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MOLECULAR PREDICTION OF DURABLE REMISSION AFTER FIRST LINE FLUDARABINE-CYCLOPHOSPHAMIDE-RITUXIMAB IN CHRONIC LYMPHOCYTIC LEUKEMIA

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INTRODUCTION AND AIM

Fludarabine, cyclophosphamide and rituximab (FCR) is the most effective regimen for the management of chronic lymphocytic leukemia (CLL), and represents the current standard for previously untreated patients who are young and in good physical conditions.[1-3] Though the majority of CLL patients receiving FCR as frontline therapy are destined to relapse, a subgroup of cases may experience durable first remission of their disease.[4-6]

Recently, an impressive array of novel effective therapies has been developed that hold the potential of increasingly individualized treatment modalities if patients' risk could be accurately characterized.[7-13] Also, in the new scenario of targeted agents for CLL, affordable treatment strategies should be patient-risk oriented as well as cost-effective and resource-saving.[14] On these bases, there is an increasing interest in identifying *a priori* patients who may maximally benefit from a single shot of FCR chemoimmunotherapy.

Over the last decade, several molecular prognostic markers capable of stratifying the outcome of CLL patients have been identified.[15-18] Some of them are widely utilized in the routine clinical practice and, individually, have consistently shown prognostic or predictive capability in the clinical setting of patients who have received FCR.[2,4,5,19,20] However, none of them has been tested either alone or in combination with other biomarkers to specifically predict the long-term benefit of chemoimmunotherapy.

In this observational retrospective study based on a large dataset of FCR-treated CLL, we show that the combination of three biomarkers of common use, i.e. immunoglobulin heavy variable (*IGHV*) gene mutation status and FISH abnormalities at chromosomes 11q and 17p, allows to segregate a subgroup of CLL patients who may achieve a durable remission after first-line FCR and experience an expected survival similar to that of the general population.

METHODS

Patients

The study collected 404 progressive and previously untreated CLL patients who consecutively received standard FCR as first-line therapy in 19 hematologic centers between 2001 and 2010. Patients must have fulfilled all of the following criteria for being actively registered in the study: i) having a diagnosis of untreated progressive CLL according to NCI or IWCLL/NCI criteria;[21,22] ii) having received first-line treatment with FCR at standard doses (i.e. no FCR lite);[1,2] iii) having received at least the first dose of the first FCR cycle according to an intention to treat approach; iv) having started FCR treatment by 2010, in order to have an adequate follow-up among alive patients; v) not receiving maintenance after FCR; and vi) having a minimal set of information, including demographic data, treatment indication (development or worsening of anemia and/or thrombocytopenia, massive or progressive or symptomatic splenomegaly or lymphadenopathy, lymphocyte doubling time, other),[21,22] Binet stage at treatment, date of FCR start, date of the last dose of FCR, number of FCR courses administered, date of progression according to NCI or IWCLL/NCI criteria,[21,22] and date of last follow-up or death. The following information were also collected for the majority of patients: pre-treatment *IGHV* mutation status, pre-treatment FISH profile (performed within 3 months before FCR treatment start), cause of death, treatment emergent second primary malignancies, date and type of next treatments after FCR.

Study design

The study was designed as a retrospective observational analysis. Patients received FCR between April 2001 and December 2010. The database was locked on June 2014. For sample size definition, we assumed a 6-years progression-free survival (PFS) of 38%, a constant hazard of

progression of 0.012 per month and a maximum follow-up of 96 months.[4,5] Given these assumptions, the sample size (n=404) has an 86% and 98% power (two-tailed alpha 5%) to identify cases in which the hazard of progression drops to zero after 5 years of follow-up if they account for 10% or 20% of the entire population, respectively.

The Reporting Recommendations for Tumor Marker Prognostic Studies (REMARK) criteria were followed throughout this study.[23] Patients provided informed consent in accordance with local IRB requirements and Declaration of Helsinki. The study was approved by the Ethical Committee of the Ospedale Maggiore della Carità di Novara associated with the Amedeo Avogadro University of Eastern Piedmont (Protocol Code 59/CE; Study Number CE 8/11).

Statistical analysis

PFS was the primary endpoint and was measured from date of treatment start to date of progression according to IWCLL-NCI guidelines (event), death (event) or last follow-up (censoring).[22] Overall survival (OS) was measured from date of initial presentation to date of death from any cause (event) or last follow-up (censoring).[22] Time to progression (TTP) was measured from date of treatment start to progression according to IWCLL-NCI guidelines (event), death (censoring) or last follow-up (censoring).[22] Response assessment was according to NCI or IWCLL-NCI guidelines.[21,22] Survival analysis was performed by the Kaplan-Meier method and compared between strata using the Log-rank test.[24]

The adjusted association between exposure variables and PFS was estimated by Cox regression.[25] Cox regression included exposure variables showing an univariate association with PFS with a significant level <0.1.[26] The proportional hazard assumption was assessed by plotting the smoothed Schoenfeld residuals against time.[27] The bias corrected c-index and calibration slope of the Cox model were calculated through the .632 bootstrap method (1000 resamplings).[28,29] The heuristic shrinkage estimator was calculated using the formula (model likelihood ratio $\chi 2$) -

(number of degree of freedom in the model)/(model likelihood ratio $\chi 2$). This approach provides an estimate of prediction accuracy of the Cox model to protect against overfitting.[26,28] The stability of the Cox model was internally validated using bootstrapping procedures.[30] In the first step, 1000 bootstrap samples were generated randomly with replacement from the original CLL population. Cox regression was applied to each bootstrap sample with the same covariates as the original modeling. The percentage of bootstrap samples for which each covariate was selected as significant in the model was then calculated. Percent of selection reflects the prognostic importance of a covariate, because it is expected that an important covariate will be selected for the majority of bootstrap samples. In the second step, 1000 additional bootstrap samples were generated randomly with replacement from the original CLL population. Cox regression was applied to each bootstrap sample with the same covariates as the original modeling. For each covariate, the mean standard deviation and confidence intervals were computed for the 1000 bootstrap replications. Smooth estimate of the hazard of progression according to the time elapsed from treatment start were estimated as previously reported.[31,32]

Compared to the Cox-fitted model, recursive partitioning for survival data with censoring has the advantage of a more objective and non arbitrary construction of a hierarchical classification of covariates.[33] The first step in recursive partitioning analysis was to find the best split of the data into two groups (nodes) by the predictor variable that captures the most information in the variability of PFS. The process was recursively repeated, so succeeding steps find the best splits of the data within each of the nodes resulting from prior splits (daughter nodes). The entire dataset was considered as the primary node. Three major steps were utilized to derive the best decision tree: *i)* growing an initial tree under the following constraints and stopping rules: *a)* split criteria of $p<0.05$ according to the log-rank test adjusted for multiple comparisons by Bonferroni; *b)* >20 patients in a node in order to be considered for splitting; *c)* >10 patients in a terminal node; *ii)* applying a pruning algorithm based on the complexity parameter (cp=0.015); and *iii)* cross-validating the best tree size. Ten-fold cross-validation was used to determine the best tree size. The best number of

splits was identified as that showing a cross-validation error lower than the smallest cross validation error + the corresponding standard error. The stability of the recursive decision tree was validated by the random survival forest method.[34] An amalgamation algorithm was used to merge terminal nodes showing homogenous PFS (further details are available in the Supplementary Appendix).[33]

Relative survival, defined as the ratio between the actuarial survival observed in the CLL cohort and the expected survival of the general Italian population matched to CLL patients by sex, age and calendar year of diagnosis, was calculated using the Ederer II method.[35] The major advantage of relative survival is that it provides a measure of the excess mortality experienced by CLL patients, irrespective of whether the excess mortality is directly or indirectly attributable to the disease. Estimates of the expected survival were calculated utilizing Italian life tables obtained from the Human Mortality Database (http://www.mortality.org/, accessed June 18, 2014).

Categorical variables were compared by Chi-square and exact tests when appropriate. All statistical tests were two-sided. Statistical significance was defined as p value <0.05. The analysis was performed with the Statistical Package for the Social Sciences (SPSS) software v.22.0 (Chicago, IL) and with R statistical package 3.1.2 (http://www.r-project.org).

