Diagnostic Tools of Waldenström's Macroglobulinemia – Best Possibilities for Non-invasive and Long-term Disease Monitoring

Diagnostické přístupy u Waldenströmovy makroglobulinemie – nejvhodnější dostupné možnosti neinvazivního a dlouhodobého monitorování nemoci

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Summary

Waldenström's macroglobulinemia (WM) is a B-cell malignancy characterized by high level of monoclonal immunoglobulin M (IgM) paraprotein in blood serum and associated with the bone marrow infiltration by malignant cells with lymphoplasmacytic differentiation. WM remains incurable advances in therapy. Most of WM cases are associated with a somatic point mutation L265P in *MYD88*. Significantly higher risk of progression from the IgM monoclonal gammopathy of undetermined significance (IgM MGUS) to WM for patients with mutated *MYD88* gene suggests that this mutation is an early oncogenic event and plays a central role in development of malignant clones. The second, most prevalent mutation in WM is found in the *CXCR4* gene and is often associated with drug resistance and aggressive disease presentation. Therefore, detection of these mutations ($MYD88^{L265P}$ and $CXCR4^{S338X}$) could be useful diagnostic and prognostic tool for the patients with WM. While detection of these mutations in bone marrow sample is common, the aim of our study was to compare sensitivity of detection of mutation from different cell fraction from peripheral blood and bone marrow. The results show possibility to describe *MYD88* and *CXCR4* mutation status even from peripheral blood sample (sensitivity for $MYD88^{L265P}$ was 100%, for $CXCR4^{S338X}$ 91%), which significantly facilitate material collection. Moreover, comparable detection sensitivity of these mutations in bone marrow and peripheral blood samples examined before and during the therapy offers a promising tool for more routine diagnostic and monitoring of disease progression.

Key words

Waldenström macroglobulinemia – hematology – neoplasms – lymphoma – mutation – *MYD88* – *CXCR4*

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Souhrn

Waldenströmova makroglobulinemie (WM) je B buněčné maligní onemocnění charakteristické vysokou hladinou monoklonálního imunoglobulinu M (IgM) v krevním séru a je spojeno s infiltrací kostní dřeně lymfoproliferativní populací maligních buněk. Navzdory pokrokům v léčbě zůstává toto vzácné onemocnění stále nevyléčitelné. Téměř všechny případy WM souvisí s přítomností somatické bodové mutace L265P v proteinu MYD88. Pacienti s touto mutací mají signifikantně vyšší riziko progrese z IgM monoklonální gamapatie nejistého významu (IgM MGUS) do WM, což nasvědčuje významnou roli této mutace v nádorové transformaci. Druhá nejčastější mutace v genu pro chemokinový receptor CXCR4 je spojena s klinickým významem, konkrétně ovlivňuje rezistenci k léčbě. Detekce zmíněných mutací (*MYD88*^{L265P} a *CXCR4*^{S338X}) se tudíž jeví být užitečným diagnostickým a prognostickým nástrojem u pacientů s WM. Zatímco vyšetření mutací z kostní dřeně je běžné, našim cílem bylo porovnat senzitivitu záchytu mutací z odlišných buněčných frakcí periferní krve a kostní dřeně. Výsledky ukazují na možnost vyšetření mutačního stavu i z periferní krve (senzitivita pro *MYD88*^{L265P} je 100 %, pro *CXCR4*^{S338X} je 91 %), což výrazně usnadňuje odběr potřebného materiálu. Navíc obdobná senzitivita záchytu mutací ze vzorků kostní dřeně a neinvazivního vyšetření periferní krve během progrese onemocnění a léčby se zdá být příslibem pro rutinní diagnostiku a monitorování onemocnění.

Klíčová slova

Waldenströmova makroglobulinemie – hematologie – neoplazmy – lymfom – mutace – *MYD88* – *CXCR4*

Introduction

Waldenström macroglobulinemia (WM) is defined as a lymphoplasmacytic lymphoma characterized by uncontrolled bone marrow (BM) infiltration with malignant cells characterised by plasmacytic differentiation and increased production of monoclonal immunoglobulin M (IgM) [1]. WM accounts for approximately 2% of hematologic neoplasms with nearly 2× higher incidence in men (7.3 in men and 4.2 in women per million in the Europe) and with a prevalence in elderly people of median age about 63–68 years in Caucasians population. Overall survival (OS) ranges from 5 to 10 years. Among the main causes of death belong disease progression, treatment complications or transformation to high-grade lymphoma [2].

Clinical course of WM is typically indolent, but there are district differences in OS [3]. Asymptomatic patients require no treatment and their survival is not different from healthy population [4]. WM patients with cytopenias, organomegaly, lymphadenopathy and IgM related complications as hyperviscosity, cryoglobulinemia, cold agglutinin disease, amyloidosis and progressive neuropathy, require a treatment [5]. Twenty-five percent of patients with WM show familial predisposition documented by the presence of B-cell malignancies or multiple cases of WM or IgM type of monoclonal gammopathy of undetermined significance (IgM MGUS) in the family [6–8].

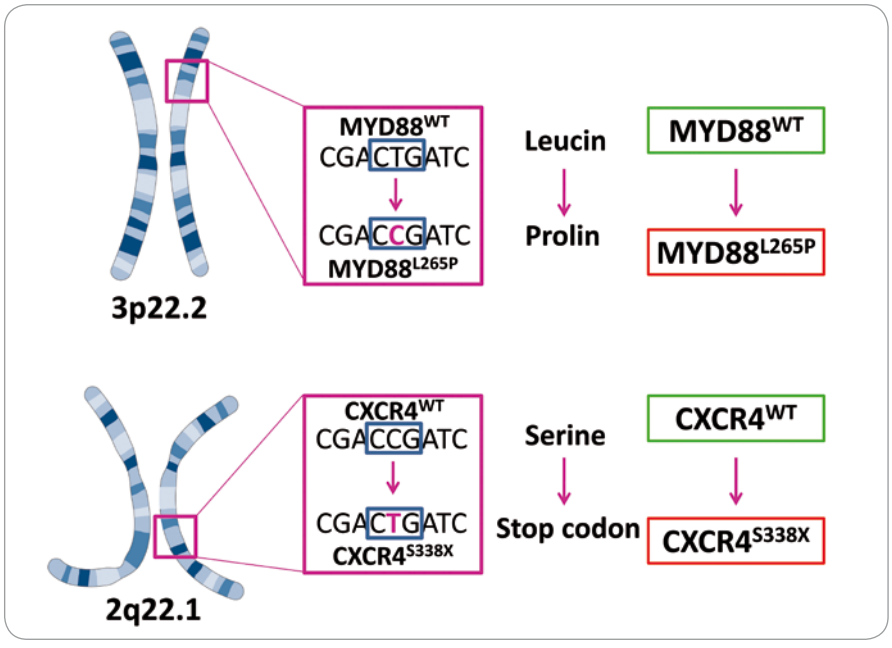

Fig. 1. Mutation *MYD88*^{L265P} and *CXCR4*^{S333X} in WM.

