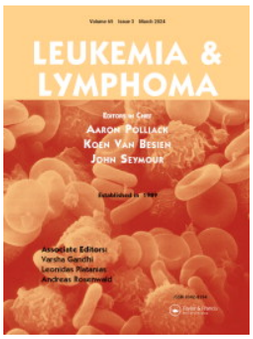

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ORIGINAL ARTICLE

Mutational landscape in Waldenström macroglobulinemia evaluated using a next-generation sequencing lymphoma panel in routine clinical practice

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ABSTRACT

Next-generation sequencing (NGS) affords comprehensive insights into the genomic landscape of lymphomas. We examined the mutational pattern in patients with Waldenström macroglobulinemia (WM) or lymphoplasmacytic lymphoma (LPL) as well as the diagnostic and clinical utility of a tailored NGS lymphoma panel. A consecutive series of 45 patients was reviewed and NGS analysis was performed as part of a routine diagnostic setup. The custom designed NGS panel assayed all coding sequences of 59 genes of known clinical significance in lymphoid neoplasms. The most frequently mutated genes were *MYD88*, *CXCR4*, *BIRC3*, *CD79B*, and *ARID1A*. Additional somatic mutations were detected in 17 genes with four mutations categorized as pathogenic or likely pathogenic. *BIRC3* and *TP53* mutations were associated with adverse clinical phenotypes. NGS performance for the $MYD88^{L265P}$ variant was 96% when compared to qPCR. In conclusion, targeted NGS provided important diagnostic and prognostic information in a routine clinical setting.

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KEYWORDS
Waldenström macroglobulinemia; next-generation sequencing; MYD88; somatic mutation; histological transformation

Introduction

Waldenström macroglobulinemia (WM) is defined by the combination of a lymphoplasmacytic lymphoma (LPL) in the bone marrow (BM) and an IgM monoclonal component [1]. $MYD88^{L265P}$ mutations (> 90% of cases) and $CXCR4^{WHIM}$ mutations (approximately 30% of cases) have been established as genetic hallmarks of WM although the genomic landscape of WM is not fully defined [2,3]. Recurring somatic mutations have also been demonstrated in *ARID1A* and *CD79* genes, along with copy number alterations including those on chromosome 6q that impact regulatory genes affecting NF-κB, BTK, BCL2, and apoptosis [2,4,5]. Furthermore, the mutational burden appears to be gradually increasing from IgM monoclonal gammopathy of undetermined significance to symptomatic WM, with $MYD88^{L265P}$ and $CXCR4^{WHIM}$ mutations being early events in the pathogenesis [6]. The presence of genetic alterations that may contribute to transformation of WM into an aggressive lymphoma has also been demonstrated [7].

Consensus reports have stated that allele-specific polymerase chain reaction (AS-PCR) and droplet digital PCR (ddPCR) provide appropriate reproducibility and sensitivity in detecting $MYD88^{L265P}$ mutation on unselected BM samples [8,9]. The most sensitive method is ddPCR with a detection rate of 96% in WM patients [10,11]. Next-generation sequencing (NGS) or Sanger sequencing is preferred when testing for *CXCR4* mutations but require a higher tumor burden than AS-PCR and ddPCR or CD19 enrichment of BM samples before analysis [8,9]. In recent years, NGS technology, including whole-genome sequencing (WGS) and whole-exome sequencing (WES) studies, have provided extensive information on molecular aberrations in B-cell lymphomas [12–15]. However, routine application of WGS or WES is limited by costs, sequencing requirements, and analysis of formalin-fixed paraffin-embedded (FFPE) samples that may hinder an optimal, uniform coverage of all coding areas. Custom-targeted panels allow sufficient and uniform sequencing depth of specific regions. We, and others, have shown that a customized NGS lymphoma panel may

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provide information that directly impacts clinical management both in relation to diagnostic precision, choice of therapy, and prediction of outcome [16–19]. However, the ability of targeted NGS to reliably detect *MYD88*^{L265P} has been brought into question by a recent study showing a high false negative rate when compared to AS-PCR [20].

Only limited real-world NGS data in WM are available. We designed a 59-gene NGS panel including all coding areas for a routine diagnostic setup in lymphoid malignancies. In this study, we report real-life data from 45 patients consecutively diagnosed with WM/LPL, including the occurrence of somatic mutations and their association with clinical variables. We also evaluated the diagnostic utility of our gene panel and proficiency in detecting *MYD88*^{L265P}.