Immunoglobulin gene mutation analysis and FISH

IGHV mutation analysis and FISH were performed at the reference laboratory of each participating center. *IGHV* mutation status was tested on tumor gDNA (5 centers) or cDNA (14 centers) collected at diagnosis or before FCR treatment start, and was assessed according to the ERIC guidelines[36] by using the BIOMED-2 primers (13 centers),[37] primers by Fais et al (5 centers),[38] or an internally developed set of primers (1 center).[39] Sequences that differed by more than 2% from their corresponding germline were considered as mutated.[15,16,36] FISH analysis was performed on nuclei extracted from fresh or frozen peripheral blood mononuclear cells collected no

more than three months before FCR treatment start. Probes (Abbott) used for FISH analysis were: LSI13 and LSID13S319, CEP12, LSIp53, and LSIATM. For each probe, at least 200 interphase cells were examined. The presence of 13q deletion, trisomy 12, 11q deletion and 17p deletion abnormalities was scored when the percentage of nuclei with the abnormality was above each laboratory internal cut offs defined as the mean plus 3 standard deviations of the frequency of normal control cells exhibiting the abnormality.[17]

RESULTS

Characteristics of the study cohort

The characteristics of the study cohort (n=404; Table 1) were consistent with those reported in CLL receiving FCR as first treatment,[2] including age (median: 61 years; ≥65 years in 33.4% of patients), gender (male in 67.8% of patients), stage (progressive Binet A in 10.6% of patients; Binet B in 59.7%; Binet C in 29.7%) and number of FCR courses (median: 6; <6 courses in 42.1% of patients). Most patients (n=336; 83.2% of the entire cohort) were evaluable for the *IGHV* mutation status (unmutated in 216, 64.3% of patients) and genomic aberrations at treatment requirement (n=317; 17p deletion in 30, 9.5% of patients; 11q deletion in 61, 19.2%; +12 in 70, 22.1%; 13q deletion in 111, 35.0%). Cases assessable for both *IGHV* mutations status and FISH (n=317) and cases lacking this molecular information (n=87) did not differ with respect to demographic features, clinical stage at FCR and treatment indication, thus excluding selection biases. The clinical outcome of the study cohort was also consistent with the outcome reported for CLL patients receiving FCR as first-line treatment.[2] Complete response was documented in 63.9% of assessed cases and partial response in 26.9% (Table 1). After a median follow-up of 70 months, 194 patients have progressed and 72 have died, accounting for a median PFS of 54.8 months and for a 5-year OS of 81.2% (median: not reached) (Figure 1A-1B).

IGHV mutation status and high-risk cytogenetics are independent predictors of PFS after front line FCR

As a preliminary step towards the construction of a model to predict remission duration in FCR-treated CLL, we assessed the impact on PFS of explanatory variables collected at baseline

before treatment initiation. By univariate analysis (Table 2), patients harboring unmutated *IGHV* genes (5-year PFS: 36.3%; median: 48.2 months) showed a significantly shorter PFS compared to *IGHV* mutated patients (5-year PFS: 58.6%; median: not reached; p=.0005). PFS was also significantly shorter in 11q deleted patients (5-year PFS: 18.4%; median: 43.5 months; p=.0106) and 17p deleted patients (5-year PFS: 10.9%; median: 22.5 months; p<.0001). Analysis of hierarchically disposed FISH abnormalities reproduced the previously described prognostic groups in this study cohort (Figure 2).[2,5]

By bivariate analysis, 17p deleted patients had a short PFS, independent of the *IGHV* mutation status (Figure 3). Conversely, the presence of 11q deletion significantly affected PFS among *IGHV* mutated patients, while it was irrelevant among *IGHV* unmutated cases (Figure 4). By multivariate analysis (Table 3), unmutated *IGHV* genes (HR: 1.65; p=.0099), 11q deletion (HR: 1.67; p=.0096) and 17p deletion (HR: 3.72; p=<.0001) maintained independent association with PFS, thus providing the rationale to utilize these molecular features in the development of a model to predict remission duration after FCR.

Most IGHV mutated patients lacking poor risk cytogenetic abnormalities remain free of progression after front-line FCR

The hierarchical order of relevance in predicting PFS among 17p deletion, 11q deletion and *IGHV* mutation status was established by recursive partitioning analysis (Figure 5A).[33] Deletion of 17p was the most predictive variable in the survival tree, followed by *IGHV* mutation status and 11q deletion. Measure of the variable importance validated the hierarchical order of relevance of the molecular lesions established by the recursive partitioning analysis and confirmed the stability of the decision tree (Figure 5B).[34] Based on the application of the amalgamation algorithm to the terminal nodes,[33] cases carrying unmutated *IGHV* genes and cases harboring 11q deletion were grouped into a single category because they shared a similar drop of the PFS curve (Figure 5A).

This approach allowed to establish a molecular model to classify CLL patients who received FCR as first treatment according to the risk of progression.

Three CLL subgroups were hierarchically classified (Figure 6A). Disease stage at FCR and treatment indication were superimposable across the three risk groups, suggesting that the differences in outcome cannot be ascribed to an unintended overtreatment of CLL patients not fulfilling the guideline recommended features of active and symptomatic CLL (Table 4). The high-risk category accounted for 9.5% of the study cohort and included patients harboring 17p deletion independent of co-occurring 11q deletion or the *IGHV* mutation status (5-year PFS: 10.9%; median: 22.5 months). The intermediate-risk category accounted for 62.1% of the study cohort and included patients harboring 11q deletion and/or unmutated *IGHV* genes in the absence of 17p deletion (5-year PFS: 37.9%; median: 51.7 months). The low-risk category accounted for 28.4% of the study cohort and comprised patients harboring mutated *IGHV* genes but lacking both 11q deletion and 17p deletion (5-year PFS: 71.6%; median: not reached). Consistent with a composition of mixed molecular profiles and lack of selection biases, cases not classifiable according to the model because of lacking FISH and/or *IGHV* mutation status (n=87) showed an intermediate clinical outcome (Figure 7). In the tree risk groups, TTP almost matched PFS, indicating that deaths without progression did not bias the survival analysis (Figure 8).

High- (17p deleted) and intermediate-risk (*IGHV* unmutated and/or 11q deleted) patients showed a constant increase of the hazard of progression over time and almost all were projected to relapse after FCR, although at a different rate: 17% per year of follow-up in the high-risk group and 10% per year of follow-up in the intermediate-risk group (Figure 6A and 9). Conversely, among low-risk patients (*IGHV* mutated without 17p or 11q deletion) the hazard of relapse plateaued at 20 months after FCR and dropped to 0 after five years of follow-up (Figure 9). Consistently, the PFS curve of low-risk patients showed a plateau starting at 5 years from FCR and most of the low-risk patients (71.6%) were projected to remain free of progression (Figure 6A). Overall, these data indicate that the combination of three biomarkers that are widely tested in the clinical practice

allows segregating a sizable subgroup of patients who may achieve a durable remission after front-line FCR.

Low- and intermediate-risk patients who received six FCR cycles showed a significantly higher chance of achieving a complete response and a longer PFS compared to patients who received less than six FCR cycles, suggesting that a full course of chemoimmunotherapy ensures a deeper disease control. The number of FCR courses did not impact on PFS of high-risk patients (Figure 10).

IGHV mutated patients lacking poor risk cytogenetic abnormalities have a near-normal life expectancy after front line FCR

Relative survival analysis was used to provide a measure of the excess mortality experienced by CLL patients treated at first-line with FCR, irrespective of whether the excess mortality is directly or indirectly attributable to the disease.[35] When the demographic effects of age, gender and year of treatment were compensated, the 5-year and 10-year survival rates of the whole cohort of patients were only 85.3% and 68.7%, respectively, of those expected in the matched normal general population ($p<.0001$) (Figure 1B).

Upon OS stratification according to the hierarchical model based on 17p deletion, 11q deletion and *IGHV* mutation status (Figure 6B), the life expectancies of high- (5-year relative survival: 60.2%; $p<.0001$) and intermediate-risk (5-year relative survival: 87.2%; $p<.0001$) patients were significantly impaired compared to that expected in the matched general population, thus indicating an excess of deaths related to the disease or treatment complications in these unfavorable CLL groups. Conversely, the life expectancy of low-risk patients was similar to that observed in the matched normal general population (5-year relative survival: 95.8%; $p=.2770$) (Figure 6B), indicating that neither the disease, nor the complications of treatment, affected survival in this favorable CLL group. Consistently, only five patients have died among low-risk patients, including

one of progressive disease, one of lung cancer and three of unrelated causes while in remission. In addition, the prevalence of treatment-emergent second primary malignancies was significantly lower in low-risk patients (3.9%, including one skin basal cell cancer, one lung cancer and one thyroid adenoma) compared to intermediate-risk and high-risk cases (12.1% and 19.0%, respectively; Table 4).

DISCUSSION

This study shows that the combination of three biomarkers that are widely tested at treatment requirement allows to segregate a subgroup of CLL patients - *IGHV* mutated without 17p or 11q deletion - who: i) accounts for a sizable fraction (28.4%) of progressive previously untreated CLL requiring treatment; ii) achieve a durable remission after first-line treatment with FCR; and iii) experience an expected survival similar to that of the general population.

