Highly sensitive *MYD88*^{L265P} mutation detection by droplet digital polymerase chain reaction in Waldenström macroglobulinemia

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SUPPLEMENTAL MATERIAL AND METHODS

Patients and samples collection

Three patient series were tested for MYD88^{L265P} mutation by ddPCR. The local series of Torino (n=110) was used as discovery series, while the Salamanca and Pisa series (23 and 15 patients, respectively) were used for validation (Figure S1). Additionally, BM from 15 mantle cell lymphomas, 10 follicular lymphomas and PB from 10 chronic lymphocytic leukemia patients were tested. BM sample was filtered through a 1ml syringe, resuspend in erythrocytes lysis buffer (NH4Cl) (1:2 dilution) and leaved 15 minutes at room temperature (lying flat at dark), then centrifuged 10 minutes at 250xg at room temperature. The cell pellet was resuspended in 10-15 ml of NH4Cl and centrifuge again 10 minutes at 250xg at room temperature. The cell pellet was finally resuspended in PBS or 0.9% NaCl (q.s.), counted and dispensed as 5-10x10^6 cells, in each tube, centrifuged 1 minute at 13000xg and stored indefinitely, as dried pellets, at -80°C for further DNA extraction. Finally, 60 plasma and 32 urine samples were collected, as well. To ensure a good quality of cfDNA, plasma samples were collected in BCT tubes (Streck) when coming from centers outside our hospital and in K3EDTA tubes, processed within 4 hours from draw, when coming from inside hospital (samples collection and storage are also listed in Table S1). Blood collected in Streck BCTs was shipped, stored and processed at room temperature. The first centrifugation was conducted at 1300×g for 13 min at 4 °C for K_3EDTA tubes (while BCTs at room temperature), after which the plasma was carefully removed and transferred to a new tube. The second centrifugation was performed at 1800×g for 10 min. The resultant plasma was collected and stored at −80 °C until analysis. Plasma samples were thawed and centrifuged at 13000 ×g for 3 min before cfDNA extraction.

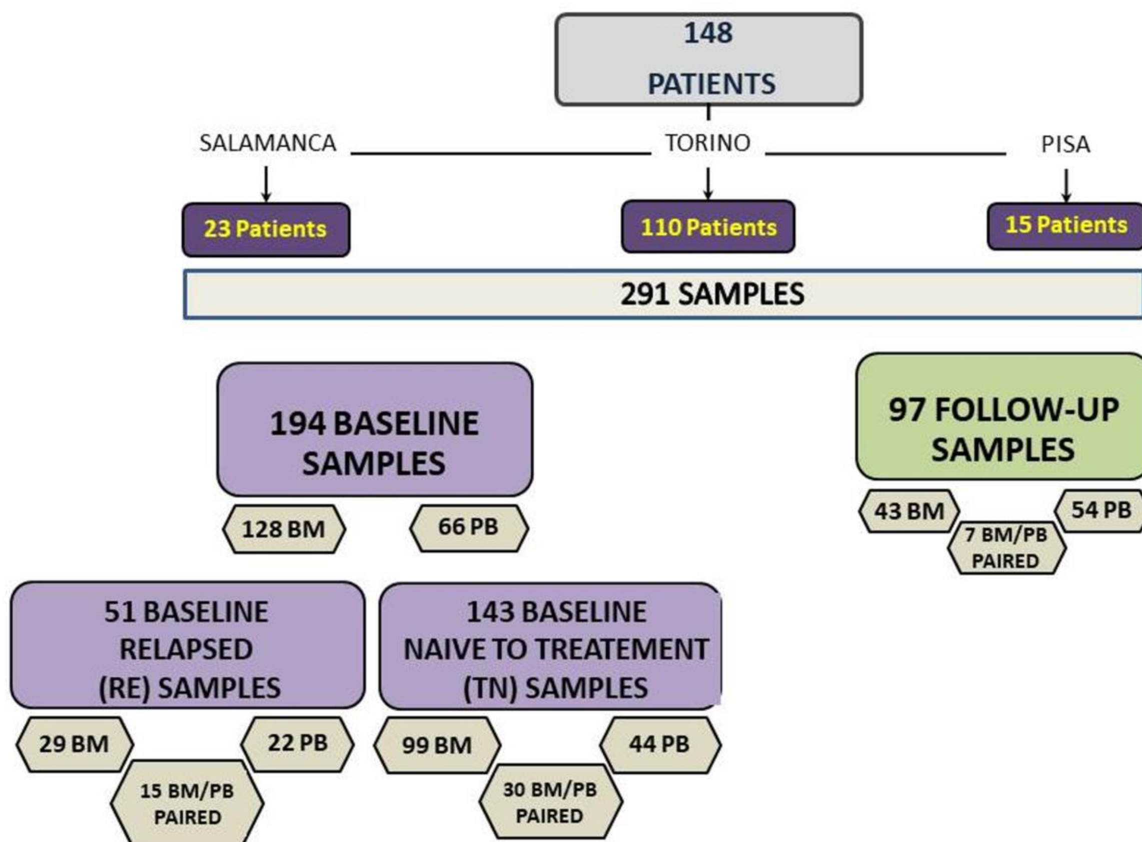

Figure S1: Overview of patients and samples characteristics, collected for MYD88^{L265P} mutation detection by ddPCR. BM:bone marrow, PB: peripheral blood, NT: naïve to treatment at baseline, RE: relapsed at baseline.