Methods

Patients and materials

The study is a single-center observational cohort study. Patients diagnosed with mature non-Hodgkin lymphoma at Herlev Hospital, Denmark, during 2020 and 2021 had NGS performed using a 59-gene panel as an integrated part of routine diagnostic workup [16,21]. In this study, only patients with WM or LPL were included ($n = 45$). Patients were diagnosed according to the revised 4th Edition WHO Classification, 2017, based on morphology and immunophenotype including flow cytometry ($n = 41$) and *MYD88* analysis of *MYD88*^{L265P} using quantitative PCR (qPCR) with the qBiomarker somatic mutation PCR assay ($n = 26$) [22].

Samples for NGS analysis were collected at the time of diagnosis or at progression/relapse. The samples included fresh frozen BM aspirate in 26 (58%) patients, FFPE BM in 14 (31%) patients, and FFPE lymph node biopsies in 5 (11%) patients. No cases with a morphological BM involvement below 10% were included. Clinical data were collected from patient records.

This study was approved by the Ethics Committee of Region Zealand, Denmark (ID: 10204209).

Next-generation sequencing

NGS analysis was performed using an in-house lymphoma panel designed at the Department of Pathology, Herlev Hospital [16,23]. The panel was designed to cover exons and consensus splice sites of the following 59 genes: *ARID1A* (NM_006015.5), *ATM* (NM_000051.3), BCL6 (NM_001706.4), *BIRC3* (NM_182962.2), *BRAF* (NM_004333.4), *BTK* (NM_000061.2), *B2M* (NM_004048.2), *BCL2* (NM_000633.2), *BCL10* (NM_003921.4), *CARD11* (NM_032415.5), CCND3 (NM_001760.4), *CCND1* (NM_053056.2), *CD58* (NM_001779.2), *CD79A* (NM_001783.3), *CD79B* (NM_001039933.2), *CDKN2A* (NM_001195132.1), *CDKN2B* (NM_004936.3), *CHD2* (NM_001271.3), *CIITA* (NM_000246.3), *CREBBP* (NM_004380.2), *CXCR4* (NM_003467.2), *EP300* (NM_001429.3), *EZH2* (NM_004456.4), *FBXW7* (NM_033632.3), FOXO1 (NM_002015.3), GNA13 (NM_006572.5), *ID3* (NM_002167.4), *IDH2* (NM_002168.3), *IRF4* (NM_002460.3), KLF2 (NM_016270.3), *KMT2A* (NM_001197104.1), *KMT2D* (NM_003482.3), *KRAS* (NM_033360.3), MAL (NM_002371.3), MEF2B (NM_001145785.1), *MYC* (NM_002467.4), *MYD88* (NM_001172567.1, NB: The variant NM_001172567.1 c.818T>C, p.(Leu273Pro) using this reference sequence corresponds to the common *MYD88* variant in WM which is commonly referred to as p.(Leu265Pro) or *MYD88*^{L265P}), *NFKBIE* (NM_004556.2), *NOTCH1* (NM_017617.4), *NOTCH2* (NM_024408.3), *NRAS* (NM_002524.4), *PAX5* (NM_016734.2), PIM1 (NM_002648.3), *PLCG2* (NM_002661.4), *POT1* (NM_015450.2), *PRDM1* (NM_001198.3), *PTEN* (NM_000314.6), *PTPN11* (NM_002834.4), *PTPRD* (NM_002839.3), *RHOA* (NM_001664.3), *REL* (NM_002908.3), *SF3B1* (NM_012433.3), *SOCS1* (NM_003745.1), *STAT6* (NM_003153.4), *TCF3* (NM_001136139.3), *TNFAIP3* (NM_001270507.1), *TNFRSF14* (NM_003820.3), *TP53* (NM_000546.5) and *XPO1* (NM_003400.3). NGS primers for 51 of the genes were available as Ion AmpliSeq On-Demand panels (2 pools), while primers for 8 genes (underscored) were ordered as custom designs in 2 separate pools to optimize coverage. Sequencing was done on the Ion Torrent S5 XL system using manual library preparation and the Ion Chef system as described by the manufacturer (Thermo Fisher Scientific, Waltham, MA). The samples were subjected to a custom-designed workflow and a custom data filtering algorithm that included single nucleotide variants, multi nucleotide variants, insertions and deletions of all exons and consensus splice sites with a variant allele frequency (VAF) > 5% and excluded common single nucleotide polymorphisms and recurrent artifacts.