These findings have potential implications for the design of clinical trials and, possibly, for overall disease management of CLL patients. Beyond FCR, significant therapeutic advances have occurred in the treatment of CLL and chemotherapy-free approaches are increasingly being developed.[7-10,12,13] Novel agents such as tyrosine kinase inhibitors show promising activity in CLL but are associated with considerable costs and are not affordable in many health care systems if applied broadly across large numbers of patients.[14,40,41] To responsibly and effectively advance the development of these new therapies, they should be targeted specifically to patient subgroups in which they can provide the greatest benefit compared to established chemoimmunotherapy regimens. Among high-risk CLL with *TP53* abnormalities, the activity of tyrosine kinase inhibitors appears significantly better than all previous pharmacologic strategies.[7-10,12,13] On these bases, though *per se* they do not assure long-lasting remissions and formal head to head comparisons with FCR or other chemoimmunotherapy combinations are lacking, tyrosine kinase inhibitors currently represent the best treatment option for *TP53* disrupted CLL patients and have been approved as front-line therapy in this molecular subgroup. Among CLL patients lacking *TP53* abnormalities, ongoing clinical trials are comparing tyrosine kinase inhibitors vs FCR as front-line treatment. Given the highly favorable outcome of *IGHV* mutated CLL lacking 17p and 11q deletion hereby reported following front-line FCR treatment, assessment of whether novel agents provides additional survival benefit in this biologic subgroup will require highly powered studies with a long follow-up.

Long-term toxicities, including treatment emergent second primary malignancies, represent a concern for patients treated with FCR.[42] In our study population, a proportion of patients developed a second primary malignancy, including 3.9% of low-risk patients, 12.1% of intermediate-risk patients and 19% of high-risk patients. The low rate of second primary tumors among low-risk patients might be explained, at least in part, by the lower requirement of salvage treatments, and thus by the low overall load of chemotherapy received by these patients. Beside being of limited proportion, second primary malignancies also did not translate into an excess mortality in low-risk patients compared to the matched general population, suggesting that the risk of death for second primary tumors, as well as other treatment complications, is not increased among low-risk patients treated with FCR.

The intermediate-risk group of patients represents a case mix warranting further stratification, as suggested by the observation that ~20% of intermediate-risk patients progress shortly after FCR, while, on the other hand, ~20% of them are projected to remain progression-free after 5 years. Additional molecular markers, including gene mutations, have proved to be effective in refining CLL prognostication when combined to cytogenetics and *IGHV* mutation status,[43,44] and may allow to fine tune the definition and composition of the intermediate-risk group.

This study reports on patients treated with FCR in the every-day life clinical practice and provides an insight into the "real-world" outcome of patients treated with FCR in the academic and community settings. Though not affected by the constrains of clinical trial inclusion criteria, this study cohort shows baseline features and outcome superimposable to those described for patients treated with FCR within the framework of a clinical study.[1,2] On these bases, though the retrospective design represents a limitation of this analysis, an external and prospective validation of our data is provided by the consistent association between PFS and *IGHV* mutation status, 17p deletion and 11q deletion in multivariable analyses from clinical trials of FCR treated CLL.[2,5]

A strong biologic rationale supports the application of this model to identify patients who may benefit mostly from FCR. Indeed, low-risk CLL patients harboring mutated *IGHV* genes, but

lacking 17p or 11q deletion, are those showing the highest sensitivity and deepest response to FCR, as documented by the high rate of minimal residual disease (MRD) eradication in this biologic subgroup of CLL.[45,46] This might stem from a lower degree of genetic complexity and lower rate of high-risk subclones that characterize *IGHV* mutated CLL.[47,48] In addition, *IGHV* mutated patients, independent of the levels of disease burden reduction achieved by treatment, generally show a slow progression rate in keeping with the lower predisposition of *IGHV* mutated CLL to proliferate in response to microenvironmental stimuli.[45] Consistently, 40% (9/22) of the low-risk patients who have progresses after FCR, did not require a second-line treatment after progression because of the indolent course of the relapsed disease, compared to only 14% (15/102) intermediate-risk patients and virtually none (1/27) of high-risk patients.

A limitation of this study is the lack of MRD data, which represent a strong independent predictor of PFS in CLL patients treated with upfront FCR.[45,46] *IGHV* mutated CLL patients have a chance of obtaining negative MRD after few FCR courses,[45] suggesting that they are the best candidates for an early discontinuation approach. According to our results, however, a full course of FCR provides additional PFS benefit to low-risk patients. This observation is consistent with the notion that most (~50%) *IGHV* mutated low-risk patients achieve MRD eradication only after completing all six cycles of FCR.[45] Studies incorporating MRD eradication monitoring along with molecular stratification might allow to derive recommendations about the optimal number of FCR courses each patient needs.

In the era of personalized medicine, the challenges of CLL treatment will involve correctly matching therapy to the unique risk profile of each individual patient. Our data support front-line FCR as a highly active option in physically fit patients with progressive CLL whose disease has a low-risk molecular profile. Novel chemoimmunotherapy approaches are in development, including less toxic combinations (i.e. bendamustine, rituximab, BR) or regimens incorporating second generation anti-CD20 monoclonal antibodies (i.e. obinutuzumab and ofatumumab).[11,49,50] Application of our model to CLL cohorts treated with new chemoimmunotherapy approaches will

allow to assess whether low-risk patients might equally benefit from BR, and whether second generation anti-CD20 monoclonal antibodies might further increase the proportion of low-risk cases that will remain progression-free on the long-term.

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TABLES

Table 1. Characteristics of the study cohort [a]

Characteristics	All cases n=404			With biomarkers n=317		
	Cases	%	Valid (%)	Cases	%	Valid (%)
Age <65 years	269	66.6	66.6	222	70.0	70.0
Age ≥65 years	135	33.4	33.4	95	30.0	30.0
Male	274	67.8	67.8	215	67.8	67.8
Female	130	32.2	32.2	102	32.2	32.2
Binet A	43	10.6	10.6	39	12.3	12.3
Binet B	241	59.7	59.7	194	61.2	61.2
Binet C	120	29.7	29.7	84	26.5	26.5
Treatment indication						
Development or worsening of anemia and/or thrombocytopenia	79	19.6	19.6	61	19.2	19.2
Massive or progressive symptomatic splenomegaly or lymphadenopathy	234	57.9	57.9	185	58.4	58.4
Lymphocyte doubling time	87	21.5	21.5	70	22.1	22.1
Other	4	1.0	1.0	1	0.3	0.3
Number of FCR courses 6	234	57.9	57.9	195	61.5	61.5
Number of FCR courses <6	170	42.1	42.1	122	38.5	38.5
IGHV						
Mutated	120	29.7	35.7	108	34.1	34.1
Unnmutated	216	53.5	64.3	209	65.9	65.9
Missing	68	16.8		-	-	-
13q deletion						
Absent	206	51.0	65.0	206	65.0	65.0
Present	111	27.5	35.0	111	35.0	35.0
Missing	87	21.5		-	-	-
Trisomy 12						
Absent	247	61.1	77.9	247	77.9	77.9
Present	70	17.3	22.1	70	22.1	22.1
Missing	87	21.5		-	-	-
11q deletion						

Absent	256	63.4	80.8	256	80.8	80.8
Present	61	15.1	19.2	61	19.2	19.2
Missing	87	21.5		-	-	-
17p deletion						
Absent	287	71.0	90.5	287	90.5	90.5
Present	30	7.4	9.5	30	9.5	9.5
Missing	87	21.5		-	-	-
Response to FCR						
Complete response	228	56.4	63.9	186	58.7	66.7
Partial response	96	23.8	26.9	75	23.7	26.9
Stable disease	12	3.0	3.3	9	2.8	3.2
Progressive disease	14	3.5	3.9	7	2.2	2.5
Not assessable	7	1.7	2.0	2	0.6	0.7
Missing	47	11.6		38	12.0	
Second treatment after FCR	158	39.1	39.1	126	39.7	39.7
No second treatment after FCR	246	60.9	60.9	191	60.3	60.3
Type of second treatment after FCR						
Alemtuzumab-based	18	11.4	13.2	15	11.9	14.3
Anti-CD20	8	5.1	5.9	6	4.8	5.7
Anti-CD20+alkylator	28	17.7	20.6	22	17.5	21.0
Anti-CD20+bendamustine	44	27.8	32.4	34	27.0	32.4
Anti-CD20+purine analogue	18	11.4	13.2	13	10.3	12.4
Second line NHL	6	3.8	4.4	5	4.0	4.8
Other	14	8.9	10.3	10	7.9	9.5
Unknown	22	13.9		21	16.7	
Second primary malignancy after FCR						
Yes	33	8.2	9.6	28	8.8	10.3
No	312	77.2	90.4	243	76.7	89.7
Missing	59	14.6		46	14.5	
Cause of death during disease course (including cause of death after disease relapse)						
Infection	12	16.7	17.4	10	19.6	20.4
Progressive disease	42	58.3	60.9	31	60.8	63.3
Second primary malignancy	5	6.9	7.2	5	9.8	10.2
Treatment complication	1	1.4	1.4	1	2.0	2.0

Other	9	12.5	13.0	2	3.9	4.1
Unkown	3	4.2		2	3.9	

[a] FCR, fludarabine-cyclophosphamide-rituximab treatment; *IGHV*, immunoglobulin heavy variable gene; NHL non Hodgkin lymphoma

Table 2. Univariate analysis of PFS and OS [a]

