
<i>CD79B</i>								
Mutated (<i>vs.</i> Wildtype)	1.43	0.81-2.53					0.76	0.38-1.49
<i>MYD88</i>								
Mutated (<i>vs.</i> Wildtype)	1.64	1.08-2.48					1.87	1.10-3.20
Cross-validated C-index			0.6		0.69		0.70	

For the multivariable model, unknown was regarded as a separate category for these variables for which some data were missing (not reported).

were 37.2% (95%-CI 31.2-43.3) and 10.1% (95%-CI 6.4-13.9), respectively. Figure 3 shows the survival outcomes presented for the anatomical location, IPI-score, and MYD88 status. The survival outcomes of COO and the other aberrations are outlined in the *Online Supplementary Figure S2* (none of these factors had a significant impact).

The IPI clearly predicted OS (Figure 3): patients with IPI scores of 0/1, 2/3, and 4/5 had an OS of 84.9% (95%-CI 76.3-94.5), 58.0% (95%-CI 50.3-66.8), and 34.4% (95%-CI 21.3-55.5), respectively. IPI also showed a significant difference in cumulative incidences of relapses (Gray's; $P=0.025$) and non-relapse mortality (Gray's; $P=0.006$). In addition to the IPI, DLBCL with IP locations had inferior outcomes (OS 47.1%, 95%-CI 36.5-60.9; PFS 41.0%, 95%-CI 30.7-54.9) compared to nodal (OS 71.2%, 95%-CI 61.4-82.4; PFS 55.7%, 95%-CI 45.3-68.6) and other extranodal sites (OS 62.6%, 95%-CI 53.9-72.7; PFS 58.1%, 95%-CI 49.4-68.2) (log-rank; $P=0.004$ and $P=0.024$). This unfavorable prognosis was particularly associated with the CNS location. Within the IP group, patients with CNS location had a significantly inferior 5-year OS of 29.9% (95%-CI 17.7-50.5) compared to 65.5% (95%-CI 50.9-84.3%) for PTL (log-rank; $P=0.003$).

With respect to the molecular markers, patients without any detected aberrancy demonstrated a good-risk profile with a superior OS (78.0%, 95%-CI 67.2-90.4, *versus* 56.3%, 95%-CI:48.6-65.2; Figure 2B) (log-rank; $P=0.010$) and PFS (65.4%, 95%-CI 53.2-80.3, *versus* 48.2%, 95%-CI 40.6-57.3; Figure 2C) (log-rank; $P=0.031$) compared to patients who had one or more aberration(s). The cumulative incidence of relapse/progression for this good-risk profile was 28.6% (95%-CI 15.8-41.4) compared to 39.3% (95%-CI 31.2-47.4) (Gray's; $P=0.155$). This good risk profile included patients with a lower ECOG-PS, age<60 years, and more GCB subtypes (Chi square; $P=0.012$, $P=0.001$, and $P=0.006$, respectively) compared to patients with one or more aberrations. Patients in the good risk category seem to be susceptible for immune-chemotherapy with enduring responses, however, the molecular background of this subgroup remains unknown. In IP-DLBCL, a total of 93.8% of the patients were classified in the risk group with ≥ 1 aberrations.

MYD88-mutated DLBCLs had a significantly inferior 5-year OS compared to DLBCL with wild-type MYD88 (log-rank; $P=0.019$; HR 1.64, 95%-CI 1.08-2.48) and significantly inferior 5-year PFS (log-rank; $P=0.049$; HR 1.46,

95%-CI 1.00-2.14). Employing competing risk analysis, *MYD88*-mutated DLBCL revealed significantly higher relapse rates (46.6%, 95%-CI 35.1-58.1) than cases with wild-type *MYD88* (33.3%, 95%-CI 26.2-40.4)(Gray's; $P=0.029$; CSH 1.62, 95%-CI 1.06-2.48), while non-relapse mortality showed no significant difference (Gray's; $P=0.832$). Mutated *CD79B* showed higher cumulative incidence for relapse/progression (56.3%, 95%-CI 37.9-74.8) *versus* wild-type *CD79B* (35.1%, 95%-CI 28.5-41.8)(Gray's; $P=0.019$, CSH: 1.82, 95%-CI 1.06-3.14), whereas no significant difference was found for OS (HR 1.43, 95%-CI 0.81-2.53).