Two most common mutations in WM cells, *MYD88*^{L265P} and *CXCR4*^{S333X}, are presented. Figure shows position, nucleotide change and amino acid change of both mutations. Single nucleotide substitution T→C at position 3p22.2 resulting switch of leucine to proline at amino acid position 265 (L265P) and leading to constitutive activation of MYD88. Single nucleotide substitution C→T at position 2q22.1 resulting in stop codon (S338X) and leading to decrease CXCR4 internalization.

IgM MGUS is a pre-malignant disorder preceding development of WM and other lymphomas. MGUS is defined as a pathological state, when BM is infiltrated by less than 10% of aberrant cells and M-protein level (IgM in case of IgM MGUS) of less than 30 g/L. Overall MGUS affects approximately 3.5% of the population over 50 years of age, the median age of patients at diagnosis is 62 years. MGUS represents a risk factor for the development of symptomatic WM, various B-cell non-Hodgkins lymphoma, multiple myeloma or primary amyloidosis (AL) with total incidence 1% per year. The potential risk of IgM MGUS lies also in the production of aberrant monoclonal IgM which may be responsible for organ damage due to the targeting of endogenous antigens or antibody deposition in tissues [9,10]. Treatment of asymptomatic IgM MGUS patients is not indicated [11].

The transition stage between IgM MGUS and WM is known as smoldering WM (SWM). SWM is a poorly de-

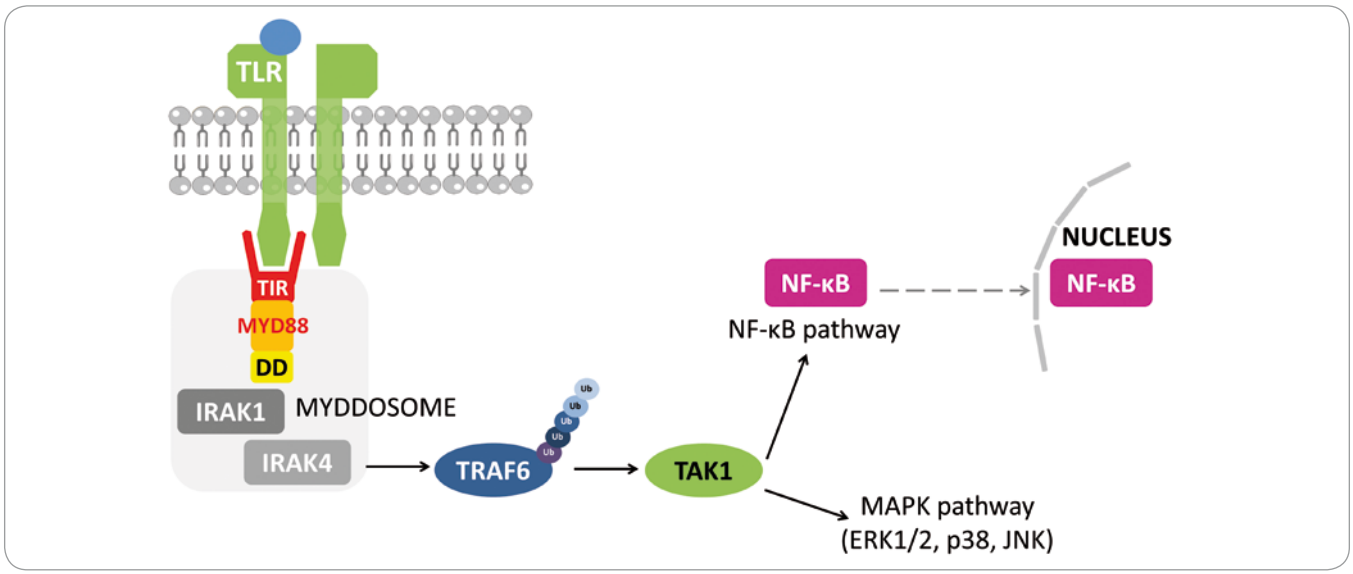

Fig. 2. MYD88 signalling pathway.

MYD88 is an adaptor protein for Toll-like receptors (TLR). Its modular structure consists of a Toll/IL-1R (TIR) domain at COOH terminus and a death domain (DD) at NH2 terminus. MYD88 binds the serine-threonine kinases IRAK and together create myddosome, responsible for polyubiquitination of TRAF6 and activation of the TAK1 protein kinase complex. TAK1 lead to activation of two different pathways, NF-κB and MAPK (ERK1/2, p38, JNK).

scribed asymptomatic phase with an increased risk of progression to symptomatic WM. A presence of a serum IgM value ≥ 3 g/dL and/or ≥ 10% BM lymphoplasmacytic infiltration with no evidence of end-organ damage (anaemia, hyperviscosity syndrome, lymphadenopathy, hepatosplenomegaly etc.) is typical for SWM. The median age at time of diagnosis is 63 years. The probability of progression to symptomatic WM is 12%, which is slightly higher than risk for IgM MGUS. Because both entities, SWM and IgM MGUS, represent a possible risk of developing the symptomatic stage, we could speculate that most WM patients have event. gone through the benign stages of IgM MGUS and SWM before developing clinical symptoms [12,13].

A broad spectrum of novel drugs is currently available for the therapy, including monoclonal antibodies, proteasome inhibitors and Bruton tyrosine kinase inhibitors. Treatment should be tailored to the individual patient while covering many clinical factors [8].