Characteristics	5-year PFS (%)	Median PFS	95% CI	p	5-year OS (%)	95% CI	p
Age <65 years	46.6	58.1	49.5-66.6	0.0617	86.0	80.9-91.1	0.0001
Age ≥65 years	42.9	46.5	36.0-56.9		71.0	61.8-80.2	
Male	42.7	51.6	43.8-59.3	0.3240	82.1	76.8-87.4	0.7575
Female	50.1	66.2	50.5-82.0		79.4	71.0-87.8	
Binet A	59.5	64.7	31.8-96.6	0.0848	94.7	84.7-100.0	0.0172
Binet B+C	43.5	54.3	48.3-60.3		79.8	74.9-84.7	
IGHV Mutated	58.6	nr	na	0.0005	88.1	80.7-95.5	0.0359
IGHV Unnmutated	36.3	48.2	43.7-52.7		78.4	71.7-85.1	
No 13q deletion	45.4	55.6	48.3-62.8	0.9041	82.6	79.5-82.7	0.1742
13q deletition	41.1	50.8	38.6-63.0		82.6	77.6-87.6	
No Trisomy 12	45.1	55.6	46.3-64.8	0.6188	84.7	81.8-87.6	0.0245
Trisomy 12	40.3	51.7	44.9-58.6		76.5	70.6-82.4	
No 11q deletion	49.4	56.9	47.1-66.6	0.0106	82.5	79.6-85.4	0.9919
11q deletion	18.4	43.5	32.2-54.7		84.3	79.5-90.1	
No 17p deletion	48.0	58.9	49.3-68.4	<0.0001	86.0	83.5-88.5	<0.0001
17p deletion	10.9	22.5	8.5-36.4		57.5	47.6-67.4	

[a] PFS, progression free survival; OS, overall survival; *IGHV*, immunoglobulin heavy variable gene; CI, confidence interval; nr, not reached; na, not applicable

Table 3. Multivariate analysis of PFS[a]

	Multivariate analysis				Internal bootstrapping validation: Bootstrap parameters (mean)			
Characteristics	**HR**	**LCI**	**UCI**	**p**	**HR**	**LCI**	**UCI**	**Bootstrap selection**
Age <65 years	-	-	-		-	-	-	39.4%
Age ≥65 years	1.23	0.87	1.75	0.2323	1.24	0.87	1.78	
Binet A	-	-	-		-	-	-	55.9%
Binet B+C	1.57	0.85	2.91	0.1474	1.72	0.88	3.40	
IGHV Mutated	-	-	-		-	-	-	88.0%
IGHV Unnmutated	1.65	1.12	2.41	0.0099	1.70	1.15	2.52	
No 11q deletion	-	-	-		-	-	-	88.9%
11q deletion	1.67	1.13	2.46	0.0096	1.67	1.16	2.56	
No 17p deletion	-	-	-		-	-	-	100%
17p deletion	3.72	2.42	5.71	<0.0001	4.04	2.59	6.31	

[a] PFS, progression free survival; *IGHV*, immunoglobulin heavy variable gene; HR, hazard ratio; LCI, lower confidence interval; UCI upper confidence interval

Shrinkage coefficient: 0.92

Discrimination: bias-corrected c-index: 0.64; optimism: 0.02

Calibration: bias-corrected calibration slope: 0.91; optimism: 0.09

Table 4. Characteristics of the three risk subgroups [a]

Characteristics	Low-risk (n=90)		Intermediate-risk (n=197)		High-risk (n=30)		p
	Cases	%	Cases	%	Cases	%	
Age							
<65 years	63	70.0	140	71.1	19	63.3	0.6901
≥65 years	27	30.0	57	28.9	11	36.7	
Gender							
Male	53	58.9	140	71.1	22	73.3	0.0974
Female	37	41.1	57	28.9	8	26.7	
Stage							
Binet A	16	17.8	22	11.2	1	3.3	0.0855
Binet B+C	74	82.2	175	88.8	29	96.7	
Number of FCR courses							
6	53	58.9	127	64.5	15	50.0	0.2635
<6	37	41.1	70	35.5	15	50.0	
Time to treatment (median)	26 months		13 months		17 months		0.0038
Treatment indication							
Development or worsening of anemia and/or thrombocytopenia	18	20.0	40	20.3	3	10.0	
Massive or progressive symptomatic splenomegaly or lymphadenopathy	54	60.0	110	55.8	21	70.0	0.4207
Lymphocyte doubling time	17	18.9	47	23.9	6	20.0	
Other	1	1.1	0	0	0	0	
IGHV							
Mutated	90	100	11	5.6	7	23.3	<0.0001
Unnmutated	0	0	186	94.4	23	76.7	
13q14 deletion							
Absent	42	46.7	139	70.6	25	83.3	<0.0001
Present	48	53.3	58	29.4	5	16.7	
Trisomy 12							
Absent	76	84.4	144	73.1	27	90.0	0.0234
Present	14	15.6	53	26.9	3	10.0	
11q deletion							

Absent	90	100	138	70.1	28	93.3	<0.0001
Present	0	0	59	29.9	2	6.7	
17p deletion							
Absent	90	100	197	100	0	0	<0.0001
Present	0	0	0	0	30	100	
Second primary malignancy after FCR							
No	74	96.1	152	87.9	17	81.0	0.0500
Yes	3	3.9	21	12.1	4	19.0	

[a] FCR, fludarabine-cyclophosphamide-rituximab treatment; *IGHV*, immunoglobulin heavy variable gene; NHL non Hodgkin lymphoma

FIGURE LEGENDS

Figure 1. Kaplan-Meier estimates of progression free survival and overall survival of the whole study cohort. Panel A. Progression free survival (PFS) of the whole study cohort. Panel B. Overall survival (OS) of the whole study cohort (blue line) relative to the expected OS in the age-, sex- and calendar year of treatment-matched general population (black line). p, p-value of the comparison between the observed survival and the expected survival.

Figure 2. Kaplan-Meier estimates of progression free survival according to hierarchically classified chromosomal abnormalities by FISH. Cases harboring 17p deletion independent of co-occurring chromosomal abnormalities are represented by the red line. Cases harboring 11q deletion, but lacking 17p deletion are represented by the purple line. Cases harboring trisomy 12 but lacking 17p and 11q deletion are represented by the yellow line. Cases harboring 13q deletion, but lacking 17p deletion, 11q deletion and trisomy 12 are represented by the blue line. Cases harboring a normal FISH karyotype are represented by the green line. p values according to the Log-rank statistics

Figure 3. Kaplan-Meier estimates of progression free survival according to 17p deletion among IGHV mutated and IGHV unmutated patients. Panel A. Progression free survival (PFS) according to 17p deletion status among patients harboring mutated (M) IGHV genes. Cases lacking 17p deletion are represented by the blue line. Cases harboring 17p deletion are represented by the red line. Panel B. Progression free survival (PFS) according to 17p deletion status among patients harboring unmutated (UM) IGHV genes. Cases lacking 17p deletion are represented by the blue line. Cases harboring 17p deletion are represented by the red line. nr, not reached; na, not applicable; p values according to the Log-rank statistics.

Figure 4. Kaplan-Meier estimates of progression free survival according to 11q deletion among *IGHV* mutated and *IGHV* unmutated patients. Panel A. Progression free survival (PFS) according to 11q deletion status among patients harboring mutated (M) *IGHV* genes. Cases lacking 11q deletion are represented by the blue line. Cases harboring 11q deletion are represented by the red line. Panel B. Progression free survival (PFS) according to 11q deletion status among patients harboring unmutated (UM) *IGHV* genes. Cases lacking 11q deletion are represented by the blue line. Cases harboring 11q deletion are represented by the red line. nr, not reached; na, not applicable; p values according to the Log-rank statistics.

Figure 5. Decision tree resulting from recursive partitioning analysis and amalgamation. Panel A. Deletion of 17p and 11q and *IGHV* mutation status were the factors selected by the algorithm to split the patient population in four terminal nodes according to their progression free survival (PFS). Presence or absence of 17p deletion independent of co-occurring 11q deletion or unmutated *IGHV* genes was the most significant covariate for the entire study population. Among patients lacking 17p deletion, the most significant covariate was the *IGHV* mutation status. Among patients lacking both 17p and unmutated *IGHV* genes, the most significant covariate was 11q deletion. Based on the application of the amalgamation algorithm to the terminal nodes, cases harboring unmutated *IGHV* genes and cases harboring 11q deletion were grouped into a single category. Covariates are represented from right to left according to their hierarchical order of relevance in splitting the parent node into daughter nodes with significantly different survival probabilities. The p value corresponds to the log-rank test adjusted for multiple comparisons. The right branch of each split represents the presence of the lesion. The left branch of each split represents the absence of the lesion. The Kaplan-Meier curves estimate the PFS of patients belonging to each terminal node. n, number of

patients in the node. Panel B. The bars represent the variable importance measure for the random survival forest model.