Despite relatively high HR, none of the other molecular aberrations was a significantly adverse prognostic factor for OS (Table 3), which can be explained by the lack of power due to the low incidence of these aberrations. For these molecular data, univariate cause-specific hazards for relapse/progression showed similar results. The nine HGBL had an OS of 50.0% (95%-CI 24.1-100) compared to 63.6% (95%-CI 57.3-70.6) (log-rank; $P=0.628$) for non-HGBL.

Prognostic significance of *MYD88* mutations in multivariable analysis

To evaluate the prognostic impact of mutated *MYD88* on survival outcomes in addition to other molecular aberrations and the IPI, the initial multivariable Cox regression model included the standard individual IPI risk factors (Model 1, Table 3A/3B). In the second model, the current WHO 2016 molecular aberrations (EBV and oncogenic rearrangements) were added. In the third model, also *MYD88* and *CD79B* mutations were included. *MYD88* mutations showed prognostic significance for OS (HR 1.87, 95%-CI 1.10-3.20) in addition to ECOG-PS (≥ 2) (HR 8.16, 95%-CI 4.90-13.59) and Ann Arbor stage (III/IV) (HR 1.84, 95%-CI 1.04-3.25). In this third model, oncogenic rearrangements, mutated *CD79B*, elevated LDH, and age (>65 years) did not have a significant impact. The performance of the IPI prognostic model was improved by adding all molecular aberrations and mutated *MYD88* and *CD79B* as risk factors, as indicated by an increase in cross-validated C-index (CVC) from 0.67 to 0.70. *MYD88* did not have a significant impact on the cause-specific survival

Table 3B. Prognostic impact of molecular aberrations and IPI risk factors on relapse/progression: univariable and multivariable analysis.

	Univariable		Cause-specific hazards (CSH) for relapse/progression					
	HR	95%-CI	Multivariable Model 1 IPI	Multivariable Model 2 (IPI + molecular aberrations WHO 2016)	Multivariable Model 3 (IPI + molecular aberrations WHO 2016 + <i>MYD88</i> + <i>CD79B</i>)	HR	95%-CI	
IPI: >2 Extranodal								
Yes (<i>vs.</i> No)	1.57	0.99-2.41	1.55	0.99-2.41	1.63	1.04-2.57	1.81	1.14-2.86
IPI: Stage								
III/IV (<i>vs.</i> I/II)	2.76	1.63-4.68	2.12	1.22-3.67	2.06	1.17-3.63	2.14	1.19-3.82
IPI: ECOG Performance Score								
>2 (<i>vs.</i> <1)	4.48	2.58-7.78	4.48	2.58-7.78	5.09	2.86-9.05	4.60	2.57-8.22
IPI: Age								
>60 (<i>vs.</i> <60)	1.14	0.75-1.74	1.11	0.71-1.72	1.14	0.73-1.79	1.12	0.71-1.77
IPI: LDH								
>Upper limit (<i>vs.</i> Normal)	0.98	0.64-1.50	0.77	0.49-1.21	0.77	0.48-1.22	0.82	0.51-1.31
<i>MYC</i>								
Rearranged (<i>vs.</i> Wildtype)	1.63	0.86-3.09			1.84	0.94-3.49	1.90	0.96-3.77
<i>BCL2</i>								
Rearranged (<i>vs.</i> Wildtype)	1.34	0.75-2.40			1.03	0.56-1.90	1.23	0.66-2.30
<i>BCL6</i>								
Rearranged (<i>vs.</i> Wildtype)	1.01	0.57-1.78			0.89	0.49-1.59	0.91	0.49-1.68
EBV Status								
Positive (<i>vs.</i> Negative)	0.79	0.36-1.71			0.66	0.29-1.49	0.79	0.34-1.86
<i>CD79B</i>								
Mutated (<i>vs.</i> Wildtype)	1.82	1.06-3.13					1.23	0.64-2.36
<i>MYD88</i>								
Mutated (<i>vs.</i> Wildtype)	1.62	1.06-2.48					1.42	0.85-2.37
Cross-validated C-index			0.63		0.63		0.64	