Before reporting, variants were reviewed manually using the Integrative Genomics Viewer version 5.01 (http://software.broadinstitute.org) to check for technical artifacts and misinterpreted variants as previously described [24]. Accepted variants were classified as pathogenic or likely pathogenic corresponding to Tier I/Tier II variants according to Guidelines for the Interpretation and Reporting of Sequence Variants in cancer or as variants of unknown significance (VUS) using data from Varsome (including data from gnomAD exomes ClinVar, information on hot spot location and bioinformatic pathogenicity scores), COSMIC (Wellcome Sanger Institute), the IARC database on

TP53 gene variations related to cancer and literature [25–27]. Polymorphisms and known benign variants were not included. In samples with no *MYD88* variant detected, the NGS raw data was manually inspected for the *L265P* variant and included in the study if detected manually with a VAF > 1%.

Statistics

Characteristics are presented as frequencies and percentages for categorical variables, and medians and ranges for continuous variables. Spearman rank correlation analysis was applied to identify linear relationships between two variables. A p value < 0.05 was considered statistically significant. Clinical correlation studies included all variants (pathogenic/likely pathogenic and VUS). Statistical analysis was performed using the R Statistical Software version 4.2.1 (Vienna, Austria). Individual mutations were categorized according to biological pathway activities and molecular interactions considered to be of major pathogenic significance. These data were descriptively reported and retrieved from the Genomic Data Commons data portal (https://portal.gdc.cancer.gov/) accompanied by a search in https://pubmed.ncbi.nlm.nih.gov/.

Results

Patient characteristics

We included 45 consecutive patients with a WM (n = 42) or LPL diagnosis (n = 3). Two patients with contemporaneous chronic lymphocytic leukemia were excluded. Median age was 73 years (range 45–87) at diagnosis. The samples used for NGS analysis were collected at primary diagnosis (n = 34) or at progression/relapse (n = 11). Median time from WM/LPL diagnosis to follow-up was 17 months (range 7–270). Two WM patients had concurrent transformation to diffuse large B-cell lymphoma (DLBCL) at the time of NGS analysis and 36 (80%) patients were treatment-naïve at the time of NGS analysis. Patient characteristics are outlined in Table 1.

Table 1. Clinical characteristics of 45 patients with WM/LPL.

Characteristics	Value	Missing (n)
Age (years), median (range)	73 (45–87)	0
Gender, male/female ratio	1.8	0
Hemoglobin (g/dL), median range	12.49 (6.93–15.79)	3
Platelet count (x10^9/L), median (range)	262 (20–697)	3
LDH (U/L), median (range)	169 (95–263)	4
β-2 microglobulin conc. (mg/L), median (range)	2.9 (1.5–11.6)	4
Monoclonal IgM conc. (g/L), median (range)	8.6 (0.5–80)	7
BM infiltration (%), median (range)	40 (10–95)	3
IPSSWM risk category, n (%):		4
Low	9 (22)	–
Intermediate	19 (46)	–
High	13 (32)	–
Extramedullary involvement, n (%)	12 (29)	4
Transformation to DLBCL, n (%)	2 (4)	0
Treatment of symptomatic disease, n (%)	20 (44)	0

BM: bone marrow; DLBCL: diffuse large B-cell lymphoma; IgM: immunoglobulin M; IPSSWM: international prognostic scoring system for Waldenström macroglobulinemia; LDH: lactate dehydrogenase; LPL: lymphoplasmacytic lymphoma; WM: Waldenström macroglobulinemia.

NGS findings and clinical implications

Somatic variants were identified by NGS in 22 different genes across the 45 patients. All cases harbored at least one variant and in 30 (67%) patients, two or more variants were detected. Frequencies of identified variants are illustrated in Figure 1. Median number of variants was 2 (range 0-8) per sample with a total of 91 variants found across all samples. The genes most frequently mutated were *MYD88* (93%), *CXCR4* (29%), *BIRC3* (11%), *CD79B* (11%), and *ARID1A* (9%).

Distribution and interpretation of somatic variants in individual patients is outlined in Figure 2. Variants categorized as benign, likely benign, or germline are filtered out in the present analysis. The relation between detected variants and the affected biological mechanisms or signaling pathways is shown in Figure 3. Sixteen (36%) patients had variants in the *ATM*, *BCL2*, *CCND1*, *CDNK2B*, IDH2, *KMT2A*, *KMT2D*, *MEF2B*, *NOTCH1*, *NOTCH2*, PAX5, *PLCG2*, *PTEN*, *PTPN11*, PTPRD, *TCF3*, or TP53 genes and the underscored genes harbored variants classified as pathogenic or likely pathogenic (Figure 2).