Nucleic acid extraction

Genomic (gDNA) from $5x10^6$ cells and cell-free DNA (cfDNA) from 1 ml of plasma, was extracted by Maxwell automatic system (MaxWell RSC, Promega), following the manufacturer recommendations. To ensure extraction quality and no contamination from gDNA, selected cfDNA samples, with different amount of WT copies, were tested with the Specimen control size ladder kit (InVivoScribe Technologies). Moreover, the amount of cfDNA was also evaluated by RnaseP gene.

ddPCR assays for MYD88^{L265P} mutation detection

Mutation detection assay was designed as reported in Figure S2A. A single set of primers (Forward FP 5'-CCTTGGCTTGCAGGT-3' and Reverse RP 5'-TCTTTCTTCATTGCCTTGT-3') was combined with two competitive probes in two assays (CSTM DDPCR HEX/FAM ASSAY BIO-RAD), one for MYD88^{L265P} mutation (MUT 5'-TGGGGATC**G**GTCGC-3') labeled with FAM and one for MYD88^{L265P} wild type (WT 5'-TGGGGATC**A**GTCGCTT-3') labeled with HEX. At first, the ddPCR assay was optimized for DNA amount and PCR conditions using the QX100 Droplet Digital PCR system (Bio-Rad Laboratories, Hercules, CA, USA). Briefly, for each replicate, 11 μl of 2X ddPCR Supermix for Probes with no dUTP (Bio-Rad Laboratories), 1.1 μl of each 20X mutation detection assay and 5.5 μl of gDNA (20 ng/μl) or cfDNA were mixed in a total 22 μl reaction volume. Droplets were generated, by a QX100 droplet generator device, from 20 μl of the reaction mix, and end-point PCR was performed on a T100 Thermal Cycler (Bio-Rad Laboratories) at following conditions: 95°C for 10 minutes, 40 cycles of 94°C for 30 seconds, 55°C for 1 minute followed by 98°C for 10 minutes. Ramp rate was set at 2.5°C/second. PCR products were loaded into the QX100 droplet reader and analyzed by QuantaSoft v1.6.6.0320 (Bio-Rad Laboratories). Samples were tested in triplicate and results expressed as merge of wells. ddPCR gate setting was performed based on the positive control sample. The cut-off for mutation was set based on the highest MYD88^{L265P} level detected within the control group of healthy samples and is indicated in figures as a dashed line. dMIQE guidelines (Minimum Information for Publication of Quantitative Digital PCR Experiments) for ddPCR experiments are listed in Table S1.[1]

ASOqPCR assay for tumor-specific IGH-VDJ rearrangement

Patient-specific IGH-VDJ rearrangements were amplified and directly sequenced from diagnostic gDNA. Sequences were analyzed using the IMGT/V-QUEST tool [http://imgt.org].[2,3] and patient-specific ASO primers and consensus probes were designed, as previously described (Figure S2B) [4]. IGH-based MRD analysis was performed according to the Euro-MRD guidelines[5].