Myeloid differentiation factor 88

A highly recurrent somatic mutation in gene *MYD88* (T>C) at the position 38182641 in chromosome 3p22.2 (Fig. 1) results in amino acid change from leu-

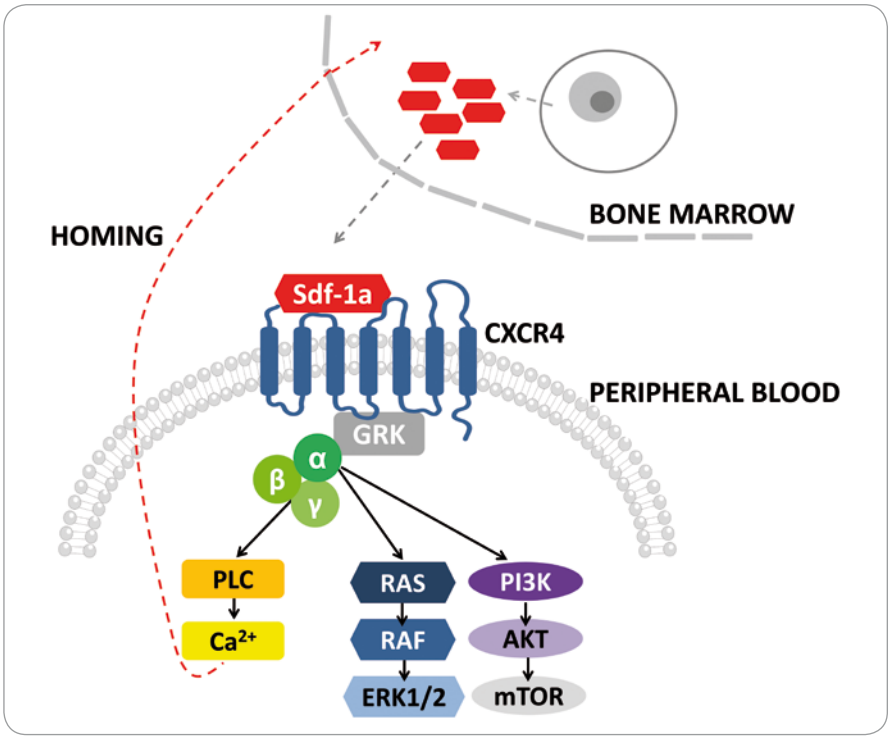

Fig. 3. CXCR4 signalling pathway.

C-X-C chemokine receptor type 4 (CXCR4) is expressed on peripheral blood cells (PB cells) – monocytes, B-cells, and naive T-cells. Stromal derived factor 1a (SDF-1a/CXCL12), ligand for CXCR4, is released by bone marrow stromal cells and binds to extracellular loops of CXCR4. G protein-coupled receptor kinase (GRK) interacts with intracellular loops of CXCR4 and diverse conformations of CXCR4 complex subunits regulate different signals. Dimer of Gβ and γ subunits regulates intracellular calcium mobilization through phospholipase C (PLC), whereas Gαtriggers PI3K/AKT/mTOR and ERK1/2 signalling therefore regulates cell survival and proliferation.

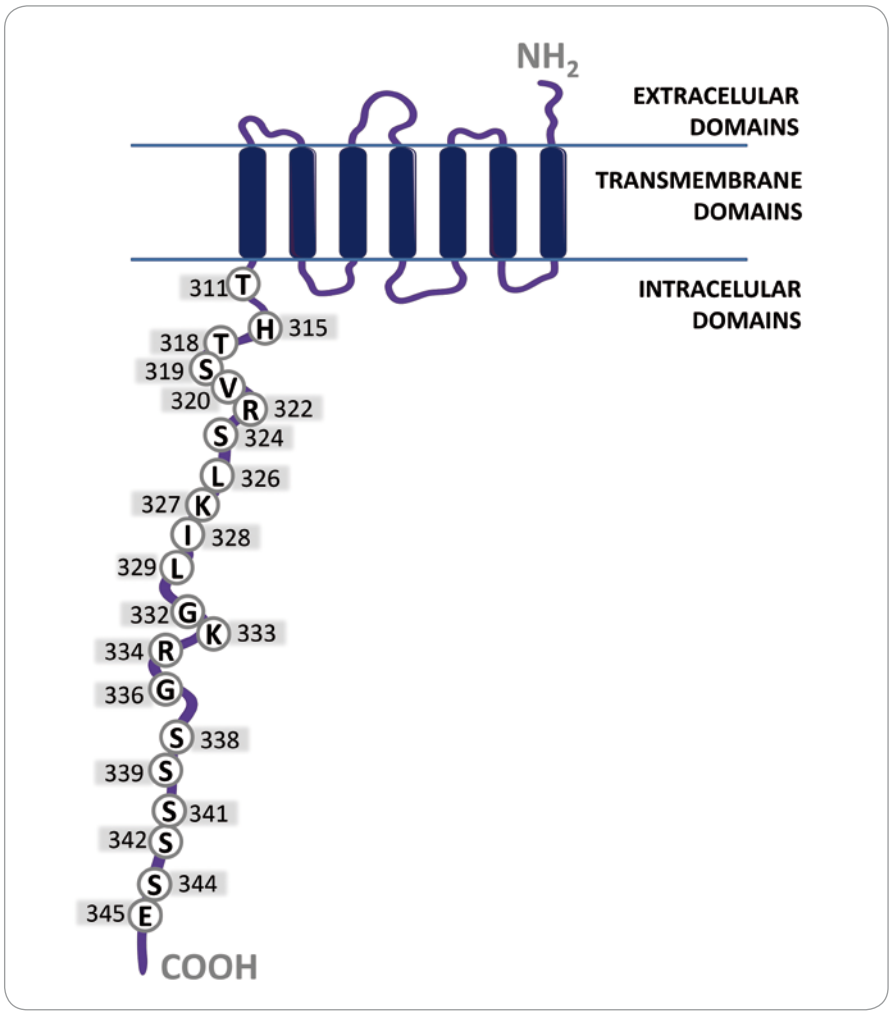

Fig. 4. Familiar mutation in CXCR4.

The table shows *CXCR4* mutation detected by Sanger sequencing in WM patients. Mutated amino acid sites at the C-terminal domain are depicted by white balls labelled with number of position and name of amino acid.

cine to proline (L265P). This mutation was first identified in WM patients by Treon et al. using whole-genome sequencing of paired tumor and normal samples [14]. The results were confirmed by multiple groups using Sanger sequencing or allele-specific polymerase chain reaction [14,15].

Myeloid differentiation factor 88 (MYD88) is recurrently mutated in 95% of WM and 50% of patients with IgM MGUS [13]. MYD88 is an essential adaptor protein in the Toll-like receptors (TLRs), interleukin (IL) -1 and IL-18 signalling pathways (Fig. 2). Modular structure of MYD88 consists of the Toll/IL-1R (TIR) domain at the C-terminus and the death domain at the N-terminus. MYD88 binds the serine-threonine kinases IRAK1 and IRAK2. After engagement of TLR receptor, IRAK1 is activated and associates with the E3 ubiquitin ligase TRAF6, which autoubiquitinates itself and the TAK1 protein kinase complex. TAK1 (MAPKKK family member) forms a complex interacting with polyubiquitin chains and lead to the TAK1 activation. Once active, TAK1 triggers two separate signalling cascades – the NF-κB and MAPK pathway. In the NF-κB axis, the inhibitory protein IκBα is phosphorylated by the IKK complex and undergoes proteasome degradation. This step allows NF-κB translocation into the nucleus and induces expression of pro-inflammatory genes. In the second scenario, the MAPK family members (ERK1/2, p38, JNK) together with activated TAK1 regulate other parts of the inflammatory response. Taken together MYD88 is essential for induction of NF-κB and MAPK signalling pathways in reaction to IL-1, IL-18, lipopolysaccharide (LPS) and stimuli engaging some of the TLR receptors [16–18].