In 26/45 (58%) patients, an additional qPCR analysis for *MYD88*^{L265P} mutation was performed. A positive qPCR analysis was in concordance with the corresponding NGS analysis in 25/26 (96%) patients. In three patients with *MYD88*^{WT} according to the NGS analysis, only one patient had a detectable *MYD88*^{L265P} by qPCR, and qPCR raw data indicated a low VAF. This patient had a low tumor cell infiltration both estimated by immunohistochemistry (15%) and by flow cytometry (6%).

The median VAF of *MYD88*^{L265P} was 16% (range 3–70) across all cases and 11.5% (range 3–44) in cases where NGS was performed on unselected BM aspirates (n = 37). Correlations between *MYD88* ^{L265P} VAF and BM infiltration estimated by immunohistochemistry and flow cytometry, respectively, are shown in Figure 4. The median BM infiltration estimated by morphology was 40% (range 10–95) and 10% (range 1–69) when

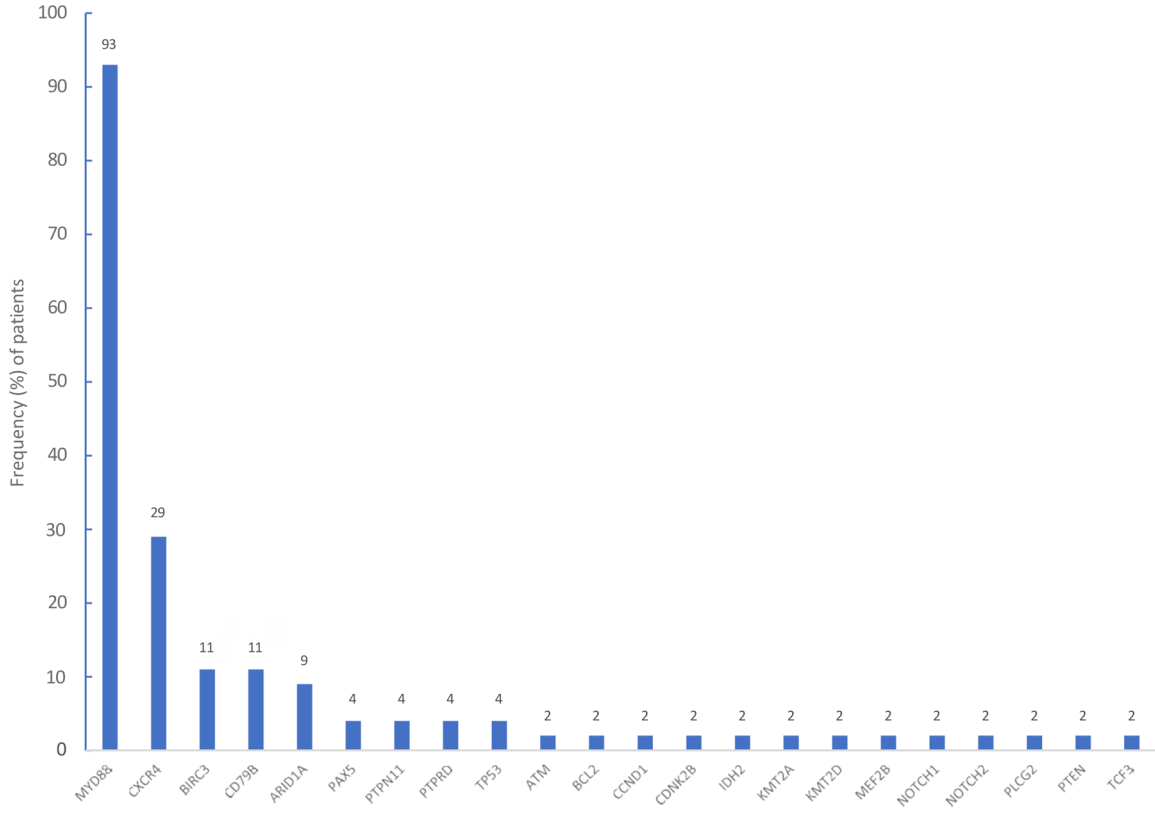

Figure 1. Frequency (%) of detected variants of pathogenic, likely pathogenic or unknown significance in 45 patients with LPL/WM.

assessed using flow cytometry. Even though no patient had a morphological BM infiltration <10%, flow cytometry analysis revealed a tumor cell infiltration below 10% in 17 (38%) patients.

