Cytogenetic and molecular abnormalities in Waldenström's macroglobulinemia patients: correlations and prognostic impact

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Abstract

While Waldenström macroglobulinemia (WM) is characterized by an almost unifying mutation in *MYD88*, clinical presentation at diagnosis and response to therapy can be widely different among WM patients. Current prognostic tools only partially address this clinical heterogeneity. Limited data compiling both molecular and cytogenetic information have been used in risk prognostication in WM. To investigate the clinical impact of genetic alterations in WM, we evaluated cytogenetic and molecular abnormalities by chromosome banding analyses (CBA), FISH and targeted NGS in a retrospective cohort of 239 WM patients, including 187 patients treated by first-line chemotherapy or immunochemotherapy. Most frequent mutations were identified in *MYD88* (93%), *CXCR4* (29%), *MLL2* (11%), *ARID1A* (8%), *TP53* (8%), *CD79A/B* (6%), *TBL1XR1* (4%) and *SPI1* (4%). The median number of cytogenetic abnormalities was two (range, 0-22). Main cytogenetic abnormalities were 6q deletion (del6q) (27%), trisomy 4 (tri4) (12%), tri18 (11%), del13q (11%), tri12 (7.5%) and del17p (7%). Complex karyotype (CK) was observed in 15% (n=31) of cases, including 5% (n=12) of highly CK (high-CK). *TP53* abnormalities (*TP53*abn) were present in 15% of evaluable patients. *TP53*abn and del6q were associated with CK/high-CK (P<0.05). Fifty-three percent of patients with hyperviscosity harbored *CXCR4* mutations. Cytogenetic and molecular abnormalities did not significantly impact time to first treatment and response to therapy. Prognostic factors associated with shorter PFS were del6q (P=0.01), *TP53*abn (P=0.002) and high-CK (P=0.01). These same factors as well as IPSSWM, tri4, *CXCR4* frameshift and *SPI1* mutations were significantly associated with lower OS (P<0.05). These results argue for integration of both cytogenetic and molecular screening in evaluation of first-line WM patients.

Introduction

Waldenström's macroglobulinemia (WM) is a rare mature B-cell lymphoproliferative disorder characterized by the presence of serum monoclonal IgM associated with bone marrow infiltration by lymphoplasmacytic cells[1]. WM is a mostly indolent but still incurable disease for which there is a wide clinical heterogeneity. Some patients never require treatment while others have symptomatic disease upon diagnosis harboring hyperviscosity syndrome, bulky adenopathy and/or profound cytopenias and rapidly relapse after first-line (1L) immunochemotherapy. Different biomarkers have been developed to predict this heterogeneity. The International Prognostic Scoring System for WM (IPSSWM) and its revised version used simple clinical and biological parameters to stratify patients into, respectively, three and five distinct risk prognostic groups that are highly correlated with overall survival (OS)[2,3]. As observed in several other B-cell malignancies, it has been suggested that acquired cytogenetic and molecular abnormalities could also provide prognostic value in WM but it is still a matter of debate. Cytogenetic abnormalities identified in WM include deletion of the long arm of chromosome 6 (del6q) (20-40%), del13q (10-15%), trisomy 18 (tri18) (10%), tri4 (8%) and deletion of the short arm of chromosome 17 (del17p) (8%)[4-6]. Only few data exist regarding the impact of cytogenetic abnormalities in WM but shorter progression-free survival (PFS) has been reported for del17p patients[4]. Next-generation sequencing (NGS) studies revealed that the most common somatic mutations were activating mutations in *MYD88* and *CXCR4* genes, present in respectively more than 90% and 30% of WM patients. Both these mutations have been associated with specific clinical and biological presentation at diagnosis[7], along with prognosis in some studies[7,8] but not confirmed in others[9], and have been considered as predictive for response to ibrutinib[10]. Other mutations have been described at a lower frequency (5-20%) in *ARID1A*, *CD79B*, *SPI1* or *TP53* genes[11-14], the last two mutations being associated with poor survival but in single and limited series. Recently, we have also reported two groups of WM according to DNA methylation patterns, related to normal memory B-cells and plasma cells profiles, and respectively significantly associated to *CXCR4* mutations and del6q[15].

Cytogenetic analyses combining chromosomal banding analysis (CBA) and fluorescent *in situ* hybridization (FISH) have been widely used in hematologic malignancies to evaluate and predict patient's prognosis. Both technics have limitations but are complementary. In B-cell lymphoproliferative disorders, such as chronic lymphocytic leukemia (CLL) and WM, one limitation of CBA was the relative difficulty in obtaining sufficient metaphases but this has been overcome by the introduction of modern cell stimulation protocols, allowing for robust CBA[16,17]. Importance of CBA has been emphasized by the association of karyotypic complexity with shorter treatment-free and OS in many hematologic malignancies and in particular CLL[18,19]. Recently, karyotypic complexity in CLL has been refined[20] demonstrating that complex karyotype (CK) comprises different subgroups with distinct prognosis, depending on the type of associated abnormalities, and that not all CK are of poor prognosis. Data regarding chromosomal abnormalities and karyotypic complexity are in contrast scarce in WM.

Immunochemotherapy (ICT), consisting in the association of an anti-CD20 monoclonal antibody and an alkylating agent, is part of the standard of care for WM patients. However, ICT use is still unsatisfactory, leading to non-complete responses and inevitable recurrence. Alternative treatments have been developed to obtain longer responses, consisting mainly in BTK inhibitors (BTKi) (ibrutinib, acalabrutinib, zanubrutinib) in monotherapy or in association with an anti-CD20 monoclonal antibody[21,29,32]. Although these latter regimens are efficient, data are lacking to choose one treatment or another according to a specific molecular or cytogenetic abnormality.

