SUPPLEMENTARY APPENDIX

# Pattern of somatic mutations in patients with Waldenström macroglobulinemia or IgM monoclonal gammopathy of undetermined significance

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## SUPPLEMENTAL METHODS

### Sample collection and cell separation

Samples were collected in untreated patients in 236/260 patients (91%) and after treatment in 24 (9%). Bone marrow mononuclear cells (BMMNCs) were separated by standard density gradient centrifugation (Lympholyte-H; CEDARLANE Laboratories Ltd). CD19+ cells were further isolated from BMMNCs by immunomagnetic adsorption on MiniMACS separation columns using an anti-CD19 antibody (Miltenyi Biotec GmbH) according to the manufacturer's recommendations. The purity of CD19+ separated cells was assessed by flow cytometry using anti-CD19 monoclonal antibodies (Becton Dickinson). CD19-depleted BMMNCs cells were used as control tissue. DNA was extracted following standard protocols for human tissue.

### Mutation analysis of MYD88 using allele-specific RT-qPCR for MYD88 (265P)

RT-qPCR based allelic discrimination assay was developed for MYD88 (L265P) mutation. For the allelic discrimination of the c.794T>C, a common forward primer (MYD88_F 5'-AATGTGTGCCAGGGGTACTTAG-3') and 2 reverse primers (MYD88_Rwt 5'-GCCTTGTACTTGATGGGGAaCA-3' and MYD88_Rmut 5'-CCTTGTACTTGATGGGGAaCG-3') were designed based on the nucleotide difference at the 3' terminal base (T or C). To prevent the amplification of the nonmatching primer, an additional nucleotide mismatch (A_C) located 3 bases from the 3' termini of the allele-specific primers was incorporated. PCR was performed on RotorGeneQ real-time analyzer on a 100-well Gene Disk (Qiagen, Milan, Italy) in two separate tubes for normal and mutated alleles. In all, 20 nanograms of genomic DNA were amplified in a 40-cycle PCR at an annealing temperature of 61 °C. All reactions were carried out in a final volume of 20 ul

containing 1X Brilliant SYBR Green QPCR master mix (Stratagene, Cedar Creek, TX, USA) and 100 nM of both forward and reverse primers. Cell lines OCI-LY19 (MYD88 wt) and OCI-LY3 (MYD88 MUT, L265P) were used to construct two different standard curves by dilution series of 7 different concentrations ranging from 40 ng/µl to 0.08 ng/µl corresponding to allele burdens ranging from 100% to 0.5%. Allele burden quantification was performed by the ratio MYD88 L265P mutated/MYD88 (mutant and wild-type alleles).

**Mutation analysis of CXCR4 using Sanger sequencing**

The C-terminal domain of the CXCR4 gene was sequenced by Sanger sequencing. The forward PCR primer 5'-CATCCTGGCTTTCTTCGCCT-3' and reverse PCR primer 5'-TTGCTGTATGTCTCGTGGTAGG-3' were designed to amplify a 572 bp fragment. PCR was carried out in a final volume of 25 µl containing 50 ng genomic DNA, 1X reaction buffer, 0.2 µM of each primer, 200 µM dNTPs, 2 mM MgCl2 and 2.5 U of HotStarTaq (Qiagen, Milan, Italy). PCR consisted of an initial denaturation step of 15 minutes at 95°C, followed by 35 cycles of 95°C for 30 seconds, 60°C for 30 seconds and 72°C for 60 seconds, with a final extension step of 10 minutes at 72°C. PCR products were purified and sequenced using BigDye® Terminator v3.1 Cycle Sequencing Kit and an ABI 3500 automatic sequencer (Applied Biosystems, Foster City, CA, USA).

Sequences were aligned to the corresponding germline RefSeqGene (NG_011587.1) using the MultAlin software after manual curation to detect variants.

## Table 1. Clinical characteristics of patients according to diagnosis

| Characteristic | IgM MGUS (n=130) | WM (n=130) |
|---|---|---|
| Age (years), median (range) | 64 (20-83) | 65 (27-86) |
| Sex (male/female),% of patients | 56/44 | 59/41 |
| Hemoglobin (g/dL), median (range) | 13.8 (10-17.4) | 12.9 (6.1-16.1) |
| Platelets (x$10^9$/L), median (range) | 241 (16-593) | 258 (34-800) |
| IgM levels (mg/dL), median (range) | 433 (101-4728) | 1240 (60-8940) |
| Serum albumin (g/dL), median (range) | 4.2 (2.9-5) | 4.1 (2.3-4.9) |
| Abnormal serum free light chain k/λ ratio, % of patients | 25 | 67 |
| $\beta_2$-microglobulin (mcg/L), median (range) | 1893 (900-6820) | 2662 (1230-11600) |
| Detectable BJ proteinuria, % of patients | 22 | 49 |
| Bone marrow involvement by IHC, %, median (range) | NA | 35 (0-90) |
| Extramedullary involvement, % of patients | NA | 23 |

NA= not applicable

**Table 2. Correlation of CXCR4 mutational status with clinical characteristics and MYD88 allele burden in WM patients**

| Characteristic | CXCR4 mutated | CXCR4 wild type | P value |
|---|---|---|---|
| Age (years), median (range) | 67 (44-83) | 65 (37-85) | >0.900 |
| Sex (male/female),% of patients | 45/55 | 66/34 | 0.121 |
| Hemoglobin (g/dL), median (range) | 12.2 (8.3-16.1) | 13.3 (8-16) | 0.113 |
| Platelets ($x10^9$/L), median (range) | 246 (69-368) | 262 (65-548) | 0.490 |
| Serum M-protein (g/L), median (range) | 1 (0.1-2.9) | 1.3 (0.2-6.3) | 0.316 |
| Serum albumin (g/dL), median (range) | 4.2 (2.5-4.8) | 4.1 (2.3-4.9) | >0.900 |
| Abnormal serum free light chain k/λ ratio, % of patients | 64 | 66 | >0.900 |
| $\beta_2$-microglobulin (mcg/L), median (range) | 2281 (1430-4360) | 2530 (1230-10465) | 0.761 |
| Detectable BJ proteinuria, % of patients | 40 | 48 | 0.776 |
| Bone marrow involvement by IHC, %, median (range) | 50 (5-90) | 30 (0-90) | 0.042 |
| Extramedullary involvement, % of patients | 20 | 19 | >0.900 |
| MYD88 allele burden (%), median (range) | 24.5 (4.3 – 93.3) | 9.4 (0.1 – 49.7) | 0.010 |

**Table 3. Mutually-adjusted effect of CXCR4 mutation and other clinical factors on risk of progression to symptomatic WM requiring treatment**

| Covariates | Hazard ratio | 95% confidence interval | P value |
|---|---|---|---|
| CXCR4 | 20.15 | 2.12-191 | 0.009 |
| Hemoglobin levels | 0.98 | 0.59-1.63 | 0.951 |
| BM infiltration % | 0.98 | 0.94-1.02 | 0.218 |
| Serum monoclonal protein | 4.76 | 1.48-15.28 | 0.009 |