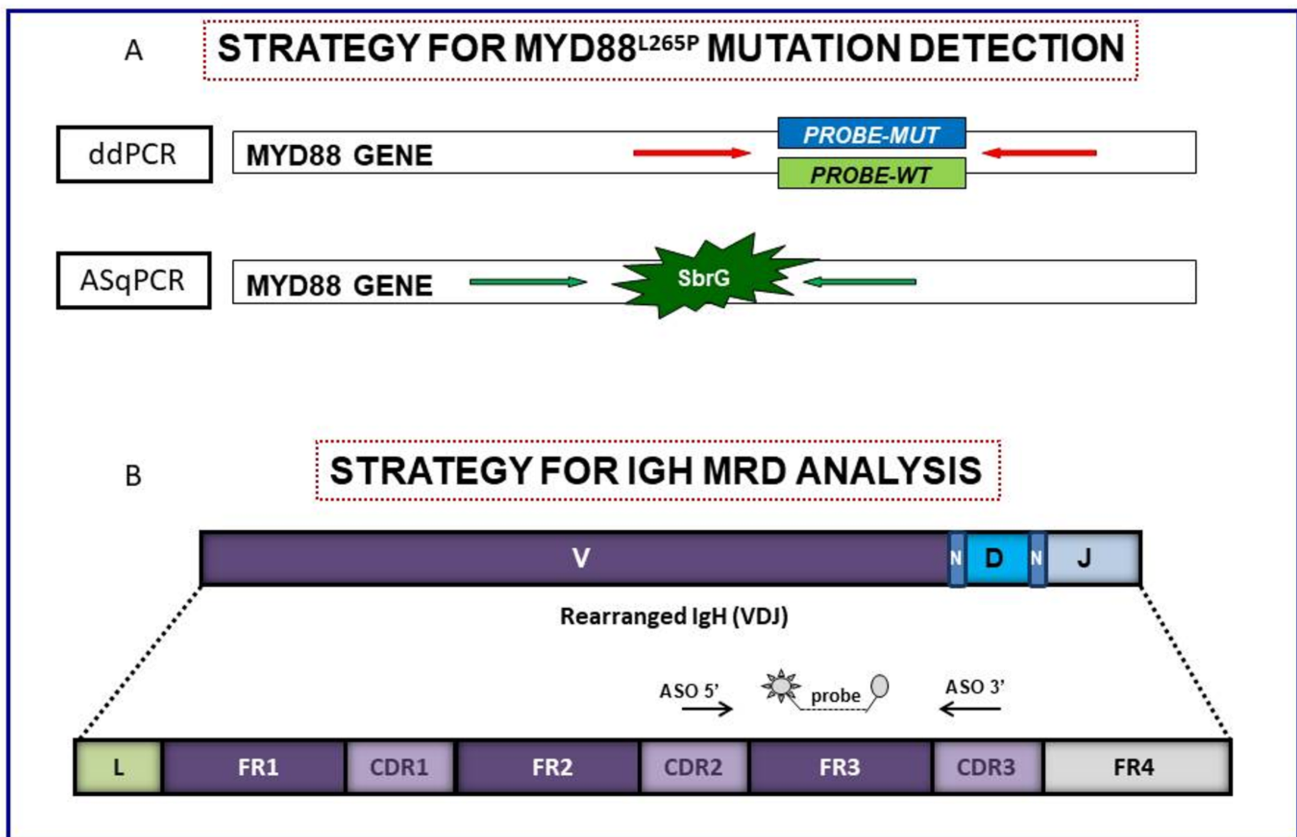

Figure S2: PCR strategies for A) $MYD88^{L265P}$ mutation detection by ddPCR and ASqPCR and B) IGH rearrangement quantification by ddPCR. ASO: allele specific oligonucleotide (clone specific primers), MUT: mutated, WT: wildtype, SbrG: Sybergreen dye.

	ITEM	IMPORTANCE	INCLUDED	COMMENTS
Experimental Design	Definition of experimental and control groups	E	Y	
	Number within each group	E	Y	
	Assay carried out by core lab or investigator's lab?	D		Investigator's lab
	Power analysis	D	Y	
Sample	Description	E	Y	
	Volume or mass of sample processed	E		10 ml of peripheral blood (PB) and 5ml of bone marrow (BM)
	Microdissection or macrodissection	E	n	n/a
	Processing procedure	E		PB were centrifuged 13 minutes at 1300Xg at +4°C, plasma was centrifuged 10 minutes at 1800Xg at room temperature. Urine sample were centrifugated 10 minutes at 200Xg at room temperature.
	If frozen - how and how quickly?	E		Plasma samples were collected on BTC tubes (StrecK) when came from outside hospital and within 4 hours from draw when came from inside hospital
	If fixed - with what, how quickly?	E	n	n/a
	Sample storage conditions and duration (especially for FFPE samples)	E		plasma and urine were stored in 1 ml aliquotes, PB and BM cells in dryed pellets both at -80°C,
Nucleic Acid Extraction	Quantification-instrument/method	E		Nanodrop 2000
	Storage conditions: temperature, concentration, duration, buffer	E		gDNA and cfDNA were stored at -20°C
	DNA or RNA quantification	E		after extraction and dilution
	Quality/integrity, instrument/method	E	Y	
	Template structural information	E		not performed
	Template modificcation (digestion, sonication, preamplification, etc.)	E		not performed
	Template treatment (initial heating or chemical debnaturation)	E		not performed
	Inibhition dilution or spike	E	n	n/a
	DNA contamination assessment of RNA sample	E	n	n/a
	Details od Dnase treatment where performed	E	n	n/a
	Manufacturer of reagents used and catalogue number	D	n	n/a
	Storage of nucleic acid: temperature, concentration, duration, buffer	E		cfDNA from plasma in 60 uL, urine in 40 ul, gDNA in 100 ul of elution buffers
ddPCR Target Information	Sequence accession number	E	n	n/a
	Amplicon location	D	n	see RTPrimerDB (ID:1529)
	Amplicon length	E		65-mer
	In silico specificity screen (BLAST, etc)	E	n	n/a
	Pseudogenes, retropseudogenes or other homologs?	D	n	n/a
	Sequence alignment	D	n	n/a
	Secondary structure analysis of amplicon and GC content	D	n	n/a
	Location of each primer by exon or intron (if applicable)	E	n	n/a
	Which splice variants are targeted?	E	n	n/a
	ITEM	IMPORTANCE	INCLUDED	COMMENTS