Recent advances in deciphering precise genomic landscape in WM have led to the proposition of a precision-guided treatment approach using *MYD88* and *CXCR4* mutational status[11]. While various studies have confirmed the relevance of this approach in WM patients receiving ibrutinib[10,29-31], other studies have shown more controversial results[32,33], either regarding the impact of *CXCR4* mutations and/or in the specific context of ICT[34]. Finally, very limited data compiling both molecular and cytogenetic information are actually available and used in risk prognostication in WM.

The aim of this study was to evaluate clinical and biological characteristics of WM patients along with cytogenetic and molecular abnormalities, the potential association of CK with recurrent mutations identified in WM and their impact on outcome.

Patients and methods

Patients

This retrospective study included 239 WM patients diagnosed from 1988 to 2020 and followed up in Pitié-Salpêtrière hospital (Paris, France). Diagnosis, treatment initiation and response criteria followed the WHO classification[1] and recommendations from the tenth International Workshop Waldenström's Macroglobulinemia (IWWM)[21]. All cases were selected based on the availability of chromosome banding analyses (CBA)/FISH and/or DNA material sufficient for evaluation of *MYD88* and *CXCR4* mutations. Written consent for bone marrow (BM) and biological analyses were obtained in accordance with the declaration of Helsinki and with ethical approval from national (CNIL 2212382) and local (CPP Ile-De-France 05/21/2014) ethics committees.

Cytogenetic analyses

CBA were successfully performed in 219 patients according to the usual techniques as previously described[4], with 12-O-tetradecanoyl-phorbol-13-acetate (28 patients) until 2006 and CpG-oligonucleotides plus interleukin 2 stimulation (191 patients). All karyotypes (K) were described according to the International System for Human Cytogenetic Nomenclature (ISCN 2020). FISH was performed on interphase nuclei and metaphases, following standard procedures and using specific probes: *ATM* (11q22), *TP53* (17p13.1) (Metasystems), *BCL2* (18q21) (Zytovision), D12Z1 (12p11-q11), D13S319 (13q14), *LAMP1* (13q34) Metasystems, D6Z1 (6p11-q11)/SEC63 (6q21)/*MYB* (6q23) Metasystems, *FIP1L1/CHIC2/PDGFRA* (4q12) Deletion/Fusion Cytocell [4].

To consider a karyotype normal, a minimum of 15 metaphases had to be examined. Single-cell abnormalities were taken into consideration only if verified by FISH analysis. A karyotype was defined as complex (CK) if ≥ 3 clonal aberrations were present in CBA, respectively. Highly CK (high-CK) and non-high-CK were respectively defined if ≥ 5 and 3 or 4 clonal aberrations were identified. CBA was performed at WM diagnosis or before the administration of any treatment in respectively 85/219 (39%) and in 99/219 (45%), with a median of 39 [1-92] months between diagnosis and karyotype in this latter situation.

Mutation analyses

Genomic DNA was extracted using the QiAmp DNA micro kit (Qiagen) from non-sorted bone marrow (BM) samples. Routine sequencing of *MYD88* L265P and *CXCR4* S338X mutations was performed by restriction fragment length polymorphism (RFLP) and allele-specific polymerase chain reaction (AS-PCR) for respectively 199 and 189 patients. Among them, 168 BM samples were analyzed by targeted sequencing, including three tumor controls pairs analyzed by whole-exome sequencing. Twenty-one recurrently mutated genes in WM or other lymphoproliferative B-cell disorders from our experience or in the literature were selected for targeted sequencing (see **Supplementary Tables S1** for genomic coordinates

of targeted regions and for the list of genes) using TWIST custom capture kit (Bioscience, San Francisco, CA). Libraries were generated with addition of paired-end adaptors (NEXTflex, Bioo Scientific, Austin, TX) before paired-end sequencing (2 x 100 bp reads) using an Illumina Novaseq6000 flow cell and the onboard cluster method (Illumina, San Diego, CA). Targeted sequencing was analyzed according to previously described algorithms with minor modifications[22]. Briefly, sequence reads were mapped to the reference genome GRCh38 using the Burrows–Wheeler Aligner (BWA) alignment tool. PCR duplicates were removed using Picard tools - Mark Duplicates (version 1.119). Local realignment around indels and base quality score recalibration were performed using GATK 3.2 (Genome Analysis Tool Kit). The number of reads containing single-nucleotide variations (SNV) and indels was enumerated using Varscan (v2.3.7). Functionally annotated variants were filtered according to the following criteria: synonymous variants and variants located outside coding regions were filtered; polymorphisms described in Kaviar, gnomAD and the 1000 Genomes Project were removed; variants with coverage < 30X and less than ten supporting reads and variants with an allelic fraction lower than 1% were filtered; the remaining variants, evaluated as candidate somatic mutations, were manually reviewed and tagged as oncogenic using different criteria based on information retrieved from the literature, sequence conservation and *in silico* prediction of effect. The mean read depth within the targeted regions was 2000X.

In total, both *MYD88* L265P and *CXCR4* S338X status were available for 189 samples, targeted NGS for 168 and both cytogenetic and targeted NGS analyses for 165.

Abnormalities of *TP53* were defined by the presence of del(17p) and/or *TP53* mutations.