Overexpression of $MYD88^{L265P}$ is supportive for survival and growth of WM cells [18]. *MYD88* mutation status can also affect treatment response. This is supported by outcomes of the clinical trial using ibrutinib in relapsed patients with WM. The trial showed major responses and fewer OS among patients with wild type MYD88 and C-X-C chemokine receptor type 4 (CXCR4) compare to patients with aberrantly activated $MYD88^{L265P}$ and either wild type CXCR4 or mutated CXCR4 (see below) [19].

Presence of overlapping pathological symptoms complicates discrimination of WM from other B-cells malignancies such as marginal zone lymphomas, IgM-secreting myeloma or follicular lymphoma. Thus, screening for *MYD88* mutational status in BM or more routinely in peripheral blood samples would greatly facilitate disease diagnosis. In patients with IgM MGUS, *MYD88* gene should be analysed to assess the risk of progression to WM. Quantitative assessment of $MYD88^{L265P}$ may provide sensitive and inexpensive method to identify residual clones in WM patients after treatment and monitor the disease progression. [17,18]. Present knowledge suggests that $MYD88^{L265P}$ plays role as an unifying event in the pathogenesis of WM but only a single mutation is unlikely to explain the malignant transformation from the premalignant to symptomatic stage [13]. In future, we will need to extend our knowledge of WM pathogenesis and aim our interest on translational research that can provide novel tools for direct use in clinic.

C-X-C motif chemokine receptor 4

While $MYD88^{L265P}$ might be considered as the founder mutation, C-X-C motif chemokine receptor 4 (CXCR4) could be one the later events that accelerate WM progression. The *CXCR4* gene is located at

chromosome 2q22.1 (Fig. 1) and encodes the G-coupled receptor for the C-X-C chemokine. It is expressed on monocytes, B-cells, and naive T-cells in PB [20,21]. The CXCR4 pathway plays a role in chemotactic activity of lymphocytes, cancer cell homing and metastasis [22], it can also promote tumor vascularization and act as a survival or growth factor [23]. The ligand for CXCR4, stromal derived factor 1a (SDF-1a/CXCL12), is released by BM stromal cells. Binding of SDF-1a to CXCR4 induces increase in the intracellular Ca^{2+} level and triggers aberrant cells to home in BM [24]. The N-terminal domain of SDF-1a binds to the extracellular domain of CXCR4 resulting in the activation of intracellular signalling pathways. The intracellular part of CXCR4 interacts with G protein-coupled receptor kinase (GRK) translating the input into multiple signalling cascades including PI3K/AKT/mTOR and ERK1/2 that play important role in cell survival and proliferation (Fig. 3) [25,26].

Somatic mutations in *CXCR4* were initially associated with cancer by study identifying nucleotide changes in WM patients [27]. This receptor is the second most commonly mutated gene in the WM cells with more than 40 mutations mostly located in the C-terminal domain (Fig. 4). About 30% of WM patients have frameshift or nonsense mutations in the *CXCR4* gene [27,28]. The most common type of *CXCR4* gene mutation in the WM patients is the S338X (C1013G) nonsense mutation causing impairment in the receptor internalization and increase in the activation of intracellular kinase pathways resulting in increased migration, adhesion, growth and survival of the aberrant WM cells (Tab. 1) [27,29]. Incidence of this variant analysed from BM was 28% in WM patients and 20% in IgM MGUS [29], nevertheless incidence from PB samples remains unknown.

Ibrutinib, the inhibitor of Bruton tyrosine kinase (BTK), is one of the small molecules approved by FDA for the treatment of WM patients. Despite high response rates and durable remissions of patients [19], disease progression can occur during ibrutinib therapy. Whereas mutated *MYD88* promotes ibrutinib efficacy [30], *CXCR4* mutations are associated with slower response kinetics and lower response rates among WM patient on ibrutinib. In other words, *CXCR4* mutations impact ibrutinib clinical activity in WM [31].

While detection of $MYD88^{L265P}$ and $CXCR4^{S338X}$ mutation in BM sample is common for WM diagnosis, the aim of our study was to compare sensitivity of detection of these mutations from different cell fraction found in PB (whole PB – PB, PB mononuclear cells – PBMC, $CD19^+$ PB cells – $CD19^+$ PB) and BM ($CD19^+$ BM cells – $CD19^+$ BM). The possibility of collection and analyzing PB instead of BM

Tab. 1. Known mutations in CXCR4.
List of 40 frame shift or nonsense mutations in the *CXCR4* gene located in C-terminal domain.

Nucleotide change	Amino acid change	Type of mutation
931_933insT	T311fs	frameshift
945_946insC	H315fs	frameshift
951_953delACCTC	T318fs	frameshift
952_953insA	T318fs	frameshift
953_954delC	T318fs	frameshift
954_956insC	S319fs	frameshift
958_960delTG	V320fs	frameshift
963_964insC	R322fs	frameshift
969_971insG	S324fs	frameshift
977_978insC	L326fs	frameshift
978_980insT	K327fs	frameshift
979_985delAGATCCT	K327fs	frameshift
982_983delAT	I328fs	frameshift
984_986insT	L329fs	frameshift
993_995insA	G332fs	frameshift
997A<T	K333X	nonsense
1000C<T	R334X	nonsense
1005_1007insT	G336fs	frameshift
1006G<T	G336X	nonsense
1012_1015delTCAT	S338fs	frameshift
1012_1013insT	S338fs	frameshift
1012_1013delT	S338fs	frameshift
1013C<A	S338X	nonsense
1013C<G	S338X	nonsense
1013_1015delATCT	S338fs	frameshift
1013_1015delATCTGTTTCCACTGAGT	S338fs	frameshift
1015_1017delCT	S339fs	frameshift
1017_1018delT	S339fs	frameshift
1020_1021delT	S341fs	frameshift
1022_1023insT	S341fs	frameshift
1024_1026delCT	S342fs	frameshift
1030_1041CTGAGTCTTCGT	S344fs	frameshift
1031_1033delCT	S344X	nonsense
1033_1035delAG	E345fs	frameshift