ddPCR Oligo nucleotides	Primer sequences and/or amplicon context sequence	E	Y	
	RTPrimerDB Identification Number	D	n	n/a
	Probe sequences	D	Y	
	Location and identity of any modifications	E	Y	
	Manufacturer of oligonucleotides	D		Integrated DNA Technologies (IDT), Coralville, IA, USA
	Purification method	D		Desalting
ddPCR Protocol	Complete reaction conditions	E	Y	
	Reaction volume and amount of cDNA/DNA	E	Y	
	Primer, (probe), Mg^{2+} and dNTP concentrations	E	Y	(ONLY FOR PRIMERS AND PROBE)
	Polymerase identity and concentration	E	n	n/a
	Buffer/kit catalougue no. and manufacturer	E		2X ddPCR Supermix for Probes (No dUTP) (cat. 186-3024), ddPCR droplet generation oil for probes (cat. 186-3005), ddPCR droplet reader oil (cat. 186-3004) (Bio-Rad Laboratories, Hercules, CA, USA)
	Exact chemical constitution of the buffer	D	n	n/a
	Additives (SYBR Green I, DMSO, etc.)	E	n	n/a
	Plates/tubes catalog no and Manufacturer	D		DG8 cartridges (cat. 186-4008), DG8 gaskets (cat. 186-3009), pierceable foil heat seals (cat. 181-4040),
	Complete thermocycling parameters	E	Y	
	Reaction setup	D	Y	
	Gravimentric or volumetric dilutions	D	Y	
	total PCR reaction volume prepared	D	Y	
	Partition number	E		> 9000 UP TO 22000
	Individual partition volume	E	Y	
	Total volume of partitions measured (effective reaction size)	E		40 μL
	Partition volume varianca/SD	D	n	
	Comprehensive details and appropriate use of controls	E	Y	
	Manufacturer of dPCR instrument	E	Y	
ddPCR validation	Optimization data for the assay	E	Y	
	Specificity	E	Y	
	Limit of detection of calibration control	E	Y	
	if multiplexing, comparison with singleplex assays	E	n	n/a

	ITEM	IMPORTANCE	INCLUDED	COMMENTS
Data Analysis	Mean copies per partition (Delta or equivalent)	E	n	n/a
	dPCR analysis program (source, version)	E	Y	
	Outlier identification and disposition	E	n	n/a
	Results of NTCs	E		All reactions shown negative NTC
	Examples of positive and negative experimental results as supplemental data	E	Y	
	Justification of number and choice of reference genes	E	n	n/a
	Description of normalization method	E	n	n/a
	Number and concordance of biological replicates	D	n	n/a
	Number and stage of technical replicates	E	Y	
	Repeatability (intra-assay variation)	E	Y	
	Reproducibility (inter-assay variation)	D	Y	
	Experimental variance or CI	E	Y	
	Statistical methods used for analysis	E	Y	
	Data submission using RDML	D	n	n/a

Table S1. Checklist for (E) essential and (D) desirable **dMIQE** information useful for ddPCR experiments. Any essential items not included (n) were not applicable (n/a) to the study.

Supplemental results

ddPCR limit of detection

LOD (0.035%) was defined using a statistical method based on binomial distribution, as previously reported[6]. However, we observed consistently positive replicates at one log lower dilution point of the standard curve 0,0035% (1 copy of MUT out of 30000 WT) (Table S2). In order to evaluate the reliability of the calculated LOD, based on the concept that the detection limit is a function of both signal strength and stability, we tested the reliability of 0.035% dilution point performing additional experiments. Ninety replicates were tested, to verify the reproducibility, for the 0,0035% and for the half log higher 0,018% dilution point (5 MUT copies in 30000 WT). While for 0.035% al replicates were positive, the level of reproducibility was 54/90 and 71/90 positive wells, respectively for 0,0035% and 0.018% dilution point (Table S2) showing inconsistently positive or negative replicates and confirming that the above calculated LOD and the experimentally detected cut-off, arising from the control group, are both reliable.

	Target %	MUT COPY NUMBER	MUT EVENT NUMBER	WT EVENT NUMBER	Accepted Droplets	RATIO (MUT/WT)
	35%	6120	3281	7178	13494	0,367
	35%	6200	3075	6705	12500	0,367
	35%	6180	3093	6972	12629	0,35
	3,5%	612	349	8094	12701	0,0275
	3,5%	632	365	8197	12892	0,0284
	3,5%	668	398	8497	13279	0,0298
	0,35%	64	38	8680	13251	0,0027
	0,35%	60	34	8098	12511	0,0026
	0,35%	52	31	8433	12938	0,0023
	0,035%	10,2	6	8369	12966	0,00045
	0,035%	6,6	4	8564	13230	0,00029
	0,035%	5,4	3	7866	12361	0,00024
	0,0035%	1,6	1	8325	13033	8,00E-05
	0,0035%	1,6	1	8835	13824	7,00E-05
	0,0035%	1,8	1	8184	12646	8,00E-05
	0,00035%	0	0	8063	12430	/
	0,00035%	0	0	8273	12777	/
	0,00035%	1,8	1	8376	12716	7,00E-05
	wt	0	0	8566	13085	/
	wt	1,8	1	8302	12848	7,00E-05
	wt	0	0	8054	12433	/
POS/ TOT Replicates	Target Ratio	MUT COPY NUMB	MUT EVENT NUMB	WT EVENT NUMB	AcceptedDroplets	RATIO (MUT/WT)
71/90	0,018%	4	180	828876	1170549	1,24E-04
54/90	0,0035%	2	76	770769	1210066	6,20E-05