CXCR4 variants were found in 13 (29%) patients and these patients had significantly lower hemoglobin level than patients with *CXCR4*^{WT} at the time of diagnosis ($p = 0.01$). However, *CXCR4* was not associated with other clinical variables such as requirement of treatment, BM infiltration, β-2 microglobulin (β2M), or IgM levels. The limited number of patients and short follow-up time did not allow for survival analysis. No *CXCR4* mutated patients were treated with a BTK inhibitor. *BIRC3* and *CD79B* variants were each present in five (11%) patients. In the patients with a *BIRC3* variant, two were of the non-WM LPL type. Of the three *BIRC3* mutated WM patients, 2/3 presented with anemia, 3/3 had elevated β2M, and 2/3 corresponded to high-risk IPSSWM. All three patients presented with IgM levels above 20 g/L. The *CD79B* mutated patients all presented with low IgM levels (≤7 g/L) and a low or intermediate IPSSWM. For *ARID1A* mutated patients, 3/4 had high levels of BM involvement, anemia, and a need for treatment.

Further somatic variants were detected in 17 other genes (Figure 2). Each of these variants was present in only 1 or 2 patients (2–4%) which did not allow for further analysis. Nevertheless, a combination of *TP53*, *MYD88*, and *CXCR4* variants was detected in two patients. Interestingly, both patients experienced histological transformation to DLBCL, 17 and 35 months after the initial WM diagnosis. An identical variant profile (*TP53*, *MYD88*, and *CXCR4*) was found in BM from the time of WM diagnosis and the DLBCL biopsy at subsequent histological transformation. A clonal relation between the DLBCL clone and the underlying WM was demonstrated in both patients. No patients with *TP53*^{WT} showed signs of clinical or histological transformation during the follow-up period.

In the patients with non-WM LPL a *MYD88*^{L265P} mutation was detected in all three cases, and 2/3 also harbored a *BIRC3* variant. No other pathogenic or likely pathogenic variants were detected in these patients. All three patients presented with BM involvement and lymphadenopathy.

Discussion

We report real-world findings in patients with WM/LPL after implementing a custom designed in-house NGS lymphoma panel of 59 genes as part of routine clinical practice. We identified recurrent somatic variants in *MYD88*, *CXCR4*, *CD79B*, and *ARID1A* consistent with