Statistical analyses

Main clinical and biological characteristics were collected at diagnosis and treatment initiation: age, hyperviscosity syndrome, lymphadenopathy, splenomegaly, hemoglobin level, platelet count, serum IgM monoclonal level, ß2-microglobulin (B2M), albumin, LDH. The International Prognostic Scoring System for WM (IPSSWM) was calculated according to previous published criteria[2]. Cytogenetic (FISH [del6q, trisomy 4, trisomy 12, del13q, del11q, del17p], chromosomal aberrations) and molecular abnormalities were analyzed as described above. Quantitative variables are presented as mean and standard deviation or median and range according to their distribution. Categorical variables are presented as numbers and related percentage. Primary and secondary endpoints were time to first treatment (TFT) for asymptomatic WM, and response to therapy, relapse rate, progression-free survival (PFS) and overall survival (OS) for the whole population. OS and PFS were calculated from the date of diagnosis/evaluation until the date of death from any cause or date of last follow up and date of progression, respectively. Variables included in univariate analyses included: age, B2M, IPSSWM, cytogenetic abnormalities (del6q, tri12, tri4, tri18, del13q, del11q, del17p), CK and high-CK, mutations (*MYD88, CXCR4, CD79B, ARID1A, MLL2, TP53, SPI1*). We excluded variables interesting less than 5 patients. Univariable Cox regression was applied to assess the prognostic significance of cytogenetic and molecular abnormalities, CK/high-CK and other prognostic factors on survival outcome. *P*-values were adjusted for age and multiplicity (Benjamini-Hochberg method). Multivariable Cox regression models were implemented to test the simultaneous effect of factors on outcomes, taking into account the relative effect of the remaining parameters. For the multivariable analysis, we considered only cases with available data for all the factors included in the model and variables that were significant ($P \leq 0.05$) in univariate analyses. Biostatistic analyses were performed with the help of GenoSplice (Paris, France). Kaplan-Meier analysis was performed to construct survival curves and the log rank test used to determine differences between groups. The X2 or Fisher's exact test were used to compare data distribution in different subgroups. The significance level of $p < 0.05$

was applied and statistical analyses were performed using the software SAS 9.3 (SAS Inc, Cary, NC) and R 3.0.2 (http://www.R-project.org).

Results

Clinical characteristics

Main clinical characteristics of the whole WM cohort are detailed in **Table 1**. The study population comprised 239 patients, including 63% of male. Median age at WM diagnosis was 65 years old (range, 28-88). WM was symptomatic at evaluation in 59% of patients. During the follow-up, 187/239 (78%) WM patients required first-line (1L) therapy. The indications for 1L therapies are listed in **Supplemental Table S2**. Those therapies consisted of chemotherapy (CT), immuno-CT (ICT), anti-CD20 monoclonal antibody alone, or other type in, respectively, 47/187 (25%), 125/187 (67%), 14/187 (7.5%) and 1/187 (0.5%) cases. ICT included dexamethasone-cyclophosphamide-rituximab (DRC), bendamustine-rituximab (BR) and fludarabine-rituximab based regimens in respectively 63/187 (34%), 36/187 (19%) and 23/187 (12%) (details regarding all the different types of treatment are provided in **Supplemental Table S3**). The median follow-up for the whole WM cohort was 6 years. Median PFS of patients receiving 1L therapy was 51 months and 5-year-OS was 91%. Forty-nine of 239 patients (20%) have died, due to WM progression or transformation, therapy-related toxicity, other causes and of unknown origin in respectively 28 (57%), 12 (24%), 6 (12%) and 3 (7%) cases (**Supplemental Table S4**).

Cytogenetic and mutational abnormalities

The distribution and frequency of gene alterations is represented in **Figure 1**. The most frequent mutations were identified in *MYD88* (93%), *CXCR4* (29%), *MLL2/KMT2D* (11%), *ARID1A* (8%), *TP53* (8%), *CD79A/B* (6%), *TBL1XR1* (4%), and *SPI1* (4%) (see **Supplemental Table S5** for details and list of mutations identified by targeted NGS). *MYD88* mutations other than the classical L265P (V217F, M232T and S243N) were found in 5 (2%) cases. Among the 7% *MYD88* wild-type (WT) cases, we observed mutations in other recurrently mutated genes such as *ARID1A, MLL2, CD79B* or *TNFAIP3*. *CXCR4* mutations were frameshift (FS) and nonsense (NS) in respectively 35 and 56% and more than half of them (55%) affected S338 amino-acid. *CXCR4* mutations affecting S338 amino-acid were mainly NS (23/27 (85%) cases) while NS mutations represented 5/21 (24%) cases of those affecting other amino-acids. Mutations identified in *CD79A/B*, *TBL1XR1*, *CARD11* (1%), *EZH2* (4%), *TNFAIP3* (3%), *NFKBIE* (2%) corresponded mainly to those previously described in other B-cell malignancies[22-27]. *SPI1* and *IKZF3* (3%) mutations were respectively hotspot Q226E and L162R[13,28].

The median number of cytogenetic abnormalities per sample was two (0-22). No cytogenetic abnormality was detected by CBA in 87/191 (40%), and by both CBA and FISH in 55/166 (33%) evaluable cases. Cytogenetic abnormalities identified by CBA and/or FISH were as follows, in decreasing order of frequency: del6q (27%), tri4 (12%), tri18 (11%), del13q (11%), tri12 (8%), del17p (7%), tri3 (6%) and del11q (5%). *TP53* abnormalities (*TP53*abn) (either del17p and/or *TP53* mutation) were present in 15% of 165 evaluable patients.

CK was observed in 15% (n=31/219) of cases, comprising three, four and ≥ five chromosomal aberrations (high-CK) in respectively 7% (n=13), 3% (n=6) and 5% (n=12) of cases. Details of the 31 CK (either non-high-CK or high-CK) are provided in **Supplemental Table S6**. Among the 31 CK, 15 (48%) included at least one trisomy (one trisomy, n=6; two, n=6; three, n=2; four, n=1), while, among the 12 high-CK, 7 (58%) included at least one trisomy (one trisomy, n=4; two, n=2; three, n=1).