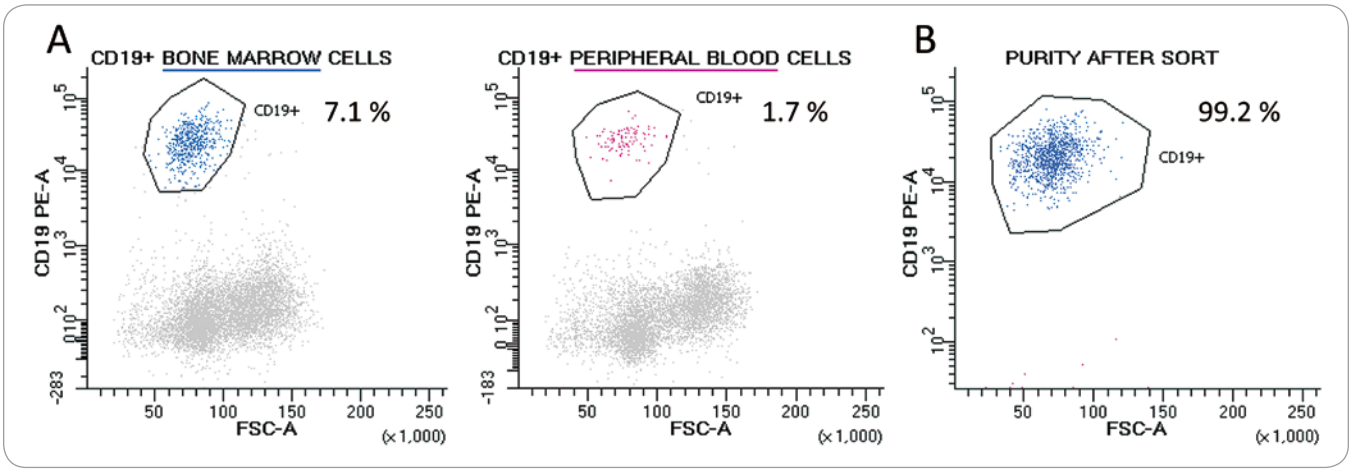

Fig. 5. Separation of the cells of interest entering molecular analyses of mutation status.

A. WM cells are characterised by expression of CD19 on their surface, therefore this marker was used for enrichment of analysed cell population with aberrant WM cells from BM (blue dots) and from peripheral blood (purple dots).

B. Purity after sorting was controlled and achieved at least 98% in all cases (blue dots). For cell staining monoclonal antibody CD19-PE (Exbio) was used.

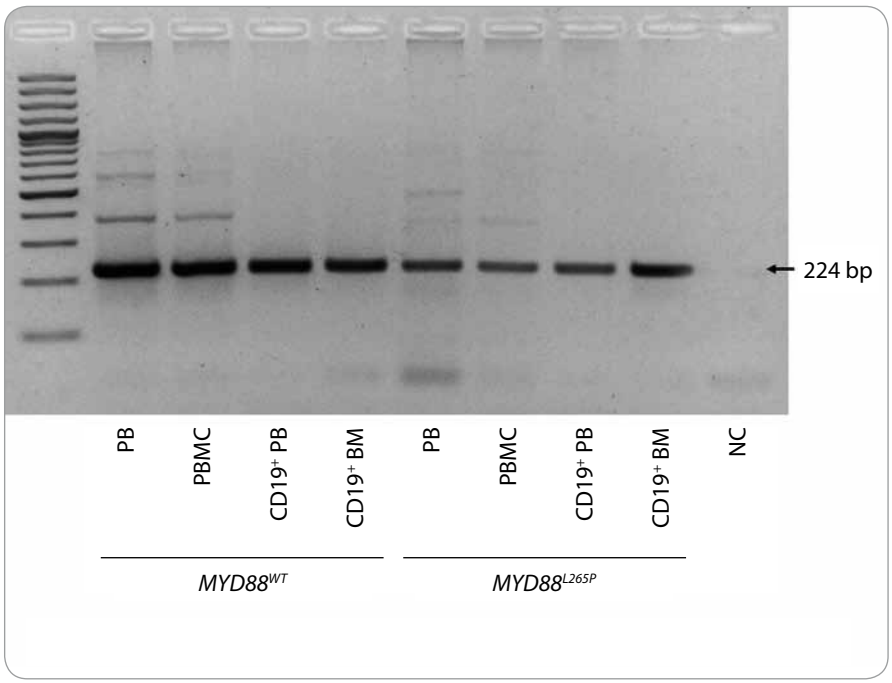

Fig. 6. Detection of *MYD88*^{L265P} in different input material.

Detection of *MYD88*^{L265P} from PB, PB mononuclear cells (PBMC), CD19$^+$ peripheral blood cells (CD19$^+$ PB) and CD19$^+$ bone marrow cells (CD19$^+$ BM) was done using allele specific PCR. All input materials were sufficient for detection of mutant allele (line 6–9).

would be significantly easier and most importantly less invasive for patients.

Material and methods

Material and samples

PB and BM samples were collected at the Department of Haematooncology of University Hospital Ostrava (Czech Republic), University Hospital Bratislava (Slovak Republic) and City Hospitals' Group in Chorzow (Poland). This study was approved by institutional ethical review boards and all individuals provided written informed consent. Mononuclear cells were separated from PB and BM by Ficoll-Plaque PLUS (GE Healthcare) gradient centrifugation. Infiltration of CD19$^+$ cells was analysed by flow-cytometry analysis using anti-CD19-PE (Exbio) antibody. The CD19$^+$ cells were sorted from PBMC and BM mononuclear cells (BMMC) by fluorescence activated cell sorting (FACS Aria III, Becton Dickinson). DNA from whole blood and PBMC were isolated by Magnesia® 16 magnetic bead extraction system (Anatolia Geneworks). DNA from the CD19$^+$ cells of PB and BM was isolated by QIAamp DNA Mini kit (Qiagen).

ASO-PCR (MYD88)

Detection of somatic mutation L265P in the *MYD88* gene was performed by the allele-specific PCR. Two forward primers were used to differentiate the mutant and the wild type alleles. Sequences of the MUT forward primer was 5'-GTGCCCATCAGAAGCGCCC-3' and the WT forward primer was 5-GTGCCCATCAGAAGCGCCT-3'. The common reverse primer was 5'-AGGAGGCAGGGCAGAAGTA-3' [32]. PCR was performed in the final volume of 20 ul with 100 ng of each primer and 50 ng of DNA using GoTaq® G2 Hot Start Polymerase (Promega). Conditions of PCR were following – 1 cycle: 94 °C for 1 min, 35 cycles: 94 °C for 30 s, 65 °C for 30 s and 72 °C for 1 min and the final extension step: 72 °C for 10 min. Final products of 224 bp were detected on 2% agarose gel.