For the multivariable model, unknown was regarded as a separate group (not reported).

(HR 1.42, 95%-CI 0.85-2.37), whilst ECOG-PS, Ann Arbor stage, and extranodal location were prognostic in this model.

Further multivariable analyses were performed to evaluate the prognostic significance of *MYD88* mutational status in comparison to the COO subtype or anatomical lymphoma location. The COO subtype did not improve the performance of models 2 and 3 (results not shown). However, the prognostic impact of model 2 was improved by adding the anatomical lymphoma location (CVC index

= 0.71, model 4, presented in the *Online Supplementary Table S1*) and outperforms model 2 (Table 3A, CVC index = 0.69, including the IPI factors and molecular aberrations of WHO 2016). Model 4 demonstrated a nearly identical prognostic performance when compared to model 3 (CVC index = 0.70, including the IPI factors, molecular aberrations of WHO 2016 and the mutational status of *MYD88* and *CD79B*). When adding the mutational status of *MYD88* and *CD79B* to model 4, the performance of this model 5 was not improved (CVC index 0.71, *Online*

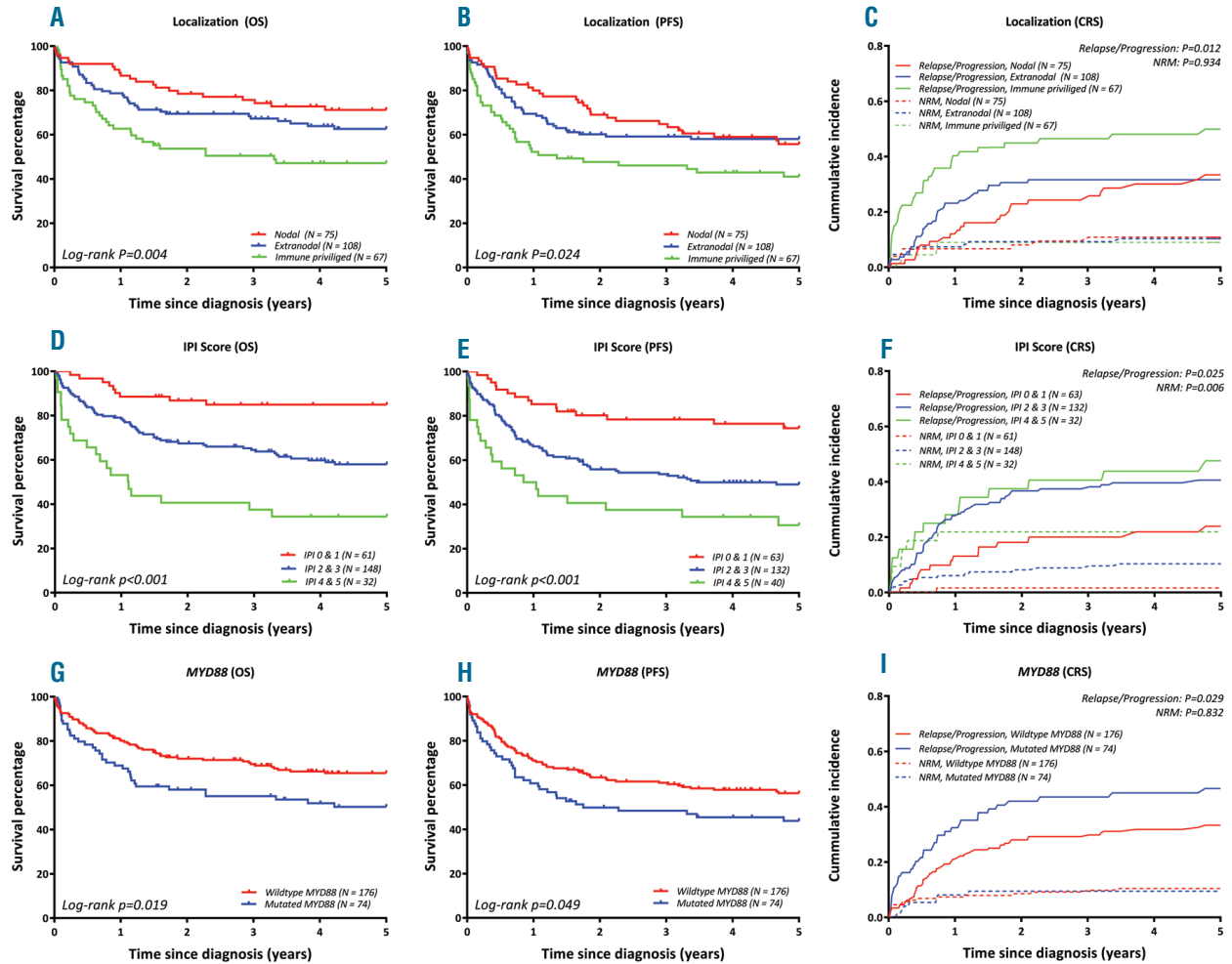


Figure 3. Prognostic significance of anatomical location, IPI Score and *MYD88* in DLBCL. OS, PFS, and cumulative incidence of relapse/progression compared to NRM (1st row: Location, 2nd row: IPI Score, 3rd row: *MYD88*). CRS: competing risk; OS: overall survival; PFS: progression free survival; NRM: non-relapsed mortality.