Correlation between cytogenetic and mutational alterations

Significant associations between different genetic alterations are synthetized in **Supplemental Tables S7**. We did not observe specific mutations or cytogenetic abnormalities associated with *MYD88* WT status. *CXCR4* S338 mutations were significantly associated with CK (*P*=0.05) and *TP53*abn (*P*=0.03), while the presence of *TP53*abn was associated with CK, either non-high-CK (*P*=0.02) or high-CK (*P*=0.0005). Del6q was associated with many other cytogenetic abnormalities (del13q, del11q, del17p, tri4) and non-high-CK/high-CK (*P*<0.0001). Del6q and *CXCR4* mutations were not exclusive in our series, as 18/51 del6q cases detected by CBA and/or FISH and explored by targeted NGS also harbored *CXCR4* mutations (S338, n=10; other amino-acid, n=8). Tri4 and tri18 were significantly associated with each other (*P*=0.02) as del17p and *TP53* mutations (*P*=0.03).

Specific comparisons between non-high-CK and high-CK (**Supplemental Tables S8**) showed that this latter harbored significantly more frequent del6q (by CBA and/or FISH, 11/12 [92%] vs. 9/19 [47%], *P*=0.02), del17p (5/12 [42%] vs. 1/19 [5%], *P*=0.02) and *TP53*abn (6/12 [50%] vs. 2/15 [13%], *P*=0.03) while no difference was observed in terms of specific gene mutation frequencies.

Of note, *TP53*abn were not equally distributed among CK subgroups as they were more frequently observed in CK without trisomies and more particularly in high-CK without trisomies. Only two *TP53*abn (n=2/14, 1 del17p, 1 *TP53* mutation) were identified in CK with trisomies (vs. 6/13 [45%] in CK without trisomies, *P*=0.07). More precisely, the two *TP53*abn were identified in high-CK with trisomies (n=2/7 vs. 4/5 in high-CK without trisomies, P=0.07) while none was observed in non-high-CK with trisomies (n=0/7 vs. 2/8 in non-high-CK without trisomies, *P*=0.15).

Correlation between genetic alterations and disease phenotype

Significant associations between genetic alterations and disease phenotype are synthetized in **Supplementary Table S9**. When comparing the population carrying an abnormality to that without the same abnormality, lymphoplasmacytic BM infiltration was significantly increased in patients with *CXCR4* mutations (56% vs. 41%, *P*=0.02), del6q (53% vs. 36%, *P* = 0.001), tri4 (58% vs. 35%, *P*=0.003) and/or non-high-CK (55% vs. 35%, *P*=0.02)/high-CK (68% vs. 43%, *P*=0.003); the presence of lymphadenopathy in those with *TP53*abn (44% vs. 21%, *P*=0.02) and *SPI1* mutations (67% vs. 23%, *P*=0.03). No significant correlation was observed between genetic alterations and IPSSWM.

In more detail, *CXCR4* mutations were also significantly associated with increased serum IgM levels (28 vs. 18 g/L, *P*=0.05), symptomatic hyperviscosity at presentation (17% vs. 6%, *P*=0.04; 53% of patients with symptomatic hyperviscosity at presentation harbored *CXCR4* mutations), and *CXCR4* mutated patients had more frequently platelet count < 100 x 10^9/L (11/48 [23%] vs. 5/120 [4%], *P*=0.0002). *TBL1XR1* mutations were associated with the lowest serum IgM levels (9.5 vs. 20 g/L, *P*=0.0007). Del6q patients had lower hemoglobin level (101 vs. 120 g/L, *P*<0.001) and tri4 lower hemoglobin (91 vs. 116 g/L, *P*=0.003) and more frequently platelet count < 100 x 10^9/L (9/27 [33%] vs. 19/183 [10%], *P*=0.001) that may reflect in both cases the increased BM infiltration described above.

Outcomes

Among the 239 WM patients of our cohort, 179 (75%) were asymptomatic at diagnosis. The median time to first therapy (TFT) for asymptomatic WM at diagnosis was 43 months (range, 7-236). Only elevated B2M was associated with shorter TFT (*P*=0.02). Cytogenetic and molecular abnormalities were not

significantly associated with TFT; only a trend for shorter TFT was observed in tri12 and del6q patients (**Supplemental Table S10**).

Among the 187 (87%) symptomatic WM who received 1L therapy, response rates were distributed as followed: 6% (n=11) complete response (CR), 11% (n=21) very good partial response (VGPR), 49% (n=91) PR, 24% (n=45) stable disease (SD) and 10% (n=19) progressive disease (PD). No significant correlation was observed between cytogenetic/mutational abnormalities and response. One hundred and two (54%) patients further experienced relapse with a median time of 55 months (range, 3-255). Univariate analyses of clinical and biological variables associated with relapse, PFS and OS are represented in **Table 2** and **Supplemental Table S10**. Prognostic factors associated with shorter PFS included del6q (*P*=0.01), *TP53*abn (*P*=0.002) and high-CK (*P*=0.01) (**Figure 2**). OS was pejoratively impacted by IPSSWM (*P*=0.002), del6q (*P*=0.027), tri4 (*P*=0.026), *CXCR4* frameshift (*P*=0.047), *SPI1* mutations (*P*=0.037), *TP53*abn (*P*=0.002) and high-CK (*P*=0.008) (**Figure 2** and **Supplemental Figure S1**). In multivariate analyses, only *TP53*abn (*P*=0.03) for PFS and IPSSWM (*P*=0.03) and *TP53*abn (*P*=0.04) for OS retained significant.