Table S2. MUT/WT ratio detected by ddPCR on 10 fold dilution standard curve. From 35% to 0.0035 % corresponding to 10500, 1050, 105, 10.5 and 1 mutated copy numbers. Each dilution point was evaluated in triplicate. Reproducibility for 1 copy (0,0035%) and 4 copies (0,018%) on 90 replicated is also reported.

Comparison of MYD88^{L265P} ddPCR versus ASqPCR assays

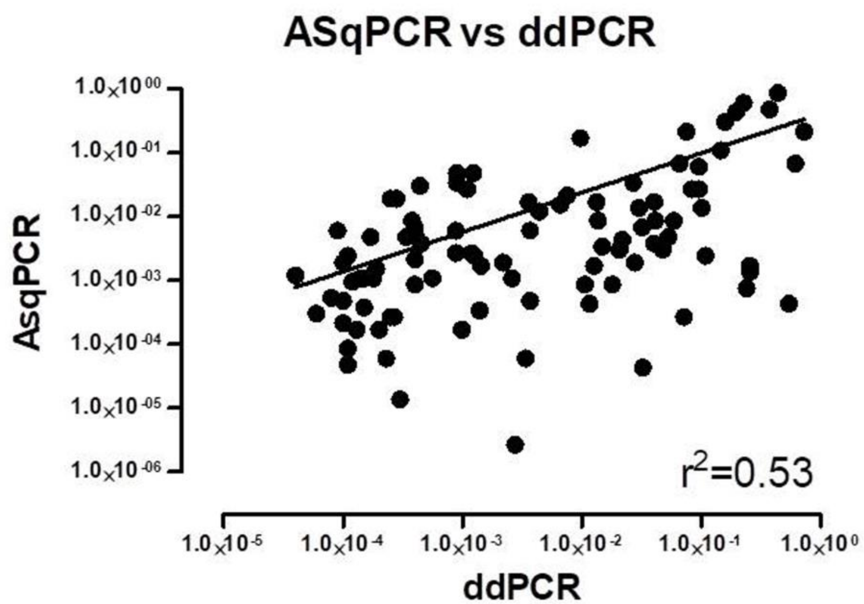

Figure S3: ddPCR vs ASqPCR concordance in 100 samples

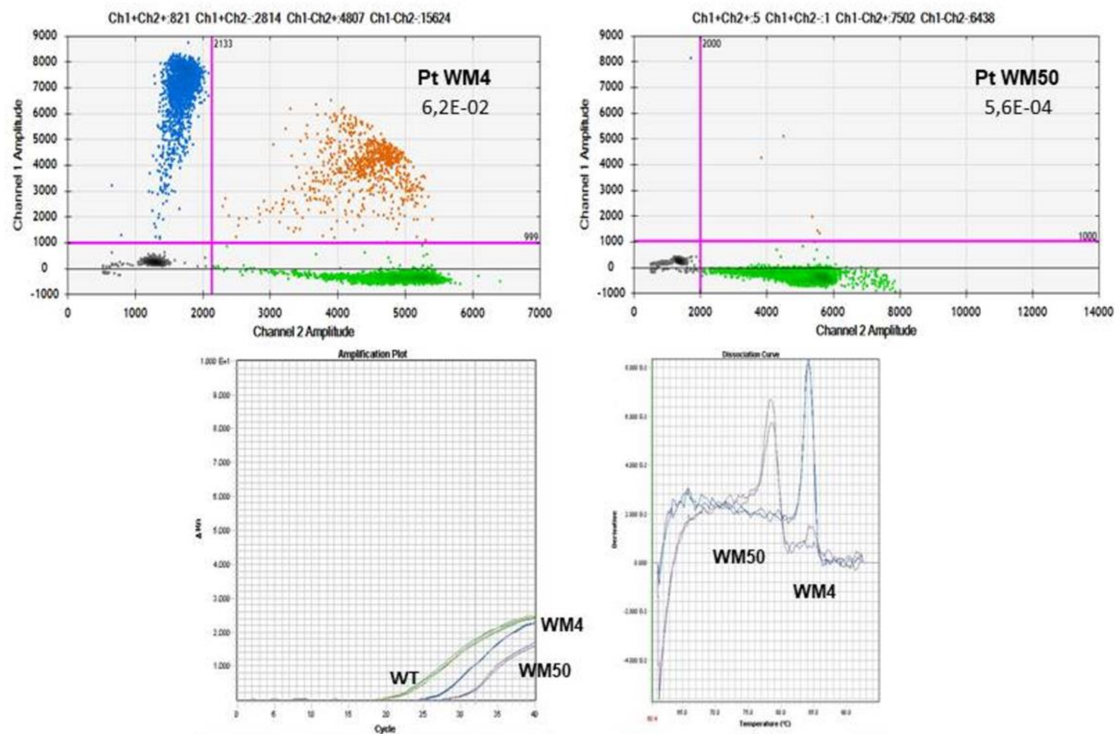

Figure S4: ddPCR versus AsqPCR. Plots, for both methods, reported from two patients with high (WM4) and low (WM50) mutation ratio.