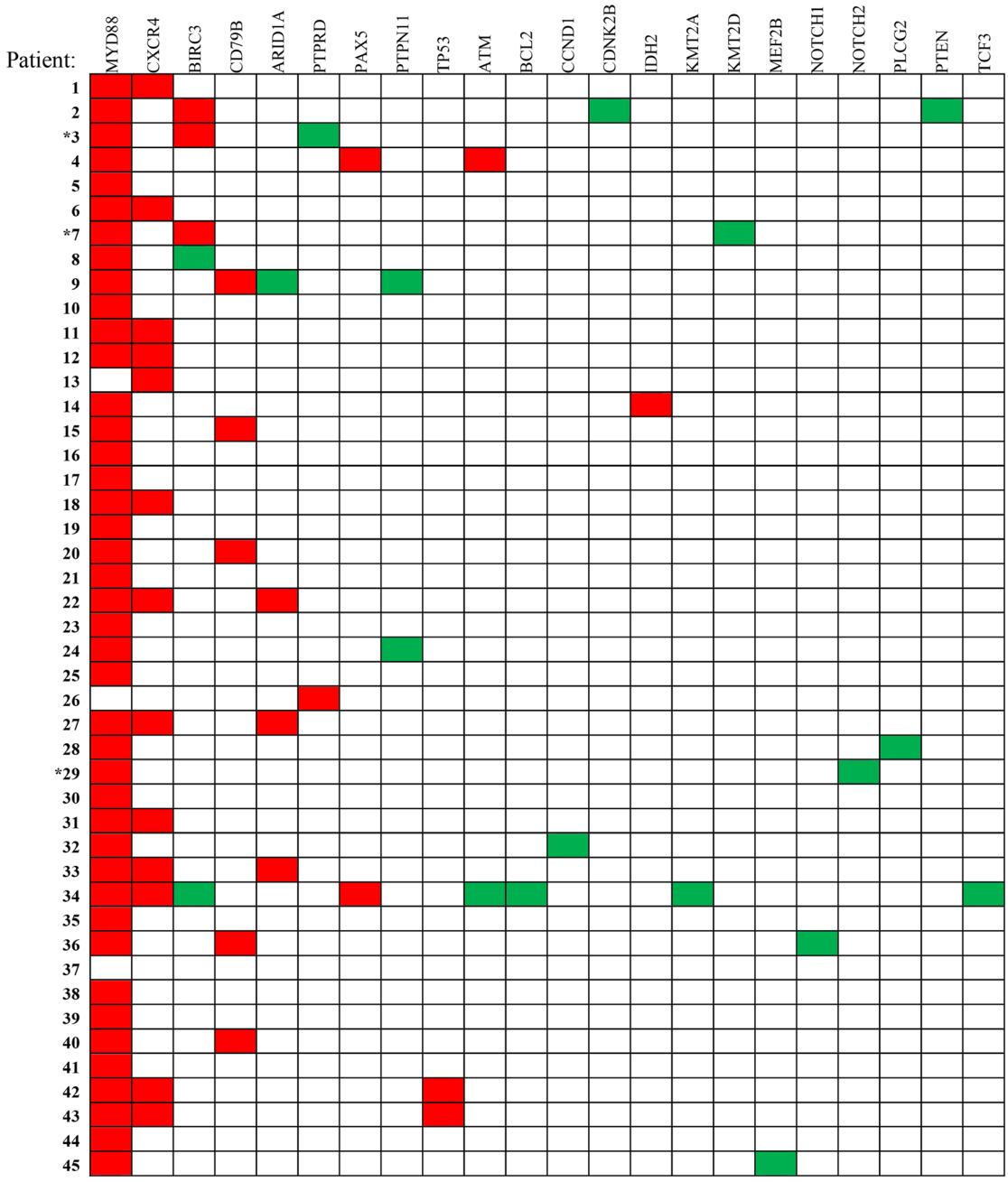

Figure 2. Heatmap plot showing the distribution of all genetic alterations across the 45 patients. Pathogenic or likely pathogenic mutations (red) and variants of unknown significance (green). Patients marked with * are non-WM LPL patients.

data reported in previous studies [3,12,28–33]. This indicates that our NGS panel is reliable in detecting the most common somatic variants of known importance to diagnostic precision and clinical management in patients with WM. The assay failed to detect *MYD88*^{L265P} in one patient where the mutation was identified by qPCR, illustrating that NGS analysis is only complementary to PCR analysis, particularly in patients with a low tumor burden. All detected *MYD88* mutations were L265P variants and no other driver mutations were found in the *MYD88* gene, despite full exon sequencing.

Current guidelines recommend a sensitive method, such as ddPCR or AS-PCR for the detection of *MYD88*^{L265P} [8,9,34]. In a study of unsorted BM samples, *MYD88*^{L265P} was found in 95% of the samples using ddPCR which confirms that it is a feasible and sensitive tool for mutation screening even in unsorted samples [10]. In another recent study, the performance of targeted NGS for detection of *MYD88*^{L265P} was compared to that of AS-PCR [20]. NGS

Pathways and mechanisms	Variants																					
	MYD88	CXCR4	BIRC3	CD79B	ARID1A	PTPRD	PAX5	PTPN11	TP53	ATM	BCL2	CCND1	CDNK2B	IDH2	KMT2A	KMT2D	MEF2B	NOTCH1	NOTCH2	PLCG2	PTEN	TCF3
NF-kB signaling	42																					
Chemokine signaling		13																				
Cell cycle regulatory genes						2		2	2	2		1	1							1		
Transcription factors							2										1					1
B-cell receptor signaling				5																		
Chromatin remodelling/DNA methylation					4									1	1	1						
NOTCH signaling																		1	1			
Inhibitor of apoptosis			5								1											
AKT/PKB signaling																					1	

Figure 3. Distribution of genetic alterations according to affected signaling pathways or mechanisms of action (values are number of mutations (total number = 91)).