As the entire cohort of WM patients received two main different types of 1L therapies (CT and ICT), we compared PFS and OS of these two subgroups (respectively, 5-year PFS, 40 vs. 50% [*P*=0.02]; 10-year OS, 76 vs. 78% [*P*=0.3]; **Supplemental Figure S1**). The repartition of main cytogenetic and molecular abnormalities was similar in these two subgroups (**Supplemental Table S11**). We then specifically analyzed prognostic factors for PFS and OS in 1L ICT patients (**Figure 3** and **Supplemental Table S12**). Del6q, high-CK, *TP53* abnormalities negatively impacted PFS (**Figure 3**) while only IPSSWM and *TP53* abnormalities were significantly associated with shorter OS in univariate analysis in this specific population. In multivariate analyses, only *TP53*abn for PFS and IPSSWM and *TP53*abn for OS retained significant (**Supplemental Table S12**).

Regarding more specifically the outcomes of CK, the pejorative impact on PFS and OS was stronger for high-CK compared to non-high-CK. High-CK was one of the most impactful prognostic factors, being associated with one of the shortest median PFS of 20 months and a 5-year OS of 75%. CK with trisomies were associated with a trend to better PFS compared to CK without trisomies (**Supplemental Figure S2**).

Nine patients experienced transformed WM in the course of the disease. For the seven patients for whom cytogenetic and molecular screening were available, all harbored either high-CK (n=4/7, 57%, all these four including del6q) and/or *TP53* abnormalities (n=6/7, 87%) (see **Supplemental Table S13** for details regarding cytogenetic and molecular abnormalities of these samples).

Discussion

Our comprehensive approach integrating CBA, FISH and targeted NGS analyses in a large cohort of WM patients offered the opportunity to study the prognostic impact of both molecular and cytogenetic abnormalities and, to our knowledge, for the first time the one of high-CK that has been recently emphasized in CLL[20]. In this series, we confirmed many of the already published WM biological features but also extended our knowledge of particular associations between different genomic characteristics and finally highlighted prognostic factors associated with those, which could be of importance in future 1L therapeutic strategies in WM patients.

Our cohort harbored main characteristics of WM real-life patients: median age of 65 years, 80% of kappa isotype, mean IgM level around 20 g/L, > 90% *MYD88* L265P, 30% *CXCR4* mutations, and respective 5-year and 10-year OS of 91% and 75%. IPSSWM strongly impacted OS (**Table 1** and **Supplemental Figure S1**) supporting the validity and reliability of our cohort. However, our study harbored notable limitations due in part to its retrospective design encompassing a long period of time, including patients with missing

data and receiving diverse therapies. Since many patients have a smoldering disease not requiring therapy, the analysis of OS performed from the date of diagnosis date can be in part misleading. More, the absence of patients treated in 1L by BTKi that have actually no marketing authorization in France for 1L WM limited the possibility to infer which therapies between ICT and BTKi will more benefit for certain cytogenetic/molecular abnormalities. Further prospective studies are thus clearly warranted to address this important question.

Chromosomal abnormalities have been little studied so far in WM and information about frequencies and characteristics of the most recurrent abnormalities is therefore of interest. In this respect, we confirmed our previous results regarding the occurrence of del6q (27%) and tri4 (12%) and their respective association with CK and tri18[4]. Frequency of del6q was slightly lower in our cohort than that historically reported in the literature (40-50%)[35,36] but was concordant with the one (28/93 [30%]) identified in symptomatic WM of a recent Spanish study[37]. Although del6q and *CXCR4* mutations have been suggested to be mutually exclusive[38], we did not observe this exclusivity in our series. This difference could be due in part by the technics used for del6q identification (CBA and/or FISH vs. RQ-PCR). Del6q and tri4 cases had more aggressive presentation with increased BM involvement, lower hemoglobin and platelet counts, and shorter PFS and OS, confirming recent published data for del6q[37]. Del6q-associated poor prognosis may be in part due to its association with CK and more particularly with high-CK as nearly all these latter (11/12, 92%) harbored del6q and plead for its potential implication in chromosomal instability. Further studies will be necessary to confirm that del6q could represent a particular group of patients with potential specific methylation profile, association with *VH3* usage, CD38 expression and enrichment in plasmacytoid lymphocytes as previously suggested[15]. We also brought more information about del17p (7%)/*TP53*abn and CK frequencies, that are confirmed to be recurrent events in WM as previously described[4,12,39]. Other trisomies than tri4 such as tri18 and tri3 were recurrent representing respectively 11 and 6% of cases.

We provided more precise characterization of CK in WM, especially high-CK, which was positively associated in our cohort with the presence of del6q and *TP53*abn. As described in CLL[20], not all CK were equivalent, as in particular (i) the pejorative impact on PFS and OS was stronger for high-CK compared to non-high-CK and (ii) CK with trisomies were associated with a lower frequency of *TP53*abn and a trend to better PFS compared to CK without trisomies. This latter point will need to be confirmed in largest cohorts of patients.

Regarding main somatic gene mutations, frequencies were relatively similar to those previously described for *CXCR4*, *ARID1A*, *CD79A/B*, *TP53* and *SPI1*[7,11-14]. No significantly relevant association with cytogenetics was observed for most gene mutations (**Supplemental Tables S5**), except for the ones described above for *TP53* and *CXCR4*. The number of mutated cases was too small for *EZH2, IKZF3, PRDM1, TNFAIP3, CARD11, ETV6* and *HIST1H1E* to draw reliable conclusions. *CXCR4* mutated patients represented around one third of the cohort and presented with increased BM infiltration, serum IgM levels and symptomatic hyperviscosity as previously described[7]. Somatic mutations in *ARID1A* were identified in 8% of patients, including NS and FS variants, but were not associated with specific clinical presentation.