Table 4. Mutated *MYD88* improved the prognostic performance of the IPI.

	Overall survival		Cause-specific hazard (CSH) for relapse/progression					
	Univariable	Multivariable	Univariable		Multivariable		Multivariable	
	HR	95%-CI	HR	95%-CI	HR	95%-CI	HR	95%-CI
IPI-score								
As continuous variable	1.73	1.45-2.08	1.77	1.47-2.13	1.45	1.21-1.73	1.47	1.22-1.76
<i>MYD88</i>								
Mutated (<i>vs.</i> Wildtype)			1.83	1.19-2.80			1.69	1.09-2.60
Cross-validated C-index		0.57		0.61		0.53		0.57

Supplementary Table S1). As such, the prognostic impact of the *MYD88* mutational status on mortality was not superior to the anatomical lymphoma location.

Next, we explored whether mutated *MYD88* could improve the prognostic performance of the currently used IPI risk model (Table 4). The inclusion of the IPI as a continuous variable (0-5 points) and the *MYD88* status in the multivariable analysis demonstrated an independent and similar impact of mutated *MYD88* (HR 1.83, 95%-CI 1.19-2.80) and IPI (HR 1.77, 95%-CI 1.47-2.13) on OS. Similar effects were observed for cause-specific survival (Table 4). For the models, OS and relapse/progression, an increase in CVC-index was observed from 0.57 to 0.61 and 0.53 to 0.57, respectively. Altogether, these multivariable survival analyses demonstrated the significant prognostic importance of mutated *MYD88*, next to (genetic) aberrations and clinical/biochemical variables, and the improvement of adding mutated *MYD88* to the prognostic performance of the IPI.