Figure 6. Kaplan-Meier estimates of progression free survival and overall survival according to the model based on 17p deletion, 11q deletion and *IGHV* mutation status. Panel A. Progression free survival (PFS). Panel B. Overall survival (OS). Cases harboring 17p deletion independent of co-occurring 11q deletion or unmutated *IGHV* genes are represented by the red line. Cases harboring unmutated *IGHV* genes and/or 11q deletion in the absence of 17p deletion are represented by the yellow line. Cases harboring mutated *IGHV* genes in the absence of 11q and 17p deletion are represented by the blue line. The black line represents the expected OS in the age-, sex- and calendar year of treatment-matched general population. nr, not reached; na, not applicable; p values according to the Log-rank statistics.

Figure 7. Kaplan-Meier estimates of progression free survival and overall survival according to the model based on 17p deletion, 11q deletion and *IGHV* mutation status, and including cases lacking biological information. Panel A. Progression free survival (PFS). Panel B. Overall survival (OS). Cases harboring 17p deletion independent of co-occurring 11q deletion or unmutated *IGHV* genes are represented by the red line. Cases harboring unmutated *IGHV* genes and/or 11q deletion in the absence of 17p deletion are represented by the yellow line. Cases harboring mutated *IGHV* genes in the absence of 11q and 17p deletion are represented by the blue line. Cases lacking biological information are represented by the green line. nr, not reached; na, not applicable.

Figure 8. Kaplan-Meier estimates of time to progression (TTP) according to the model based on 17p deletion, 11q deletion and *IGHV* mutation status. Cases harboring 17p deletion independent of co-occurring 11q deletion or unmutated *IGHV* genes are represented by the red line. Cases harboring unmutated *IGHV* genes and/or 11q deletion in the absence of 17p deletion are

represented by the yellow line. Cases harboring mutated *IGHV* genes in the absence of 11q and 17p deletion are represented by the blue line. nr, not reached; na, not applicable; p values according to the Log-rank statistics.

Figure 9. Hazard of progression in relation to the time elapsed from FCR treatment start. Cases harboring 17p deletion independent of co-occurring 11q deletion or unmutated *IGHV* genes are represented by the red line. Cases harboring unmutated *IGHV* genes and/or 11q deletion in the absence of 17p deletion are represented by the yellow line. Cases harboring mutated *IGHV* genes in the absence of 11q and 17p deletion are represented by the blue line.

Figure 10. Kaplan-Meier estimates of progression free survival (PFS) according to number of FCR courses among patient belonging to the low-, intermediate- and high- risk subgroups. Cases that received 6 courses are represented by the blue line. Cases received <6 courses are represented by the red line. nr, not reached; na, not applicable; p values according to the Log-rank statistics.

FIGURES

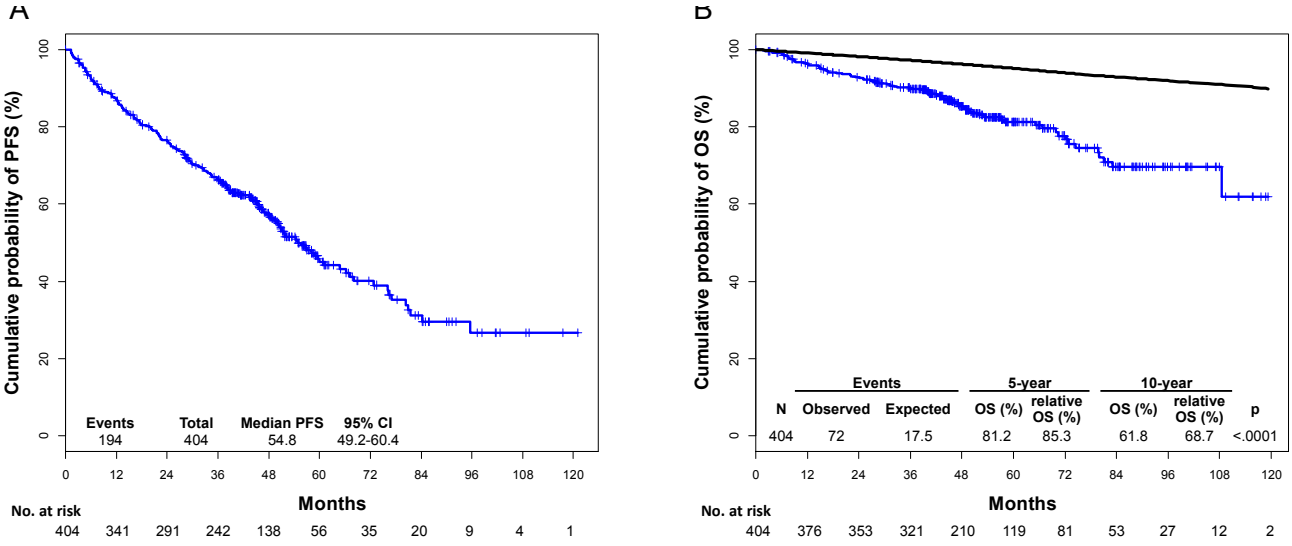


Figure 1

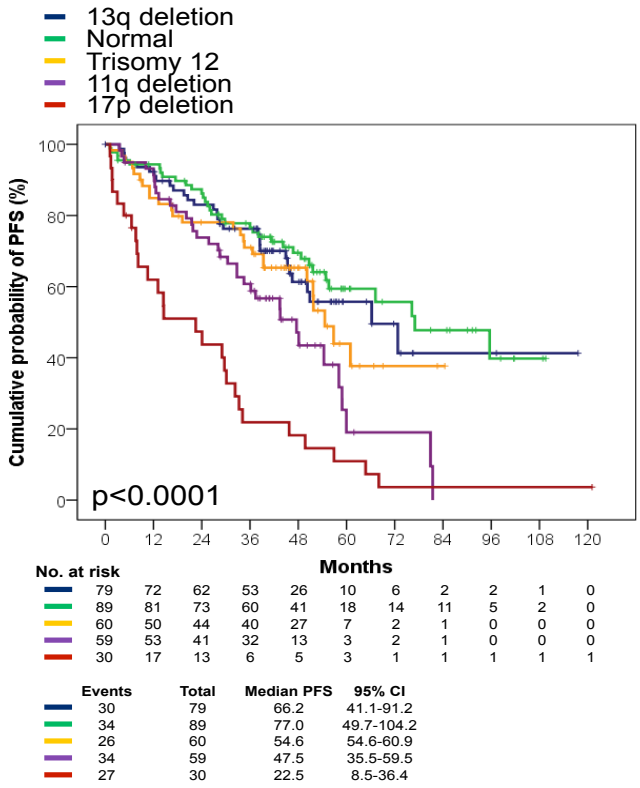


Figure 2

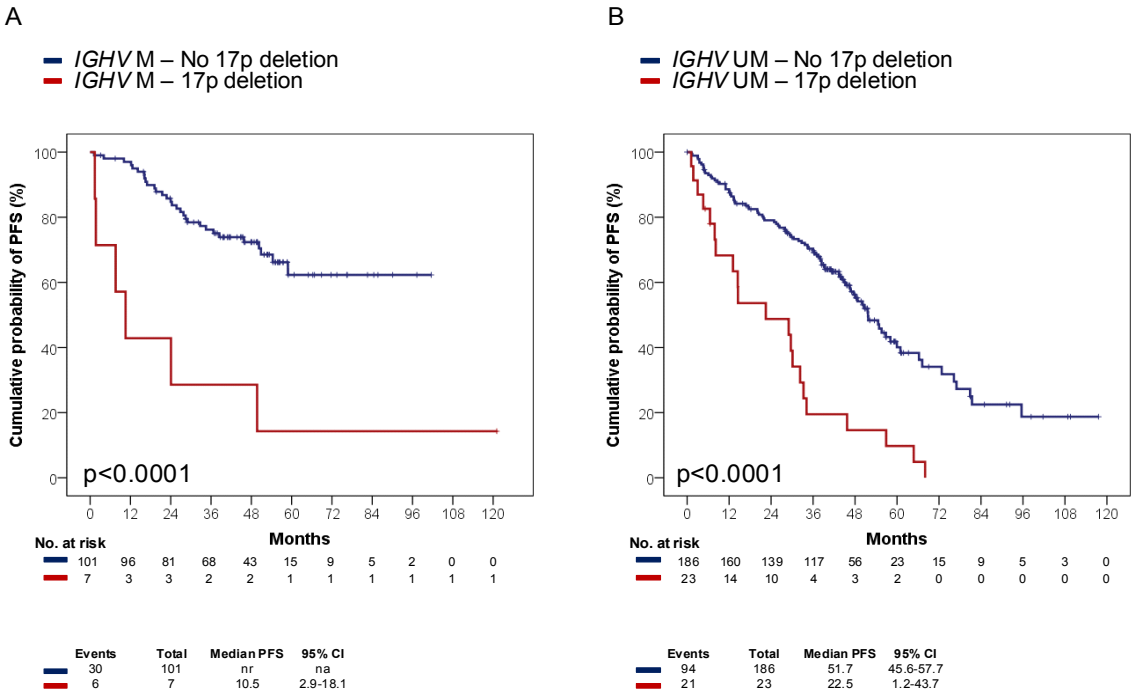
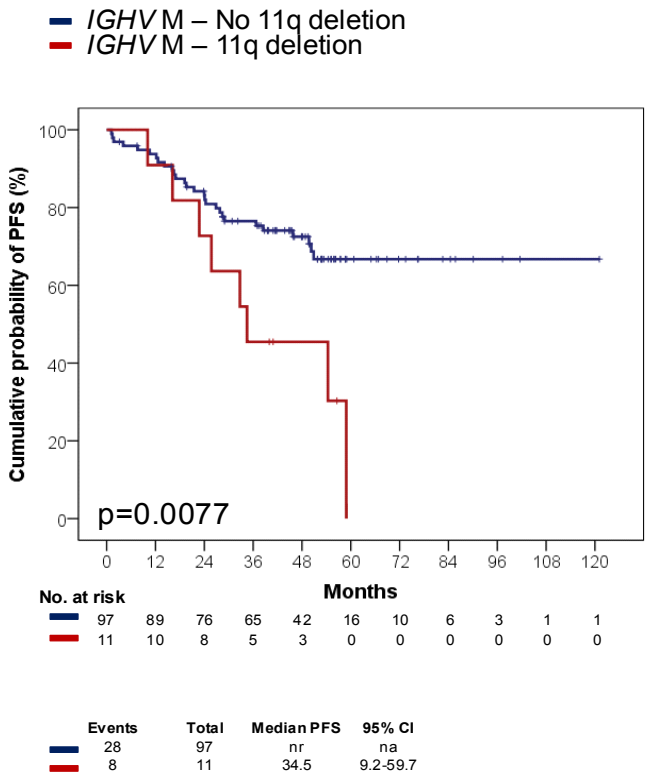