Interestingly, various gene mutations were associated with outcome. We did observe a trend for shorter PFS for non-*MYD88* L265P cases but this was not confirmed if we integrated others (non-L265P) *MYD88* mutated cases in the analysis. Definitive conclusions are difficult to draw in this particular population regarding the small number of patients. The poor prognosis of *SPI1* mutations was confirmed as

previously suggested[13]. *CXCXR4* S338 and/or NS mutations did not affect outcomes in our cohort while *CXCR4* FS have a slight significant pejorative impact on OS (**Supplemental Figure S1**). These results are different from what has been described in WM patients treated with BTKi ibrutinib for whom the presence of *CXCR4* NS mutations impacted survival outcomes [40,41]. Further studies will be necessary to interrogate the hypothesis that BTKi and ICT may be beneficial specifically for *CXCR4* FS and NS respective populations. Interesting information is also provided in this WM cohort receiving 1L (I)CT regarding the pejorative role of *TP53*abn for PFS and OS in uni- and multivariate analyses, emphasizing its potential predictive value for considering 1L targeted therapies in this specific population as described in CLL. This pejorative impact on PFS and OS was confirmed in the group of patients who specifically received 1L ICT (n=125) in uni- and multivariate analyses (**Figure 3** and **Supplemental Table S12**). The negative impact of del6q and high-CK on PFS was also confirmed in this specific group of patients in univariate but not multivariate analysis (**Supplemental Table S12**).

Finally, as WM is frequently an asymptomatic disease at diagnosis, prognostic factors predicting evolution to symptomatic disease are of importance. Only B2M was significantly associated with shorter TFT in our cohort and we failed to demonstrate any significant association between cytogenetic/molecular abnormalities and TFT. Larger studies in asymptomatic WM may be necessary to fully explore these potential associations.

In conclusion, our work is one of the first to describe a cohort of WM patients treated by (I)CT with comprehensive analysis of both cytogenetic and molecular abnormalities, providing important information for prognosis prediction and therapy selection. In particular, a negative impact of del6q, *TP53*abn and high-CK has been demonstrated on both PFS and OS. Prospective studies are warranted to confirm these prognostic factors, the potential role of 1L targeted therapies in high-risk WM and the use of both cytogenetic and molecular screening for guiding therapeutic strategy between BTKi and ICT in 1L WM patients.

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Contribution statement: DK, FNK and DRW designed the research, analyzed data and wrote the manuscript. DK, CB, LS, JC, EC and DRW performed experiments. DK, NG, CBG, MB, CB, FD, EC, SS, VL, FNK and DRW recruited patients. All authors reviewed and approved the manuscript.

Conflict-of-interest disclosure: No relevant COI

Data availability statement: The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Figures legends

Figure 1. Mutational and cytogenetic analyses of Waldenström macroglobulinemia (WM) samples by chromosome banding analysis (CBA), FISH and/or targeted sequencing. Each column represents a patient sample and each row a mutated gene (italic) or cytogenetic abnormality. The percentage of each mutated gene or cytogenetic abnormality in the whole cohort are indicated on the right of the grid. Complex karyotypes (CK) are represented at the top for highly CK (high-CK) and non-high-CK in respective dark blue and light blue. Colored box: presence; white box: absence; light grey box: not available.

Figure 2. Respective progression free-survival (PFS) and overall survival (OS) for the following recurrent molecular/cytogenetic abnormalities in the entire cohort: *MYD88*/*CXCR4* status (A, B), del6q (C, D), *TP53*abn (E, F), non-high-CK and high-CK (I, J). Abbreviations: abn, abnormalities; CA, cytogenetic abnormalities; high-CK, highly complex karyotype; mut, mutated; WT, wild-type.

Figure 3. Respective progression free-survival (PFS) and overall survival (OS) for the following recurrent molecular/cytogenetic abnormalities in patients who received first-line immunochemotherapy (ICT): *MYD88*/*CXCR4* status (A, B), del6q (C, D), *TP53*abn (E, F), non-high-CK and high-CK (I, J). Abbreviations: abn, abnormalities; CA, cytogenetic abnormalities; high-CK, highly complex karyotype; mut, mutated; WT, wild-type.

Supplemental Figures legends

Figure S1. Progression free-survival (PFS) (A) and overall survival (OS) (B) of WM patients who received either 1L chemotherapy (CT, n=47) or immunochemotherapy (ICT, n=125).

Figure S2. Progression free-survival (PFS) (A) and overall survival (OS) (B) of the whole WM cohort and respective PFS/OS for the following recurrent molecular/cytogenetic abnormalities: *CXCR4* mutated vs. WT (C, D), *CXCR4* WT vs. nonsense (NS) vs. frameshift (FS) mutations (E,F), *ARID1A* (G,H), *SPI1* (I,J), *MLL2* (K,L), tri4 (M,N), CK with/without trisomies (O,P) and IPSSWM (Q,R).