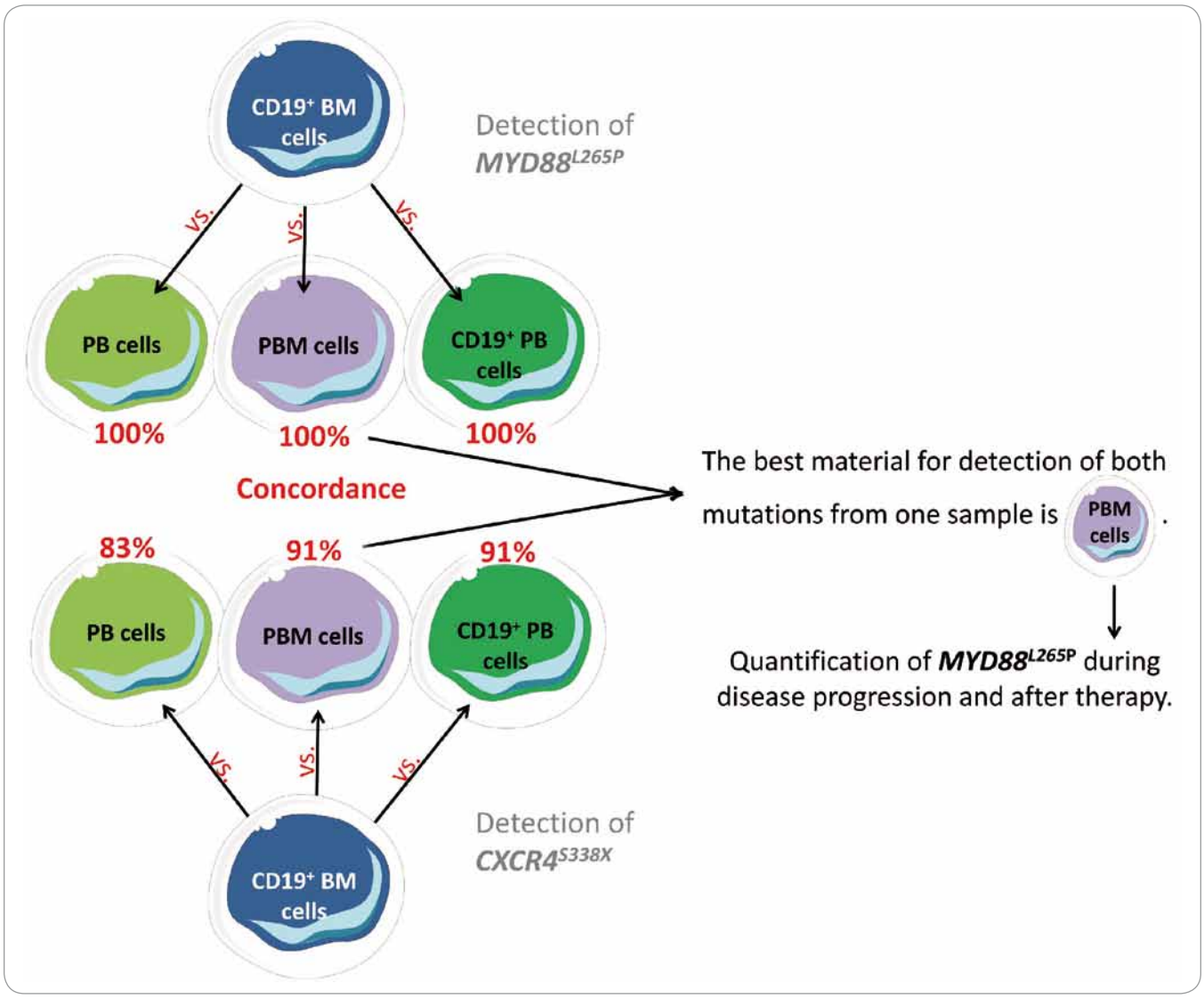

Fig. 7. Scheme showing comparison of ASO-PCR sensitivity detecting *MYD88*^{L265P} and *CXCR4*^{S333X} from different input material.

Use of different input material for detection of *MYD88*^{L265P} shows 100% concordance (red numbers in %) and 83–91% concordance for detection of *CXCR4*^{S333X} (red numbers in %). Although analysis of peripheral blood mononuclear cells and CD19$^+$ PB cells shows same results, PB mononuclear cells (PBM cells) were chosen as best material for detection of both mutations from same material as well as for detection of *MYD88*^{L265P} during therapy and disease progression, because of simpler sample processing. PB cells (light green), PB mononuclear cells (violet), CD19$^+$ PB cells (dark green) and CD19$^+$ BM cells (blue).

Real time ASO-PCR (CXCR4)

Detection of somatic mutation S338X in the *CXCR4* gene was performed by the real-time allele-specific PCR. The common forward primer was 5′-TTTCTTCCACTGTTGTCTGAACC-3′. Two reverse primers were used to differentiate the mutant and WT allele. Sequence of the MUT reverse primer was 5′-GACTCAGACTCAGTGGAAACAGATG-3′ and the WT reverse primer 5′ GACTCAGACTCAGTGGAAACAGAAC-3′ [29]. PCR was performed using TaqMan Gene Expression Master Mix (Applied Biosystems). The probe was tagged by 6FAM-MGB1 and its sequence was 5′-TATGCTTTCCTTGGAGCCA-3′. StepOne Real Time PCR system (Applied Biosystems) was used for detection of mutations. Conditions of reaction were following – 1 cycle: 95 °C for 10 min, 50 cycles: 95 °C for 15 s and 60 °C for 1 min.

Quantification of *MYD88*^{L265P}

Continuous quantification of *MYD88*^{L265P} during disease development and treatment was performed by qPCR from PB samples. Set of three primers was used, common forward primer with sequence 5′-CCTTGTACTTGATGGGGATCA-3′, mutated reverse primer 5′-CCTTGTACTTGATGGGGATGG-3′ for detection of *MYD88*^{L265P} and WT reverse primer 5′-CCTTGTACTTGATGGGGATGG-3′ as a control of amplification [15]. For each experiment concentration line was prepared using mutant DNA diluted to final concentrations – 100, 50, 25, 12.5, 6.25, 3.12,