Figure 3

A


IGHV M – No 11q deletion
IGHV M – 11q deletion
Cumulative probability of PFS (%)
100
80
60
40
20
0
p=0.0077
0 12 24 36 48 60 72 84 96 108 120
Months
No. at risk
97 89 76 65 42 16 10 6 3 1 1
11 10 8 5 3 0 0 0 0 0 0
Events Total Median PFS 95% CI
28 97 nr na
8 11 34.5 9.2-59.7

B

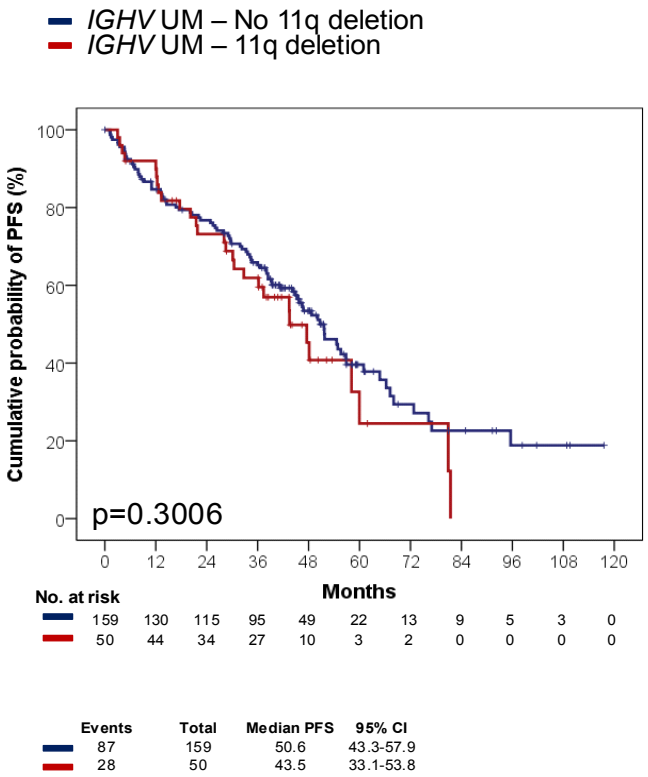
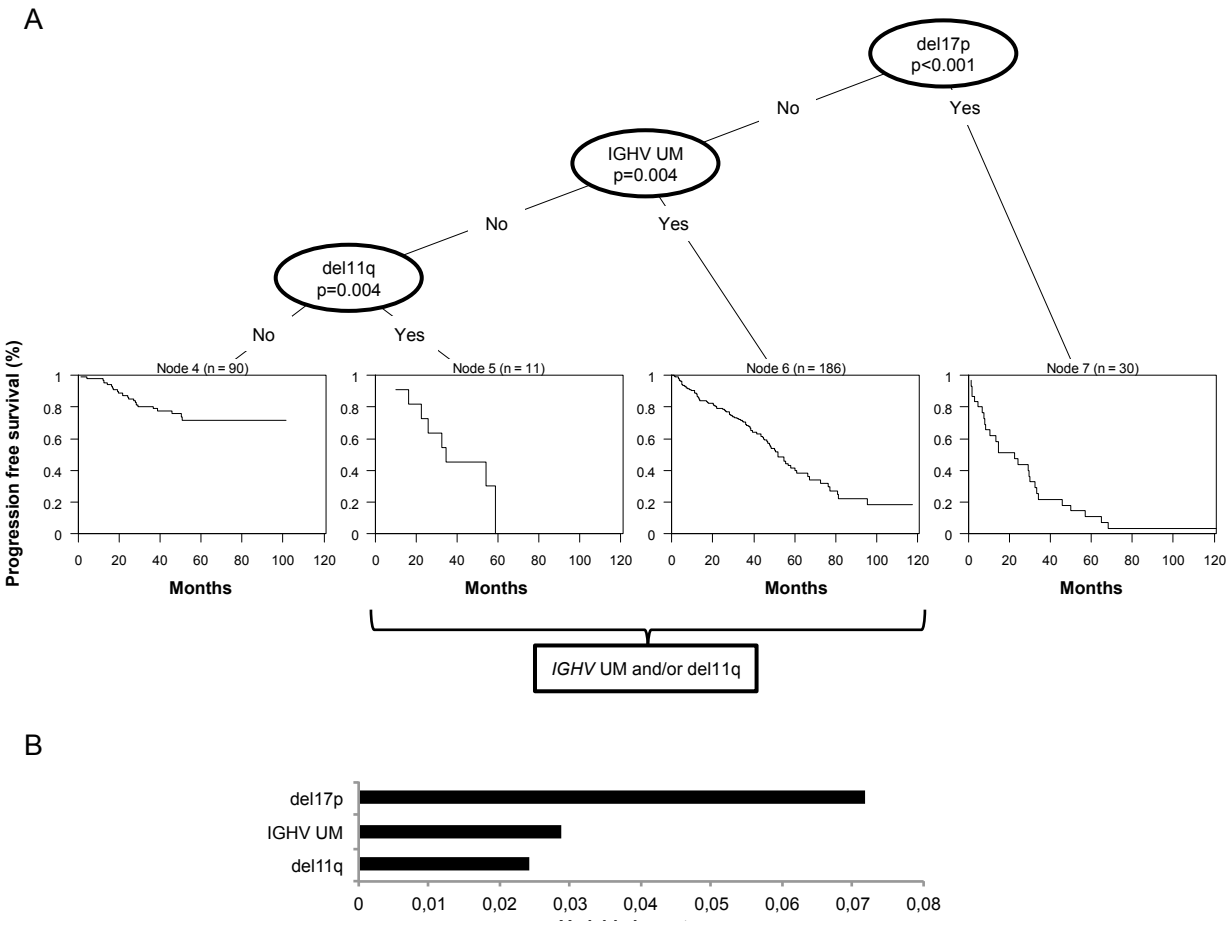

IGHV UM – No 11q deletion
IGHV UM – 11q deletion
Cumulative probability of PFS (%)
100
80
60
40
20
0
p=0.3006
0 12 24 36 48 60 72 84 96 108 120
Months
No. at risk
159 130 115 95 49 22 13 9 5 3 0
50 44 34 27 10 3 2 0 0 0 0
Events Total Median PFS 95% CI
87 159 50.6 43.3-57.9
28 50 43.5 33.1-53.8

Figure 4


A
del17p
p<0.001
No
Yes
IGHV UM
p=0.004
No
Yes
del11q
p=0.004
No
Yes
Node 4 (n = 90)
Node 5 (n = 11)
Node 6 (n = 186)
Node 7 (n = 30)
Progression free survival (%)
Months
IGHV UM and/or del11q