MYD88^{L265P} ddPCR on ctDNA

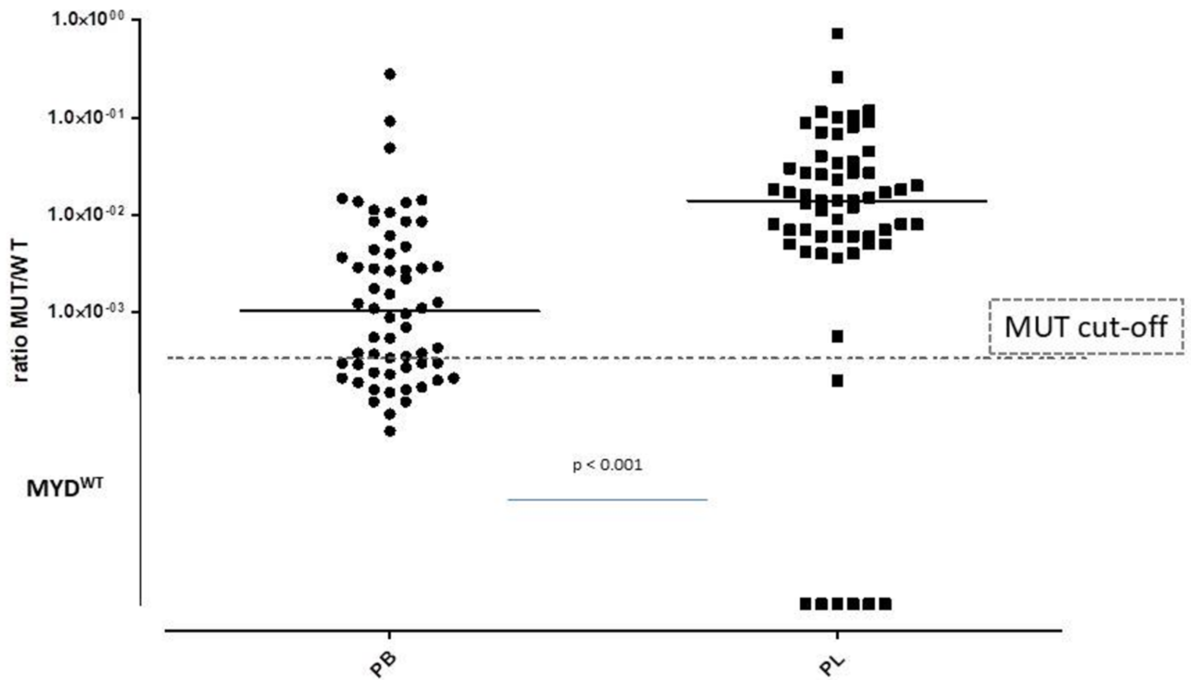

Figure S5: MYD88^{L265P} MUT/WT ratio in PB/ctDNA from PLASMA (PL) paired samples from 60 patients. Dashed line shows the negativity cut-off depicted based on MUT/WT ratio within the control samples group.