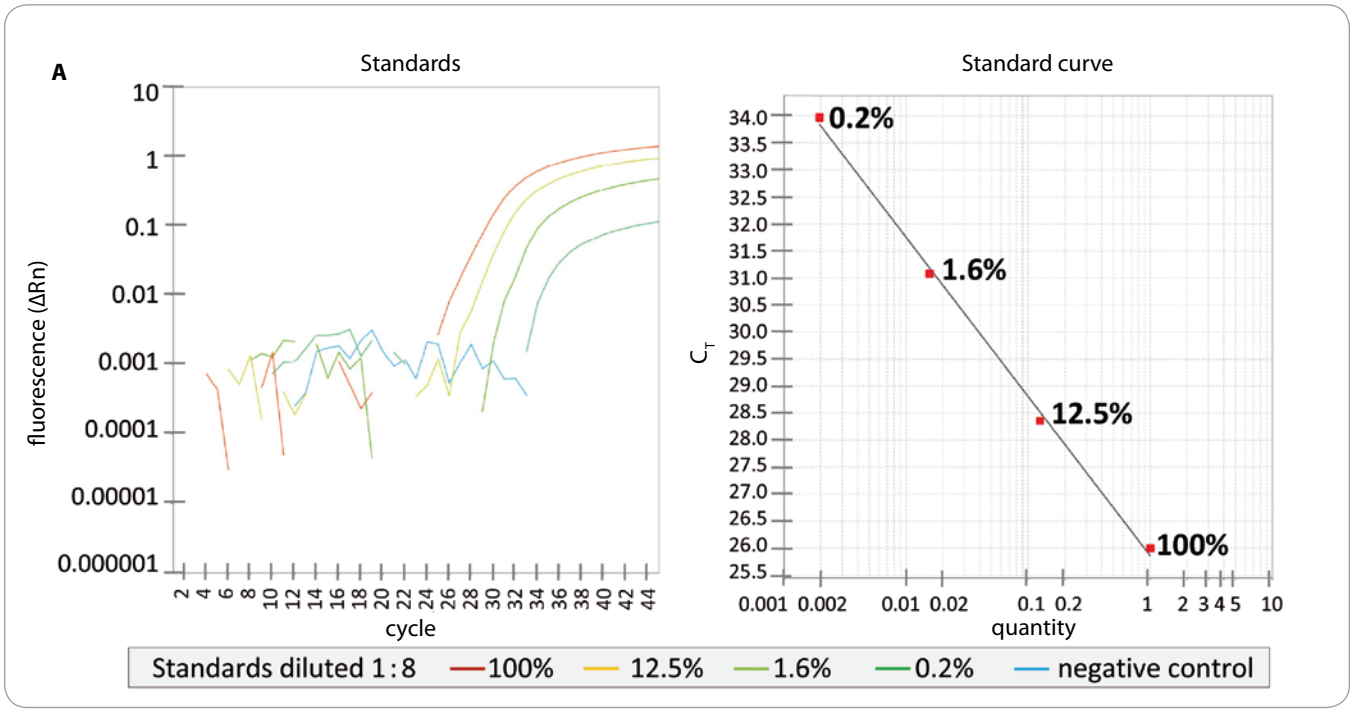

Fig. 8. Quantification of *MYD88*^{L265P} from PB mononuclear cells during therapy and disease progression.

A. Standards were prepared from *MYD88*^{L265P} BM cells with dilution coefficient 1 : 8 (100%, 12.5%, 1.6%, 0.2% of *MYD88*^{L265P} positive sample).

1.56 and 0%. Final volume of each reaction was 20 µl, containing 300 nM primers, 200 nM probe and 20 ng of DNA. Experiments were performed using TaqMan Gene Expression Master Mix and StepOne Real Time PCR system (Applied Biosystems) with following conditions – 1 cycle: 95 °C 10 min, 50 cycles: 95 °C for 15 s and 60 °C for 1min.

Results

Comparison of different input material for detection of *MYD88*^{L265P} and *CXCR4*^{S338X} mutations

The aim of this study was to compare the sensitivity of PCR detection of *MYD88*^{L265P} and *CXCR4*^{S338X} in different cell fraction of PB and BM. And to choose the best material for routine diagnostics in the view of reproducibility and patients comfort.

Aberrant WM cells are characterised by expression of specific surface markers. Specifically, immunophenotype of WM cells is associated with high level of CD19, CD20, CD22, CD25, CD27 and IgM and compare to the other B-cell non-Hodgkin lymphomas they lack expression of CD5, CD10, CD11c and CD103 [13,33,34]. The fluorescence activated cell sorting (FACS) is a common technique to separate cells of interest even if the infiltration is less than 5%. The use of FACS for separation of clonal WM plasma cells might be problematic as their immunophenotype is similar to the healthy plasma cells CD19$^+$/CD56$^-$/CD138$^+$ [10]. For the purpose of this study, the CD19$^+$ cells were separated to enrich for the WM cells in analysed samples (Fig. 5). The alternative way for cell separation based on the immunophenotype is the magnetic activated cell sorting (MACS) that is recommended when infiltration of malignant cells is > 5%. Nevertheless the purity of magnetic separation is significantly lower than fluorescence based separation [35], which is the main reason for using FACS in our study (purity of sort was > 98% in all cases; Fig. 5).

BM and PB samples of WM (27) and IgM MGUS (10) patients were tested for presence of *MYD88*^{L265P} and *CXCR4*^{S338X} mutations. Allele specific PCR for detection of studied mutation was done from four different cell fractions (PB, PBMC, CD19$^+$ PB, CD19$^+$ BM) in each patient. Although the sensitivity is different (bands on agarose gels have different intensity), the concordance is relatively high (Fig. 6). When the results of allele specific PCR detecting *MYD88*^{L265P} in CD19$^+$ BM are compared with those in PB, PBMC or CD19$^+$ PB the concordance is 100% in all cases. In other words, when the mutation was present in BM it was always detected also in other cell fractions (PB, PBMC, CD19$^+$ PB) and in case of absence of mutation all cell fractions were negative. For *CXCR4*^{S338X} the concordance is slightly lower, CD19$^+$ BM vs. CD19$^+$ PB or PBMC show 91% concordance, in case of CD19$^+$ BM vs. PB concordance was 83% (Fig. 7).

Based on our results we choose PBMC as the best cell fraction to determinate mutation in both studied genes. PBMC shows 100% concordance with BM samples when detecting *MYD88*^{L265P} and 91% concordance in case of *CXCR4*^{S338X}. Collection of blood is much more comfortable for patients and analysis of both mutations (*CXCR4*^{S338X}, *MYD88*^{L265P}) from the same material is money and time-saving for purpose of routine examination. Summarising schema of our result suggesting a laboratory guide for future analyses is presented in Fig. 7.