Tables

Table 1. Main characteristics of the whole WM cohort		
	n (%)	
Total	239 (100)	
Median age at WM diagnosis, years (range)		65 (28-88)
Sex, male	155/239 (63)	
Clinical and biological parameters at evaluation		
Adenopathy ≥ 1,5 cm	57/230 (25)	
Splenomegaly	32/230 (14)	
Hemoglobin, mean in g/dL (range)		11.5 (4.2-16.3)
Hemoglobin < 11,5 g/dL	114/233 (49)	
Platelets, mean (G/L)		242 (26-630)
Platelets < 100 G/L, yes (%)	78/229 (34)	
IgM, mean in g/L (range)		18 (2-78)
Isotype, kappa	184/232 (80)	
Bone marrow infiltration, mean in % (range)		44 (10-100)
Requiring first-line therapy	187/239 (78)	
IPSSWM		
Low	43/156 (27)	
Intermediate	39/156 (25)	
High	74/156 (48)	
Type of first-line therapies		
Alkylating agents	47/187 (25)	
Immunochemotherapy (ICT)	125/187 (68)	
Anti-CD20 monotherapy	14/187 (7)	
Other	1/187 (1)	
Cytogenetic abnormalities by CBA and/or FISH		
del6q	60/219 (27)	
tri4	27/219 (12)	
del13q	25/219 (11)	
tri18	25/219 (11)	
tri12	17/219 (8)	
del17p	16/219 (7)	
del11q	11/219 (5)	
complex karyotype (CK)	31/219 (14)	
highly CK (high-CK)	12/219 (5)	
missing data	20/239 (8.5)	
Mutations		
MYD88	186/199 (93)	
L265P mutation	181/186 (97)	
Other *MYD88* mutations	5/186 (3)	
CXCR4	48/168 (29)	
S338	27 (55)	
Other	21 (45)	
***TP53* abnormalities** (del17p/*TP53* mutation)	26/170 (15)	
Abbreviations: CBA, chromosome banding analysis; IPSS, international prognostic scoring system; WM, Waldenström macroglobulinemia		

Table 2. Uni- and multivariate analyses of variables associated with PFS and OS in the entire cohort.

	PFS						OS					
	Univariate (n=187)			Multivariate* (n=121)			Univariate (n=239)			Multivariate* (n=136)		
	HR	*IC 95%*	*P*	HR	*IC 95%*	*P*	HR	*IC 95%*	*P*	HR	*IC 95%*	*P*
B2M	-	-	NS				-	-	NS			
IPSSWM	-	-	NS				3.70	1.47-9.10	0.002	3.06	1.26-5.96	0.03
Del6q	2.24	1.17-4.29	0.01	-	-	NS	2.02	1.07-3.83	0.027	-	-	NS
Tri4	-	-	NS				2.38	1.08-5.22	0.026	-	-	NS
CK	-	-	NS				-	-	NS			
High-CK	3.29	1.23-8.79	0.01	-	-	NS	3.44	1.30-9.10	0.01	-	-	NS
***TP53* abn**	3.19	1.48-6.85	0.002	2.9	1.08-5.33	0.03	2.95	1.43-6.07	0.002	2.41	1.19-4.83	0.04
***CXCR4* FS mutations**	-	-	NS				2.56	1.00-6.66	0.047	-	-	NS
***SPI1* mutations**	-	-	NS				4.10	1.01-12.4	0.037	-	-	NS

*For multivariable analyses, we considered only variables that were significant ($P \leq 0.05$) in univariate analyses and cases with available data for all the factors included in the model.

Abbreviations : B2M, beta2microglobulin; CI, confidence interval; CK, complex karytotype; high-CK, highly CK; HR, hazard ratio; IPSSWM, international prognostic scoring system for Waldenström macroglobulinemia; NS, non significant; PFS, progression-free survival; OS, overall survival.