reproducibly detected single nucleotide variants at allele frequencies of ≥5% but only identified *MYD88*^{L265P} in 66% of cases whereas AS-PCR had a much higher detection rate of 94%. The median VAF for *MYD88*^{L265P} was 5.95% (range 0.5–86.5) and the median BM infiltration rate was 30% (range 2–95). Only low levels of BM involvement represented a significant predictor of false negative *MYD88*^{L265P}. However, even at higher levels of BM involvement the false negative rate was still 19% which challenges the diagnostic utility of targeted NGS analysis or suggests that BM infiltration may have been overestimated. In our NGS assay, raw data were manually inspected for the L265P variant to increase the sensitivity in patients with <10% tumor cells estimated by flow cytometry and no detectable *MYD88* variant (VAF < 5%). Additionally, the median value for morphological BM infiltration was 40%, which may explain a higher detection rate. There was a significantly better correlation between *MYD88* ^{L265P} VAF and tumor burden determined by flow cytometry when compared to morphological evaluation even when the NGS analysis was performed on the same FFPE material as the morphological BM estimate. These results imply that the estimation of BM involvement may be subject to uncertainty and methodological aspects regarding assessment of BM involvement should be considered and thoroughly described in NGS studies.

The frequency of *CXCR4*, *ARID1A*, and *CD79B* variants found in our study was in accordance with previous reports [2,28,32,33]. However, associations between clinical variables and individual variants are limited by the size of our patient cohort. Of note, *BIRC3* variants were detected in 11% of the total cohort and 7% of the patients with WM. This frequency is higher than what has been previously reported [35]. *BIRC3* is a gene that encodes the cellular inhibitor of apoptosis protein 2 (cIAP2), and in chronic lymphocytic leukemia, *BIRC3* variants have been shown to confer a high-risk phenotype [36]. Two of the five variants detected in *BIRC3* in our study were classified as VUS, and these included a missense variant and an in-frame two amino acid deletion in the RING finger domain. As they were not functionally characterized or previously described they were classified as VUS in the clinical report. However, both variants were absent from controls in gnomAD, and because the RING finger domain is a critical functional domain known as a hot spot for *BIRC3* variants in CLL they were included in the clinical association study [37]. Intriguingly, patients with *BIRC3* variants presented with anemia and high levels of β2M and IgM with the majority corresponding to high-risk IPSSWM. Further studies investigating the pathogenetic role and clinical implications of *BIRC3* variants in WM are warranted.

Pathogenic or likely pathogenic variants were found in four additional genes *TP53*, *PAX5*, *PTPRD*, and *IDH2*. *TP53* alterations are associated with poor outcome in most B-cell lymphomas [38]. Remarkably, only two patients in our WM cohort harbored a *TP53* variant and both patients developed clonally related histological transformation to DLBCL. The *TP53* variant was found concurrently with both *MYD88* and *CXCR4* variants. The remaining 43 patients with *TP53*^{WT} showed no evidence