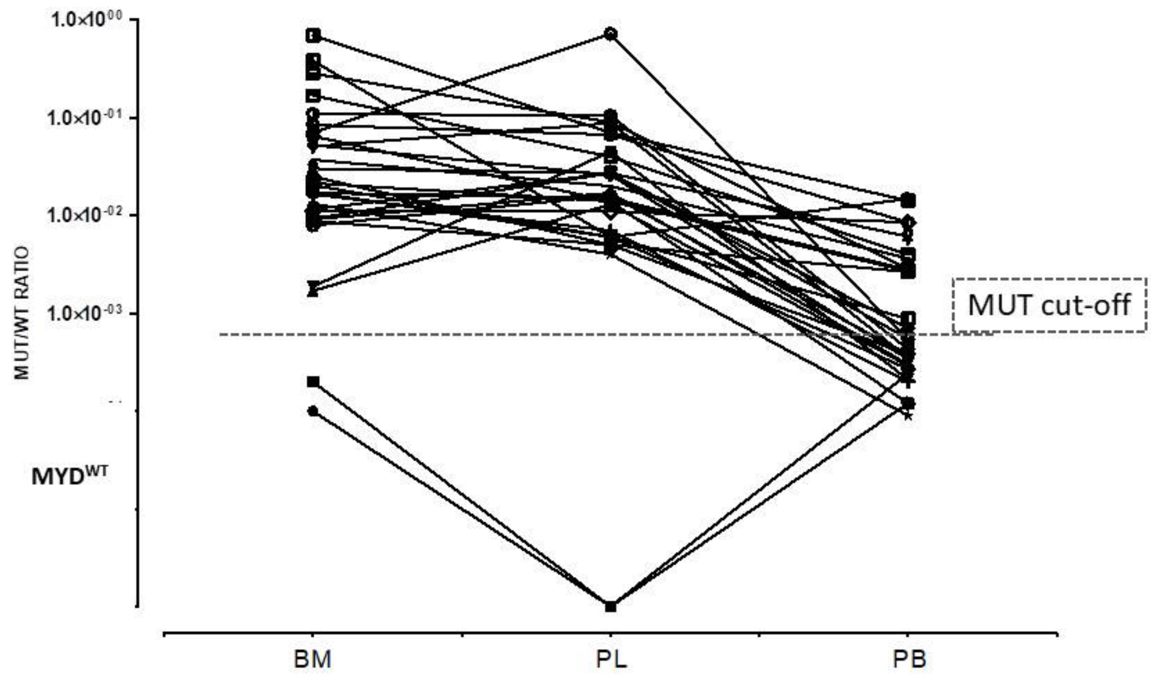

Figure S6: MYD88^{L265P} MUT/WT ratio in BM-PLASMA-PB paired samples from 32 patients. Dashed line shows the negativity cut-off depicted based on MUT/WT ratio within the control samples group.

MRD monitoring in WM by MYD88^{L265P} ddPCR

(N= 52) Selected patients chacteristics	
WM	45 (86,6%)
IgG LPL	6 (11,5%)
LPL amyloid tumor	1 (1,9%)
Sex, F	28 (53,8%)
Median age (years, range)	67 (24-81)
Median β2 microglobulin (mg/l, range)	2,61 (1-6)
Median BM infiltration	
BM biopsy	40% (0-95%)
BM flow cytometry	10% (0-44%)
Organomegaly	
Splenomegaly	7 (13,4%)
Adenopathies	13 (25%)
First line treatment	
W&W	11 (21,2%)
RTX single agent	2 (3,8%)
Chlorambucil	6 (11,5%)
RCD-like	13 (25%)
FCR-like	3 (5,8%)
RBendamustine-based	6 (11,5%)
Bortezomib-based	8 (15,4%)
Others	3 (5,8%)

Table S4. Abbreviations: WM, Waldenström Macroglobulinemia; LPL, lymphoplasmacytic lymphoma; F, female; BM, bone marrow; W&W, watch & wait; RTX, rituximab; RCD, rituximab-cyclophosfamide-dexamethasone;

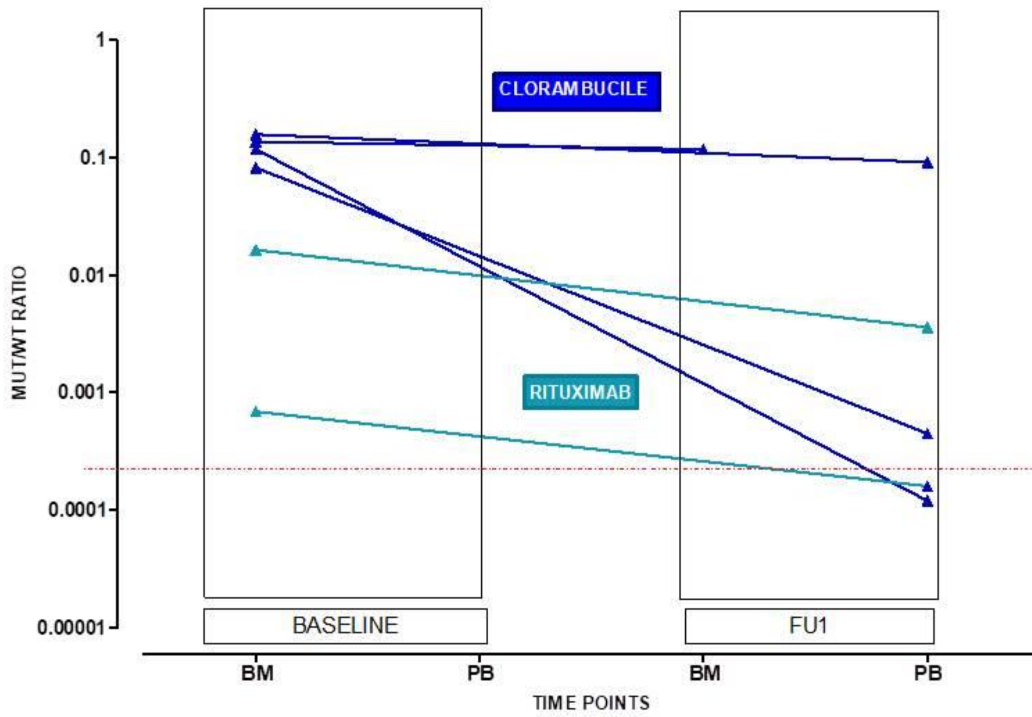

Figure S7A. MYD88[L265P] mutation monitoring in NT patients, after Chemo-therapy (chlorambucil or rituximab alone as first therapy).