Figure 1

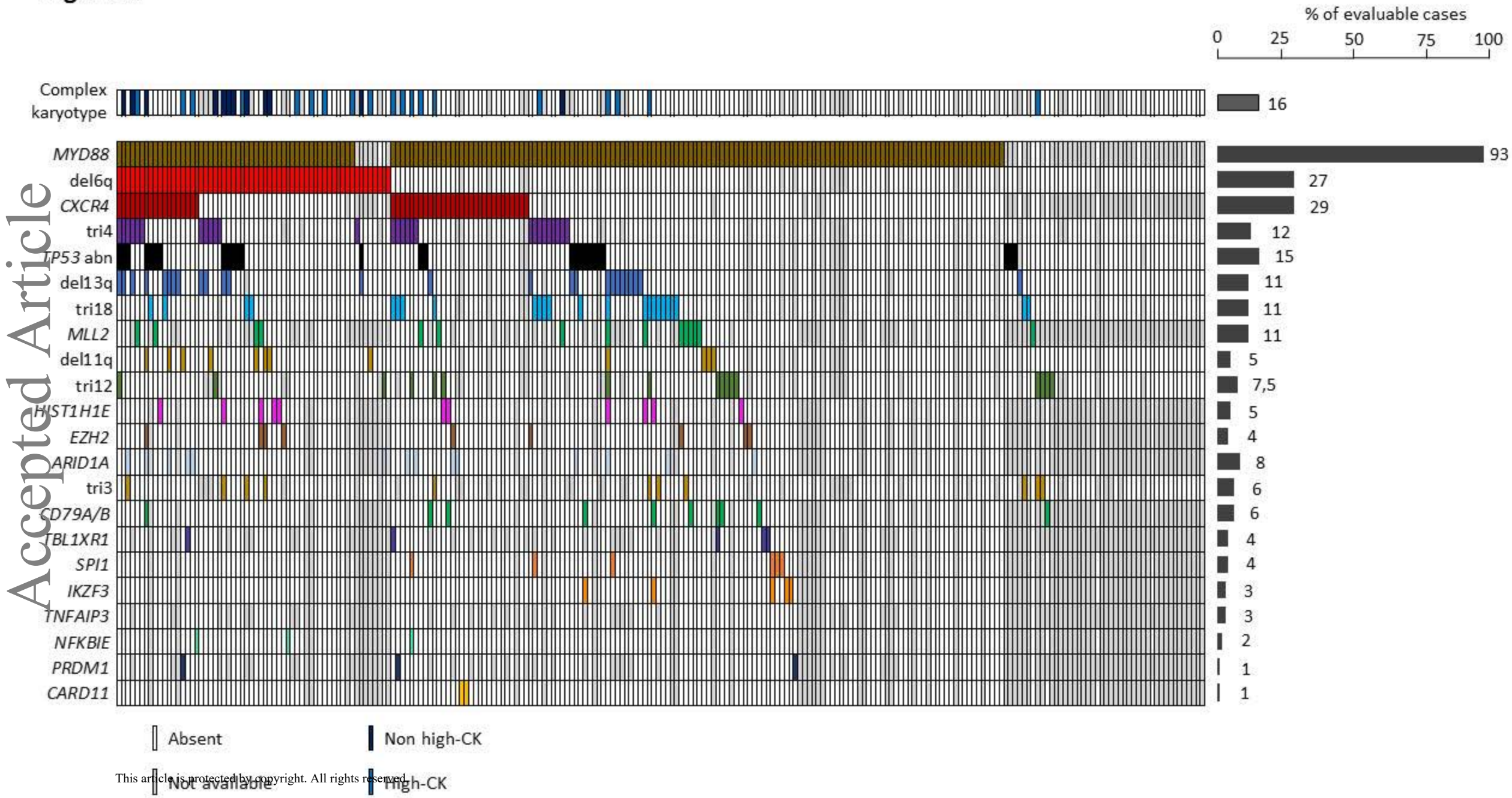

Figure 2 **Entire cohort**

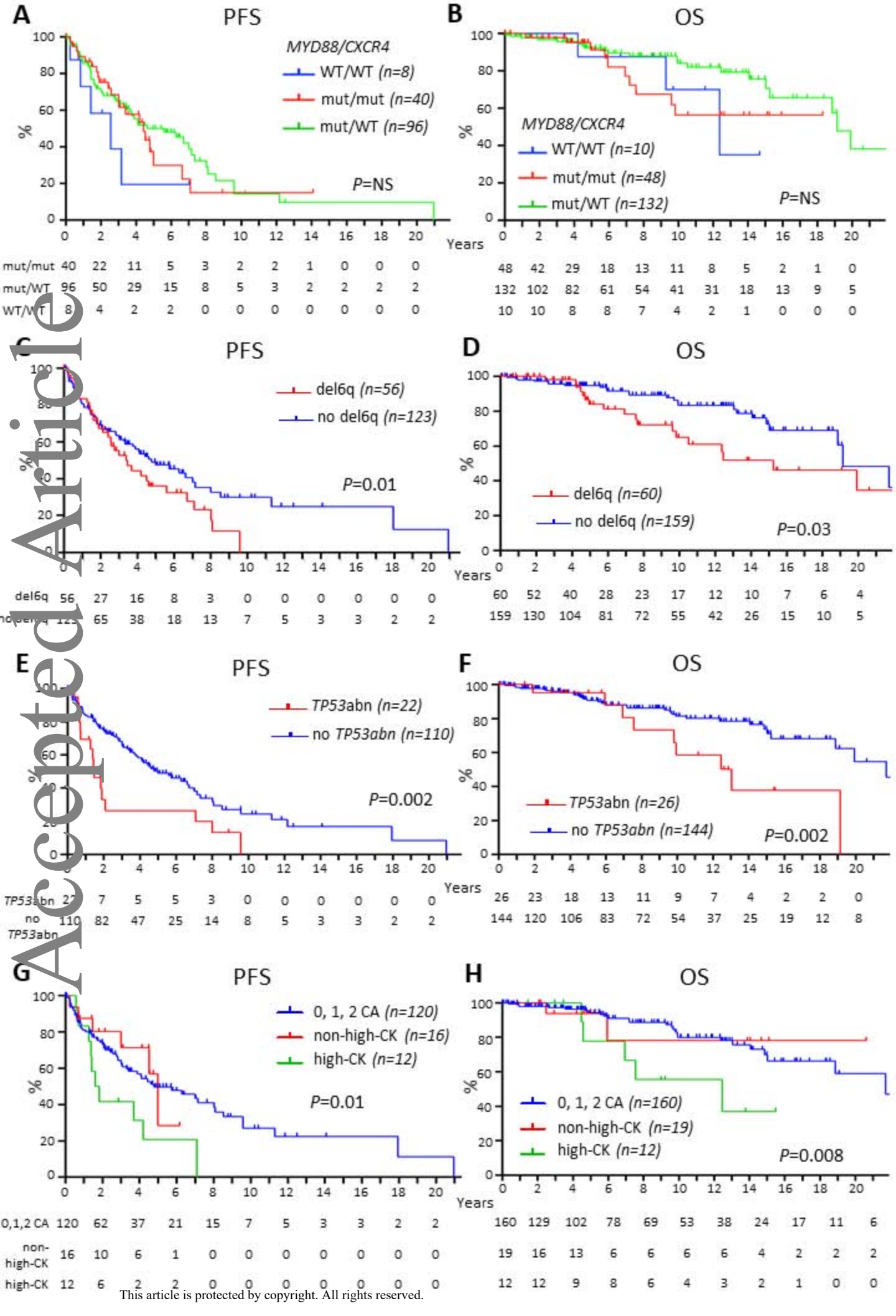

Figure 3 **ICT group**