To evaluate possible confounding of the impact of mutated *MYD88* and with the outcomes by anatomical lymphoma location, we performed a sensitivity analysis for OS on the cohort stratified by the anatomical lymphoma location, including CNS involvement. For patients with CNS involvement (N=35), *MYD88* had an unadjusted HR of 1.94 (95%-CI 0.77-4.90) in the univariable analysis. For patients without CNS involvement (N=215), *MYD88* did not have a significant impact on OS with an adjusted HR of 1.81 (95%-CI 0.96-3.42), when applying the multivariable analysis as described for model 3 (Table 3B). Although not statistically significant, the adjusted HR for this subgroup was similar to the original HR for the entire cohort.

Discussion

To the best of our knowledge, this is the first study evaluating the clinical significance of mutated *MYD88* and *CD79B* in DLBCL, in addition to the oncogenic drivers that are currently included in the WHO classification 2016 (EBV status and *MYC*, *BCL2*, and *BCL6* rearrangements), the IPI risk factors, and well-defined anatomical locations.

The strength of this study is the large number of patients with good clinical annotation and complete molecular analysis (N=198). In addition, our study shows that the incorporation of the mutational status of *MYD88* into a clinical/biochemical risk score as the IPI is feasible. An increase in the predictive performance of the IPI risk model as is illustrated by an increase in the CVC-index, suggests that this model can be improved by the introduction of molecular aberrations. However, while interpreting the results, we have encountered several limitations. *MYD88*-mutated DLBCL more often had extranodal location, older age (and thus a high IPI), and non-GCB subtype. Therefore, these patients were more frequently subjected to palliative care. Possibly the interaction between treatment and mutated *MYD88* has not been tested as more data is needed. We present an average effect over different treatment modalities. Since the reported frequencies and survival outcomes are similar to previous reports in the literature, our cohort appears to be representative for the target population.^{3,7-9,13} To investigate the prognostic significance of mutated *MYD88* adjusted for the IPI for the entire cohort, we considered PCNSL as advanced dis-

ease stage, although it is not common practice to apply the IPI in PCNSL patients. Additionally, our cohort is enriched for IP locations. Therefore, a sensitivity analysis was performed excluding PCNSL patients, demonstrating that the adjusted HR of *MYD88* for OS was similar to the entire cohort. This indicates that our results are not affected by confounding by CNS localisation. Hence, we believe that our data corroborate the clinical relevance of mutant *MYD88* for the diagnostic classification and prognostication of DLBCL and support implementation of *MYD88* mutational analysis in routine diagnostics. The simplicity and accessibility to examine *MYD88* mutations and associated low costs permit an efficient timely implementation. In addition, *CD79B* mutations were prognostic in the univariate analysis, but when adjusted for other aberrations in the multivariable analysis the prognostic importance disappeared. This finding may be explained by the prominent overlap between *MYD88* and *CD79B* mutations, as 78.2% of mutated *CD79B* had co-occurring *MYD88* mutations.

An important result of our study is the recognition of the prominent mutual exclusivity between the presence of mutations in *MYD88* and/or *CD79B*, *MYC*, *BCL2*, and *BCL6* rearrangements, and EBV infection, indicating that *MYD88* and/or *CD79B*-mutated tumors present a distinct DLBCL subcategory. In accordance with a large meta-analysis and two other studies,^{30,40,41} *MYD88* L265P mutations were preferentially found in specific anatomical sites (e.g. testis and CNS) and were significantly associated with non-GCB subtypes, older age, and poor OS. However, the published literature has studied neither explicitly analysed IP sites, nor evaluated the interaction of *MYD88* mutations with EBV status or oncogenic rearrangements in multivariable analysis. Other NGS studies have recently demonstrated high frequencies of mutated *MYD88* (15-18%) in large cohorts of DLBCL.^{1,2,15,42-44} Besides a certain association of mutated *MYD88* with poor OS (e.g. in non-GCB DLBCL), cluster analysis of multiple genes indicated distinct DLBCL subentities, including mutated *MYD88* as an important classifier for NF- κ B pathway activation. Again, these NGS studies did not take into account specific anatomical sites or investigated the interaction and prognostic significance of mutated *MYD88* in correlation with the EBV status or *MYC*, *BCL2*, and *BCL6* rearrangements.