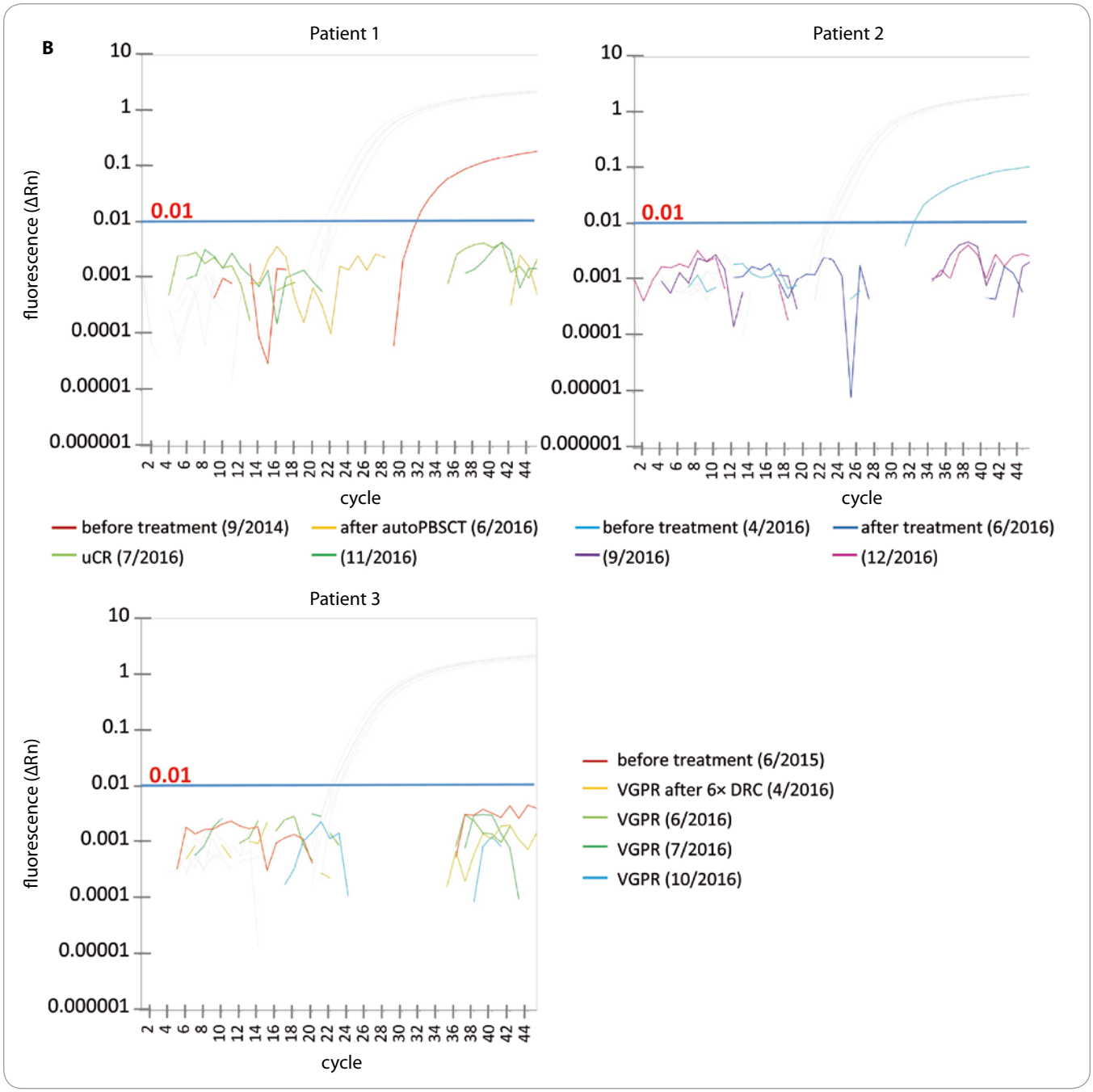

Fig. 8 – continuing. Quantification of *MYD88*^{L265P} from PB mononuclear cells during therapy and disease progression.

B. Presence of *MYD88*^{L265P} was detected before treatment initiation and subsequently in 1 to 4 month intervals from PB mononuclear cells. *MYD88*^{L265P} was present in patient 1 and patient 2 before treatment initiation, patient 3 was *MYD88*^{WT}. Level of *MYD88*^{L265P} cells in all samples of all three analysed patients during therapy was decreased under detection level of used method (qPCR).

C. Sensitivity of detection of *MYD88*^{L265P} using qPCR was 0.1%.

autoPBSCT – autologous peripheral blood stem cell transplantation, uCR – uncertain complete response, VGPR – very good partial response, DRC – dexamethasone, rituximab and cyclophosphamid

Abundance of *MYD88*^{L265P} during therapy correlates with the clinical status

We analysed presence of somatic mutations *MYD88*^{L265P} and *CXCR4*^{S338X} in 37 WM and IgM MGUS patients. Comparison of the detection sensitivity from different input materials shows PMBC as the best choice for the analysis of both mutations. This cohort of PBMC sample was used for quantification of *MYD88*^{L265P} and set of samples from three patients collected at the time of diagnosis and during therapy was used. Patients #1 and #2 were *MYD88*^{L265P} positive at the time

of diagnosis (#1 Ct = 31; #2 Ct = 32), whereas patient #3 was negative. Data shows undetectable (Ct > 45) amount of *MYD88^{L265P}* in all three patients after initiation of the therapy (Fig. 8B). This trend is in compliance with the treatment response (Fig. 8). Patient #1 achieved uncertain complete response (uCR) after autologous PB stem cells transplantation (autoPBSCT) and patient #3 achieved very good partial response (VGPR) after six cycles of DRC.

Conclusion

MYD88^{WT} is connected to differences in clinical and biological characteristics. Patients with the *MYD88^{WT}* gene were characterised by lower level of IgM, higher lactate dehydrogenase level, less somatic hypermutations in the Immunoglobulin Heavy Chain Variable gene (IGHV). But these differences do not have any impact on time to first therapy, response to treatment or progression-free or overall survival [36]. Absence of *MYD88* mutation in WM patient is associated with a female predominance, splenomegaly, gain of chromosome 3, and CD27 expression [37]. Presence of MYD88 mutations in IgM-MGUS patients relates to significantly higher levels of IgM and more frequent Bence-Jones proteinuria at the time of diagnosis. Moreover, it positively correlates with a risk of progression from the IgM monoclonal gammopathy of undetermined significance (IgM MGUS) to WM [32]. Based on our results, we were able to introduce detection of both *MYD88^{L265P}* and *CXCR4^{S338X}* mutations from PB mononuclear cells as a routine diagnostic method in our lab.

Monitoring of minimal residual disease in monoclonal gammopaties is becoming an attractive topic with high future potential. We collected PB samples from three patients during treatment and analysed *MYD88^{L265P}* using qPCR. Although sensitivity of detection for qPCR is not sufficient to detect less than 0.1% of aberrant cells, the results correlate with treatment response (Fig. 8). As flow cytometry analysis is a conventional strategy to detect aberrant cell clones, monitoring of the *MYD88* mutation status in WM patients during therapy seems to be effective and easy way for detection of MRD. Potential improved method for future analysis based on PCR is a digital droplet quantitative PCR (ddPCR) with a sensitivity of 5×10^5 that is suitable for identification of *MYD88^{L265P}* in the MRD samples, where the aberrant cells are rare [38].

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