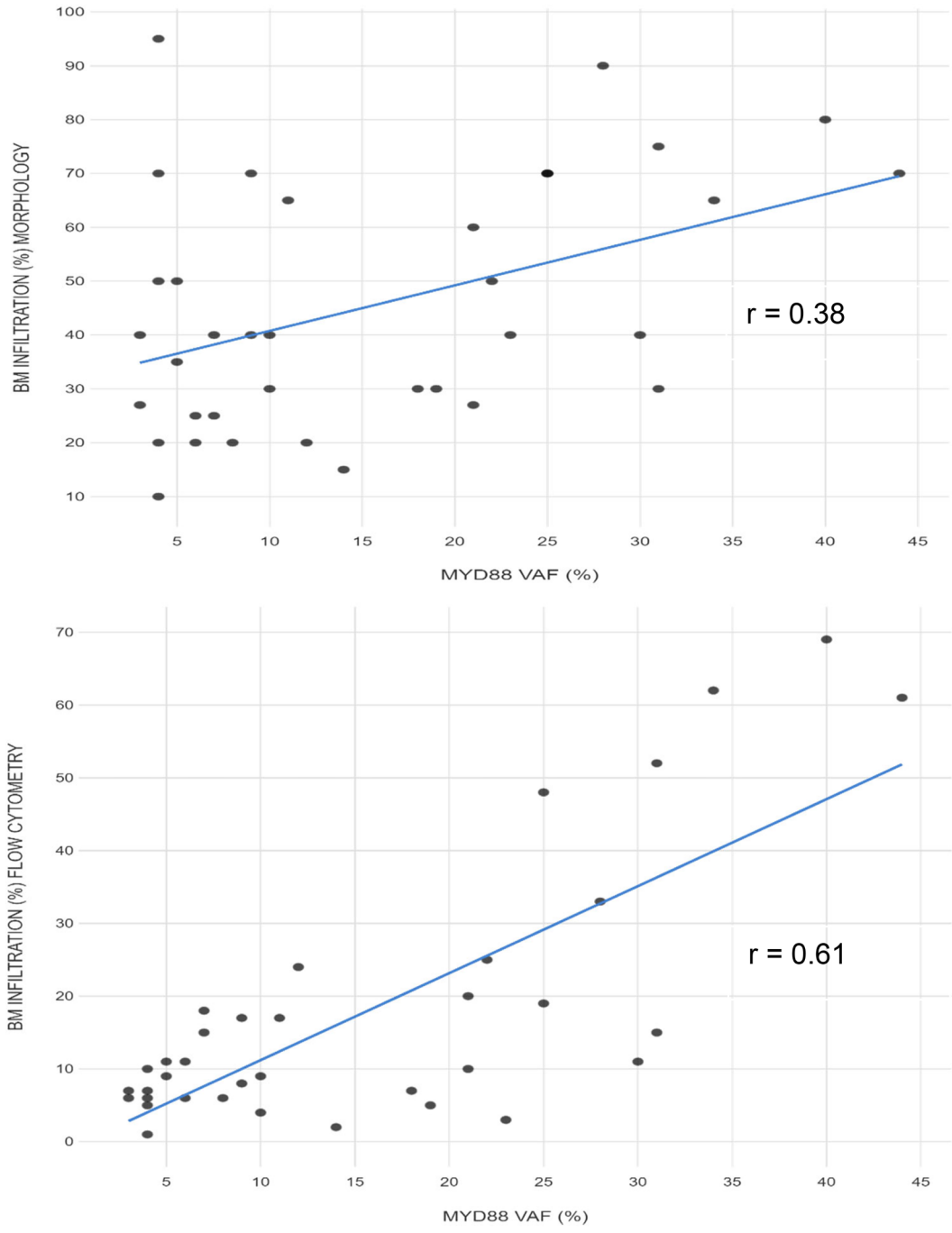

Figure 4. Scatterplots illustrating correlations between *MYD88* allele burden and BM infiltration estimated by morphology and flow cytometry.

of clinical or histological transformation. Other NGS studies have identified *TP53* variants in 2–10% of WM patients at the time of diagnosis, which is in concordance with our findings [2,29,32]. *TP53* alterations have been linked to shorter survival, independent of other adverse prognostic factors, in patients with WM [29,35]. Larger studies investigating the clinical impact of *TP53* variants in WM are warranted, including its role in the biological process of transformation. The *PAX5* gene has a role in transcriptional regulation of *MYD88*-driven B-cell lymphomas and *PAX5* variants have been observed in most *MYD88*-mutated diseases, including WM [39]. In addition, the tumor suppressor gene *PTPRD* may be implicated in the transformation of WM to DLBCL [7]. *IDH2* variants, which are found in 20% of patients with acute myeloid leukemia, have only been occasionally reported in patients with WM [40]. *IDH2* variants are associated with clonal hematopoiesis of indeterminate potential (CHIP) in the elderly population, and it is likely that the *IDH2* mutation found in

our study reflects CHIP [41]. Due to the low number of cases with *PAX5*, *PTPRD*, and *IDH2* variants, it was not possible to assess clinical implications of these aberrations. However, *IDH2* variants represent targetable genomic aberrations, currently approved for the treatment of AML [42].

Our study has some limitations. The detection rate of variants is influenced by the coverage depth and sensitivity of the assays as well as the degree of BM infiltration in the assessed samples [43]. For inclusion of samples in NGS analysis, CD19 selection of cells is recommended [8]. In our study, all BM samples were unsorted, and a significant part of the series had BM infiltration of less than 10% when estimated by flow cytometry which may have impacted the results. The clinical associations presented are based on few patients and are therefore descriptive only.

In conclusion, the use of a customized NGS panel is a feasible and robust diagnostic tool in a routine clinical setting in WM patients, but with limitations in patients with low BM infiltration. Our data contribute to the description of the mutational landscape in WM, including a potential role of *BIRC3* variants as an adverse prognostic marker and *TP53* variants as a driver in the genetic evolution of histological transformation to DLBCL.

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Disclosure statement

The authors report there are no competing interests to declare.

Author contributions

SØ contributed to research design, the acquisition of data, analysis of data, and wrote the manuscript. LS, MFB, MØP, PN, EH, and LMRG contributed to research design, analysis of data, and provided NGS data or pathology review. THN contributed to research design, collection of data, and analysis of data. TH and LM contributed to research design and interpretation of data. All authors critically revised the article and approved the submitted version.

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Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request, and with the appropriate approvals of the Danish Research Ethics Committee.

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