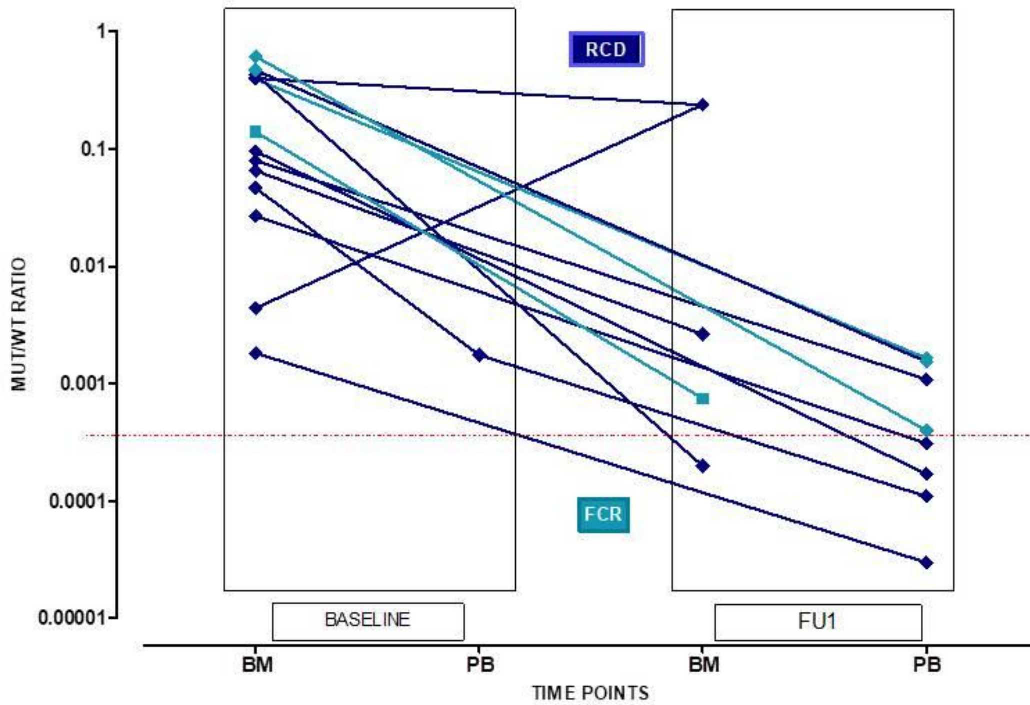

Figure S7B. MYD88^{L265P} mutation monitoring in NT patients after standard R-Chemo-therapy. RCD: Rituximab, Cyclophosphamide, Dexamethasone; FCR: Fludarabine, Cyclophosphamide, Rituximab.

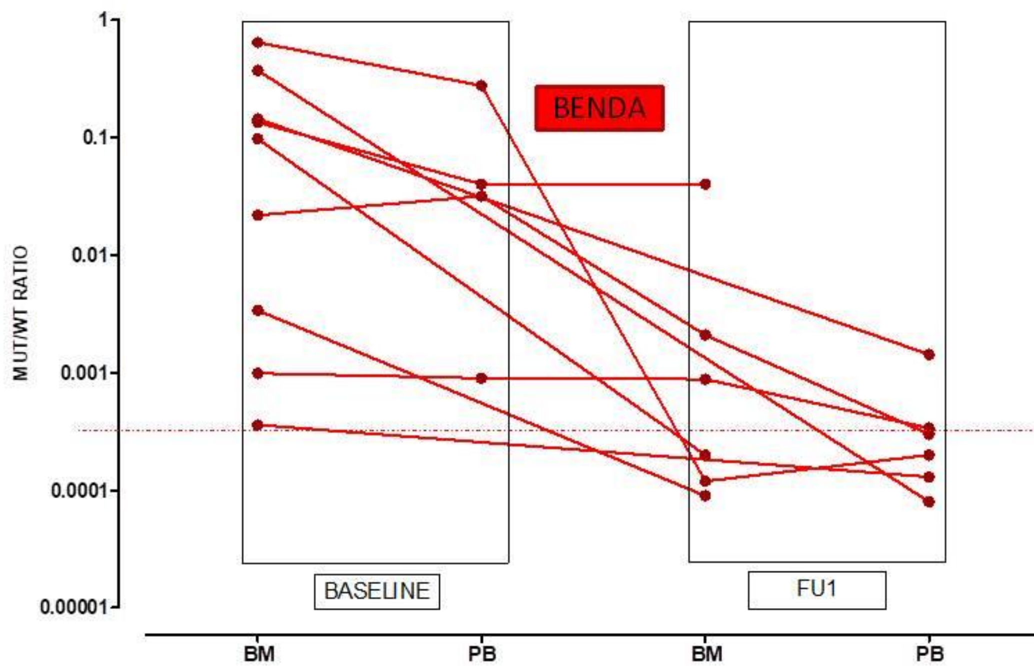

Figure S7C. MYD88^{L265P} mutation monitoring after Rituximab-Bendamustine based therapy.

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