B
del17p
IGHV UM
del11q
0
0,01
0,02
0,03
0,04
0,05
0,06
0,07
0,08

Figure 5

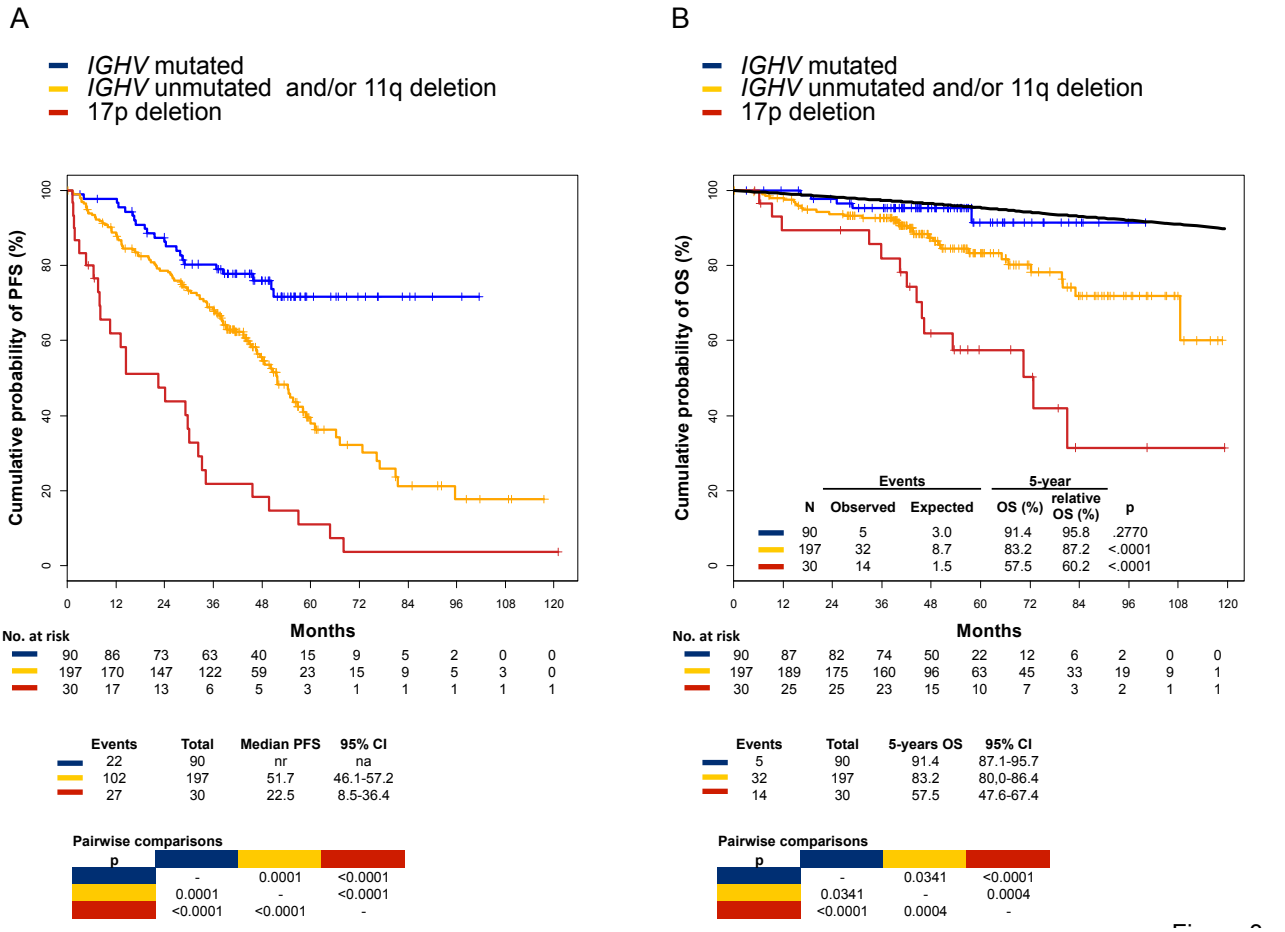

A
IGHV mutated
IGHV unmutated and/or 11q deletion
17p deletion
Cumulative probability of PFS (%)
Months
No. at risk
90 86 73 63 40 15 9 5 2 0 0
197 170 147 122 59 23 15 9 5 3 0
30 17 13 6 5 3 1 1 1 1 1
Events Total Median PFS 95% CI
22 90 nr na
102 197 51.7 46.1-57.2
27 30 22.5 8.5-36.4
Pairwise comparisons
p
- 0.0001 <0.0001
0.0001 - <0.0001
<0.0001 <0.0001 -
B
IGHV mutated
IGHV unmutated and/or 11q deletion
17p deletion
Cumulative probability of OS (%)
Events 5-year
N Observed Expected OS (%) relative OS (%) p
90 5 3.0 91.4 95.8 .2770
197 32 8.7 83.2 87.2 <.0001
30 14 1.5 57.5 60.2 <.0001
Months
No. at risk
90 87 82 74 50 22 12 6 2 0 0
197 189 175 160 96 63 45 33 19 9 1
30 25 25 23 15 10 7 3 2 1 1
Events Total 5-years OS 95% CI
5 90 91.4 87.1-95.7
32 197 83.2 80,0-86.4
14 30 57.5 47.6-67.4
Pairwise comparisons
p
- 0.0341 <0.0001
0.0341 - 0.0004
<0.0001 0.0004 -

Figure 6

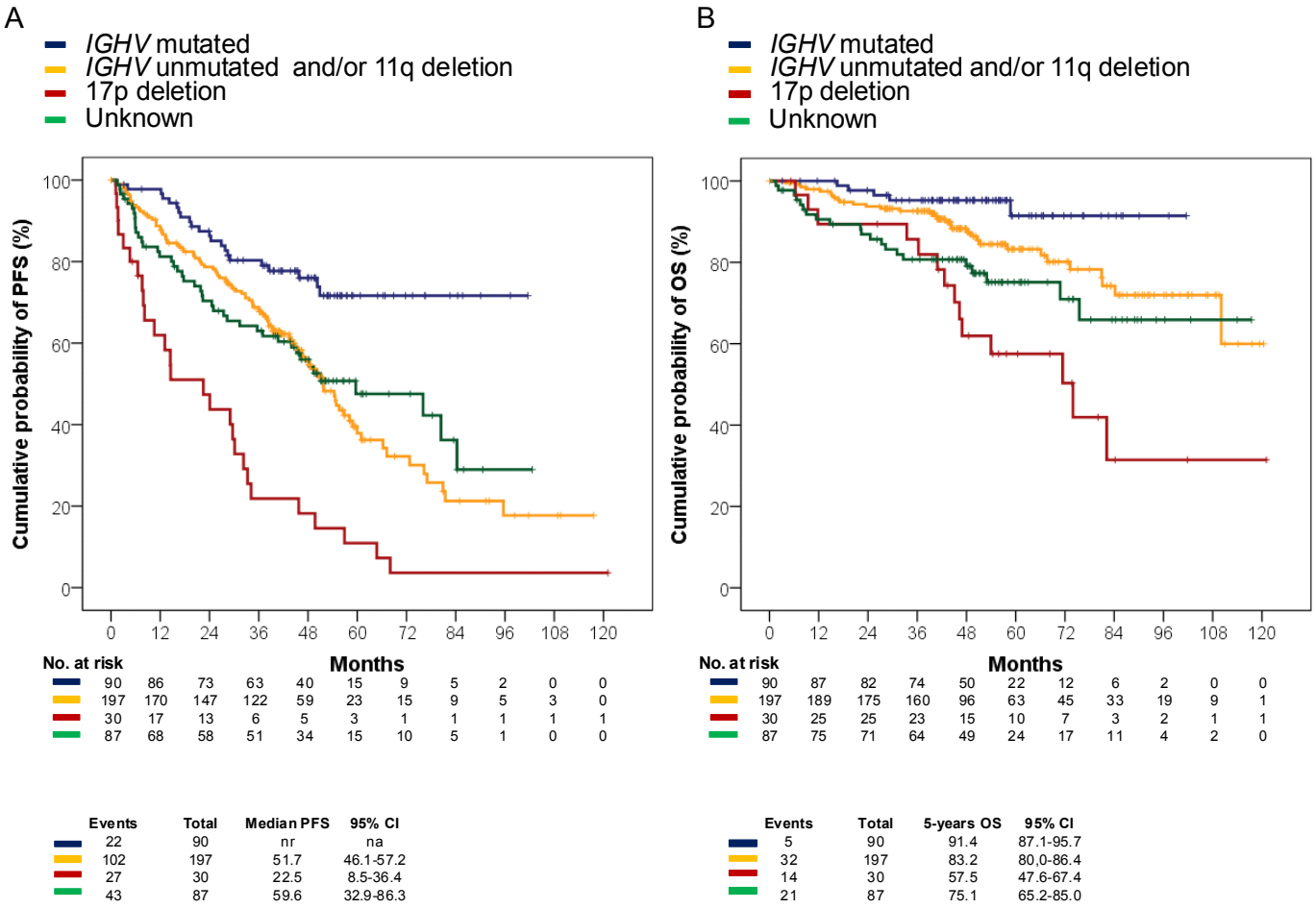
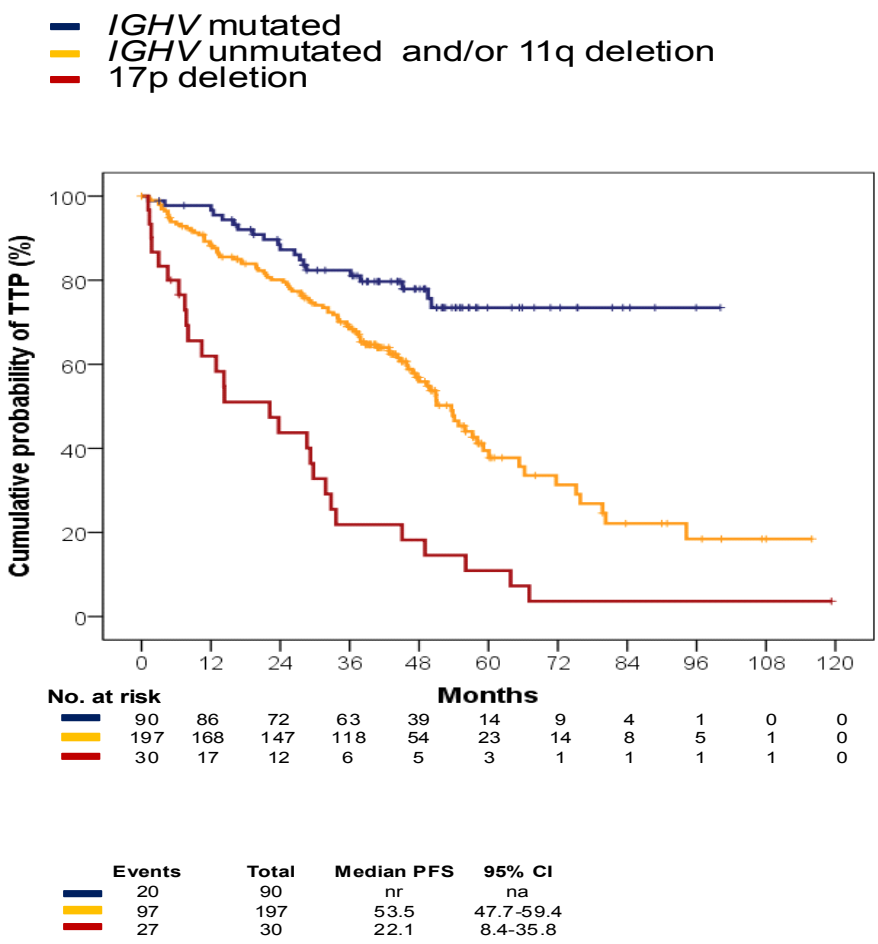