In this context, our study adds important new knowledge by demonstrating *MYD88* mutations as an adverse prognostic factor for OS and relapse/progression in a multivariate analysis that takes all major known clinical and WHO classification-defined risk factors into account. This insight does not only show that the incorporation of the mutational status of *MYD88* into a clinical/biochemical risk score as the IPI is feasible, but also highlights the importance of assessing *MYD88* at the time of diagnosis for an optimal classification and patient counselling. An increase in the predictive performance of the IPI risk model, as is illustrated by an increase in the CVC-index, formally suggests that this model can be improved by the introduction of molecular aberrations. However, the prognostic impact of the *MYD88* mutational status on the presented multivariable models was not superior to anatomical lymphoma location. Whether the *MYD88* mutational status outperforms the predictive performance of anatomical lymphoma location in the described prognostic models needs further validation in an external cohort. Of note,

no difference was found for non-relapse mortality, indicating that mutated *MYD88* is a lymphoma-specific poor prognostic factor. Routine diagnostic assessment of *MYD88* mutations is likely to gain decisive importance for DLBCL since several approaches may therapeutically target *MYD88*.^{45,46} Several studies have indicated that DLBCL with mutated *MYD88* and/or *CD79B* are more sensitive to Bruton's Tyrosine Kinase (BTK)-inhibitors.⁴⁶⁻⁴⁸ As such, the objective analysis of *MYD88* mutations will not only improve diagnostic classification and prognostication, but might also enable patient selection for precision medicine such as treatment with BTK-inhibitors. However, the predictive significance of mutated *MYD88* with or without *CD79B* mutations needs to be validated in upcoming clinical trials, including precision medicine targeting the BCR and TLR cascades.

Finally, as a corollary of this study, we identified a novel good risk DLBCL group characterized by the absence of detected genetic aberrations. These DLBCL appeared to be highly sensitive to standard immune-chemotherapy as a first-line treatment. Future studies, employing a larger NGS targeted gene panel, may elucidate the genetic drivers in this group. We anticipate that there might be a parallel with the study of Chapuy *et al.*,¹⁵ which identified a good-risk DLBCL group harbouring mainly aberrations in epigenetic pathways.

Studies by Rossi *et al.* and Kurtz *et al.*,^{49,50} have analysed liquid biopsies in DLBCL demonstrating that the mutational load in circulating-free tumor DNA obtained by NGS technologies reliably mirror the mutational profiles of DLBCL tissues, including mutated *MYD88*. Additionally, digital droplet PCR techniques enable the quantification of low amounts of mutated *MYD88* in any

physiological fluid.⁵¹ Further investigation is needed to determine whether the analysis of mutated *MYD88* in liquid biopsies prior to and during therapy will be significantly predictive for the treatment response and to establish its specificity and sensitivity.

Conclusion

The present study demonstrates that the presence of *MYD88* and *CD79B* mutations is almost mutually exclusive with EBV infection and *MYC*, *BCL2*, and *BCL6* rearrangements, indicating distinctive molecular DLBCL subgroups that can be readily appreciated in clinical practice. Mutant *MYD88* showed its prognostic importance for inferior survival outcomes, even next to other genetic and clinical prognosticators and IPI. Additionally, patients lacking all analysed aberrancies represented a novel risk group with superior survival outcomes. Taken together and after validation in an independent cohort, these results provide a rationale for including *MYD88* mutational analysis in the routine diagnostics of DLBCL, to improve classification and prognostication, as well as to guide future treatment strategies.

Acknowledgments

The authors would like to thank the involved research technicians, data managers and physicians for their contributions to this manuscript.

Funding

This study was supported in part by research funding from 'Egbers Stichting AMC Foundation', 'Stichting Fonds Oncologie Holland', and Lymph&Co (JSV, SFS, RAG, AHC, WK, MS, MJK, and STP).

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