A
IGHV mutated
IGHV unmutated and/or 11q deletion
17p deletion
Unknown
Cumulative probability of PFS (%)
100
80
60
40
20
0
0 12 24 36 48 60 72 84 96 108 120
Months
No. at risk
90 86 73 63 40 15 9 5 2 0 0
197 170 147 122 59 23 15 9 5 3 0
30 17 13 6 5 3 1 1 1 1 1
87 68 58 51 34 15 10 5 1 0 0
Events Total Median PFS 95% CI
22 90 nr na
102 197 51.7 46.1-57.2
27 30 22.5 8.5-36.4
43 87 59.6 32.9-86.3
B
IGHV mutated
IGHV unmutated and/or 11q deletion
17p deletion
Unknown
Cumulative probability of OS (%)
100
80
60
40
20
0
0 12 24 36 48 60 72 84 96 108 120
Months
No. at risk
90 87 82 74 50 22 12 6 2 0 0
197 189 175 160 96 63 45 33 19 9 1
30 25 25 23 15 10 7 3 2 1 1
87 75 71 64 49 24 17 11 4 2 0
Events Total 5-years OS 95% CI
5 90 91.4 87.1-95.7
32 197 83.2 80,0-86.4
14 30 57.5 47.6-67.4
21 87 75.1 65.2-85.0

Figure 7



	Events	Total	Median PFS	95% CI
	20	90	nr	na
	97	197	53.5	47.7-59.4
	27	30	22.1	8.4-35.8

Figure 8

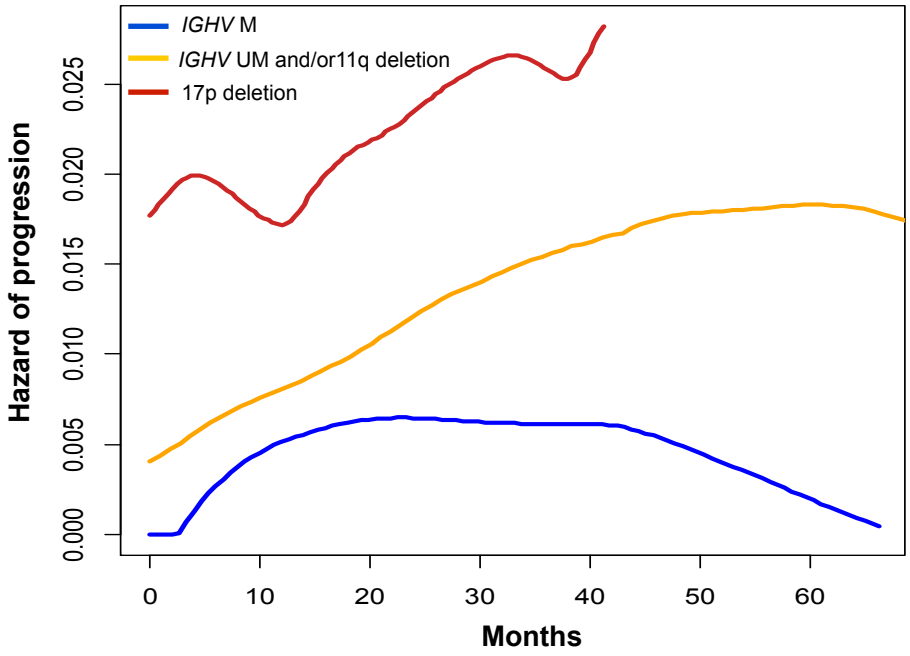


Figure 9

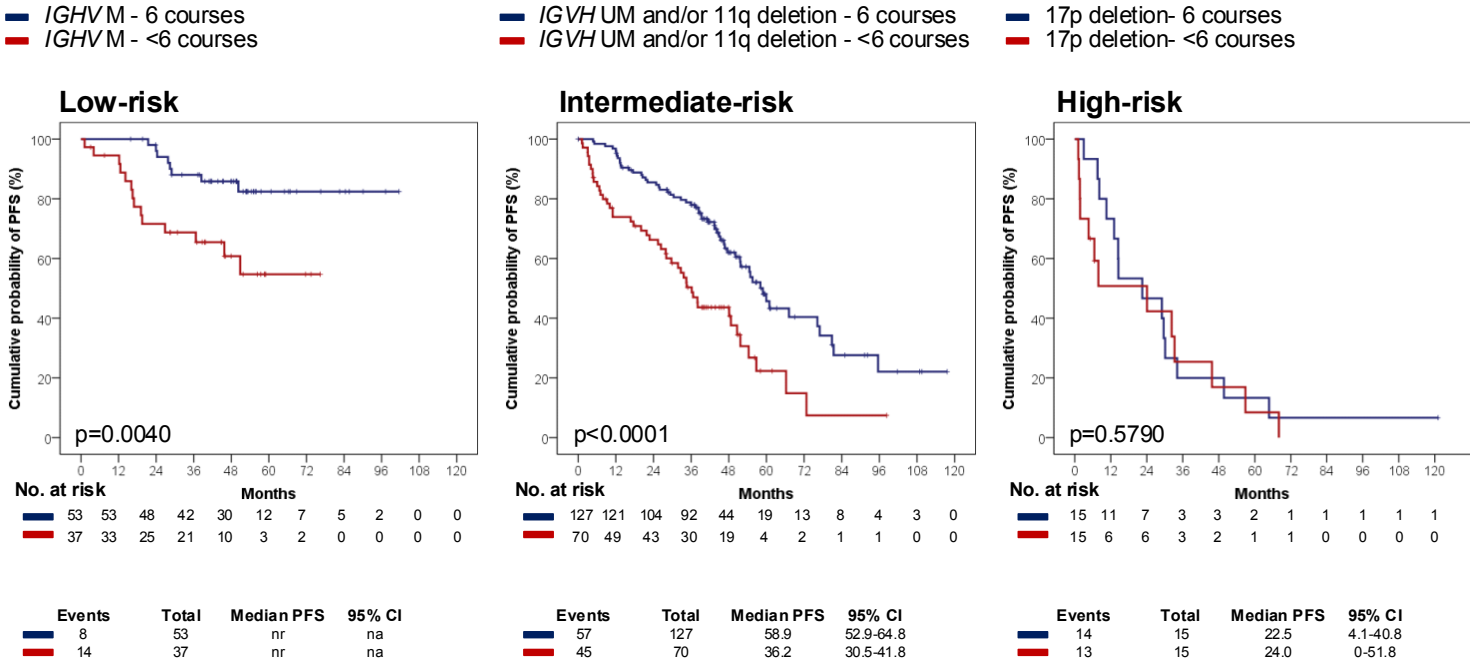


Figure 10